Hematopoietic stem cell-derived products for cancer immunotherapy

Development and characterization of immunotherapeutic strategies to boost graft-versus-tumor immunity

Soley Thordardottir
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Development and characterization of immunotherapeutic strategies to boost graft-versus-tumor immunity

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General introduction and outline of this thesis
Chapter 1

**General introduction**

Over the past decades it has been demonstrated that the immune system has an indispensable critical role in tumor control. This has consequently driven the development of novel strategies that mediate tumor clearance by initiating new or boosting existing immune responses against the malignant cells. An example of one of the first successful immunotherapeutic strategies that has been developed is allogeneic stem cell transplantation (alloSCT). AlloSCT is a powerful cellular immunotherapy for patients with aggressive hematological malignancies, and to date, is the only possible curative option for many patients. The therapeutic effect is mediated by lymphocytes, mainly T cells and natural killer (NK) cells, originating from the donor graft that recognize the patient’s tumor cells as foreign and subsequently eradicate them; in brief called the graft-versus-tumor (GVT) effect. However, in a significant number of patients the induction or reactivation of these immune responses is inadequate, contributing to disease progression or relapse. This illustrates the urgency to develop novel interventions to initiate and/or boost more potent GVT immunity and prevent tumor relapse post-alloSCT. This thesis describes the pre-clinical development of immunotherapeutic strategies that could potentially be applied as adjuvant therapy post-alloSCT to improve its therapeutic efficacy. Furthermore, these strategies could potentially also be applied in a non-transplant setting of hematological malignancies or solid cancers. We describe and characterize ex vivo-generation of different dendritic cell (DC) subsets and NK cells from CD34+ hematopoietic stem and progenitor cells (HSPCs). These HSPC-derived cell products could be used for DC vaccination and adoptive NK cell transfer, respectively. Furthermore, we explore the potential of CLEC12A, an endocytic receptor expressed by DCs and other myeloid cells, as a receptor for in vivo-targeted vaccination strategies. This chapter provides an introduction to the studies presented in this thesis.

**Normal hematopoiesis**

Hematopoiesis represents the developmental process of hematopoietic stem cells (HSCs) into mature and functional blood cells, such as erythrocytes, thrombocytes and leukocytes. Each of these different cell lineages have their unique function: erythrocytes transport oxygen throughout the body, thrombocytes are important components of blood clotting, and leukocytes protect us against pathogens. Hematopoiesis in adults occurs mainly in the bone marrow, where HSCs reside, the cells that are the apex of the hematopoietic hierarchy. HSCs are capable of self-renewal to maintain a small pool of HSCs throughout our life-span. Furthermore, they give rise to multi-potent progenitor cells committed to more specific blood cell lineages (Figure 1). In the classical model of hematopoiesis, blood cells belong to either of two fundamental branches/lineages: the lymphoid branch or the myeloid branch. In this classical model, the lymphoid branch includes T, B and NK cells, while the myeloid branch gives rise to relatively short-lived cells...
such as monocytes, granulocytes, DCs, megakaryocytes/thrombocytes and erythrocytes. However, recent research shows that this segregation between lymphoid and myeloid is not black and white, and that the mature blood cells can originate from more than one type of progenitor cell. The choices in cell fate are tightly regulated by external cues from the environment, where cytokines play the most pivotal role. After interactions with cell-surface cytokine receptors, subsequent downstream signaling regulates expression and activity of lineage-specific transcription factors, thereby determining the fate of the cell.

Figure 1. Schematic overview of hematopoiesis. Diagram showing the classical representation of how HSCs develop into the different mature blood cells via intermediate hematopoietic progenitor cells. Dashed lines indicate alternative routes of differentiation of HSCs towards mature blood cells. HSC, hematopoietic stem cell; MPP, multi-potent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-macrophage progenitor. Figure adapted from Kroeze and Koorenhof-Scheele.

Hematological malignancies

Hematological malignancies are a group of neoplasms that affect the formation and function of the different blood cells. Due to malignant transformation of cells of the hematopoietic lineage, either in the bone marrow or in peripheral lymphatic systems,
the cells gain growth advantage and multiply uncontrollably, resulting in disturbance of normal hematopoiesis and function of normal blood cells. As a result, patients may suffer from fatigue and anemia, excessive bleeding and bruising, and recurrent infections. In total, new cases of hematological malignancies account for ~8% of all new cancer diagnoses in Europe, with an estimated 230,000 patients diagnosed per year.8,9 Hematological neoplasms are mainly a disease of the elderly, and since the general population is growing and aging, it is therefore expected that the incidence rates will rise exponentially in the forthcoming years.10

Diagnosis of hematological malignancies is based on morphology, histopathology, immunophenotype, clinical features and underlying genetic defects. According to the world health organization, malignancies are primarily stratified according to lineage as myeloid cancers, lymphoid cancers, cancers with myeloid and lymphoid lineage markers or histiocytic/dendritic cell cancers.11 Further classifications are based on whether the neoplasm rises from early progenitors (e.g. acute myeloid leukemia (AML), lymphoblastic lymphomas/leukemias) or more mature blood cells (e.g. (Non-)Hodgkin lymphoma, multiple myeloma), and whether the disease progression is fast (acute) or slow (chronic). The prognosis for patients with hematological malignancies depends not only on the type of disease and etiology, but also on the patient age and their morbidity. Older patients (>65 years) have a much poorer prognosis and lower survival rate.12,13 These factors also influence the choice of treatment. Standard treatments include chemotherapy and radiation therapy, which aims to reduce tumor burden and induce long-term remission. These therapies are often combined with hematopoietic stem cell transplantation (SCT) to reconstitute a new hematopoietic system. The HSCs can be obtained either from the patient itself (autologous) or from a relative or an unrelated donor (allogeneic). As allogeneic SCT (alloSCT) is the setting of this thesis, it is presented in more details below. Besides these standard treatments, several new and more tumor-specific drugs, associated with lower toxicity, have been developed in the past decades. For example, the first-line treatment currently used for chronic myeloid leukemia (CML) consists of tyrosine kinase blockers (e.g. Imatinib, Nilotinib and Dasatinib) that inhibit the BCR-ABL oncoprotein.14,15 Since the introduction of those drugs, the 10-year overall survival for CML has increased from 10-20% to 80-90%.16,17 Another example is treatment of B cell lymphomas and other CD20-expressing malignancies which has been revolutionized with the development of anti-CD20 monoclonal antibodies, such as Rituximab.18,19 Furthermore, hypomethylating agents (HMA), such as decitabine and azacitidine, are increasingly being used as (additive) treatment for patients with myelodysplastic syndrome (MDS) and AML, not only because they are well tolerated and have direct pro-apoptotic effects on tumor cells, but also because of their immunomodulatory effects.20,21

Despite progresses with these new and more specific targeted drugs, alloSCT is the most effective therapy for patients with (very) poor risk hematological malignancies. Apart from reconstituting the patient with a new healthy immune system, the aim of alloSCT is
to induce specific immune responses against the tumor cells of the patient.\textsuperscript{22,23} Actually, alloSCT can be considered the most widely applied and, to date, the most successful cellular immunotherapy against cancer, with over 15,000 transplantations performed yearly in Europe.\textsuperscript{24}

**AlloSCT as therapy for hematological malignancies**

Originally, the aim of SCT was to replenish the patient with a new healthy hematopoietic system, facilitating the delivery of higher dosages of chemo- and radiation therapy that would otherwise have been impossible. Without SCT, patients would have suffered from prolonged bone marrow aplasia. It soon became apparent though, that when patients were transplanted with allogeneic HSCs, an additional tumor eradication took place which was independent of the high-dose chemo/radiation therapy.\textsuperscript{25,26} This therapeutic effect was attributed to donor-derived allo-reactive immune cells, mainly T cells and NK cells, that recognized hematological tumor cells of the patient and eliminated them.\textsuperscript{23} Unfortunately, development of this potent GVT effect is tightly linked to graft-versus-host disease (GVHD), a life-threatening complication where the immune competent cells of the graft attack and damage healthy tissues of the patient. Development of GVHD is linked to the pre-transplant conditioning regimen, which induces tissue damage and pro-inflammatory cytokine cascades that subsequently results in trafficking of allo-reactive T cells towards inflamed GVHD-prone organs.\textsuperscript{27} Therefore, to reduce GVHD, less intensive conditioning regimens \textit{(i.e.} reduced-intensity and non-myeloablative conditioning regimens\textit{)} and T cell depletion strategies have been developed and implemented in many alloSCT protocols. These protocols have also allowed older patients with high co-morbidity scores to be treated with alloSCT, patients that previously were unfit to receive alloSCT. Unfortunately, the GVHD prophylaxis regimens are associated with higher relapse rates, as the development and/or intensity of the GVT immunity is insufficient to eliminate the residual tumor cells.\textsuperscript{28} It is therefore important to continue working on improving this therapeutic strategy and to develop new adjuvant therapies that could be combined with alloSCT to selectively boost the potency of the GVT immune responses, while limiting the incidence and severity of GVHD. The overall aim is to thereby increase disease-free and overall survival of transplanted patients.

For alloSCT, the HSCs can be obtained from three different sources: bone marrow (BM), umbilical cord blood (UCB) or peripheral blood (PB) after mobilization of the HSCs to the PB using \textit{e.g.} G-CSF (granulocyte colony-stimulating factor). Nowadays, mobilized PB collection is most common, as it can be obtained with minimal discomfort for the donor, has high numbers of HSCs and is associated with faster hematologic recovery.\textsuperscript{29} A very important aspect in alloSCT is to select a donor that has a similar human leukocyte antigen (HLA) genotype as the patient, to minimize the risk of graft rejection and GVHD. Preferable donors are relatives, \textit{e.g.} a sibling, as the chances of HLA matching are higher.
However, many patients do not have a HLA-matched relative. Those patients then receive transplantation either from a HLA-matched unrelated donor, obtained from the stem cell registry, or from a related donor that is partially HLA-matched or haplo-identical. Notably, with the development of GVHD prophylaxis protocols mentioned above, the use of haplo-identical donors has increased in the past decade.30

**GVT effector cells and targeted antigens**

The principal task of the immune system is to distinguish self from non-self (foreign) antigens, and to recognize altered (mutated or damaged) self-antigens. In this regard, HLA molecules (major histocompatibility complex (MHC) class I and II) are very important, as they present the non-self/altered antigens on the cell surface for recognition by the appropriate T cells. All nucleated cells express MHC class I (HLA-A, HLA-B, HLA-C) molecules, which present cytoplasm-derived peptides and intracellular pathogens. On the other hand, MHC class II (HLA-DR, HLA-DQ, HLA-DP) expression is mainly restricted to hematopoietic cells such as macrophages, DCs and B cells. Extracellular antigens are presented in MHC class II. MHC class I and II molecules are recognized by CD8 and CD4 positive T cells, respectively. These HLA molecules and presented antigens play a major role in the pathogenesis and therapeutic effect of alloSCT. Donor-derived T cells recognize antigens, in the context of HLA, that differ between recipient and donor cells. This can result in their activation and subsequent attack of the recipient cells expressing the antigen, which can be healthy tissues and/or the malignant cells. This is the basis of the GVHD and GVT effect, respectively, and is primarily mediated by CD8⁺ cytotoxic T lymphocytes (CTLs).

The magnitude and specificity of the T cell activation depends on the extend of mismatching between donor and recipient. In a full HLA-matched setting, the primary immunological targets of allo-reactive T cells are minor histocompatibility antigens (MiHAs). These MiHAs are HLA-presented peptides derived from polymorphic genes that differ between donor and recipient.31,32 Patient-specific MiHAs may therefore be regarded as foreign to the donor immune T cells, and strong high-affinity T cell responses against MiHA-expressing cells can occur. If the MiHAs are exclusively expressed by hematopoietic cells, the MiHA-specific T cell responses specifically eliminate the hematopoietic cells of the patient, including the residual malignant cells (GVT effect). However, other MiHAs have a ubiquitous expression pattern and are hence also expressed by healthy tissues of the patient. When allo-reactive T cells are directed against these broadly expressed MiHAs, GVHD can occur. Therefore, strategies for separating GVHD from GVT aim at specifically targeting hematopoietic-restricted MiHAs, for example with DC vaccination or adoptive transfer of MiHA-specific T cells. Interesting target MiHAs in this respect include HA-1, HA-2, LRH-1, ARHGDIB and UTA2-1.33

Other important antigens in anti-tumor T cell immunity post-alloSCT are tumor antigens.34,35 Tumor antigens can be of different origin, but the general nominator is that
they are highly or specifically expressed by the tumor cells, while healthy cells have no or low expression of the particular antigen. Tumor-associated antigens (TAAs) are relatively restricted to tumor cells (e.g., overexpressed antigens, cancer-testis antigens and oncofetal antigens), whereas tumor-specific antigens (TSAs) are unique to tumor cells. TSAs are also called neoantigens, and arise for example due to somatic point mutation within the mutant clone. Overexpressed TAAs derived from self-proteins that have low expression on normal tissues are often considered to have lower immunogenicity than MiHAs or TSAs, due to potential immunotolerance. Despite this, studies have demonstrated that TAA-specific T cell responses can be induced with in vivo vaccination, without considerable toxicity.38

Interesting therapeutic TAA-candidates in the context of hematological malignancies are WT1, PRAME, Proteinase 3, RHAMM, MAGE and NY-ESO1.34,39 Interesting TSA-candidates are however more difficult to apply in current cancer vaccination strategies since they are often patient-specific and difficult to identify. Furthermore, studies indicate that hematological malignancies have a lower mutational load than many solid cancers, such as melanoma, resulting in fewer therapeutically-applicable TSAs.35

Besides CD8+ tumor-reactive T cells, the second key player in GVT immunity is the cytotoxic CD56+ NK cell.40 NK cells are innate immune cells that recognize and eliminate stressed and infected cells, but spare healthy cells. In contrast to T cells, NK cell activity does not require antigen-priming, so they can therefore rapidly kill targeted cells. The regulation of NK cell activation is dependent on the sum of signals they receive via a variety of activating and inhibitory receptors expressed on their surface.41,42 The most characterized inhibitory receptors are killer immunoglobulin-like receptors (KIRs) and the C-type lectin receptor NKG2A. The main ligands for these receptors are the ubiquitously expressed MHC class I molecules. In homeostasis, engagement of NK inhibitory receptors by MHC class I molecules prevents NK cells from becoming activated, thereby protecting healthy tissues from NK cell-mediated lysis. Tumor cells may lose or downregulate the expression of MHC class I molecules, thereby providing less or no inhibitory signal to NK cells. As a result, NK cells can become activated and mediate a response against the targeted cell. This is known as “missing-self”-mediated activation.42 On the other hand, NK cells can become activated when the signals via their activating receptors are stronger than the signals via inhibitory receptors. NK cells express numerous activating receptors including natural cytotoxicity receptors (NCRs, including NKp30, NKp44, NKp46), the C-type lectin receptors NKG2D and NKG2C, Fc receptor CD16, activating KIRs and DNAM-1. While some healthy cells express the ligands for these receptors, their expression is primarily increased during cell stress, including infection or DNA damage, which can trigger the activation of NK cells. This is commonly referred to as “stress-induced self” recognition.42

NK cells are one of the first immune cells to repopulate after alloSCT.43-45 Early repopulation has been associated with reduced relapse rates, indicating that allo-reactive NK cells have a specific anti-tumor activity post-transplantation.46-50 Importantly, these


studies have shown that the allo-reactive NK cells do not cause GVHD. Furthermore, donor-derived NK cells may even suppress induction and/or severity of GVHD. The potential of NK cells to eliminate tumor cells has particularly been demonstrated in the alloSCT setting where patients are transplanted with a HSC-graft from a haplo-identical or other partially HLA-matched donor. In this setting, NK cell allo-reactivity was mainly dependent on reduced inhibitory signaling, as inhibitory KIRs on the donor NK cells did not match with their cognate HLA molecule on the recipient cells. The lack of KIR engagement allows the NK cells to kill the tumor cell. On the other hand, in the context of full HLA-matching, NK tumor-reactivity may depend on enhanced signaling via activating receptors, resulting in activation of the NK cells and subsequent cytotoxicity of target cells. In this regard, activating KIRs have been shown to play a role. The KIR gene family consists of many highly polymorphic genes, which are inherited as haplotype A or B from each parent. The main differences between those two haplotypes, is that B haplotypes have more genes encoding activating KIRs than A haplotypes. Recent studies into these KIR genetics have shown that alloSCTs from HLA-matched donors with KIR B/x genotype (containing one or two B haplotypes) are associated with lower relapse rate and improved progression-free survival of transplanted patients.

Cytotoxic CD8⁺ T cells and NK cells kill their targeted malignant cells via several mechanisms. This includes release of cytotoxic granules containing perforin and granzymes, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent cytotoxicity and Fas ligand-induced apoptosis. Moreover, the activated NK cells and T cells secrete cytokines, such as interferon (IFN)-γ, which further boosts the anti-tumor immune response by directly affecting the tumor cells and by promoting recruitment and activity of other immune effector cells. IFN-γ-secreting CD4⁺ T-helper (Th) T cells also contribute to GVT immunity. CD4⁺ T cells are generally thought to be less effective killers than CD8⁺ T cells, but they provide important help to the other immune effector cells. They stimulate antigen-presenting cells (APCs) for priming of CD8⁺ T cells, as well as secrete interleukin (IL)-2, which is an important cytokine for proliferation and cytotoxic functionality of CD8⁺ T cells and NK cells. Additional immune cells that participate in the GVT effect, but are not further discussed here, include NKT cells and γδ T cells.

Boosting anti-tumor immunity with immunotherapy

To enhance the cure rate of patients with hematological malignancies both in the transplant and non-transplant setting, it is essential to improve current strategies and/or develop new therapies to prime and boost anti-tumor immune responses and to create immunological memory for sustained remission. Currently, multiple different immunotherapeutic strategies are being investigated in this regard, either as standalone interventions or combined with other immunotherapeutic treatments or anti-cancer drugs. These include non-cellular interventions such as systemic treatment with tumor-
targeting antibodies, immunomodulatory antibodies or immunostimulatory cytokines, and cellular-based approaches such as adoptive transfer of tumor-reactive T cells and NK cells. Furthermore, anti-cancer vaccination strategies, either non-cellular (using various antigen formulations) or cellular (using DCs), are being explored. Here below, DC-based vaccines and adoptive NK cell therapy is introduced in more detail, as these therapeutic approaches were the focus of this thesis.

**DC-based vaccination**

**DC biology and therapeutic potential**

DCs are the most potent APCs and play a pivotal role in determining the nature and magnitude of innate and adaptive immune responses. DCs are uniquely positioned in peripheral tissues, where they continuously sample the environment for non-self and altered antigens. The DCs subsequently process these antigens into smaller peptides as they mature and migrate towards secondary lymphoid organs. There, the DCs present the captured antigens to T cells in the context of MHC class I and II molecules, thereby inducing a cellular immune response. When compared to other APCs, such as macrophages, DCs are extremely efficient as they provide strong co-stimulatory signals needed for initial priming of naïve T cells. Furthermore, DCs excel at cross-presentation, where extracellular antigens (such as from dying infected cells) are presented in MHC class I molecules to CD8+ CTLs. DCs also orchestrate naïve and memory B cell responses, and can activate innate immune cells such as NK and NKT cells. Thus, DCs can orchestrate all the different aspects of the immune response, which makes them very attractive targets and tools for immunomodulatory strategies to improve tumor-reactive immune responses.

The role of DCs is though more complicated than described above as they have a bilateral function, whereas on one hand they induce productive T cell responses under inflammatory conditions, they on the other hand maintain tolerance against self-antigens under steady state. When immature DCs present tissue antigens to T cells in the absence of appropriate co-stimulation, peripheral tolerance is induced resulting in T cell anergy and/or development of suppressive IL-10 secreting regulatory T cells. Moreover, presence of immature DCs within tumors has been associated with tumor progression and suppressed anti-tumor immune response development. Efficient maturation of the DCs is therefore of utmost importance to facilitate induction of productive anti-tumor immune responses. During DC maturation, tightly controlled series of events occur that are initiated by a spectrum of environmental and endogenous stimuli. In this respect, DCs express numerous receptors, that recognize different components of pathogens, such as proteins, lipids, carbohydrates, RNA (ribonucleic acid) and DNA (deoxyribonucleic acid). These receptors are collectively called pattern recognition receptors (PRRs), and include Toll-like receptors (TLRs), C-type lectin receptors, NOD-like receptors and RIG-I-like receptors. The TLRs are the most studied PRRs, where 10 different ones (TLR1-10),
have been characterized in human DCs. Upon stimulation/maturation via PRRs, DCs downregulate endocytic and phagocytic receptors, upregulate chemotactic receptors (that guide migration to lymph nodes) and co-stimulatory molecules such as CD80, CD86 and CD40, increase transport of peptide-MHC complexes to the surface, and synthesize and secrete a variety of cytokines. In the context of DC vaccination, synthetic TLR ligands can be used to mimic the “natural” pathogen-induced maturation pathways of DCs. Thereby immune responses can be skewed towards productive pro-inflammatory and cytotoxic T cell and NK cell responses.

**DC subsets**

DCs are a heterogenous population of cells, that comprise many different subsets which differ in ontogeny, phenotype, function and localization. Due to the low frequency of these cells, characterization and classification of different DC subsets has remained difficult. Furthermore, their phenotype is subject to changes dependent on location, environmental cues and maturation status, which further hampers the characterization and categorization. Despite these challenges, detailed insight has been gained about DCs since their discovery. The DC subsets found in human PB are broadly divided into two major categories: myeloid DCs (mDCs; also frequently referred to as conventional or classical DCs) and plasmacytoid DCs (pDCs) (Figure 2). mDCs can further be subdivided into two populations based on their differential expression of the surface molecules BDCA1 (CD1c) and BDCA3 (CD141). Although of low frequency, these three DC subsets (BDCA1+ mDCs, BDCA3+ mDCs, and pDCs) can also be identified in steady state in other lymphoid organs, such as lymph nodes and spleen, and also in some other peripheral tissues.

![Figure 2. Human dendritic cells (DCs).](image)

In peripheral blood and lymphoid tissue, three major populations of DCs can be identified, plasmacytoid DCs (pDCs), BDCA1+ myeloid DCs (mDCs) and BDCA3+ mDCs. These three DC subsets are considered the “bona fide” DC subsets. Other cells that can be classified as DCs include Langerhans cells, which are the most abundant DCs in the epidermis. Furthermore, monocytes can differentiate into DCs under certain circumstances, such as inflammation (inflammatory DC) or in vitro with GM-CSF and IL-4 (monocyte-derived DC (MoDC)).
Recent reports have delineated the developmental pathway of these different DC subsets from CD34+ HSPCs in humans. These studies indicate that terminally differentiated DCs arise after series of lineage-restricted transitions from progenitor cells located in the BM (Figure 3). A study by Lee et al. described that these transitions go via a monocyte-DC progenitor (MDP) that has the capacity to develop into monocytes or the common DC progenitor (CDP). They further show that the CDP is restricted to generate the three major DC subsets mentioned above, by giving rise to pDCs and pre-mDCs that migrate through blood and replenish the pool of DCs in lymphoid organs and peripheral tissue. Breton et al. further described that the pre-mDC is an immediate precursor of both BDCA1+ mDCs and BDCA3+ mDCs, but not of pDCs or monocytes, and can be found in BM, UCB, PB as well as tonsils. This pre-mDC is negative for CD34 and lineage markers (including BDCA1 and BDCA3), while positive for CD45RA, CD135 (Flt3), CD116 (GM-CSF receptor) and CD117 (SCF receptor). Furthermore, the pre-mDC expresses a high level of HLA-DR and intermediate level of CD123 (IL-3 receptor). Like for other immune cells, distinctive growth factors and transcription factors guide the development of DCs from hematopoietic progenitor cells. These growth factors include Flt3-ligand (Flt3L), SCF, GM-CSF and IL-3, which act via their respective surface receptor for modulating transcription and lineage commitment. In particular, Flt3L has been established as the key cytokine driving DC development and homeostasis. For example, studies both in humans and mice have shown that over-expression or injection of Flt3L markedly increases the numbers of circulating DC subsets and pre-mDCs. Despite the recent advances in delineating human DC development, more understanding of the transcriptional network and dependency of different progenitors and DC subsets on distinctive cytokines is required, particularly in humans.

DCs that reside in lymphoid tissues are generally referred to as “resident” DCs. While, DCs that traffic from peripheral tissue into lymph nodes, carrying antigens that they have sampled in the periphery, are generally referred to as “migratory” DCs. These migratory DC subsets found in peripheral tissues are less characterized than DCs in lymphoid organs, with the exception of skin DCs. In skin, the different subsets listed above can be found, in addition to a special type of DC, called Langerhans cell (LC) (Figure 2). LCs were for many years considered to be the classical DCs, however, studies during the past decade showed that in steady-state this population can self-renew and is independent of adult hematopoiesis, and is therefore more similar to a macrophage than DC. Nevertheless, upon inflammatory stimuli or after SCT, LCs can also be generated from circulating bone marrow precursors. Whether these precursors are shared with the other DC subsets, BDCA1+ mDCs and BDCA3+ mDCs, remains to be elucidated. Interestingly, two recent studies indicate that BDCA1+ mDCs may have a potential LC-precursor function, as BDCA1+ mDCs, when exposed to transforming growth factor (TGF)-β ex vivo, can differentiate into LCs.
Chapter 1

In general, inflammatory conditions can induce major changes to the DC compartment. For example, upon inflammation, monocytes can adopt dendritic morphology and antigen-presentation functions. Transcriptome analysis showed that these DCs, referred to as monocyte-derived DCs (MoDCs) or inflammatory DCs (Figure 2), are distinct from steady-state lymphoid organ and blood DCs. Their functional contribution in vivo remains to be determined. In vitro however, MoDCs represent the best studied DC subset so far, as they can be readily generated from isolated monocytes by culturing them with the cytokines GM-CSF and IL-4. So far, most DC-based clinical trials have utilized MoDCs.

Besides ontogeny and location, other attributes differ between the DC subsets that influence their functional characteristics. Differential expression of PRRs is one such attribute, which conveys distinct maturation signals to the DC, and determines their activation status and the type of immune response generated. The two main PRRs expressed by pDCs are TLR7 and TLR9. TLR7 recognizes single-stranded RNA from RNA viruses, while TLR9 senses unmethylated DNA with CpG motifs derived from bacteria and viruses. In response to such ligands, pDCs secrete large amounts of type I interferons (IFN-α/β), which gives pDCs a critical role in induction of anti-viral immunity. The mDC subsets have a broader range of PRR expression than pDCs, which facilitates their response to a broader range of pathogens. Upon TLR-stimulation, BDCA1+ mDCs and BDCA3+ mDCs can produce IL-12, which is a key cytokine for induction of cytotoxic CD8+ T cells, IFN-γ-secreting CD4+ T cells and anti-tumor responses. For induction of anti-tumor immunity, cross-presentation of tumor-derived exogenous antigens to CD8+ T cells is another key feature of DCs in which PRRs are involved. BDCA3+ mDCs are considered the most efficient cross-presenting DC subset in humans, which is related to their selective expression of the C-type lectin CLEC9A (DNGR1), a receptor that mediates recognition and uptake of necrotic cells. However, cross-presentation capacity has also been demonstrated in vitro for both BDCA1+ mDCs and pDCs. Besides individual contribution of the DC subsets to induction of immune responses, studies show that pDCs and mDCs can interact and thereby synergistically enhance each other’s stimulatory capacity. This synergistic cross-talk has been shown to enhance the development of anti-tumor immune responses. Therefore, given the distinct functions of each subset and their potential to enhance each other’s stimulatory capacity via cross-talk, it is very interesting and promising to develop therapeutic strategies for cancer patients that involve these different DC subsets.
General introduction and outline of this thesis

Figure 3: Proposed model of dendritic cell (DC) development from hematopoietic progenitor cells. During DC development, first a granulocyte, monocyte and DC progenitor (GMDP) differentiates into a monocyte and DC progenitor (MDP), which then gives rise to a common DC progenitor (CDP). This CDP can develop into plasmacytoid DC (pDC) or pre-myeloid DC (mDC). Pre-mDCs migrate from the bone marrow into blood and peripheral tissue, where they produce the two major mDC subsets, BDCA1+ and BDCA3+ mDCs.81,82

Utilizing DCs for induction of anti-tumor immunity

Numerous different therapeutic vaccination strategies are under thorough investigation for evaluating their potential to induce productive anti-tumor immunity. All these strategies revolve around utilizing the exceptional capacity of DCs to present antigens and provide co-stimulation to T cells, in particular CD8+ T cells. The desired outcome elicited by the vaccines is priming and expansion of CD8+ CTLs that can eliminate the malignant cells, and formation of long-lived memory T cells that can rapidly generate new cytotoxic effector T cells in the case of relapse. These T cells responses can be directed against tumor antigens such as TAAs or TSAs.70 Furthermore, in the setting of MiHA-mismatched alloSCT, DCs presenting hematopoietic-restricted MiHAs can be utilized to selectively boost GVT immunity.
Anti-cancer vaccines can broadly be classified as *ex vivo*-loaded, non-targeted or *in vivo*-targeted vaccines. The first class involves infusing patients with DCs that are matured and loaded with antigens (TAAs, TSAs or MiHAs) *ex vivo*. As natural DC subsets in PB are scarce (less than 0.5% of white blood cells), so far the majority of clinical trials has applied *ex vivo*-generated MoDC vaccines. Monocytes for DC generation can easily be isolated in high numbers from the patient, or from the stem cell donor in the case of alloSCT. After *ex vivo* differentiation of monocytes into MoDCs, maturation is induced by a combination of pro-inflammatory cytokines and/or synthetic TLR ligands. Thereafter, the mature DCs are loaded with the desired antigen (*e.g.* tumor antigen or MiHA) by 1) incubation with MHC class I- and/or class II-binding peptides, 2) incubation with tumor lysate, or 3) transfection with RNA encoding tumor antigens or MiHAs which will be synthesized into proteins and processed by the DCs themselves. Clinical studies in patients with solid tumors and hematological malignancies have concluded that vaccination with *ex vivo*-loaded DCs is feasible, safe and can induce expansion of tumor-specific CD4+ and CD8+ T cells. Moreover, vaccination of alloSCT recipients has also been demonstrated to be safe without induction or aggravation of GVHD. However, despite induction of tumor-specific T cell responses, the number of vaccinated patients showing tumor regression and objective clinical responses is low. This illustrates that further optimizations of this strategy are warranted to improve the therapeutic potency. Multiple parameters could affect the clinical response/therapeutic efficacy, including DC maturation stimuli and duration, antigen loading strategy, route of administration, and dosing and frequency of the vaccine infusions. Furthermore, the subtype of DCs could also matter. We and others postulate that primary DC subsets, such as pDCs and mDCs, might be more potent stimulators of anti-cancer immune responses than MoDCs. In this regard, few clinical trials have recently explored the use of pDCs or BDCA1+ mDCs isolated from PB of melanoma and prostate cancer patients for therapeutic vaccination. An alternative approach, particularly for vaccination of alloSCT recipients, is the generation of different DC subsets from HSPCs obtained from SCT donors. We have established and investigated this strategy in chapters 2-4, where we describe novel *ex vivo* culture systems for generation of different subsets of DCs, including BDCA1+ mDCs, BDCA3+ mDCs, and pDCs, from CD34+ HSPCs. We demonstrate the potential of these DC subtypes to initiate and boost tumor-reactive T cell and NK cell responses *ex vivo*. It would be very interesting to explore vaccination with antigen-loaded DCs of different subtypes as post-transplantation therapy for alloSCT patients. It has the promise to selectively boost tumor-reactive T cell and NK cell responses, without evoking severe GVHD.

Besides studies that focus on *ex vivo*-loaded DC vaccines, multiple studies explore strategies that harness the DCs *in vivo* for induction of anti-tumor immunity. On one hand, these include non-targeted vaccination strategies, where for example formulations of tumor antigen (commonly long peptide) and adjuvant are infused. These formulations are captured randomly by DCs or other APCs at the injection site or in draining lymph
nodes, leading to in vivo antigen processing and presentation. On the other hand, multiple studies investigate efficacy of in vivo-targeted vaccines, that are delivered specifically to (a subset of) DCs by coupling or fusing antigens to antibodies directed against endocytic receptors expressed on the surface of DCs. In this regard, multiple DC-specific receptors are being characterized and evaluated for their effectiveness as DC targets, including DEC-205, DC-SIGN, mannose receptor, CLEC9A and Langerin. Many factors need to be considered when selecting the most appropriate receptor for targeting, dependent on the desired outcome. This includes for example the expression pattern of the targeting receptor on the different DC subsets, their ability to deliver antigens to intracellular compartments for processing and loading of peptides onto MHC, and the type of immune responses (e.g. Th1, Th2, cellular, humoral) that are initiated upon targeted delivery of antigens. In chapter 5, we describe such characterization for the C-type lectin receptor CLEC12A, an endocytic receptor expressed by all human DC subsets and monocytes. We show that CLEC12A is a promising targeting receptor, as targeted antigens were efficiently processed and presented in both MHC class I and II, leading to activation of antigen-specific CD4+ and CD8+ T cells.

Adoptive NK cell therapy

Immunotherapeutic strategies utilizing the potent cytolytic capacity of NK cells against tumor cells are being increasingly investigated both in the setting of solid and hematological cancers. The activation and cytolytic function of NK cells is regulated by signals they receive via inhibitory and activating receptors expressed on their surface. The principle of adoptive NK cell transfer is to infuse NK cells that get activated and target the tumor cells due to the absence of inhibitory signals (i.e. missing-self) and/or by infusing NK cells with high expression of activating ligands. The missing self-mediated activation can be promoted by using allogeneic NK cells, and for that reason, the majority of clinical trials to date have utilized allogeneic NK cells for adoptive transfer. In this setting, NK cells are generally isolated or enriched from PB of a relative or unrelated donor. Subsequently, NK cells are infused into the patient right away, overnight stimulated with IL-2 and/or IL-15, or expanded ex vivo for up to three weeks in the presence of IL-2 to obtain higher numbers of cells for infusion. Clinical studies investigating these approaches have demonstrated that infusion of allogeneic NK cells is safe, but yet no firm conclusion on clinical efficacy has been drawn. Limited clinical efficacy of PB NK cells has been associated with low numbers and poor activation/maturation status. Furthermore, low purity and contamination with allogeneic T cells has hampered their use. Few trials have also investigated the use of autologous NK cells, though in that setting an inhibitory KIR-HLA engagement is likely still present, requiring powerful NK cell activation prior to infusion.

An alternative approach for obtaining NK cells for adoptive transfer is to generate them ex vivo from HSPCs. In this regard, our group has developed a novel good
manufacturing practice (GMP)-compliant manufacturing procedure for generation of NK cells from CD34+ HSPCs isolated from UCB. Using this procedure, high numbers of NK cells can be generated.\textsuperscript{128,129} The feasibility and safety of using allogeneic UCB-derived NK cells has recently been demonstrated in a phase I clinical trial in elderly AML patients who were ineligible for SCT.\textsuperscript{130} Before NK infusion, patients received cyclophosphamide and fludarabine (Cy/Flu) lympho-depleting chemotherapy which prevents immediate rejection of the allogeneic NK cells. Furthermore, Cy/Flu conditioning promotes cytokine availability for the transferred NK cells.\textsuperscript{131} Infusion of UCB-derived NK cells in combination with Cy/Flu conditioning was well tolerated, and did not induce GVHD nor other toxicity. Notably, 2 out of 4 patients with minimal residual disease (MRD) before treatment showed promising response as they became temporarily MRD negative.\textsuperscript{130} It would also be very interesting to explore the use of \textit{ex vivo}-generated NK cells in the transplantation setting, since early repopulation and high NK cell numbers after transplantation have been associated with reduced relapse rates.\textsuperscript{46-50} Furthermore, NK cells also provide protection against viral, bacterial and fungal infections in transplanted patients.\textsuperscript{132-134} In the alloSCT setting, it would be most ideal to inject NK cells of stem cell donor origin, to avoid rejection and allow longer persistence. Thereby, multiple infusions of NK cells would be feasible. Furthermore, pre-conditioning with lympho-depleting chemotherapy might not be required when donor-derived NK cells are used. For this, the NK cells would need to be generated from CD34+ HSPCs isolated from G-CSF-mobilized PB or BM, as these stem cell sources are more commonly used for transplantation of patients than UCB. In \textbf{chapter 6}, we describe the optimization of the before-mentioned culture system for generation of NK cells from G-CSF-mobilized PB and BM HSPCs. Infusion of these HSPC-derived NK cells post-transplantation has the potential to induce long-term GVT efficacy and protection against infectious complications post-alloSCT.
Outline of this thesis

AlloSCT has the potential to cure patients with hematological malignancies by clearing residual disease. However, alloSCT is still associated with significant morbidities, such as development of the detrimental GVHD and opportunistic infections, that affect the patient’s quality of life and outcome. Furthermore, a significant portion of patients experiences relapse of the malignancy. For these reasons, development of new (adjuvant) therapeutic strategies are needed to improve the outcome and long-term relapse-free survival of transplanted patients. In this respect, new strategies aim to boost GVT immunity, while preventing adverse events such as GVHD and infections. In this thesis, we describe the pre-clinical development of such immunotherapeutic strategies, including DC vaccination and NK cell adoptive transfer.

In chapter 2, we aimed to develop a culture protocol for generating immunostimulatory DCs from CD34+ UCB HSPCs to use as a vaccine for patients with hematological malignancies. We describe a step-wise ex vivo culture protocol using specific combinations of different cytokines and growth factors, that facilitates the generation of high numbers of phenotypically mature DCs. Within this culture system, primarily CD1a+ mDCs and LC-like DC subsets were generated, that could efficiently induce allogeneic and MiHA-specific T cell responses in vitro. Furthermore, cytomegalovirus (CMV)-loaded UCB-derived DCs induced proliferation of CMV-specific CD8+ cytotoxic T cells, both in vitro and in vivo. However, this culture system did not support the generation of other DC subsets, such as pDCs and BDCA3+ mDCs. As we believe these subsets may be superior for induction of anti-tumor immunity, we explored alternative culture protocols. In chapter 3, we describe our discovery that inhibition of the aryl hydrocarbon receptor (AhR), using the antagonist StemRegenin 1 (SR1), was essential for differentiation of pDC, BDCA1+ mDC and BDCA3+ mDC subsets from CD34+ HSPCs ex vivo. We demonstrated how these subsets could be generated in high numbers from both UCB and G-CSF mobilized PB CD34+ HSPCs. Furthermore, we assessed their phenotype and function, which was comparable to their naturally occurring counterparts in blood. In chapter 4, we describe further optimization of this novel SR1-based culture protocol for generation of higher amounts of the different DC subsets and adaptations to GMP-culture conditions. Importantly, we demonstrated the superior capacity of SR1-generated HSPC-derived pDCs and BDCA1+ mDCs in stimulating tumor-reactive T cell and NK cell responses.

In addition to the ex vivo-generated DC approach, we pursued the idea to move towards an off-the-shelf vaccine for stimulating anti-tumor immunity in chapter 5. In this regard, we investigated the potential of targeting DCs in vivo via endocytic receptors. We show that CLEC12A, a C-type lectin receptor expressed by all the different DC subsets, is an attractive target. We demonstrate that CLEC12A targeting does not negatively affect DC maturation nor function, and that antigens delivered via CLEC12A were efficiently presented to CD4+ and CD8+ T cells. Importantly, we observed that CLEC12A-targeted
delivery of MiHAs resulted in efficient antigen cross-presentation and activation of MiHA-specific CD8+ T cells.

Our group has previously established a GMP-compliant cytokine-based ex vivo culture protocol for generation of high numbers of NK cells from UCB CD34+ HSPCs. As recent advances have shown that AhR does not only play a role in DC development, but also regulates homeostasis and differentiation of various other immune cells,135 we examined the effect of SR1 addition during ex vivo generation of NK cells. In chapter 6, we demonstrate that SR1 not only enhances expansion of CD34+ HSPCs, but also potentiates the differentiation of these cells into NK cells. As a result, high numbers of NK cells could be generated from BM and G-CSF mobilized PB CD34+ HSPCs. Furthermore, we demonstrated that these NK cells were highly active and efficiently targeted and lysed malignant hematopoietic cells. Finally, as these NK cells have the potential to be used for adoptive transfer post-transplant, we showed that NK cell proliferation and function was not inhibited by cyclosporine A (CsA), an immunosuppressive drug often used after alloSCT.

In chapter 7 the findings described in this thesis are summarized and in chapter 8 they are discussed in the context of potential future implications.
References

Chapter 1


General introduction and outline of this thesis

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General introduction and outline of this thesis
Ex vivo generation of interstitial and Langerhans cell-like dendritic cell subset-based vaccines for hematological malignancies

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Abstract

Autologous, patient-specific, monocyte-derived dendritic cell (MoDC) vaccines have been successfully applied in the clinical studies so far. However, the routine application of this strategy has been hampered by the difficulties in generating sufficient numbers of DCs and the poor DC vaccine quality due to pathology or prior treatment received by the patients. The immunotherapeutic potential of other subsets of DCs has not been thoroughly investigated due to their rarity in tissues and difficulties associated with their ex vivo generation. The high expansion and differentiation potential of CD34+ hematopoietic stem and progenitor cells (HSPC), isolated from umbilical cord blood (UCB), into different DC subsets make them an attractive alternative DC source for cancer immunotherapy. Therefore, the aim of this study was to generate a large number of different DC subsets from CD34+ HSPCs and evaluate their functionality in comparison with MoDCs. Our culture protocol generated a clinically relevant number of mature CD1a+ myeloid DCs and CD207+ Langerhans cell (LC)-like DC subsets from CD34+ HSPCs with >95% purity. Both DC subsets exhibited a cytokine profile that favor cytotoxic T cell responses. Furthermore, UCB-DCs and UCB-LCs demonstrated superior induction of proliferation of both allogeneic as well as viral antigen-specific CD8+ T cells, both in vitro and in vivo. Additional studies revealed that UCB-DCs and UCB-LCs can efficiently expand minor histocompatibility antigen (MiHA) HA-1-specific cytotoxic T cells in the peripheral blood of leukemia patients and prime MiHA HA-1-specific and HA-2-specific cytotoxic T cells in vitro. These pre-clinical findings support the pharmaceutical development of the described culture protocol for clinical evaluation.
Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia occurring in adults.\(^1\) Despite improvements in chemotherapy and supportive care, AML has still a very poor prognosis resulting in a 5-year overall survival rate of only 24\%.\(^2\) One of the main causes for this bad prognosis is relapse of the disease after reaching complete remission with classical polychemotherapy due to persistence of chemo-resistant malignant cells during remission.\(^3,4\) Although, allogeneic hematopoietic stem cell transplantation together with donor lymphocyte infusion (DLI) after polychemotherapy is a curative option, the treatment-related mortality and morbidity issues, and lack of suitable donors limit its routine use in elderly AML patients.\(^5,6\) Therefore, alternative treatment options are urgently needed to improve the prognosis of AML patients.

Evidence for immunosurveillance of AML and susceptibility of leukemia cells to both T and NK cell attack has paved way for the development of new immunotherapeutic strategies.\(^7\) Currently, interleukin (IL)-2 administration\(^8,9\), monoclonal antibody therapy\(^9\) vaccination with peptides derived from leukemia-associated antigens (LAA)\(^10,11\) and cellular therapies\(^12\) are being explored as adjuvant therapies to eradicate minimal residual disease (MRD) or to induce anti-leukemic immunity to control relapse of malignant disease. Among these cellular therapies, dendritic cell (DC)-based immunotherapy holds a great potential as an adjuvant cancer therapy in the setting of MRD to boost the patient’s own immune system to eradicate AML cells and induce immunological memory that protects against disease recurrence.

DCs are the most potent antigen presenting cells identified to date. The unique capability of DCs to activate naïve T cells and orchestrate primary immune responses make them an ideal candidate for cellular immunotherapy.\(^13\) The invention of the protocol to generate considerable numbers of DCs from monocytes \textit{in vitro} boosted the enthusiasm for the clinical application of DC-based vaccines.\(^14,15\) Since then, numerous clinical trials have evaluated the safety, feasibility and clinical efficacy of DC-based vaccines derived from patient’s own monocytes or G-SCF mobilized CD34\(^+\) hematopoietic stem and progenitor cells (HSPCs) in cancer patients.\(^14,16–22\) Although, the published results of clinical trials have clearly demonstrated that monocyte-derived DC (MoDC)-based cancer vaccination strategy is feasible, safe and can induce potent anti-cancer immune responses, the clinical responses observed so far are limited.\(^23,24\) In addition, the routine application of this strategy has been hampered mainly by a) the difficulties associated with the generation of sufficient numbers of DCs for vaccination purposes and b) poor DC vaccine quality due to pathology or prior treatment received by the patients.\(^12\)

Although DCs are composed of multiple subsets with specialized functions, the vast majority of clinical studies conducted so far have used MoDCs. The physiological standing of this subset and whether it is an ideal subset for cancer immunotherapy is currently unclear. Alternatively, CD34\(^+\) HSPCs can be expanded and differentiated \textit{ex vivo} into various types of myeloid DCs that resemble CD14\(^+\) dermal DCs, CD1a\(^+\) interstitial or blood
DCs, BDCA3⁺ blood DCs and CD207⁺ Langerhans cells (LCs). So far, granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood HSPC from cancer patients have been used for generation of autologous DC vaccines.

Tumor-associated antigens (TAAs) usually trigger weak immune responses in contrast to potent allogeneic responses directed against non-self major histocompatibility complex (MHC). These allogeneic immune responses are mediated by MHC class II-restricted CD4⁺ T cells that can promote bystander antigen-specific cytotoxic T lymphocyte (CTL) responses. In DC vaccination strategy, allogeneicity could therefore be exploited to promote immunogenicity towards tumor antigens by vaccinating cancer patients with TAA-loaded DCs generated from partial HLA-matched donors.

In this study, we explored umbilical cord blood (UCB) as an alternative source of HSPCs for generation of allogeneic DC-based vaccines for AML. By combining the established approaches that either induced enormous expansion of DC precursors or proper DC differentiation, we have developed a robust cytokine-based culture protocol that generates clinically relevant numbers of mature CD1a⁺ myeloid DCs and CD207⁺ LC-like DCs from CD34⁺ HSPCs. Furthermore, we compared the phenotype, cytokine secretory profile and allogeneic, as well as, viral antigen-specific T cell stimulatory capacity of generated UCB-DCs and UCB-LCs with MoDCs. Most importantly, model minor histocompatibility antigen (MiHA) HA-1-specific CTL expansion and HA-1 and HA-2 antigen-specific CTL priming capability of UCB-DCs and UCB-LCs was studied using the peripheral blood of leukemia patients or healthy donors, respectively.

Materials and Methods

Isolation of CD34⁺ cells from UCB
UCB samples from full-term deliveries were obtained via the cord blood bank of the Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands, after written informed consent. Mononuclear cells were isolated from UCB by density gradient centrifugation using Ficoll-hypaque gradient (Healthcare, WI, USA). CD34⁺ cells were purified from mononuclear cells, using the human CD34⁺ magnetic cell sorter (MACS) MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer’s recommendations.

Generation of MoDCs from buffy coats and UCB
MoDCs were generated in 7-8 days from plastic adherent monocytes isolated from healthy donor buffy coats (Sanquin blood foundation, Nijmegen, the Netherlands) or UCB using 800 IU/mL GM-CSF (granulocyte-macrophage colony-stimulating factor) and 500 IU/mL interleukin (IL)-4 (both Immunotools, Friesoythe, Germany) in X-VIVO-15 medium (Lonza, Verviers, Belgium) supplemented with 2% human serum (HS, PAA laboratories, Pasching, Austria) (Figure 1). Maturation was induced by adding IL-6 (20 ng/mL), IL-1β
(5 ng/mL), tumor necrosis factor (TNF)-α (20 ng/mL; all from Immunotools) and PGE2 (prostaglandin E2, 1 µg/mL, Pharmacia & Upjohn, Puurs, Belgium) and culturing for two additional days (Figure 1).

Figure 1. Culture scheme for generation of UCB-DCs and UCB-LCs from UCB-CD34+ cells and MoDCs from peripheral blood or UCB-derived monocytes. UCB-CD34+ cells were first expanded for 7 days in medium (IMDM or X-VIVO) supplemented with 10% FCS or HS and Flt3L, SCF, IL-3 and IL-6 (F536) cytokines. At day 7, IL-3 and IL-6 in the expansion medium were replaced with TPO (FST). At day 11, DC precursors were differentiated in medium supplemented with GM-CSF and IL-4. During the last four days of differentiation TNF-α was also added. LC medium was additionally supplemented with TGF-β1. At day 18, both UCB-DCs/UCB-LCs were matured with IL-1β, IL-6, TNF-α and PGE2. Alternatively, MoDCs were differentiated from plastic adherent monocytes in medium supplemented with GM-CSF and IL-4, and were matured with IL-1β, IL-6, TNF-α and PGE2.

Generation of UCB-DCs and UCB-LCs

Isolated CD34+ cells from UCB were expanded for 7 days by seeding them at 0.2 x 10^6 cells/mL in Iscove-modified Dulbecco medium (IMDM, GIBCO Invitrogen, CA, USA) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Colbe, Germany), Flt3L (FMS-like tyrosine kinase 3 ligand, 100 ng/mL), SCF (stem cell factor, 100 ng/mL), IL-3 (20 ng/mL) (all R&D Systems, Abingdon, UK) and IL-6 (20 ng/mL) (Figure 1). During the additional 4 days of expansion, IL-3 and IL-6 in expansion medium was replaced with TPO (thrombopoietin, 20 ng/mL). During the expansion period, medium was refreshed every other day. Expanded cells were differentiated into either UCB-DCs with medium supplemented with GM-CSF (800 IU/mL) and IL-4 (500 IU/mL) or UCB-LCs with medium supplemented with TGF-β1 (transforming growth factor β1, 10 ng/mL, ImmunoTools) in addition to GM-CSF and IL-4 (Figure 1). During the last 3 days of UCB-DC or UCB-LC differentiation, TNF-α (20 ng/mL) was also added to the media. The cell density was maintained at 0.25 x 10^6 cells/mL and 0.5 x 10^6 cells/mL during expansion.
and differentiation, respectively. Immature UCB-DCs and UCB-LCs were matured using the cytokine combination listed above for MoDCs.

**Flow cytometric analysis of mature DCs**

Mature MoDC, UCB-DC and UCB-LC cultures were analyzed by flow cytometry (FC500, Beckman Coulter, Fullerton, CA, USA) after staining with monoclonal antibodies conjugated with fluorescein Isothiocyanate (FITC): CD1a (clone: HI149, Becton Dickinson), CD40 (clone: HB-7, Becton Dickinson), CD80 (clone: MAB104), CD86 (clone: BU63, DAKO), hCCR7 (clone: 150503, R&D Systems) and HLA-DR (clone: Immu357); phycoerythrin (PE): CD83 (clone: HB15a), CD40 (clone: MAB89), CD207 (clone: DCGM4), CD11c (clone: BU15); electron coupled dye (ECD): HLA-DR (clone: Immu358); cyanine-5 (Cy5)-conjugated: CD11c (clone: BU15); and cyanine-7 (Cy7)-conjugated: CD14 (clone: RMO52) and CD11b (clone: Bear1). All monoclonal antibodies were from Beckman Coulter, unless stated otherwise. The data was analyzed using Kaluza analysis software (Beckman Coulter).

**Cytokine release and quantification**

Immature UCB-DCs and UCB-LCs were plated at $5 \times 10^4$ cells/mL/well in a 24-well plate (Corning Costar, Amsterdam, the Netherlands) and stimulated with or without Resiquimod (R848) (5 µg/mL, Alexis biochemicals, Lausen, Switzerland) and Poly I:C (Polyinosinic-polyctydyllic acid, 20 µg/mL, Sigma-Aldrich, St. Louis, USA) in IMDM with 10% FCS. After 24 hours, supernatant was collected for quantification of pro-inflammatory, T-helper 1 (Th1) and Th2 cytokine production by UCB-DCs/UCB-LCs and stored at -20°C until evaluation by Luminex assay or enzyme-linked immunosorbent assay (ELISA). The concentration of IL-1β, IL-4, IL-6, IL-10, TNF-α, GM-CSF and interferon (IFN)-γ was quantified using the Human Cytokine Ten-Plex panel kit (Invitrogen, Carlsbad, CA, USA) and IL-12 was quantified using the Human IL-12p70 ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA, USA) following the methods provided by the manufacturer.

**Allogeneic mixed leukocyte reaction (MLR)**

Allogeneic T cell stimulatory capacity of mature MoDCs, UCB-DCs and UCB-LCs was tested in a carboxyfluorescein diacetate succinimidyl ester (CFSE)-based MLR assay. Allogeneic peripheral blood mononuclear cells (PBMCs) labeled with 5 µM CFSE (Molecular Probes Europe, Leiden, the Netherlands) were used as responder cells. Responder cells ($0.5 \times 10^5$) were co-cultured in triplicate for a week with $5 \times 10^4$, $1 \times 10^5$ or $5 \times 10^3$ stimulator cells (MoDCs, UCB-DCs or UCB-LCs) to get responder to stimulator cell ratios of 1:10, 1:50 and 1:100. On days 4 and 7, culture samples were subjected to flow cytometric analysis after staining with anti-CD4 (PE-Cy5, Clone: 13B8.2, Beckman Coulter) and anti-CD8 (PE-Cy7, clone: SFC121Thy2D3, Beckman Coulter) antibodies to evaluate allogeneic CD4+ and CD8+ T cell proliferation.
Expansion of cytomegalovirus (CMV)-specific CD8+ effector T cells

*In vitro:* CD8+ T cell were isolated from CMV-positive donor buffy coats by positive selection with a magnetic cell sorting device (Miltenyi Biotec) and were stimulated with CMV pp65 peptide-loaded (5 µM, TPRVTGGGAM, RPHERNGFTVL) MoDCs, UCB-DCs or UCB-LCs at a ratio of 10:1 (T cell:DC) in 1 mL IMDM with 10% FCS in a 24-well plate (Corning Costar). On day 4, IL-2 (50 IU/mL, Immunotools) and IL-15 (5 ng/mL, Immunotools) were added to the cultures. On day 0, 4 and 7 post-stimulation, the frequency of CMV-specific CD8+ T cells was quantified after staining the samples with PE-labeled HLA-B7 tetramers presenting the corresponding CMV pp65 peptide. Tetramers were kindly provided by Prof. F. Falkenburg (Dept. of Hematology, Leiden University Medical Center, Leiden, the Netherlands).

*In vivo:* Twenty-four, 6-week-old female NOD-SCID-IL-2Rγ-/- mice were intraperitoneally (IP) injected with 3.5 x 10^6 peripheral blood lymphocytes (PBLs) isolated from a CMV-specific CD8+ T cell-positive healthy donor buffy coat. Four hours after PBL injection, 4 groups of 6 mice were injected with PBS or CMV peptide-loaded MoDCs, UCB-DCs or UCB-LCs (0.2 x 10^6 cells, IP). Additional PBS or CMV peptide-loaded MoDCs, UCB-DCs or UCB-LCs (0.1 x 10^6 cells, IP) injections were given to the respective groups on days 7 and 14 post-PBL injection. On day 21, mice were sacrificed, peripheral blood and spleen samples were collected, cell numbers were counted and the frequency of CMV-specific CD8+ T cells was quantified by flow cytometry. This animal experimental protocol was reviewed and approved by the institutional animal ethics committee of Radboud university medical center Nijmegen (Permit Number: 2011-178).

Expansion of MiHA-specific T cells *ex vivo*

PBMC samples of HA-1-specific CD8+ T cell-positive AML patients were stimulated with HLA-matched, HA-1 negative, MoDCs, UCB-DCs or UCB-LCs loaded with HA-1 peptide (5 µM, VLHDDLLEA) at a ratio of 10:1 in 2 mL IMDM supplemented with 10% HS in 24-well plates (Costar Corning). Non-peptide-loaded MoDCs, UCB-DCs or UCB-LCs were included as negative controls. At day 4, culture medium was supplemented with 50 IU/mL IL-2 and 5 ng/mL IL-15 (Immunotools). At day 7, cells were harvested, counted and the percentage of HA-1-specific CD8+ T cells was determined by flow cytometry after HA-1-specific tetramer and CD8 staining.

Induction of MiHA-specific T cells *ex vivo*

CD8+ T cell were isolated from HLA-A2+ healthy donor PBMCs and were stimulated with HLA-A2-matched, HA-1-negative or HA-2-negative MoDCs, UCB-DCs or UCB-LCs loaded with HA-1 peptide (5 µM, VLHDDLLEA) or HA-2 peptide (5 µg/mL, YIGEVLVSV) at a ratio of 10:1 in 2 mL IMDM supplemented with 10% HS in 24-well plates (Costar; Corning). Non-peptide-loaded MoDCs, UCB-DCs or UCB-LCs were included as negative controls. At day 4, culture medium was supplemented with 50 IU/mL IL-2 and 5 ng/
mL IL-15 (Immunotools). At day 7, cells were harvested, counted and the percentage of MiHA-specific CD8+ T cells was determined by flow cytometry after MiHA-specific tetramer and CD8 staining.

**Statistical analysis**
The data were analyzed by one-way or two-way ANOVA with Bonferroni post-hoc correction using GraphPad Prism software.

**Results**

**High numbers of DCs and LCs can be generated from UCB CD34+ cells**
UCB-DC and UCB-LC precursors were expanded for seven days in the medium supplemented with Flt3L, SCF, IL-3 and IL-6. On day 7, IL-3 and IL-6 in expansion medium were replaced with TPO and cells were further expanded for 4 additional days (Figure 1). During the total expansion period of 11 days, UCB-CD34+ cells expanded on average 467-fold (Figure 2A). Expanded precursor cells were washed and resuspended in medium containing GM-CSF and IL-4 without or with TGF-β1 to differentiate them into either UCB-DCs or UCB-LCs, respectively. Addition of TNF-α during the last four days of differentiation accelerated the process of differentiation of both immature UCB-DCs and UCB-LCs. Subsequently, immature UCB-DCs and UCB-LCs were matured by culturing them in medium containing IL-1β, IL-6, TNF-α and PGE2. During the total culture period of 20 days, the mean increase in total cell number was 648-fold and 338-fold for mature UCB-DCs and UCB-LCs, respectively (Figure 2A).

**Mature UCB-DCs and UCB-LCs show phenotypic resemblance to MoDCs**
UCB-derived CD34+ cells gradually lost the expression of stem cell markers CD34 and CD133, and expressed myeloid lineage marker CD33 upon expansion (Figure 2B and Supplementary Figure 1A). A part of the expanded cells also expressed CD14 and CD11c, the markers expressed on monocytes and DCs (Figure 2B and Supplementary Figure 1A). More than 60% of the expanded cells expressed MHC class II cell surface receptor HLA-DR on their surface (Figure 2B and Supplementary Figure 1A).

Both immature UCB-DCs and UCB-LCs expressed comparable levels of the myeloid DC differentiation marker CD1a and other markers expressed on DCs such as CD11c, HLA-DR, CD40, CD80, CD86 and CD83 (Figure 2C and Supplementary Figure 1B). However, UCB-LCs expressed higher levels of the LC-specific marker CD207 when compared to UCB-DCs (Figure 2C and Supplementary Figure 1B). Only a very low percentage of UCB-DCs and UCB-LCs expressed the monocyte marker CD14, indicating a proper DC/LC differentiation (Figure 2C, Supplementary Figure 1B and Supplementary Table 1).
Mature UCB-DCs and UCB-LCs generated from CD34+ cells phenotypically resembled the MoDCs (Figure 2D and Supplementary Figure 1C) and expressed DC maturation marker CD83 and co-stimulatory markers CD80 and CD86 to the same extent as that of MoDCs. UCB-DCs and UCB-LCs expressed no or low levels of the monocyte marker CD14 and high levels of CD11b, CD11c, HLA-DR and CD40 (Figure 2D and Supplementary Figure 1C). The expression levels of lymph node homing chemokine receptor CCR7 were comparable between all three DC subsets evaluated (Figure 2D and Supplementary Figure 1C). The purity of mature UCB-DCs and UCB-LCs, quantified based on CD11c+CD83+ expression, reached greater 95% and it was slightly higher compared to mature MoDCs (Figure 2D and Supplementary Figure 1C)

**UCB-DCs and UCB-LCs produce low levels of pro-inflammatory, but high levels of Th1 cytokines and induce strong allogeneic MLR**

The cytokine profile expressed by DCs upon activation gives an indication on the magnitude and the type of the immune response initiated. The DC subset/s producing cytokines that favor the differentiation of Th0 cells into Th1 cells are preferred for cancer immunotherapy. Therefore, the cytokine profile expressed by UCB-DCs and UCB-LCs was compared to that of MoDCs. The data revealed that UCB-DCs and UCB-LCs produced...
relatively lower levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF-α, but higher levels of the Th1 cytokine IL-12β when compared to MoDCs (Figure 3A). Among the other cytokines measured, UCB-DCs and UCB-LCs produced slightly lower levels of IL-4 when compared to the levels produced by MoDCs (Figure 3A).

Next, mature UCB-DCs, UCB-LCs or MoDCs were used as stimulator cells and PBMCs isolated from healthy donor blood were used as responder cells in allogeneic MLR. Both mature UCB-DCs and UCB-LCs induced equal (at E:T ratios 1:10 and 1:50) or significantly higher (at E:T ratio 1:100, P<0.001) proliferation of both CD4+ and CD8+ T cells in an allogeneic MLR when compared to mature MoDCs (Figure 3B).

Figure 3. UCB-DCs and UCB-LCs exhibit cytokine profiles that favor Th1 immune responses. (A) Pro-inflammatory (IL-1β, IL-6 and TNF-α), Th2 (IL-4 and IL-10) and Th1 (IL-12β) cytokine production by UCB-DCs and UCB-LCs in comparison with MoDCs upon stimulation with Resiquimod (R848) (5 µg/mL) and Poly I:C (20 µg/mL, Sigma-Aldrich, St. Louis, USA) was quantified using Luminex and ELISA. The data shown are the mean ± SE of three independent donors tested. The data was analyzed by one-way ANOVA with bonferroni correction. *, P<0.05. (B) Allogeneic T cell stimulatory capacities of UCB-DCs and UCB-LCs in comparison with MoDCs were tested in a MLR. In vitro generated UCB-DCs, UCB-LCs or MoDCs were used as stimulators for CFSE-labeled PBMCs that were isolated from healthy donor buffy coats at a ratio of 1:100, 1:50 or 1:10 ratio (DC:PBMC). Five days later, the proliferation of CD8+ and CD4+ T cells was analyzed by flow cytometry. This is a representative result (mean ± SE) of three experiments. The data was analyzed by two-way ANOVA with bonferroni correction. ***, P<0.001.

UCB-DCs and UCB-LCs efficiently activate memory CTLs both in vitro and in vivo
The antigen-specific memory CTL activation capacity of UCB-DCs and UCB-LCs was evaluated in vitro by co-culturing HLA-A2-matched PBMCs containing either CMV-specific CD8+ T cells or HA-1-specific CD8+ T cells with CMV or HA-1 peptide-loaded UCB-DCs or UCB-LCs. In these assays autologous MoDCs loaded with the respective peptide were included as a control. The absolute number of antigen-specific T cells was
determined at day 7 post co-culture. UCB-DCs induced proliferation of both CMV and HA-1 specific CD8+ T cells as efficiently as MoDCs (Figure 4A,B). Although, there were no marked phenotypic differences in expression pattern of either maturation or co-stimulatory markers between UCB-DCs, UCB-LCs and MoDCs, notably UCB-LCs induced the highest level of HA-1-specific T cells proliferation in comparison to UCB-DCs and MoDCs (Figure 4B).

Figure 4. UCB-DCs and UCB-LCs effectively activate and induce proliferation of both viral antigen-specific and MiHA HA-1-specific CD8+ T cells. (A) The absolute numbers of CMV-specific CD8+ T cells in the cultures restimulated in vitro with CMV pp65 peptide-loaded MoDCs, UCB-DCs or UCB-LCs. CD8+ T cells were isolated from CMV-positive HLA-B7+ donor buffy coats and were stimulated with DC subsets loaded with or without CMV pp65 peptide for 4 days. (B) The absolute numbers of HA-1-specific CD8+ T cells in the cultures restimulated with HA-1 peptide-loaded MoDCs, UCB-DCs or UCB-LCs. PBMC samples of HA-1-positive HLA-A2+ AML patients were stimulated with DC subsets loaded with or without HA-1 peptide for 7 days. The absolute numbers of CMV-specific or HA-1-specific CD8+ T cells were quantified by flow cytometry after staining with respective tetramers and CD8 antibody. The data shown are the mean ± SE that are corrected for background proliferation induced by the respective non-pulsed DC control from two independent experiments (CMV-specific T cell restimulation) or four AML patient PBMCs tested (HA-1-specific T cell restimulation). (C) The percentage of CMV-specific CD8+ T cells in the peripheral blood and spleen of CMV+ donor PBL-engrafted NOD-SCID-IL2Rγ-/- mice were injected IP with 3.5 x 10^6 PBL isolated from a HLA-B7+ CMV+ donor buffy coat. Four hours after PBL injection, four groups of 6 mice received either PBS or CMV pp65 peptide-loaded MoDCs, UCB-DCs or UCB-LCs (2 x 10^5, IP). Additional booster injections of PBS or CMV pp65 peptide-loaded DC subsets (1 x 10^5, IP) were given on day 7 and 14. At day 21, mice were sacrificed, blood and spleen samples were collected to evaluated the frequency of CMV-specific CD8+ T cells by flow cytometric technique after staining the samples with mouse CD45, human CD45 and human CD8 and CMV tetramer. The data shown are mean ± SE of six mice tested per group.

In order to evaluate the antigen-specific memory CTL activation capacity of UCB-DCs and UCB-LCs in vivo, NOD-SCID-IL-2Rγ−/− mice were engrafted with PBLs isolated from a HLA-matched CMV antigen-specific T cell-positive healthy donor blood and were injected with CMV peptide-pulsed UCB-DCs, UCB-LCs or MoDCs. After three rounds
of vaccination with CMV peptide-pulsed DCs/LCs, the frequency of CMV-specific T cells in peripheral blood and spleen of NOD-SCID mice was evaluated by flow cytometry (Supplementary Figure 2). These data demonstrated that the efficiency of induction of CMV-specific T cell proliferation of both UCB-DCs and UCB-LCs was equivalent to that of MoDCs (Figure 4C).

**UCB-DCs and UCB-LCs efficiently prime MiHA specific CD8⁺ T cells**

Tumor antigen-specific T cell priming capacity of UCB-DCs and UCB-LCs was evaluated using the MiHAs HA-1 and HA-2. For this, MoDCs, UCB-DCs and UCB-LCs were generated from HLA-A*0201-positive, HA-1-negative or HA-2-negative UCB units. The DCs/LCs were pulsed with the corresponding peptide and were used to stimulate unprimed CD8⁺ T cells isolated from HLA-matched MiHA⁺ healthy donor PBMCs. In these experiments, MiHA peptide-pulsed autologous MoDCs and MoDCs generated from plastic adherent monocytes of UCB were included as a controls. The data showed that both peptide pulsed UCB-DCs and UCB-LCs can efficiently prime HA-1-specific and HA-2-specific CD8⁺ T cells in vitro (Figure 5A,B).

![Figure 5](image_url)

**Figure 5.** UCB-DCs and UCB-LCs efficiently prime HA-1-specific and HA-2-specific T cells. (A-B) The bar diagrams show the absolute numbers of HA-1-specific (A) and HA-2-specific (B) CD8⁺ T cells primed by MoDCs, UCB-DCs or UCB-LCs pulsed with respective peptide. CD8⁺ T cells were isolated from HLA-A2⁺ healthy donor PBMCs and were primed with HA-1-negative or HA-2-negative, HLA-A2⁺ peptide-pulsed DC subsets. After 7 days of priming, the frequency of HA-1-specific or HA-2-specific CD8⁺ T cells in the cultures was quantified by flow cytometry after staining the samples with respective tetramer and CD8 antibody. Representative data of four experiments are shown.

**Animal serum-free culture conditions also generates DCs from UCB CD34⁺ cells**

In order to make the UCB-DC culture protocol suitable for clinical application, subsequent attempts were made to generate UCB-DCs in the presence of pooled HS instead of FCS using the cytokine mixtures listed in Figure 1. The replacement of FCS with HS resulted
in higher expansion rates (Figure 6A). The phenotype of the expanded pre-DCs was only marginally affected by the replacement of FCS with HS in the expansion medium (Figure 6B). However, the percentage of UCB-DCs expressing the MHC class II marker (HLA-DR), co-stimulatory marker (CD86) and DC maturation (CD83) was moderately reduced by the replacement of FCS with HS (Figure 6C). Most importantly, although UCB-DCs generated in the medium supplemented with HS were less mature, they met the accepted release criteria of >70% purity for therapeutic DC products and they did not greatly differ in their allogeneic T cell stimulatory capacity when compared to UCB-DCs generated in the medium supplemented with FCS (Figure 6D). These data demonstrate that the UCB-DC vaccines can be efficiently generated in animal serum-free culture conditions.

Figure 6. High numbers of functional UCB-DCs can be generated in animal serum-free culture conditions. (A) The fold increase of UCB-DCs cultured in medium supplemented with either FCS or pooled HS during expansion, differentiation and maturation is shown. (B-C) The phenotypic characteristics of precursor UCB-DCs (B), and mature UCB-DCs (C) analyzed by FACS at day 8 and 17, respectively, are shown. (D) Allogeneic T cell stimulatory capacity of FCS-UCB-DCs and HS-UCB-DCs was tested in a MLR. In vitro generated UCB-DCs were used as stimulators for CFSE-labeled PBMCs that were isolated from healthy donor buffy coats at a ratio of 1:100, 1:100 or 1:10 ratio (DC:PBMC). Five days later the proliferation of CD8+ and CD4+ T cells was analyzed by flow cytometry. The data shown are the mean ± SE of two independent donors tested.
Chapter 2

Discussion

Despite of the progress made in the field of modern treatment for leukemia, the outlook for the majority of patients with AML has remained dismal with poor long term survival. Therefore, need for alternative therapies remains high to improve the prognosis for patients with AML. A broad array of immunotherapeutic strategies are under active investigation as adjuvant therapy to improve the overall survival of AML patients by eliminating residual leukemic cells following standard therapy. DC-based vaccination is an attractive immunotherapeutic strategy, due to its ability to induce leukemia-specific T cell responses, that offers the desired anti-tumor effects with minimal toxicity. In AML, DC vaccines are generated either by differentiating myeloid leukemia cells into AML-derived DCs or differentiating monocytes isolated from AML patients in remission to MoDCs. Phase I clinical trials conducted so far have demonstrated that DC vaccines are safe and can induce immunological responses in AML patients. However, the feasibility in terms of obtaining sufficient AML-DC vaccines and the clinical responses induced by AML-DC vaccines have been limited. The aim of this study was therefore to evaluate the possibility to generate clinically relevant doses of potent AML-reactive T cell inducing DC and LC vaccines from UCB-CD34+ cells. Here we report that clinically relevant doses of mature CD1a+ myeloid DC and CD207+ LC-like DCs can be generated from CD34+ HSPC. Most importantly, the generated UCB-DCs and UCB-LCs efficiently expanded MiHA HA-1-specific cytotoxic T cells in the peripheral blood of leukemia patients and also primed MiHA HA-1-specific and HA-2-specific cytotoxic T cells. In the final part of this study, an attempt was made to generate UCB-DCs in animal serum free culture conditions. The replacement of FCS with clinically acceptable pooled HS in UCB-DC expansion medium enhanced the expansion rate of DC precursors and did not affect the phenotype of the DC precursors. However, the use of HS in the DC differentiation and maturation medium had a marginal negative effect on the purity of mature UCB-DCs.

The key role played by DCs in inducing immunity has been the rationale for the application of DC-based vaccines in cancer immunotherapy. DCs, comprised of heterogeneous populations of HSPC-derived cells, have the unique ability to activate naïve T cells and direct the subsequent immune response towards a Th1, Th2, Th17 or T regulatory (Treg) profile. The distinct capacity of DCs to induce immune responses or tolerance depends not only on their activation or maturation state, but also on the DC subset involved. Human DCs can be categorized into two major types depending on their lineage: 1. The myeloid DCs (mDCs, also called classical or conventional DCs) and 2. Plasmacytoid DCs (pDCs). The DC subsets reside in the tissue where they are poised to capture antigen, or circulate in the blood. So far, human DC subsets in skin and blood are the best characterized, although DCs also can be found in other tissues. Skin DCs comprise of three types of mDCs, the epidermal LCs, the dermal interstitial CD1a+ DCs and CD14+ DCs. Blood DCs comprise of two subsets of BDCA2+ pDCs, (CD2^high^ pDCs and CD2^low^ pDCs) and three subsets of CD11c+ mDCs (CD1c+ mDCs, CD141+ mDCs and
CD16+ mDCs). Among mDCs, LCs and CD141+ mDCs possess the most robust CD8+ T cell stimulatory capacity, followed by the CD1a+ interstitial DCs, making them the most preferred subsets of DCs for cancer immunotherapy.\cite{27,39} Due to rarity of LCs and CD141+ mDCs in the tissues and difficulties with their generation \textit{ex vivo}, the majority of the studies have used autologous \textit{ex vivo}-generated MoDCs (that is, interstitial DC and CD1c+ blood DC equivalent), while only few trials have used mDCs derived from CD34+ precursors. Nevertheless, it has been suggested that DCs generated from CD34+ cells are more potent inducers of anti-tumor T cell responses than MoDCs.\cite{15,42} The favorable effect of DCs generated from CD34+ cells was attributed to contaminating LCs in the DC vaccines prepared from CD34+ cells. In light of the above findings, we aimed at generating LCs, dermal DC equivalent, CD141+ mDCs and pDCs from CD34+ cells isolated from UCB. Notably, we succeeded in generation of higher numbers of mature LC (UCB-LC)-like and dermal DC equivalent (UCB-DC) with high purity and high levels of expression of co-stimulatory molecules in contrast to the previously used protocol for generation of DCs from CD34+ cells.\cite{42} Phenotypically both UCB-LCs and UCB-DCs expressed typical myeloid DC markers (CD1a, CD11c, HLA-DR, CD40, CD80, CD83, CD86) with the exception that UCB-LCs expressed higher levels of Langerin (i.e. CD207). Unlike previous report, UCB-LCs did not express e-cadherin or lacked expression of CD11b and CD52.\cite{27} These phenotypic differences may in part be attributed to the discrepancies in the source of CD34+ cells (UCB in the current study vs. G-CSF mobilized) and the addition of IL-4 to the current UCB-LC differentiation medium. We observed the expression of LC marker Langerin in a small fraction of UCB-DCs cultured with GM-CSF, IL-4 and TNF-\alpha without TGF-\beta1. The presence of TNF-\alpha in UCB-DC differentiation medium may explain this finding, as exposure of DC precursors to proinflammatory cytokines such as TNF-\alpha during their differentiation is known to induce Langerin expression.\cite{29} In line with previous reports, UCB-DCs and UCB-LCs were slightly better than MoDCs in stimulating allogeneic, as well as tumor-reactive T cells \textit{in vitro}\cite{27} and all the three studied subsets of DCs pulsed with MiHA peptide exhibited comparable HA-2-specific T cell priming capacity. So far, the ability of LCs to cross-present exogenous antigens to CD8+ T cells via MHC class I pathway and induce very potent CTL responses makes them an attractive vaccine target for cancer immunotherapy,\cite{43} whether that also holds true for UCB-LCs still needs to be investigated.

Despite of the ample success achieved so far with DC-based cancer vaccines; one of the main problems remains the limited number of DCs available for immunotherapy.\cite{44} As DCs play a pivotal role not only in the initiation of CTL responses but also in the maintenance of CTLs, it may be crucial to repeatedly vaccinate cancer patients with DC vaccines.\cite{45} So far the general maximum dose of DC vaccines applied has been 30 x 10^6 DC per injection,\cite{46} whether this is the maximum optimal dose for DC vaccines has yet to be investigated. Therefore, we aimed at generating a high number of DCs for multiple DC vaccination rounds. The mature UCB-LC/UCB-DC yields obtained in the current protocol...
ranged between $34 \times 10^6$ and $65 \times 10^6$ UCB-CD34+ cells. According to our previous report, on average $2 \times 10^6$ CD34+ cells can be isolated from an UCB unit. Thus, the current protocol will generate on average $6.8 \times 10^8$ and $1.3 \times 10^9$ mature UCB-LCs and UCB-DCs, respectively, from one UCB unit. Those achieved numbers should be sufficient for multiple vaccinations of one or even multiple patients. The possibility to freeze aliquots of expanded precursors or fully mature DCs provide an additional advantage for the preparation of our off-the-shelf DC vaccine batches that could be applied to a group of patients at multiple time-points.

Routinely applied DC vaccines in cancer immunotherapy have been generated from autologous monocytes, but reports indicate that wide scale application of such DC vaccines suffers from a very high inter-patient variability and sometimes also the poor vaccine quality. Alternatively, DCs can be generated from G-CSF-mobilized peripheral blood CD34+ cells. However, these DCs are not preferred for cancer immunotherapy due to their polarization towards the Th2 response. Furthermore, G-CSF treatment for mobilization of CD34+ cells poses an extra burden on the patients. In order to overcome these limitations associated with autologous DC vaccines, allogeneic DCs have been considered as an attractive alternative. The major advantage of using allogeneic DCs is the feasibility of large scale production of more standardized DC vaccines in terms of phenotype and maturation status, that can be applied to all patients. In addition, HLA-matched allogeneic DC vaccines are reported to induce strong anti-tumor immune responses and clinical responses in cancer patients. In the current study, we explored UCB as a source of CD34+ cells for generation of allogeneic DCs.

UCB is an extremely attractive source of HSPCs not only for clinical transplantation, but also for the generation of a multitude of cell therapy products due to their strong multi-lineage expansion and differentiation potential. Unlike other allogeneic HSPC sources, UCB do not pose any risk to the donor and are readily available from the cord blood banks worldwide. As cord blood banks have large numbers of HLA-typed HSPCs available, there is a higher probability of finding a partially HLA-matched donor for generation of DC vaccines for immunotherapeutic purposes. A combination of UCB-derived HSPCs for transplantation and DCs generated from the same donor may provide an appealing new treatment option for patients suffering from hematological malignancies. There are few reports on the DC generation from UCB-derived CD34+ cells. However, the current study supersedes the number, purity and maturation status of the generated UCB-DCs. Most importantly, this study is the first to describe the preclinical evidence for the suitability of UCB-DCs for either induction or reactivation of MiHA-specific T cells that are of clinical significance in transplanted patients suffering from hematological malignancies.

The type and the concentration of the serum present in the DC culture medium is known to affect the expression pattern of key DC and LC identification markers such as CD1a and CD207. Therefore, we initially generated UCB-DCs and UCB-LCs in medium
containing FCS. Although, some clinical trials have used FCS for DC generation\textsuperscript{59,60}, in order to prevent immune responses against xenoserum constituents and transfer of xenoinfections, DCs generated in serum-free or HS-supplemented medium are preferred. Hence, we subsequently replaced FCS by pooled HS in UCB-DC/UCB-LC generation medium and demonstrated the feasibility of the current UCB-DC culture protocol for clinical application. However, the feasibility of UCB-LC culture protocol could not be demonstrated because of lack of expression of the LC-specific marker Langerin on the cells cultured in the absence of FCS. The identification of key components in FCS that induce Langerin expression is crucial for further development of UCB-LCs for the clinical application.

In conclusion, a very high number of mature DCs can be generated from CD34\textsuperscript{+} HSPCs for clinical evaluation using the current protocol. Both allogeneic, as well as tumor-reactive T cell stimulatory capacity exhibited by the mature UCB-DC make them a good vaccine candidate for leukemia. These pre-clinical findings support the further pharmaceutical development of the described culture protocol for clinical evaluation.
References


Chapter 2

A  The phenotypic characteristics of precursor UCB-DCs/UCB-LCs

B  The phenotypic characteristics of immature UCB-DCs and UCB-LCs
Supplementary Figure 1. (A-D) The phenotypic characteristics of precursor, immature and mature UCB-DCs/UCB-LCs next to the mature MoDCs analyzed in terms of percentages by flow cytometry are shown. A representative donor data is shown.
Supplementary Figure 2. CMV-antigen specific T cell stimulatory capacity of UCB-DCs and UCB-LCs in comparison with MoDCs in vivo. (A) The experimental scheme. (B) Engraftment of human CD3+ T cells in peripheral blood of NOD-SCID-IL2Rγ-/- mice. The data shown are mean ± SE of six mice tested per group. NOD-SCID-IL2Rγ-/- mice were injected IP with 3.5 x 10^6 PBLs isolated from HLA-B7+ CMV+ donor buffy coat. Four hours after PBL injection, four groups of 6 mice were treated with either PBS or CMV pp65 peptide-loaded MoDCs, UCB-DCs or UCB-LCs (2 x 10^5, IP). Additional booster injections of PBS or CMV pp65 peptide-loaded DC subsets (1 x 10^5, IP) were given at day 7 and 14. At day 21, mice were sacrificed, blood and spleen samples were collected to evaluate the frequency of CMV-specific T cells by flow cytometric technique after staining the samples with mouse CD45, human CD45, human CD8 and CMV tetramer.

Supplementary Table 1. The percentages of CD1a and CD14-positive cells in immature and mature UCB-DC and UCB-LC cultures analyzed by flow cytometry at day 18 and 21, respectively are presented.

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The aryl hydrocarbon receptor antagonist StemRegenin 1 promotes human plasmacytoid and myeloid dendritic cell development from CD34+ hematopoietic progenitor cells

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Chapter 3

Abstract

The superiority of dendritic cells (DCs) as antigen-presenting cells has been exploited in numerous clinical trials, where generally monocyte-derived DCs (MoDCs) are injected to induce immunity in patients with cancer or infectious diseases. Despite promising expansion of antigen-specific T cells, the clinical responses following vaccination have been limited, indicating that further improvements of DC vaccine potency are necessary. Pre-clinical studies suggest that vaccination with combination of primary DC subsets, such as myeloid and plasmacytoid blood DCs (mDCs and pDCs, respectively), may result in stronger clinical responses. However, it is a challenge to obtain high enough numbers of primary DCs for immunotherapy, since their frequency in blood is very low. We therefore explored the possibility to generate them from hematopoietic stem and progenitor cells (HSPCs). Here, we show that by inhibiting the aryl hydrocarbon receptor with its antagonist StemRegenin 1 (SR1), clinical-scale numbers of functional BDCA2⁺BDCA4⁺ pDCs, BDCA1⁺ mDCs and BDCA3⁺DNGR1⁺ mDCs can be efficiently generated from human CD34⁺ HSPCs. The ex vivo-generated DCs were phenotypically and functionally comparable to peripheral blood DCs. They secreted high levels of pro-inflammatory cytokines such as IFN-α, IL-12 and TNF-α and upregulated co-stimulatory molecules and maturation markers following stimulation with TLR ligands. Furthermore, they induced potent allogeneic T cell responses and activated antigen-experienced T cells. These findings demonstrate that SR1 can be exploited to generate high numbers of functional pDCs and mDCs from CD34⁺ HSPCs, providing an alternative option to MoDCs for immunotherapy of patients with cancer or infections.
Introduction

Dendritic cells (DCs) are specialized in capturing, processing and presenting antigens to T cells, and thereby play a crucial role in initiating and shaping immune responses. The prominent role of DCs in T cell activation is the rationale for DC-based immunotherapy of cancer and infectious diseases, while their tolerogenic potential is being exploited in the recently developing field of DC-based therapy for auto-immune diseases. In cancer, DC vaccination therapy aims to induce tumor-specific T cell responses, and to develop immunological memory to control tumor relapse. So far, the vast majority of DC vaccination studies have been performed with DCs differentiated ex vivo from monocytes (MoDCs). This strategy has been reported to induce the expansion of tumor-specific T cells in the majority of patients, however only a fraction of the patients develop clinical responses. Various strategies to improve the potency of DC-based vaccines are being investigated, such as using natural occurring DCs from peripheral blood or combining multiple DC subsets in one vaccine to provide cross-talk.

DCs form a heterogeneous population of cells, comprising several subsets with different phenotypes and functional properties. In human blood two main DC subsets can be defined; CD11c+ myeloid DCs (mDCs) and CD11c-CD123+BDCA2+BDCA4+ plasmacytoid DCs (pDCs). The mDCs can be further divided into BDCA1(CD1c)+ and BDCA3(CD141)+DNGR1+ cells. These pDC and mDC subsets express a diverse set of pattern recognition receptors, including Toll-like receptors (TLRs), and secrete different cytokines and induce diverse T cell responses following activation by pathogens. mDCs are for example broadly responsive to microbial stimulation, where they upregulate co-stimulatory molecules and secrete cytokines such as interleukin (IL)-12 and tumor necrosis factor (TNF)-α following activation. On the contrary, pDCs play a crucial role in initiating anti-viral immunity by secreting high levels of type I interferons (IFN-α/β) in response to TLR7 and TLR9 ligation by viruses. Additional studies indicate that pDCs and mDCs interact during the development of immune responses. For example, it has been shown that activated pDCs strongly enhance the ability of mDCs to stimulate potent anti-tumor cytotoxic T lymphocyte (CTL) responses. Therefore, combining these naturally occurring DCs appears to be an attractive strategy to exploit for DC-based therapy. However, since the frequency of pDCs and mDCs in the blood is very low, it is a challenge to obtain high enough numbers for immunotherapy. It would therefore be advantageous if high numbers of DCs, which are phenotypically and functionally similar to blood pDCs and mDCs, could be generated from CD34+ hematopoietic stem and progenitor cells (HSPCs).

Recent findings indicate that the aryl hydrocarbon receptor (AhR) not only regulates toxic effects of environmental contaminants, but also plays a role in modulating hematopoiesis and the immune system. For instance, Boitano et al. reported that StemRegenin 1 (SR1), a small molecule inhibitor of AhR, promotes the ex vivo expansion of human CD34+ HSPCs that are able to effectively engraft immunodeficient mice.
Furthermore, differentiation of Langerhans cells and monocytes in vitro from HSPCs was inhibited by the addition of the AhR agonist VAF347. In light of these data, we investigated whether it was possible to generate DC subsets from CD34+ HSPCs by supplementing SR1. We observed that SR1 explicitly and simultaneously induced the ex vivo differentiation of pDCs, BDCA1+ and BDCA3+ mDCs. Importantly, SR1 induced the generation of high numbers of all these DC subsets, high enough to be included in clinical vaccination studies. Furthermore, these different DC subsets were phenotypically and functionally comparable to their blood counterparts and potently stimulated antigen-specific T cell. Therefore, our SR1 culture system not only allows detailed study of DC differentiation and molecular regulations in vitro, but it also offers the opportunity to evaluate the in vivo efficacy of a combined pDC and mDC vaccine in patients with cancer, viral infections or auto-immune conditions.

Materials and Methods

Ex vivo generation of DCs from CD34+ hematopoietic progenitor cells

Umbilical cord blood (UCB) was obtained at birth after normal full-term delivery with written informed consent from the cord blood bank of the Radboud university medical center (Nijmegen, The Netherlands). Mononuclear cells from UCB were isolated by Ficoll-Hypaque (1,077 g/mL; GE Healthcare, Uppsala, Sweden) density gradient centrifugation. Next, CD34+ cells were isolated from mononuclear cells using anti-CD34 immunomagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany). A small aliquot of CD34+ cells was obtained from stem cell donors after the CliniMACS (Miltenyi Biotech) selection procedure of granulocyte colony-stimulating factor (G-CSF)-mobilized blood after written informed consent. The purity of the isolated CD34+ cells from UCB and G-CSF-mobilized blood was 71±12% (n=9) and 90±5% (n=3), respectively. CD34+ cells were either used freshly or cryopreserved until use.

CD34+ cells were plated at 10^4-10^5 cells/mL in 24-well tissue culture plates (Corning Costar, NY, USA) in Glycostem Basal Growth Medium (GBGM, Glycostem Therapeutics, s’Hertogenbosch, The Netherlands) with 100 ng/mL of the following cytokines: thrombopoietin (TPO), stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (Flt3L; all three from Cellgenix, Freiburg, Germany) and IL-6 (ImmunoTools, Friesoythe, Germany). Additionally, CD34+ cells were cultured in the presence of 1 μM SR1 (Cellagen Technology, CA, USA), which was identified as the optimal dose in dose titration experiments (see Supplementary Figure 1). The AhR agonist VAF347 (Exclusive Chemistry, Obninsk, Russia) was used in the concentrations 0.05-50 nM. Corresponding concentrations of DMSO (dimethyl sulfoxide, Merck, Darmstadt, Germany) were used as control. Fresh medium containing cytokines and compounds was added every 2-4 days, and the cells were split upon confluence and transferred to 6-well plates (Corning Costar) if needed. Cells were cultured at 37°C, 95% humidity and 5% CO2. The total number of viable cells
was determined by trypan blue exclusion counting on day 7, 14 and 21. At each time-point a sample was taken for flow cytometry analysis, and absolute numbers of DCs were calculated by multiplying the frequency of DCs with the absolute number of total cells generated from $10^5$ CD34+ cells.

**Isolation of peripheral blood (PB)- and HSPC-DCs**

PB-DCs were isolated from buffy coats (Sanquin blood bank, Nijmegen, The Netherlands) of healthy individuals after written informed consent. First, PBMCs were purified using Ficoll-Hypaque density centrifugation. Subsequently, non-DCs were depleted from PBMCs by negative selection with anti-CD3/CD14/CD19 magnetic beads (Becton Dickinson, Franklin Lakes, NJ, USA) or with Pan-DC enrichment kit (Miltenyi Biotech). Next the negative fraction was labeled with anti-BDCA1, anti-BDCA3, anti-BDCA4 (all from Miltenyi Biotech), anti-CD14, anti-CD20 (both from Biolegend, CA, USA) and anti-CD19 (Dako, Glostrup, Denmark). Finally, pDCs, BDCA1+ mDCs and BDCA3+ mDCs were sorted on an EPICS Elite cell sorter (Beckman Coulter, Fullerton, CA, USA) or FACS Aria (Becton Dickinson) as BDCA4+BDCA1-CD14+CD19-, BDCA1+BDCA3-CD14-CD19-CD20- and BDCA3+BDCA1-CD14-CD19-CD20- cells, respectively.

HSPC-DCs were sorted from the whole bulk of cultured cells at day 21 of culture. First, the cultured cells were incubated with 10-20% human serum (HS; PAA Laboratories, Austria) at 4°C to block Fc receptors. Next, the cultured cells were stained with anti-CD123 (Biolegend), anti-CD14 (Beckman Coulter), anti-BDCA2 (Miltenyi Biotech), anti-BDCA1 and anti-BDCA3 antibodies and sorted with the FACS Aria for pDCs (CD14 CD123hiBDCA2+ cells), BDCA1+ mDCs (CD14 CD123lowBDCA1+BDCA3- cells) and BDCA3+ mDCs (CD14 CD123low BDCA3+BDCA1- cells). Purity of isolated PB- and HSPC-DCs was >90%.

**Flow cytometry**

Immunophenotypical analysis was performed by flow cytometry, where cells were first washed with PBS/0.5% BSA and subsequently stained with appropriate antibody concentrations for 30 min at 4°C. To determine the phenotype and maturation state of DCs, following antibodies and isotype controls were used: anti-BDCA1, anti-BDCA2, anti-BDCA3 (all from Miltenyi Biotech), anti-CD83, anti-CD123, anti-lineage cocktail (CD3, CD14, CD16, CD19, CD20, CD56) (all from Becton Dickinson), anti-CD11c, anti-CD14, anti-CD80, anti-CD86, anti-HLA-DR, mouse IgG1, mouse IgG2b (all from Beckman Coulter), anti-DNGR1, anti-CCR7, mouse IgG2a (all from Biolegend,) and anti-CD86 (Dako). To evaluate T cell proliferation in mixed lymphocyte reaction (MLR), cells were stained with anti-CD3, anti-CD8 (both from Beckman coulter) and anti-CD4 (Biolegend). For all analysis, live cells were gated based on forward scatter and side scatter characteristics. Additionally, in some experiments, SYTOX blue stain (Invitrogen, CA, USA) was used to further exclude dead cells from analysis. Furthermore, cell doublets
were excluded based on signal pulse height and width. Acquisition was performed with the Coulter FC500 flow cytometer or CyAn ADP analyzer and data analysis was performed with Kaluza or CXP analysis software (all from Beckman Coulter).

**DC stimulation with TLR ligands**

Sorted HSPC-derived pDCs, BDCA1+ mDCs and BDCA3+ mDCs were resuspended in IMDM (Iscove-modified Dulbecco medium, Invitrogen) supplemented with 10% fetal calf serum (FCS, Integro, Zaanadam, The Netherlands), 1% penicillin and streptomycin (PS; MP Biomedicals, Ohio, USA) and seeded in a 96-well round bottom plate (Corning Costar). 10 ng/mL IL-3 or 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; both from ImmunoTools) was additionally added to the medium for survival of the pDCs and mDCs, respectively. Next, pDCs were stimulated with 3.4 μg/mL CpG ODN 2216 (CpG-A) or CpG ODN 2006 (CpG-B, both from Enzo Life Sciences, NY, USA) whereas mDCs were stimulated with 20 μg/mL Poly I:C (Polyinosinic:polycytidylic acid, Sigma-Aldrich, St. Louis, USA) and 5 μg/mL R848 (Resiquimod, Enzo Life Sciences). After overnight stimulation, IFN-α, IL-6, TNF-α and IL-12 concentrations were measured in the supernatant by ELISA according to manufacturer’s instructions (Human IFN-α Module set ELISA (Bender MedSystems GmbH, Vienna, Austria); Human IL-6 ELISA Ready-Set-Go (eBioscience); PeliPair human TNF-α ELISA reagent set (Sanquin, Amsterdam, The Netherlands); IL-12 ELISA (Pierce Endogen, Rockford, IL, USA)) and phenotypical maturation was evaluated by flow cytometry.

**Allogeneic MLR**

HSPC-pDCs and mDCs were harvested and washed after overnight stimulation with 3.4 μg/mL CpG-B or 10 μg/mL Poly I:C, respectively. Subsequently, they were co-cultured with allogeneic PBMCs from healthy donors that were labeled with 1.25 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes Europe, Leiden, The Netherlands) as described previously. Co-cultures were performed at a 1:10 ratio (DC:PBMCs) in IMDM supplemented with 10% FCS and 1% PS in 96-well round bottom plates in triplicate. After 4-5 days of co-culture, supernatant was collected for cytokine analysis (Th1/Th2/Th17 cytometric bead array, Becton Dickinson) and T cell proliferation was quantified by flow cytometry by evaluating the CFSE dilution within the CD3+CD4+ and CD3+CD8+ T cell populations.

**Antigen-specific T cell activation**

CD8+ T cells specific for the minor histocompatibility antigen LRH-1 or HA-1 were isolated from PBMCs obtained from patients that had chronic myeloid leukemia and were treated with allogeneic stem cell transplantation and pre-emptive donor lymphocyte infusion as previously described. Sorted HSPC-DCs from HLA-B7+ or HLA-A2+ donors were loaded with 1 μM LRH-1 peptide (TPNQRQNV, IHB-LUMC peptide synthesis synthesis
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facility, Leiden, The Netherlands) or HA-1 peptide (VLHDDLLEA, IHB-LUMC peptide synthesis facility), respectively. After 1 hour incubation at 37°C, LRH-1-specific CD8+ CTL culture RP1 or CD8+ HA-1-specific T cell bulk containing 38% HA-1 tetramer positive T cells were added. At the same time, 5 μg/mL R848 was added for the maturation of pDCs or 5 μg/mL R848 and 10 μg/mL Poly I:C for the maturation of mDCs. Co-cultures were performed at 1:1 ratio (104 DCs with 104 T cells/well) in replicates of three or six in 96-well round bottom plates in 200 μl IMDM supplemented with 10% HS, 1% PS and 10 ng/mL IL-3 or 800 IU/mL GM-CSF for pDCs and mDCs, respectively. After 24 hour incubation, IFN-γ production was measured by ELISA (Pierce Endogen).

**RNA isolation and real-time quantitative RT-PCR (qRT-PCR)**

Total RNA was extracted from PB-DCs, HSPC-DCs and total PBMCs with the Quick-RNA MinPrep isolation kit (Zymo Research, CA, USA) according to the manufacturer’s protocol. cDNA synthesis and PCR amplification were performed as previously described.20 qRT-PCR reactions were run on an ABI 7900-HT real time PCR system (Applied Biosystems, Bleiswijk, The Netherlands) with 2 μl of cDNA, 1xSYBR green PCR master mix (Invitrogen) and 300 nM of following primers: TLR3-Fw: AGTTGTCATCGAATCAAATTAAAG TLR3-Rv: CATTGTTCAGAAAGAGGCCAAAT TLR4-Fw: 5'-GGCATGCTGTGCTGAGTT-3', TLR4-Rv: 5'-CTGCTACAACAGATACTACAAGCACACT-3', TLR7-Fw: 5'-TGCC ATCAAGAAAGTTGTGCT-3', TLR7-Rv: 5'-GGAATGTAGAGGTCTGGTTGAAGAG-3', TLR8-Fw: CGGAATGAAAAATTAGAACAACAGAA TLR8-Rv: GAACCAGAT ATTAGCAGAAATGC TLR9-Fw: 5'-TGAAGACTTCCAGCCCAACTG-3', TLR9-Rv: 5'-TGCACGTTCACAGGTTGT-3', phorphobilinogen deaminase (PBGD)-Fw: 5'-GGCAATGCGCAGAAACTG-3', PBGD-Rv: 5'-GGGTACCCACGCGAATCAC-3'. PBGD primers were purchased from Eurogentec (Maastricht, The Netherlands). mRNA expression of TLRs in DCs is depicted as ΔΔCt values and was quantified relative to mRNA expression in total PBMCs, which was set at 1. ΔΔCt was calculated as follows: 2^(-ΔCt_{pDCs} – ΔCt_{PBMCs}) in which ΔCt was normalized for PBGD by calculating ΔCt = Ct_{target gene} – Ct_{PBGD} per sample.

**Statistics**

Statistical analysis was performed using GraphPad Prism 5.0. One-tailed paired Student’s t-test or one-way or two-way ANOVA with repeated measures (RM), followed by Bonferroni post-hoc test, was used, as indicated in figure legends. P-values <0.05 were considered significant.
Results
The AhR antagonist SR1 induces \textit{ex vivo} differentiation of pDCs and mDCs from human CD34\textsuperscript{+} HSPCs

To simultaneously generate sufficient numbers of pDCs and mDCs for use in DC vaccination therapy, we studied the effect of inhibiting AhR during \textit{ex vivo} DC differentiation. Therefore, UCB CD34\textsuperscript{+} HSPCs were cultured for three weeks with the early acting cytokines TPO, SCF, Flt3L and IL-6 \textsuperscript{23-25}. Additionally, we cultured the cells with 1 μM SR1 or DMSO as control. Interestingly, addition of SR1 promoted the emergence of pDCs (CD11\textsuperscript{c} CD123\textsuperscript{hi} BDCA2\textsuperscript{+} cells), BDCA1\textsuperscript{+} mDCs (Lin1- HLA-DR\textsuperscript{-}BDCA1\textsuperscript{-}BDCA3\textsuperscript{-} cells) and BDCA3\textsuperscript{+} mDCs (Lin1 HLA-DR\textsuperscript{-}BDCA1 BDCA3\textsuperscript{+} cells) (Figure 1A). After three weeks of culture, the frequency of these DC subsets was significantly higher in cultures with SR1 compared to control conditions; 2.9\% vs. 0.04\% for pDCs, 4.6\% vs. 0.5\% for BDCA1\textsuperscript{+} mDCs and 1.1\% vs. 0.1\% for BDCA3\textsuperscript{+} mDCs (Figure 1B). The average yield after three weeks of culture with SR1 starting from 10\textsuperscript{5} CD34\textsuperscript{+} HSPCs was 3.8 x 10\textsuperscript{6} pDCs, 5.3 x 10\textsuperscript{6} BDCA1\textsuperscript{+} mDCs and 1.2 x 10\textsuperscript{6} BDCA3\textsuperscript{+} mDCs (Figure 1C and Supplementary Table 1).

Figure 1. Effect of AhR antagonist SR1 on differentiation of pDCs, BDCA1\textsuperscript{+} mDCs and BDCA3\textsuperscript{+} mDCs from HSPCs. (A-C) UCB or (D-F) G-CSF-mobilized CD34\textsuperscript{+} cells were cultured for three weeks with 1 μM SR1 or 0.01% DMSO (control) after which the occurrence of pDCs and mDCs was evaluated by flow cytometry. (A,D) Dot plots show the percentage of pDCs (CD123\textsuperscript{hi}), BDCA1\textsuperscript{+} mDCs and BDCA3\textsuperscript{+} mDCs within the total population of live cells. pDCs are gated as CD11\textsuperscript{c} cells and mDCs as lineage marker -, HLA-DR\textsuperscript{+} cells. mDCs are further defined as single positive for either BDCA1 or BDCA3. (B,E) Frequency within total cultured cells, and (C,F) total yield from 10\textsuperscript{5} UCB CD34\textsuperscript{+} cells of pDCs, BDCA1\textsuperscript{+} mDCs and BDCA3\textsuperscript{+} mDCs. Data are depicted as mean ± SEM of 3-5 independent donors tested. Statistical analysis was performed with one-tailed paired Student’s t-test. *P<0.05, **P<0.01.
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Furthermore, SR1 promoted the differentiation of DC subsets from CD34+ cells obtained from peripheral blood of G-CSF-mobilized donors (Figure 1D). The average frequency of DCs in these SR1-cultures was 4.7%, 3.8% and 0.9% for pDCs, BDCA1+ and BDCA3+ mDCs, respectively (Figure 1E), which is comparable to the frequency obtained from UCB CD34+ cells. The expansion potential of G-CSF-mobilized blood CD34+ HSPCs was lower than that of UCB CD34+ cells, resulting in average DC yields of 0.6 x 10^6, 0.5 x 10^6 and 0.1 x 10^6 from 10^5 CD34+ cells (Figure 1F and Supplementary Table 2).

Figure 2. Time kinetics and AhR involvement in pDC differentiation. For panels A-C, CD34+ UCB cells were cultured for three weeks with 1 μM SR1 or 0.01% DMSO (control). Total cell number was determined at day 7, 14 and 21 and the frequency of pDCs was evaluated at each time point by flow cytometry. (A) Fold increase of total nucleated cells in the cultures. (B) Frequency of pDCs (CD123hi cells) at each time point and (C) total number of pDCs generated from 10^5 CD34+ HSPCs. For panels D-E, CD34+ UCB cells were cultured for three weeks with 1 μM SR1 and titrated concentrations of VAF347. At day 21, (D) the frequency of pDCs within the total cultured cells was evaluated by flow cytometry and (E) the fold increase of total nucleated cells in the culture was determined on day 7, 14 and 21. Data are expressed as mean ± SEM of results from 5 (A-C) or 2 (D-E) UCB donors. Statistical analysis was performed using two-way RM ANOVA (A-C) or one-way RM ANOVA (D) followed by a Bonferroni post-hoc test. *P<0.05, **P<0.01, ***P<0.001.

During the three weeks culture period the total nucleated cells continuously expanded (Figure 2A). Analysis of pDC differentiation in time revealed that the maximum frequency of pDCs was already reached at day 14 (Figure 2B). However, the absolute number of pDCs was highest on day 21 (Figure 2C). The antagonizing effect of SR1 on AhR signaling is mediated through direct binding and inhibition of AhR.18 To proof a similar effect of SR1 in our experiments, we evaluated if the SR1-induced DC differentiation was indeed AhR-dependent by adding the AhR agonist VAF347. The frequency of pDCs was partially decreased by addition of 5 nM VAF347 to the SR1-cultures, but 50 nM of VAF347 completely blocked the differentiation of HSPCs into pDCs (Figure 2D).
of VAF347 to the SR1-cultures did not inhibit the proliferation of total cells (Figure 2E). Collectively, these data demonstrate that the AhR pathway is a negative regulator of DC differentiation, and that by using SR1 together with early acting cytokines, high numbers of pDCs, BDCA1+ and BDCA3+ mDCs can be generated from UCB and G-CSF-mobilized HSPCs ex vivo.

**SR1-generated HSPC-DCs have phenotypical and functional characteristics of PB-DCs**

Next, we assessed the phenotype and function of DCs generated in the presence of SR1 (Figure 3A). Similar to their naturally occurring counterparts in blood, we observed that the three different HSPC-DC subsets were negative for the monocyte marker CD14, while they all expressed HLA-DR. On the other hand, their expression of CD11c was diverse; HSPC-pDCs were negative for CD11c, while DNGR1'BDCA3+ mDCs expressed CD11c. In contrast, only a fraction of the HSPC-BDCA1+mDCs expressed CD11c. Similar to their in vivo counterparts, the HSPC-pDCs selectively expressed BDCA2, while the HSPC-BDCA3+ mDCs uniquely expressed DNGR1. HSPC-pDCs additionally expressed BDCA4, CD4, CD45RA and CD62L (supplementary Table 3) similarly as PB-pDCs.11,26

To further characterize the HSPC-DCs, we isolated pDCs, BDCA1+ mDCs and BDCA3+ mDCs by cell-sorting on day 21 of culture and evaluated their TLR expression. PB-DCs were sorted from healthy individuals for comparison. In line with published results9,27, we observed that both HSPC-pDCs and PB-pDCs had the highest expression of TLR7 and TLR9 (Figure 3B). In addition we observed that BDCA3+ mDCs, derived from HSPCs or PB, had the highest expression of TLR3 of all the different DC subsets. However, the expression of TLR3 was in general lower in HSPC-DCs than PB-DCs. TLR8 expression was highest in BDCA1+ mDCs, followed by BDCA3+ mDCs and lowest in pDCs. Similarly, the TLR4 expression was highest in BDCA1+ mDCs, however the expression was in generally low as it is depicted relative to total PMBCs, which contain high proportion of TLR4-expressing monocytes.

Subsequently, phenotypical maturation and cytokine secretion by sorted HSPC-DCs in response to TLR stimulation was analyzed. For that reason, HSPC-pDCs were cultured overnight with two different CpG oligonucleotide ligands, CpG-A or CpG-B, while HSPC-mDCs were stimulated with a combination of Poly I:C and R848. In addition, IL-3 and GM-CSF was added to all conditions for pDCs and mDCs respectively, as we observed that it enhanced their survival and activation (data not shown). Directly following sorting and before overnight culture, the DCs did not express CD80, CD86, CD83 or CCR7 (data not shown) and had intermediate levels of HLA-DR (Figure 3A). However, as seen in Figure 4A, the majority of HSPC-DC subsets upregulated co-stimulatory molecules and maturation markers upon TLR stimulation, and all of them highly expressed HLA-DR. We additionally observed that in the presence of IL-3 or GM-CSF alone (medium), the DCs expressed low levels of CD80, CD86, CD83 and CCR7 (Figure 4B and Supplementary Figure 2), but highly upregulated them upon TLR-ligation. The only exception was CD80
AhR inhibition promotes DC differentiation on BDCA1⁺ mDCs, which was expressed to the same level with or without TLR stimulation (Figure 4B and Supplementary Figure 2).

Figure 3. Phenotype of DCs derived from HSPCs in SR1 cultures. (A) CD34⁺ UCB cells were cultured for three weeks with 1 μM SR1 after which the expression of HLA-DR, CD11c, CD14 and the DC specific markers, BDCA1, BDCA2, BDCA3 and DNGR1 on HSPC-DCs was evaluated by flow cytometry. Dot plots are representative of cultures from at least 5 different UCB donors and gated on live cells or live cells and CD14⁻ cells as indicated in the figure. (B) pDCs, BDCA1⁻ and BDCA3⁻ mDCs were sorted by flow cytometry from SR1 cultures at day 21 (HSPC-DCs n=4) or peripheral blood (PB-DCs, n=3). mRNA was extracted and expression of TLR3, TLR4, TLR7, TLR8 and TLR9 was determined by qRT-PCR. Expression was normalized to the housekeeping gene phosphobilinogen deaminase (PBGD), and is shown relative to that of PBMCs, which was set at 1.
Figure 4. Phenotypical maturation of HSPC-DCs with TLR agonists. pDCs, BDCA1+ and BDCA3+ mDCs were sorted by flow cytometry from SR1-cultured CD34+ cells at day 21 of culture and stimulated overnight with TLR agonists. Expression of maturation and co-stimulatory molecules was evaluated by flow cytometry. (A) Histograms show expression of CD80, CD86, CD83, CCR7 and HLA-DR (black line) versus isotype control (grey line) for pDCs stimulated with CpG-B and BDCA1+ and BDCA3+ mDCs stimulated with R848 and Poly I:C. (B) The mean fluorescent intensity (MFI) of CD80, CD86, CD83, CCR7 and HLA-DR for non-TLR stimulated and TLR-stimulated pDCs, BDCA1+ and BDCA3+ mDCs. All conditions were supplemented with IL-3 or GM-CSF for survival of pDCs and mDCs, respectively. Data depict mean ± SEM of results from four (pDCs), two (BDCA1+ mDCs) or one (BDCA3+ mDCs) UCB donor.

Finally, we assessed the cytokine secretion by TLR-stimulated HSPC-DCs. HSPC-pDCs activated with CpG-A secreted high levels of IFN-α and moderate amounts of TNF-α and IL-6, while CpG-B-activated HSPC-pDCs secreted both IL-6 and TNF-α,
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but no IFN-α (Figure 5A). This is in line with previous reports on responses of PB-pDCs to different classes of CpG.28,29 Furthermore, HSPC-pDCs also responded to R848 by secreting IFN-α and upregulating co-stimulatory molecules (data not shown). HSPC-BDCA1+ mDCs secreted TNF-α in response to TLR stimulation (Figure 5B) but no IL-12, while HSPC-BDCA3+ mDCs secreted both TNF-α and IL-12 (Figure 5C). Collectively, these data demonstrate that pDCs, BDCA1+ mDCs and BDCA3+ mDCs generated ex vivo by culturing CD34+ HSPCs with SR1 display similar phenotype and functional properties as PB-DCs.

Figure 5. Cytokine secretion by HSPC-DCs in response to TLR agonists. pDCs, BDCA1+ and BDCA3+ mDCs were sorted by flow cytometry from SR1-cultured CD34+ cells at day 21 of culture and stimulated overnight with TLR ligands. Concentrations of cytokines in the supernatant was measured by ELISA. (A) Concentrations of IFN-α, IL-6 and TNF-α secreted by pDCs seeded at 10⁵ cells/mL. Data depict mean ± SEM of 4 independent UBC donors. Statistical analysis was performed using one-way RM ANOVA followed by a Bonferroni post-hoc test, comparing stimulated cells (CpG-A/CpG-B) with non-stimulated cells (medium). **P<0.01, ***P<0.001. (B) Concentration of TNF-α secreted by BDCA1+ mDCs seeded at 5 x 10⁵ cells/mL. Data show mean ± SEM of 2 independent donors. (C) Concentration of TNF-α and IL-12 secreted by BDCA3+ mDCs seeded at 1.8 x 10⁵ cells/mL. Data of 1 out of 3 independent experiments is shown and bars are depicted as mean ± SD.

HSPC-DCs are potent inducers of allogeneic T cell responses

To determine the T cell stimulatory capacity of HSPC-derived DCs, we performed allogeneic MLR assays where HSPC-pDCs, BDCA1+ mDCs and BDCA3+ mDCs were matured overnight and subsequently co-cultured with CFSE-labeled PBMCs from allogeneic donors for 4-5 days. All the different HSPC-DC subsets induced robust proliferation of both CD4+ and CD8+ T cells (Figure 6A,B). In addition, allogeneic T cells stimulated with HSPC-DCs secreted both IFN-γ and IL-2 (Figure 6C,D), while IL-4, TNF-α and IL-17 were non-detectable and the concentrations of IL-10 were low in the
supernatant (data not shown). These data show that the SR1-induced HSPC-DCs are potent inducers of allogeneic T cell responses.

**Peptide-loaded HSPC-DCs induce activation of antigen-experienced T cells**

In order to extend our observations of potent antigen-specific T cell activation by HSPC-DCs, we determined the level of IFN-γ produced by CD8+ T cells recognizing the minor histocompatibility antigen LRH-1 or HA-1. Upon stimulation by HLA-B7+ HSPC-DCs loaded with LRH-1 peptide, LRH-1-specific CD8+ CTLs secreted significantly higher levels of IFN-γ than in response to non-peptide-loaded DCs (Figure 7A). Furthermore, concurrent maturation of the DCs with TLR ligands significantly enhanced the ability of the HSPC-DCs to activate IFN-γ secreting CD8+ T cells. Similarly, HA-1+ HLA-A2+ HSPC-DCs loaded with extra HA-1 peptide were used as stimulators of HA-1-specific T cells. Activation of HA-1-specific T cells was higher in response to HSPC-DCs loaded with the HA-1 peptide than to HSPC-DCs without exogenous peptide (Figure 7B), and the T cell activation was additionally potentiated by TLR-induced maturation of DCs. These data demonstrate that peptide-loaded HSPC-DCs can effectively activate antigen-experienced T cells.

**Discussion**

DC-based vaccination is an attractive strategy to boost T cell immunity in patients with cancer and infectious diseases. So far, the vast majority of clinical trials have been performed with MoDCs. However, due to sub-optimal clinical effects, further improvements of DC vaccine potency are essential. One strategy is to use primary DC subsets isolated from blood, such as pDCs, BDCA1+ mDCs and BDCA3+ mDCs. Recently, it has been shown that pDCs can recognize, process and cross-present foreign antigens to CD8+ T cells, a quality important for generation of effective anti-tumor responses. 30-32 A seminal study by Tel et al. showed that some metastatic melanoma patients that were vaccinated with antigen-loaded pDCs, could induce antigen-specific CD4+ and CD8+ T cell responses and anti-tumor immunity.7 Pre-clinical studies have shown that pDCs induce anti-tumor immunity not only by direct priming and activation of tumor-specific T cells, but also by the high amounts of type I interferons they secrete, which enhances cross-priming capacity of other DC subsets, and induces activation of NK cells.33-35 In addition, pDCs also interact with mDCs through cell-cell contact, such as by the CD40-CD40L axis, further enhancing the T cell stimulation capacity of mDCs.16 The therapeutic potential of this cross-talk between DC subsets has been investigated in murine tumor models. For instance, Nierkens et al. published that immunization of mice with mDCs activated in the presence of pDCs facilitated tumor clearance due to enhanced cross-priming capacity of the mDCs.9 Furthermore, Lou et al. reported that immunization of mice with a combination of activated pDCs and mDCs resulted in increased levels of antigen-
Figure 6. T cell stimulation capacity of HSPC-DCs in allogeneic mixed lymphocyte reactions. HSPC-DCs from SR1-cultured UCB CD34+ cells were sorted by flow cytometry at day 21 and stimulated overnight with TLR ligands (CpG-B for pDCs and Poly I:C for BDCA1+ and BDCA3+ mDCs). Subsequently the DCs were used as stimulators of CFSE-labeled PBMCs from healthy donors at a ratio of 1:10 (DC:PBMCs). Five days later the proliferation of T cells was analyzed by flow cytometry and IL-2 and IFN-γ measured in the supernatant by cytometric bead array. (A) Dot plots depict the proliferation of CD3+CD4+ and CD3+CD8+ T cells of one representative PBMC donor out of four tested. (B) Average CD3+CD4+ and CD3+CD8+ T cell proliferation of four different PBMC donors. (C-D) Average concentration of IL-2 (C) and IFN-γ (D) in the supernatant of co-cultures with four different PBMC donors. Data are expressed as mean ± SEM and show the results of one representative experiment out of two experiments conducted with different HSPC donors. Statistical analysis was performed with one-tailed paired Student’s t-test. *P<0.05, **P<0.01.
specific CD8+ T cells and enhanced anti-tumor response compared with immunization with either DC subset alone.15 These studies indicate that combination of pDCs and mDCs for DC-based immunotherapy would result in more favorable responses.

Our novel SR1-based culture method demonstrates simultaneous generation of high amounts of pDCs, BDCA1+ and BDCA3+ mDCs from UCB and G-CSF-mobilized CD34+ HSPCs. We demonstrated that the HSPC-derived DCs were phenotypically and functionally similar to their naturally occurring counterparts found in blood. The HSPC-pDCs expressed both BDCA2 and BDCA4 which are surface-antigens specifically found on pDCs.26 Furthermore, they highly expressed TLR7 and TLR9 and responded to stimulation with corresponding TLR-ligands by secreting high amounts of IFN-α, IL-6 and TNF-α and upregulating co-stimulatory molecules and maturation markers. The HSPC-derived BDCA3+ mDCs exclusively expressed DNGR1, a C-type lectin receptor which mediates antigen uptake and cross-presentation by BDCA3+ mDCs.36,37 These DCs responded to R848 and Poly I:C-stimulation by secretion of both TNF-α and IL-12 and phenotypical maturation. BDCA1+ mDCs on the other hand did not secrete IL-12 in

Figure 7. Antigen-specific T cell activation by HSPC-DCs. (A) LRH-1+ HSPC-DCs from HLA-B7+ donor were sorted from SR1-cultured G-CSF-mobilized CD34+ cells by flow cytometry at day 21 and loaded with or without 1 μM LRH-1 peptide. Subsequently, the DCs were matured with TLR ligands as depicted in the figure in medium supplemented with IL-3 or GM-CSF for pDCs and mDCs, respectively. Next, the DCs were co-cultured with CTLs specific for LRH-1 (RP1) at an 1:1 ratio. 24 hours later the concentration of IFN-γ was measured in the supernatant by ELISA. (B) Similarly, HLA-A2+, HLA-1+ HSPC-DCs were loaded with 1 μM HA-1, matured with TLR ligands and used as stimulators for HA-1-specific CD8+ T cell bulk. Bars depict mean values ± SD. Statistical analysis was performed using one-way RM ANOVA followed by a Bonferroni post-hoc test. *P<0.05, **P<0.01, ***P<0.001.
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response to these TLR-ligands, which is in contrast to what has been described in the literature for their in vivo counterparts. Furthermore, only a portion of HSPC-BDCA1+ mDCs expressed CD11c, while BDCA1+ mDCs in blood all express CD11c. The lack of IL-12 secretion by BDCA1+ mDCs and heterogenous CD11c expression might indicate that they are more immature than the other two DC subsets found in the culture, and require an additional maturation stimulus with for example GM-CSF. Nevertheless, the BDCA1+ mDCs did potently activate antigen-specific T cells secreting high levels of IFN-γ, just as the other two DC subsets, indicating that their immunostimulatory capacity is not impaired.

Previously, various in vitro culture systems have been developed for understanding the molecular regulations of DC development from human HSPCs and for generating DCs for immune-based therapies. Recently, Poulin et al. reported that functional BDCA3+DNGR1+ mDCs could be generated from human HSPCs, by first expanding HSPCs for 7-11 days and then differentiating them with Flt3L, SCF, GM-CSF and IL-4 in medium supplemented with FCS for an additional 12-14 days. On the other hand, Blom et al. were the first to describe the ex vivo generation of pDCs, where Flt3L alone was sufficient for inducing the differentiation of pDCs from CD34+CD45RA- hematopoietic stem cells. Later, Chen et al. reported that TPO acts in synergy with Flt3L in expanding and differentiating CD11c CD123+ pDCs from CD34+ HSPCs in vitro. Another group later described that culturing G-CSF-mobilized CD34+ HSPC with TPO and Flt3L additionally resulted in the differentiation of CD11c CD123+, CD1c/b+ and DNGR1+ DCs. However, their numbers of DCs generated were generally low, and the supposed pDC population showed low CD123 expression and secreted relatively low levels of IFN-α. Furthermore, the DNGR1+ mDCs found in their culture did not express BDCA3. According to our DC culture protocol, we would be able to simultaneously generate 75 x 10^6 pDCs, 100 x 10^6 BDCA1+ mDCs and 25 x 10^6 BDCA3+ mDCs under serum-free conditions, given that on average 2 x 10^6 CD34+ cells can be isolated from one UCB unit. Moreover, since our culture protocol can also be applied to G-CSF-mobilized blood CD34+ cells, the clinical application of SR1-induced HSPC-DCs can be expanded to both autologous and allogeneic settings. From 10 x 10^6 G-CSF-mobilized CD34+ cells, 50 x 10^6 pDCs, 50 x 10^6 BDCA1+ mDCs and 10 x 10^6 BDCA3+ mDCs could be generated. To the best of our knowledge this is the highest reported number of BDCA2’BDCA4+ pDCs, BDCA1+ mDCs and BDCA3’DNGR1+ mDCs that can be simultaneously generated ex vivo from CD34+ HSPCs, and additionally exceeds the numbers that can be isolated from peripheral blood. However, additionally to an initial CD34+ HSPC selection, our expansion protocol requires a 3-week culture in a clean room facility and several GMP-grade cytokines and SR1. But, paralleled isolation of BDCA4+ pDC and BDCA1+ mDC requires expensive magnetic bead isolation procedures. Furthermore, magnetic beads for isolating BDCA3’DNGR1+ mDCs are currently not available. In addition, generating DCs ex vivo from HSPCs offers additional advantages, for example the possibility to manipulate the cells
during differentiation, such as silencing their expression of co-inhibitory molecules. Such modulations could even result in stronger anti-tumor responses, as it has been published that siRNA silencing of the co-inhibitory molecules PD-L1 and PD-L2 on MoDCs augments expansion and function of antigen-specific T cells.43

In this study, the different DC subsets were generated by culturing CD34+ HSPCs with Flt3L, SCF, TPO, IL-6 and the AhR antagonist SR1. The AhR has been extensively studied through the years, in AhR knockout mice and in the context of activation by environmental contaminants. These studies demonstrate that AhR is involved in hematopoiesis and modulates immune responses in various mouse models of infectious and auto-immune diseases.44 Notably, HSPCs, DCs and T cells express high levels of AhR compared to other immune cells.45 Studies focused on T cell and DC functionality show that AhR activation differentially modulates their function, depending on the source of the cells and the AhR ligands used.46 Furthermore, AhR function has shown to regulate the balance between proliferation and quiescence of hematopoietic stem cells (HSCs). Young AhR knockout mice have increased numbers of HSCs with higher proliferation rate than stem cells from wild type mice. This is also reflected in higher numbers of white blood cells in bone marrow, spleen and blood of AhR knockout mice.47-50 The same effect was reported for human HSPCs by Boitano et al., demonstrating that culturing CD34+ cells with SR1 strongly promotes their proliferation rate.18 With a comparable culture protocol, we now demonstrate for the first time that inhibition of human AhR promotes the differentiation of human pDC and mDC subtypes from CD34+ HSPCs. In line with our findings, Liu et al. recently reported that AhR knockout mice have a higher frequency and absolute numbers of pDCs in spleens and lymph nodes compared to wild type mice.51 In contrast, they found no differences in the proportion and absolute numbers of CD11c<sup>hi</sup>CD11b<sup>+</sup>I-A<sup>b</sup> mDCs. Vorderstrasse et al. similarly reported in 2001, that the number of splenic CD11c<sup>hi</sup> mDCs was not significantly different between AhR knockout and wild type mice.52 These findings suggest that DC differentiation might be differently regulated by AhR in mice and humans. Since there is also increasing evidence for dysregulation of DC subtypes in distinct auto-inflammatory diseases, such as Rheumatoid Arthritis, Systemic Lupus Erythematosus and Psoriasis53-55, it is also tempting to speculate whether interfering with the AhR pathway could dampen the mDC and pDC activation status in these diseases.

In conclusion, we show that SR1 strongly promotes differentiation of BDCA2<sup>+</sup>BDC4<sup>+</sup> pDCs, BDCA1<sup>+</sup> and BDCA3<sup>+</sup>DNGR1<sup>+</sup> mDCs from CD34+ HSPCs. The SR1-induced HSPC-DCs are phenotypically and functionally comparable to their naturally occurring counterparts in blood and induce strong antigen-specific T cell responses. This culture system allows the generation of clinical-scale numbers of pDCs and mDCs relevant for DC-immunotherapy of cancer patients and patients with chronic infectious diseases. Furthermore, this protocol can be used for investigating molecular pathways driving aberrant DC development and function which has been shown to be instrumental for the pathogenesis of various auto-immune disorders.
Acknowledgements

We thank Willemijn Hobo and Anniek van der Waart for technical support and Rob Woestenenk and Gaby Derksen for assistance in flow cytometry. We also thank Nina Karthaus-Tel and Jurjen Tel (Department of tumor Immunology, Nijmegen, The Netherlands) for advice and generously providing TLR4, TLR7, TLR8 and TLR9 primers.
Chapter 3

References


AhR inhibition promotes DC differentiation


Supplementary Figure 1. Titration of SR1. UCB CD34+ cells were cultured with TPO, SCF, Flt3L and IL-6 as well as titrated concentration of SR1 (0.1-2μM) and the frequency of pDCs (CD11c CD123hiBDCA2+) was evaluated by flow cytometry at day 21. Data are expressed as mean ± SEM of two UCB donors.

Supplementary Figure 2. Phenotypical maturation of HSPC-DCs. pDCs, BDCA1+ and BDCA3+ mDCs were sorted by flow cytometry from SR1-cultured CD34+ cells at day 21 of culture and stimulated overnight with TLR agonists. Expression of CD80, CD86, CD83, CCR7 and HLA-DR was evaluated by flow cytometry and is depicted as % positive cells for non-TLR stimulated and TLR-stimulated pDCs, BDCA1+ and BDCA3+ mDCs. All conditions were supplemented with IL-3 or GM-CSF for survival of pDCs and mDCs, respectively. Data depict mean ± SEM of results from four (pDCs), two (BDCA1+ mDCs) or one (BDCA3+ mDCs) UCB donor.
AhR inhibition promotes DC differentiation

### Supplementary Table 1.
Fold increase, frequency and absolute numbers of pDCs, BDCA1+ and BDCA3+ mDCs at day 21 from different UCB donors cultured with 1 μM SR1.

<table>
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<th>fresh/frozen CD34+ cells</th>
<th>Fold increase of total cells</th>
<th>pDCs (%)‡</th>
<th>pDCs (x10⁶)†</th>
<th>BDCA1+ mDCs (%)‡</th>
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*Analysed at day 19
†Within total cultured cells. pDCs are gated as CD11c- cells and mDCs as Lin HLA-DR+ cells
‡Generated from 10⁵ CD34+ cells

### Supplementary Table 2.
Fold increase, frequency and absolute numbers of pDCs, BDCA1+ and BDCA3+ mDCs at day 21 from different G-CSF-mobilized donors cultured with 1 μM SR1.

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†Within total cultured cells. pDCs are gated as CD11c- cells and mDCs as Lin HLA-DR+ cells
‡Generated from 10⁵ CD34+ cells
### Supplementary Table 3. Expression levels of BDCA2, BDCA4, HLA-DR, CD62L, CD4, CD45RA, CD11b and CD11c on HSPC-pDCs.

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HSPC-pDCs (CD123hi cells) from SR1 cultured UCB CD34+ cells were analysed for the expression of BDCA2, BDCA4, HLA-DR, CD62L, CD4, CD45RA, CD11b and CD11c by flow cytometry. Expression is calculated as percentage positive HSPC-pDCs out of total HSPC-pDCs.
AhR inhibition promotes DC differentiation
Hematopoietic stem cell-derived myeloid and plasmacytoid DC-based vaccines are highly potent inducers of tumor-reactive T cell and NK cell responses \textit{ex vivo}.

Soley Thordardottir, Nicolaas Schaap, Elja Louer, Michel G. D. Kester, J. H. Frederik Falkenburg, Joop H. Jansen, Timothy R. D. Radstake, Willemijn Hobo and Harry Dolstra

\textit{Oncoimmunology}. 2017;6(3):e1285991
Abstract

Because of the potent graft-versus-tumor (GVT) effect, allogeneic stem cell transplantation (alloSCT) can be a curative therapy for hematological malignancies. However, relapse remains the most frequent cause of treatment failure, illustrating the necessity for development of adjuvant post-transplant therapies to boost GVT-immunity. Dendritic cell (DC) vaccination is a promising strategy in this respect, in particular where distinct biological functions of naturally occurring DC subsets, i.e. myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), are harnessed. However, it is challenging to obtain high enough numbers of primary DC subsets from blood for immunotherapy due to their low frequencies. Therefore, we present here an ex vivo Good Manufacturing Practice (GMP)-compliant cell culture protocol for generating different DC subsets from CD34+ hematopoietic stem and progenitor cells (HSPCs) of alloSCT donor origin. High numbers of BDCA1+ mDCs and pDCs could be generated, sufficient for multiple vaccination cycles. These HSPC-derived DC subsets were highly potent in inducing anti-tumor immune responses in vitro. Notably, HSPC-derived BDCA1+ mDCs were superior in eliciting T cell responses. They efficiently primed naïve T cells and robustly expanded patient-derived minor histocompatibility antigen (MiHA)-specific T cells. Though the HSPC-pDCs also efficiently induced T cell responses, they exhibited superior capacity in activating NK cells. pDC-primed NK cells highly upregulated TRAIL and possessed strong cytolytic capacity against tumor cells. Collectively, these findings indicate that HSPC-derived DC vaccines, comprising both mDCs and pDCs, may possess superior potential to boost anti-tumor immunity post alloSCT, due to their exceptional T cell and NK cell stimulatory capacity.
Introduction

Allogeneic stem cell transplantation (alloSCT) can be a curative treatment for patients with hematological malignancies. The therapeutic effect, the so called graft-versus-tumor (GVT) effect, can be attributed to donor-derived CD8+ T cells directed against antigens expressed on the recipient tumor cells, including minor histocompatibility antigens (MiHAs) and tumor associated antigens (TAAs).1,2 In addition, multiple reports demonstrate that NK cells also contribute to GVT immunity, as a high number of NK cells in stem cell grafts3, as well as early NK cell repopulation4,5, have been associated with reduced relapse rates. Still, tumor relapse remains a leading cause of treatment failure, illustrating the necessity to develop potent adjuvant post-transplant immunotherapies in order to boost GVT immunity, and improve clinical outcome after alloSCT.

Dendritic cell (DC) vaccination is an attractive approach to induce and boost GVT responses, as DCs are the most powerful antigen-presenting cells.6,7 Exploiting DC vaccines, T cell responses directed against hematopoietic-restricted MiHAs or TAAs can be specifically boosted, thereby enhancing GVT activity without evoking potentially life-threatening graft-versus-host disease (GVHD). In vivo, multiple blood DC subsets exist, each with their distinct phenotypic and functional characteristics.8,9 These subsets can broadly be divided into two groups, myeloid and plasmacytoid DCs (mDCs and pDCs, respectively), where mDCs can be further subdivided into BDCA1+ mDCs and BDCA3+ mDCs.10 mDCs are in the front line of defense against fungi and bacteria, which they detect via different pattern-recognition receptors, including Toll-like receptors (TLRs). They possess high antigen processing and presenting capacity and secrete interleukin (IL)-12, which makes them highly effective in promoting proinflammatory interferon (IFN)-γ secreting T helper (Th)-1 and cytotoxic T lymphocyte (CTL) responses.8,9,11,12 In contrast, pDCs play a key role in the detection and control of viral infections, primarily due to their specialized production of type I IFN (IFN-α/β) following TLR7 and TLR9 stimulation.13 Their secretion of IFN-α provides an immunostimulatory environment for both innate and adaptive immune cells. Notably, IFN-α and contact-dependent interactions potentiate DC-mediated activation, IFN-γ secretion and cytolytic capacity of NK cells.14-18 Furthermore, studies have indicated that the different DC subsets act synergistically via cross-talk, thereby potentiating each others’ stimulatory capacity.19-23 Together, these characteristics render vaccines composed of both mDCs and pDCs highly attractive for the induction of broad anti-tumor immune responses.

Recently, we reported an innovative ex vivo culture protocol to efficiently generate high numbers of functional BDCA1+ mDCs, BDCA3+ mDCs and pDCs from CD34+ hematopoietic stem and progenitor cells (HSPCs).24 We discovered that inhibition of the aryl hydrocarbon receptor, using the antagonist StemRegenin 1 (SR1), was essential for DC subset differentiation. In this paper, we describe optimizations of this SR1-based culture protocol, where culture conditions were improved for generation of higher amounts and better differentiated mDC and pDC subsets. In addition, culture conditions were adapted...
to Good Manufacturing Practice (GMP). Notably, this protocol allows the generation of the different DC subsets from CD34+ HSPCs derived from the original donor grafts. Importantly, we demonstrated the superior capacity of HSPC-derived mDCs and pDCs in stimulating tumor-reactive T cells and NK cells. While the generated mDCs were better T cell stimulators, the cultured pDCs were superior in inducing cytotoxic NK cell responses. Cumulatively, HSPC-derived DC subset (HSPC-DC) vaccines hold great promise for future application as post-transplant therapy to selectively boost GVT immunity and improve relapse-free survival.

Materials and Methods

Patient and donor material
For generation of HSPC-DCs and monocyte-derived DCs (MoDCs), peripheral blood mononuclear cells (PBMCs) were obtained from leukapheresis material of granulocyte colony-stimulating factor (G-CSF)-mobilized stem cell donors. CD8+ T cells used for priming assays were isolated from the same material. For T cell expansion assays, PBMCs were obtained from patients with hematological malignancies who developed HA1-specific CD8+ T cell responses following alloSCT.25 Samples were collected 6-31 months after alloSCT. Patient (Pt) 1 and 2 did not receive additional donor lymphocyte infusion post-transplant, whereas Pt 3 and 4 received a donor lymphocyte infusion 16 and 2 months prior to the sample date, respectively. Because no apheresis material of the corresponding transplant donors was available, HSPC-DCs and MoDCs used for expansion of these patient-derived HA1-specific T cells were generated from allogeneic HLA-A2:HA1+ donors (from a third party apheresis). For isolation of NK cells, healthy donor buffy coats were obtained from Sanquin blood bank. Cellular material was obtained in accordance with the Declaration of Helsinki and institutional guidelines and regulations (CMO 2013/064).

Ex vivo generation of HSPC-DCs and MoDCs
CD34+ cells were isolated from G-CSF-mobilized PBMCs using anti-CD34 microbeads (Miltenyi Biotec, catalog# 130-046-702). Purity and viability was evaluated by flow cytometry. CD34+ HSPCs were either used directly for HSPC-DC generation, or cryopreserved in 50% human serum (HS; PAA Laboratories), 42.5% IMDM (Iscoce-modified Dulbecco medium, GIBCO Invitrogen, catalog# 21980-032) and 7.5% DMSO (dimethyl sulfoxide, Sigma-Aldrich) and thawed at a later time-point for HSPC-DC generation. To generate HSPC-DCs, CD34+ HSPCs were cultured for 14-20 days in Cellgro DC medium (GMP-compliant, Cellgenix, catalog# 20801-0500), supplemented with 1 µM SR1 (Cellagen Technology, catalog# C7710-5) as previously described.24 The medium was additionally supplemented with 2% HS and 50 µg/mL ascorbic acid (AA; Centrafarm) during the entire culture period. During the first 7 days of culture, the medium was supplemented with Flt3L (FMS-like tyrosine kinase 3 ligand), SCF (stem
cell factor) and TPO (thrombopoietin), 100 ng/mL each (all from Immunotools, catalog# 11343305, 11343328 and 11344865, respectively). For the remaining culture period, the concentrations of SCF and TPO were reduced to 50 ng/mL. Alternatively, HSPC-DCs were generated in a two-step protocol: after 7-13 days expansion as described above, cells were harvested, washed and reseeded in Cellgro DC medium containing 800 IU/mL GM-CSF (granulocyte-macrophage colony-stimulating factor) and 500 IU/mL IL-4 (both from Immunotools, catalog# 11343125 and 11340048, respectively) and cultured for 7 additional days. At day 0, the CD34+ HSPCs were seeded at 1 x 10^5 - 2 x 10^6 cells/mL, while after day 7 their density was adjusted to 1 x 10^6 - 1.5 x 10^6 cells/mL every 2-3 days with growth factor-supplemented medium. Total number of viable cells was determined by trypan blue exclusion counting at fixed timepoints during the culture period. At the end of the culture, samples were taken for flow cytometric analysis, and absolute numbers of DCs were calculated by multiplying the frequency of DCs with the absolute number of total cells generated from 1 x 10^6 CD34+ cells. For functional experiments, BDCA1+ mDCs and pDCs were sorted from HSPC-DC cultures by labeling the cells with anti-BDCA1 (clone L161) and anti-CD123 (clone 6H6) antibodies (both from Biolegend), respectively, followed by fluorescence-activated cell sorting (FACS) using the FACS Aria (BD Biosciences). The sorted DC subsets were in all cases used directly (“fresh”) for functional assays, except in assays described in Figure 2A,B, where cryopreserved/thawed DCs were used for day 7 re-stimulation of primed MiHA-specific T cells.

MoDCs were generated in 7 days from plastic adherent monocytes in Cellgro DC medium supplemented with 2% HS, 800 IU/mL GM-CSF and 500 IU/mL IL-4, as previously described.26

**DC maturation and cytokine release**

Sorted HSPC-DC subsets were resuspended in IMDM supplemented with 10% fetal calf serum (FCS, Integro) and matured overnight with TLR-ligands. pDCs were stimulated with 3.2 µg/mL CpG-A (ODN 2216) or 5 µg/mL R848 (Resiquimod; both from Enzo Life Sciences, catalog# ALX-746-005 and ALX-420-038, respectively) in medium supplemented with IL-3 (10 ng/mL, Immunotools, catalog# 11340035), while BDCA1+ mDCs were stimulated with a combination of 5 µg/mL R848 and 20 µg/mL Poly I:C (Polynosinic:polycytidylic acid, Sigma-Aldrich, catalog# P0913), referred to as RPI:C, in the presence of GM-CSF (800 IU/mL) and IL-4 (500 IU/mL). MoDCs were matured with RPI:C or conventional cytokine mixture (cytomix) containing 5 ng/mL IL-1β, 15 ng/mL IL-6, 20 ng/mL tumor necrosis factor (TNF)-α and 1 µg/mL PGE2 (prostaglandin E2), as previously described.27 Cell culture supernatants were analyzed with ELISA for IFN-α (pan-specific, MabTech, catalog# 3425-1H-20), IL-12p70 (eBioscience, catalog# 14-8129 (standard), 16-8126 (coating Ab) and 13-7129 (detection Ab)) and TNF-α (Sanquin, catalog# M9323) levels, according to manufacturer’s instructions.
Chapter 4

Flow cytometry
Cells were pre-incubated on ice with total human IgG (Nanogam, Sanquin) to block Fc-receptors, and subsequently stained with appropriate antibody combinations. HSPC-DCs were phenotyped with anti-BDCA1/CD1c (clone L161), anti-BDCA2/CD303 (clone 201A), anti-BDCA3/CD141 (clone M80), anti-BDCA4/CD304 (clone 12C2), anti-DNGR1/Clec9A/CD370 (clone 8F9), anti-HLA-ABC (clone W6/32), anti-CD11c (clone 3.9), anti-CD15 (clone W6D3), anti-CD80 (2D10), anti-CD83 (clone HB15e), anti-CD86 (clone IT2.2), anti-CD123 (clone 6H6, all from Biolegend), anti-HLA-DR (clone Immu357), anti-CD11b (clone Bear1), anti-CD14 (clone RMO52), anti-CD34 (clone 581, all from Beckman Coulter), anti-PD-L1/CD274 (clone MIHI1) and anti-CD123 (clone 7G3, both from BD Bioscience). NK cell activation was determined with anti-TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)/CD253 (clone RIK-2), anti-CD3 (clone UCHT1), anti-CD56 (clone HCD56) and anti-CD69 (clone FN50, all from Biolegend). Relevant isotype control antibodies were used from Biolegend. MiHA-specific T cells were detected by staining with anti-CD3 (clone UCHT1, Biolegend), anti-CD8 (clone 3B5, Invitrogen) and peptide/major histocompatibility complex (MHC)-tetramers (HA1.A2: VLHDDLLEA28, UTA2-1.A2: QLLNSVLTL29, HA8.A2: RTLDKVLEV30), as previously described.27,31,32 Acquisition and data analysis was performed with a Gallios flow cytometer and Kaluza software (both from Beckman Coulter). Dead cells were excluded using Sytox blue (Invitrogen, catalog# S34857), 7AAD (Sigma, catalog# A9400) or eFluor780 fixable viability dye (eBioscience, catalog# 65-0865-14).

MiHA-specific T cell assays
In T cell priming experiments, CD8+ T cells were isolated from MiHA-negative HLA-A2+ G-CSF-mobilized PBMCs from male donors using CD8 microbeads (Miltenyi Biotec, catalog# 130-045-201). For HA1-specific T cell expansion assays, PBMC samples obtained from alloSCT patients containing HA1-reactive CD8+ T cells were used. For these assays, HSPC-DCs and MoDCs were generated from HLA-A2+ MiHA+ donors. Matured MoDCs, HSPC-derived BDCA1+ mDCs and HSPC-derived pDCs were harvested, loaded with 5 µM MiHA-peptide (HA1, UTA2-1 or HA8, from LUMC-IHB peptide facility, the Netherlands or ThinkPeptides), washed and subsequently co-cultured with the isolated CD8+ T cells or patient PBMCs in IMDM/10%HS at a ratio of 1:0.2 to 1:0.1 (T cell/PBMC:DC), as previously described.27,31 For priming assays, a minimum of 9 x 10^6 CD8+ T cells were used per DC subset. At days 2 and 5, IL-2 (50 IU/mL, Proleukin®, Chiron) and IL-15 (5 ng/mL, Immunotools, catalog# 11340155) were added to the co-cultures. At day 7, cells were counted and analyzed by flow cytometry, and when indicated, re-stimulated with the respective DC subset. For re-stimulation, cryopreserved/thawed DCs were used. To determine functionality, DC-stimulated T cells were rechallenged overnight with 5 µM MiHA peptide in the presence of anti-CD107a (BD Bioscience, clone H4A3), followed by
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an intracellular staining for IFN-γ (BD Bioscience, clone B27) and CD137 (Biolegend, clone 4-1BB), as described previously.27,31

**CTL activation**

CD8+ T cell clones specific for HA1.A2 and cytomegalovirus (CMV).A2 were isolated from patient 4 and a healthy donor, respectively, and expanded following protocols previously described.33 Mature DCs were harvested, washed, seeded in triplicate in 96-well round-bottom plate (Corning Costar, catalog# 3799, 10^4 DCs/well) in IMDM/10%FCS and loaded with 1 µM HA1 or CMV (NLVPMVATV^44, LUMC-IHB peptide facility) peptide. After 1 hour incubation at 37°C, HA1- or CMV-specific CTLs were added at 1:1 ratio. After 24 hours co-incubation, supernatant was harvested and IFN-γ production was measured by ELISA (Thermo Scientific, catalog# M700A (coating Ab) and M701B (detection Ab)).

**NK-DC co-culture**

NK cells were isolated using the untouched NK cell isolation kit (Miltenyi Biotec, 130-092-657), resulting in >87% NK cell purity (CD56+CD3- cells). HSPC-derived BDCA1+ mDCs or pDCs were resuspended in IMDM/10%FCS, whereupon 10 x 10^3, 2.5 x 10^3 or 1 x 10^3 cells were seeded per well in 96-well round-bottom plates. Subsequently, BDCA1+ mDCs were matured using GM-CSF/IL-4 or GM-CSF/IL-4/RPI:C, while pDCs were matured with IL-3, IL-3/CpG-A or IL-3/R848, as described above. Next, 5 x 10^4 freshly isolated allogeneic NK cells were added, resulting in NK:DC ratios of 1:0.2, 1:0.05 or 1:0.02. After 40 hours co-culture, CD69 and TRAIL expression on NK cells was evaluated by flow cytometry. Besides, a 4 hour cytotoxicity assay was performed with 1 µM carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled^35 Daudi tumor cells in the presence/absence of 40-hour DC-primed NK cells at a NK:Daudi ratio of 1:0.5. Subsequently, cells were harvested and stained with 7AAD, followed by quantification of viable Daudi cells using the FC500 flow cytometer (Beckman Coulter). The percentage of specific killing was calculated as follows:

\[
100 - (\frac{\text{absolute number of viable CFSE^+ Daudi cells co-cultured with NK cells}}{\text{absolute number of viable CFSE^+ Daudi cells cultured alone}}) \times 100.
\]

**Statistics**

Statistical analysis was performed using GraphPad Prism 5.0. Student’s t-test or one-way ANOVA, followed by Bonferroni post-hoc test, was used as indicated. P-values <0.05 were considered significant.
Figure 1. Generation of high numbers of BDCA1+ mDCs, BDCA3+ mDCs and pDCs from HSPCs. G-CSF-mobilized CD34+ HSPCs (either freshly isolated or thawed) were cultured for 14-20 days in Cellgro DC medium supplemented with 1 µM SR1, 2% HS and 50 µg/mL AA in a one- or two-step protocol. In the one-step protocol, HSPCs were cultured for 14-20 days in the presence of Flt3L, SCF and TPO (FST), while in the two-step protocol, HSPCs were expanded for 7-13 days with FST, then washed and reseeded in medium containing GM-CSF and IL-4 (G4) and subsequently cultured for 7 additional days. (A) Schematic overview of the two different culture protocols, the FST culture (one-step protocol) and G4 culture (two-step protocol). (B) Cumulative fold expansion of total cells at the end of 14-20 days culture, where cells were expanded for 7-9 days (filled circles) or 12-13 days (open circles), followed by 7 day differentiation. Each dot represents an independent donor, lines indicate the mean value (n=11). (C-E) After 14-20 days culture, the frequencies of BDCA1+ mDCs, BDCA3+ mDCs and pDCs were determined by flow cytometry. (C) Representative plots showing percentage of BDCA1+ mDCs, BDCA3+ mDCs and pDCs within total cultured cells. Gating strategy is depicted in Supplementary Figure 2. (D) The frequencies of BDCA1+ mDCs, BDCA3+ mDCs and pDCs within total cultured cells of 9-11 different donors. (E) Numbers of BDCA1+ mDCs, BDCA3+ mDCs and pDCs generated from 1 x 10^6 CD34+ cells. (D-E) Each dot represents an independent donor, lines indicate the mean value (n=9-11). (F-G) Surface expression of BDCA1, HLA-DR, CD11c and CD14 on HSPC-DCs in FST and G4 cultures was determined by flow cytometry. (F) Dot plots from one representative donor. (G) Mean fluorescence intensity (MFI) of HLA-DR and CD11c within the BDCA1+ mDC population. Data are depicted as mean ± SEM of 7 independent donors. Statistical analysis was performed using paired student’s t-test. * p < 0.05, ** p < 0.01, *** p < 0.001.
**Results**

**A GMP-compliant cell culture protocol for generation of high numbers of BDCA1+ mDCs and pDCs from HSPCs**

The overall aim of this study was to generate a DC vaccine composed of both mDC and pDC subsets and to examine its potential to boost both anti-tumor T cell and NK cell responses. The first objective was to establish culture conditions compliant with GMP, where sufficient numbers of well differentiated and functional DC subsets were generated from HSPCs for eventual clinical application. Therefore, we first modified our recently established SR1-based culture protocol, where the different HSPC-DC subsets were generated under serum-free conditions in GBGM medium supplemented with FLT3L, SCF, TPO, IL-6 and SR1. Our first optimization steps were to omit IL-6 from the cytokine cocktail and to switch to the widely available GMP-compliant Cellgro DC medium from Cellgenix, supplemented with 2% HS and AA (50 µg/mL). We observed that IL-6 inhibited in vitro DC differentiation, while AA and HS had a positive effect on DC-generation (Supplementary Figure 1). As mDCs generated with the previously published protocol showed low CD11c expression and limited IL-12 secretion upon TLR stimulation, we next investigated whether the HSPC-DC generation protocol would benefit from an additional mDC-differentiation boost at the end of the culture. For this purpose, we compared a two-step protocol, where HSPCs were first expanded with Flt3L, SCF and TPO (FST) for 7-13 days and then differentiated for one week in the presence of GM-CSF and IL-4 (G4 culture), to the standard one-step protocol where HSPCs were cultured for 14-20 days in the presence of FST (FST culture, Figure 1A). After 14-20 days, FST-cultured cells had expanded 133-fold in average, while G4-cultured cells expanded only 82-fold in average (Figure 1B). The frequencies of the different DC subsets were determined in both cultures as depicted in Supplementary Figure 2. We observed a significant increase in the frequency of BDCA1+ mDCs in G4 cultures compared to FST cultures, while pDC differentiation and/or survival was significantly reduced in G4 cultures (Figure 1C,D). Nevertheless, low frequencies of pDCs were still detectable in short-term expanded G4 cultures, although these pDCs exhibited lower BDCA2 expression than their FST-cultured counterparts (Figure 1C and Supplementary Figure 2D). The absolute number of generated BDCA1+ mDCs was similar for FST and G4 cultures, where both protocols resulted in generation of 18 x 10^6 BDCA1+ mDCs in average from only 1 x 10^6 CD34+ HSPCs (Figure 1E). Notably, the one-step protocol resulted in almost ten-fold higher absolute numbers of pDCs, where in average 16 x 10^6 pDCs were generated from only 1 x 10^6 CD34+ HSPCs (Figure 1E). Although of low occurrence, BDCA3’DNGR1+ mDCs were detected in both cultures (Figure 1C,E). Important for clinical implementation, we observed that cryopreserved/thawed HSPCs performed equally well in terms of expansion and DC differentiation as freshly isolated CD34+ HSPCs (Supplementary Figure 3). Flow cytometric analysis showed that BDCA1+ mDCs expressed high HLA-DR in both cultures, while the expression of CD11c increased upon G4 culture, suggestive of a more differentiated phenotype (Figure
1F,G). Notably, the majority of the BDCA1+ mDCs were negative for the monocytic marker CD14 (Figure 1F).

Based on these data, we continued with the G4 culture for the generation of BDCA1+ mDCs, and the FST culture for pDC generation. For further characterization, sorted G4-generated BDCA1+ mDCs and FST-generated pDCs were matured overnight with TLR-ligands and evaluated for phenotypical maturation and cytokine secretion. Our previous studies on SR1-derived HSPC-DCs showed that the HSPC-mDCs express TLR3, TLR4, TLR7 and TLR8, while the HSPC-pDCs express TLR7 and TLR9. To optimally stimulate BDCA1+ mDCs, we used a combination of the TLR3 agonist Poly I:C and TLR7/8 agonist R848, as simultaneous stimulation with these two agents has shown a synergistic effect. For stimulation of ex vivo-generated pDCs we used either R848, which induces both IFN-α secretion and upregulation of maturation markers, or the TLR9 agonist CpG-A, known to induce secretion of large amounts of IFN-α. Importantly, we observed high IL-12p70 secretion and TNF-α by the matured BDCA1+ mDCs (Supplementary Figure 4A). Additionally, they upregulated co-stimulatory and HLA molecules upon TLR-stimulation (Supplementary Figure 4B). Similarly, the HSPC-derived pDCs showed a mature phenotype and secreted high amounts of IFN-α and TNF-α (Supplementary Figure 4A,B). Moreover, when the DCs were stimulated in the bulk of total cultured cells, high levels of IL-12p70 and IFN-α could be detected, indicating that the total bulk culture could potentially be used as a vaccine (Supplementary Figure 4C).

In conclusion, these data demonstrate that using the two-step culture we can generate a higher frequency of well differentiated, IL-12 producing BDCA1+ mDCs, while the FST culture is most optimal for generation of high numbers of IFN-α producing pDCs.

**BDCA1+ HSPC-DCs efficiently prime naïve MiHA-specific T cells**

After establishing the optimal culture conditions for generation of HSPC-derived DC subsets, we examined their T cell and NK cell stimulatory capacity. To evaluate the capacity of HSPC-DCs in priming naïve antigen-specific T cells, sorted HSPC-derived BDCA1+ mDCs and pDCs were matured, loaded with MiHA peptide and co-cultured with autologous purified CD8+ T cells from MiHA-negative donors. For these assays, we used three HLA-A2 restricted and hematopoietic-specific MiHAs named HA128, UTA2-129 and HA830. At day 7, a clear population of MiHA-specific CD8+ T cells could be observed upon BDCA1+ mDC stimulation (Figure 2A and Supplementary Figure 5), which efficiently expanded upon an additional week of stimulation with MiHA-loaded BDCA1+ mDCs (Figure 2A,B). After overnight peptide rechallenge, BDCA1+ mDC-primed MiHA-specific T cells upregulated the activation marker CD137 and were highly positive for both IFN-γ and the degranulation marker CD107a (Figure 2C). In contrast, no MiHA-specific CD8+ T cells could be detected in pDC-stimulated cultures (Figure 2D). These data demonstrate that the HSPC-derived BDCA1+ mDCs are highly effective in priming and activating CD8+ T cells from the naïve repertoire.
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Figure 2. HSPC-derived BDCA1+ mDCs efficiently prime and activate naïve MiHA-specific T cells. HSPC-DCs were generated as described in Figure 1, and subsequently BDCA1+ mDCs were sorted from G4 culture and pDCs from FST culture. Next, BDCA1+ mDCs were stimulated with R848 and Poly I:C (RPI:C) in the presence of GM-CSF and IL-4, while pDCs were stimulated with R848 in the presence of IL-3. (A-B) Purified CD8+ T cells from MiHA-negative HLA-A2+ donors were cultured for two consecutive weeks with autologous MiHA peptide-loaded TLR-matured BDCA1+ mDCs. Cells were screened for the presence of MiHA-specific CD8+ T cells using flow cytometry on days 7 and 14. Density plots (A) and absolute MiHA-specific CD8+ T cell numbers (B). (C) BDCA1+ mDC-primed and expanded MiHA-specific T cells of donor B were overnight rechallenged with or without 5 μM HA1-peptide at day 14, followed by intracellular staining for CD137, IFN-γ and CD107a. (D) Purified CD8+ T cells from MiHA-negative HLA-A2+ donor were cultured for one week with MiHA-loaded TLR-matured pDCs. Presence of MiHA-specific CD8+ T cells was determined by flow cytometry on day 7. (A,C-D) The numbers in the dot plots represent the percentage of tetramer-positive cells within CD3+CD8+ T cells.

BDCA1+ HSPC-DCs and pDCs possess superior capacity to expand and activate CD8+ T<sub>em</sub> cells

Next, we assessed the capacity of the HSPC-derived DC subsets, in comparison to autologous MoDCs, to expand and activate effector memory CD8+ T (T<sub>em</sub>) cells. In these assays, we used PBMCs from four patients with a hematological malignancy who developed HA1-specific CD8+ T cell responses after alloSCT, and stimulated them for one week with TLR-matured HA1 peptide-loaded HSPC-DCs or MoDCs, generated from unrelated third-party HLA-A2‘HA1 donors. Interestingly, HSPC-derived BDCA1+ mDCs and pDCs showed superior capacity to induce expansion of MiHA-specific T<sub>em</sub> cells,
Figure 3. HSPC-derived BDCA1+ mDCs show superior capacity to expand and activate antigen-experienced CD8+ T cells. HSPC-DCs were generated as described in Figure 1, and subsequently BDCA1+ mDCs were sorted from G4 culture and pDCs from FST culture. Next, the BDCA1+ mDCs, pDCs and autologous MoDCs were activated with respective TLR-ligands as indicated in the figure, in the presence of GM-CSF and IL-4 (MoDCs and BDCA1+ mDCs) or IL-3 (pDCs). Thereafter, TLR-matured HA-1 peptide-loaded DCs were co-cultured for one week with patient PBMCs at a 1:0.1 ratio (PBMC:DC) (n=4). The HSPC-DCs and MoDCs used for expansion of patient HA1-specific CD8+ T cells were from unrelated third party donor apheresis products, as there was no apheresis material available of the corresponding transplant donors. The frequency of HA1-specific CD8+ T cells at day 0 was <0.02% in Pt. 1-3 and 0.61% in Pt. 4. (A) Density plots showing the percentage of HA1-specific CD8+ T cells at day 7. The numbers in the dot plots depict the percentage of positive cells within CD3+CD8+ T cells. (B) Absolute HA1-specific CD8+ T cell numbers after one week co-culture.
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evident by higher frequencies and absolute numbers of HA1-tetramer+ CD8+ T cells as compared to MoDC stimulation (Figure 3A-C). In three out of four patients, the BDCA1+ mDCs were more efficient than pDCs in boosting T cell expansion. These responses were highly specific, as no expansion of HA1-tetramer+ CD8+ T cells was seen with non-loaded DCs (Figure 3A and Supplementary Figure 6A). Additionally, the expanded T cells exhibited strong effector functionalities, as reflected by the high percentage of IFN-γ+CD137+ CD8+ T cells from patient 4 after overnight HA1 peptide rechallenge (Figure 3D). To explore their T cell stimulatory capacity further, we stimulated established CTL clones with peptide-loaded HSPC-DCs or MoDCs. Interestingly, the pDCs showed better potency for boosting IFN-γ production by HA1 and CMV effector CTLs than mDCs or MoDCs (Figure 4). Furthermore, TLR-stimulated bulk of total culture HSPC-DCs also efficiently activated HA1 and CMV effector CTLs (Supplementary Figure 6B). No IFN-γ secretion was observed in DC:T cell co-cultures in the absence of HA1 or CMV peptide (Supplementary Figure 6B). In summary, these data demonstrate the superior capacity of HSPC-derived BDCA1+ mDCs in expanding tumor-reactive CD8+ Tem cells, while the HSPC-derived pDCs appear to exhibit stronger potency to induce effector functions of antigen-experienced T cells.

Figure 4. HSPC-derived pDCs show superior capacity to induce IFN-γ secretion by CD8+ Tem cells. HSPC-DCs were generated and activated as described in Figure 3. TLR-matured DCs were seeded in triplicate and loaded for 1 hour with 1 µM HA1 or CMV peptide at 37°C. Next, without washing, CTLs specific for HA1 or CMV were added at 1:1 ratio and co-cultured for 24 hours. Concentrations of IFN-γ were determined by ELISA. Results are depicted as mean ± SD of a representative experiment (HA1 CTL: n=2, CMV CTL: n=1). Statistical analysis was performed by one-way ANOVA, followed by Bonferroni post-hoc test * p < 0.05, ** p < 0.01, *** p < 0.001.

< with DCs. (C) Fold expansion of HA1-specific CD8+ T cells, calculated by dividing the number of HA1-specific T cells at day 7 with the number of HA1-specific T cells at day 0. (D) 7-day DC-stimulated PBMCs of Pt. 4 were rechallenged overnight with 5 µM HA1-peptide and subsequently stained intracellular for CD137 and IFN-γ. As control, a small portion of cultured cells from all conditions was pooled and cultured overnight without HA1-peptide (No peptide). Numbers in the dot plots indicate the percentage of IFN-γ+ CD8+ T cells within CD3+CD8+ T cells. Pt. = patient. N.d. = not determined.
HSPC-pDCs superiorly induce activation of cytolytic NK cells

As there is accumulating evidence for the important role of DC-mediated NK cell activation in anti-tumor immunity, we next assessed the NK cell stimulatory capacity of HSPC-derived DC subsets. Therefore, NK cells were co-cultured with sorted allogeneic HSPC-derived BDCA1⁺ mDCs or pDCs in the presence of different DC maturation stimuli. After 40 hours of co-culture, a DC dose-dependent increase in CD69 expression on the NK cells was observed, where TLR-matured BDCA1⁺ mDCs and pDCs induced the highest expression (Figure 5A,B). In contrast, TRAIL expression on NK cells was primarily induced by pDCs (Figure 5A,C), especially by CpG-A-activated pDCs. TRAIL-expression induced by R848-activated pDCs varied between donors, and was high in experiment 1 (Figure 5A), but low in experiments 2 and 3 (Figure 5C). This may possibly have correlated with the respective IFN-α secretion from the stimulated pDCs, which varied in response to R848 (19.5 fg/pDC in experiment 1, <0.1 fg/pDC in experiments 2-3), as studies indicate that TRAIL upregulation on NK cells is dependent upon signaling via type I IFN receptor. Interestingly, NK cells co-cultured at a lower ratio with non-TLR activated pDCs (in the presence of IL-3) also highly expressed TRAIL, despite very limited IFN-α secretion by non-TLR activated pDCs (<0.1 fg/pDC).

Importantly, NK-mediated cytotoxicity was greatly enhanced following DC priming, in particular with CpG-A-activated pDCs (Figure 6). NK cells co-cultured with as few as 2% pDCs (ratio 1:0.02) in the presence of CpG-A, killed over 88% of the Daudi cells compared to <30% killing by non-primed NK cells. In addition, correlating with the TRAIL expression, we observed efficient Daudi killing following co-culture of NK cells with IL-3-cultured pDCs. In control conditions, where NK cells were cultured without DCs in the presence of the different DC maturation stimuli, expression of CD69 and TRAIL remained low and Daudi killing was also low (Figure 5B,C and Figure 6). Furthermore, no Daudi killing was observed by DC:Daudi co-cultures in the absence of NK cells (data not shown). These findings show that HSPC-derived DCs can activate and potentiate cytolytic capacity of NK cells, where in particular pDCs possess superior capability for boosting NK tumor reactivity.
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Figure 5. HSPC-pDCs superiorly induce activation of cytolytic NK cells. HSPC-DCs were generated as described in Figure 1, and subsequently BDCA1+ mDCs were sorted from G4 culture and pDCs from FST culture. Next, the different HSPC-DCs were seeded and medium was added containing respective TLR-ligands and cytokines as indicated in the figure. Without washing, allogeneic NK cells were added to the DCs at 1:0.2, 1:0.05 or 1:0.02 ratio (NK:DC). As control, NK cells were cultured without DCs in the absence (control=ctrl) or presence of the different DC maturation cocktails. By flow cytometry, expression of CD69 and TRAIL on NK cells was determined after 40 hours co-culture. (A) Histograms depicting expression of CD69 and TRAIL on DC-primed NK cells of experiment 1 at a NK:DC ratio of 1:0.2. (B-C) The percentage and mean fluorescent intensity (MFI) of CD69 (B) and TRAIL (C) on NK cells of experiments 2 and 3 at a NK:DC ratio of 1:0.2, 1:0.05 or 1:0.02. (A-C) Data shown are from three independent experiments using three different HSPC-DC and three different NK cell donors. NK cell purity was 97%, 87% and 97% for experiments 1, 2 and 3, respectively. Exp. = Experiment.
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Discussion

Although alloSCT can be a curative therapy for patients with hematological malignancies, high relapse rates remain the leading cause of treatment failure. DC vaccination using a combination of different DC subsets may be an attractive adjuvant treatment to boost GVT immunity. We and others\textsuperscript{49} postulate that vaccination with multiple DC subsets could not only robustly activate tumor-antigen reactive T cells, but also alloreactive NK cells, the two key effector populations involved in tumor cell elimination post alloSCT.

Previously, we reported a novel protocol for the generation of BDCA\textsuperscript{1+} mDCs, BDCA3\textsuperscript{+} mDCs and pDCs from CD34\textsuperscript{+} HSPCs, using the aryl hydrocarbon receptor antagonist SR1 in combination with Flt3L, SCF, TPO and IL-6.\textsuperscript{24} Here, we developed an optimized GMP-compliant culture protocol, where high amounts of mDCs and pDCs can be generated from G-CSF-mobilized CD34\textsuperscript{+} cells obtained from alloSCT donors. Notably, omission of IL-6 from the culture, as well as supplementation with AA and HS, had positive impact on ex vivo DC differentiation. Furthermore, an additional differentiation boost at the end of the culture, where GM-CSF and IL-4 were added instead of Flt3L, SCF and TPO (named G4 culture) resulted in a higher frequencies of BDCA\textsuperscript{1+} mDCs with high CD11c expression. However, this protocol was less ideal for pDC generation, as GM-CSF and IL-4 inhibited pDC differentiation, as also described by others.\textsuperscript{50} Therefore, we favored generation of
HSPC-mDCs using the G4 culture, and generation of HSPC-pDCs using the FST culture. Cumulatively, these changes allow ex vivo generation of clinically relevant dosages of HSPC-derived BDCA1+ mDCs and pDCs which, importantly, produce high amounts of IL-12 and IFN-α upon TLR stimulation. We envision that these immunostimulatory HSPC-derived DC subsets, presenting MiHAs and/or TAAAs, might be a potent adjuvant therapy for patients with hematological malignancies post alloSCT.

As these HSPC-derived DC subsets could be suitable for pre-emptive or therapeutic vaccination post-alloSCT, we evaluated their stimulatory capacity. Importantly, we observed DC-subset specific characteristics of our cultured HSPC-DC subsets similar to what has been described in literature.\textsuperscript{8-10} Ex vivo-generated BDCA1+ mDCs were more efficient in priming naïve T cells and subsequent expansion of MiHA-specific T\emph{em} cells than pDCs. These characteristics are likely related to the higher co-stimulatory phenotype of BDCA1+ mDCs and their secretion of IL-12p70.\textsuperscript{8,11} Previously, we and others have demonstrated that DC-induced MiHA-specific T\emph{em} cells have efficient killing capacity against malignant cells of hematopoietic origin expressing the respective MiHA.\textsuperscript{31,51-53} Notably, HSPC-derived pDCs were superior in inducing IFN-γ secretion by T\emph{em} cells, and additionally promoted superior NK cell activation and cytotoxicity. CpG-A activated pDCs, secreting high levels of IFN-α, showed the strongest NK cell activation. IFN-α is a potent NK cell modulator, and is known to enhance both TRAIL expression and cytolytic capacity of NK cells.\textsuperscript{46-48,54} Interestingly, we observed that non-TLR activated HSPC-pDCs also induced high TRAIL expression and NK cell-mediated cytotoxicity. In the presence of IL-3 alone, no IFN-α was detectable in those co-cultures (data not shown). However, studies indicate that cell-dependent contact with NK cells may enhance the maturation and IFN-α secretion capacity of pDCs.\textsuperscript{55} This may account for the reciprocal activation of NK cells observed.

Nowadays, the majority of alloSCT patients receive hematopoietic stem cells obtained from leukapheresis products of G-CSF-mobilized donors.\textsuperscript{56} As the yield of CD34+ cells is often sufficient for transplantation, it would be possible to cryopreserve ≤5% of the donor graft (i.e. max. 3 x 10^6 total cells or 25 x 10^6 CD34+ HSPCs) for eventual DC vaccine generation. Using the optimized HSPC-DC-generation protocol, the FST culture would result in average in 180 x 10^6 BDCA1+ mDCs and 160 x 10^6 pDCs from 10 x 10^6 G-CSF-mobilized CD34+ HSPCs, and the G4 culture would result in average in 180 x 10^6 BDCA1+ mDCs from 10 x 10^6 HSPCs. Those achieved numbers are considerably higher than with the previously published protocol,\textsuperscript{24} and could be sufficient for ≥9 repetitive vaccinations. Importantly, by generating the DCs from a portion of the donor stem cell graft, these vaccines are obtained without extra apheresis burden for the alloSCT donor. Moreover, during their differentiation, HSPC-derived DCs can be modified to further augment their stimulatory capacity. For example, immature DCs could be silenced for co-inhibitory molecules such as PD-L1 and PD-L2, by transfection with small interfering RNAs. We have previously demonstrated that PD-L-silenced MoDCs show superior potential in
activating tumor-reactive T cells.\textsuperscript{27,57-59} Additionally, HSPC-DCs could be transfected with mRNA of the tumor target antigen (MiHA or TAA), to provide long-lasting presentation of multiple antigenic epitopes.\textsuperscript{58,60-62}

Further pre-clinical studies are warranted to investigate whether enrichment of the different DC subsets will be needed or whether the whole bulk of cultured cells can be used as vaccine. Apart from the different DC subsets generated from the CD34\textsuperscript{+} HSPCs, the culture contained a heterogenous mixture of monocytic and myelocytic cells (defined by CD14, CD15, CD11b and CD34 expression; Supplementary Figure 7), but not allogeneic T cells nor B cells (data not shown). Of the total cultured cells, \~25\% and \~50\% within the FST culture and G4 culture, respectively, were HLA-DR negative cells (Supplementary Figure 7A,B), which may potentially suppress DC-mediated activation of T cells and NK cells. However, Haar \textit{et al.} recently demonstrated that HSPC-derived HLA-DR\textsuperscript{−} cells obtained with their culture system did not suppress T cell proliferation, but rather induced T cell proliferation.\textsuperscript{63} Within the HLA-DR negative fraction we could identify a population of cells double positive for CD15 and CD11b (Supplementary Figure 7D,E), a phenotype associated with myeloid-derived suppressor cells.\textsuperscript{64} Since we observed these cells primarily in the long (i.e. >19 days) G4 cultures (7-18\% of total cultured cells), we favor the shorter culture procedure (i.e. <16 days), where the frequency of HLA-DR\textsuperscript{−}CD15\textsuperscript{+}CD11b\textsuperscript{+} cells was <4\% out of total cultured cells. Shorter culture procedure is also more cost-effective and requires less handling.

It also remains to be investigated which vaccination and TLR stimulation scheme is most optimal for HSPC-DC vaccination. For repeated vaccinations, the HSPC-DCs will need to be cryopreserved. Whether it would be better to cryopreserve the DCs before or after TLR maturation needs to be established. At the same time, the optimal protocol for TLR stimulation, i.e. which GMP-grade TLR ligands and duration of stimulation will need to be investigated. Furthermore, there is accumulating evidence that the different DC subsets potentiate each other’s stimulatory capacity via cross-talk.\textsuperscript{19-23} For instance, Nierkens \textit{et al.} described that vaccination of mice with mDCs activated in the presence of pDCs resulted in enhanced cross-priming capacity of the mDCs and subsequently augmented anti-tumor immune responses.\textsuperscript{19} Furthermore, Lou \textit{et al.} demonstrated that immunization of mice with a combination of activated mDCs and pDCs resulted in increased levels of antigen-specific CD8$^+$ T cells and facilitated better tumor clearance compared with immunization with either DC subset alone.\textsuperscript{20} It could therefore be favorable to stimulate and/or infuse the different DC subsets simultaneously. However, it could also be more beneficial to harness the distinct features of the different HSPC-derived DC subsets by independent vaccination with mDCs and pDCs, by infusing the different DC subsets at different locations, and/or at different time-points.

In conclusion, we have developed a GMP-compliant culture protocol where high numbers of different DC subsets, with unique characteristics and functionalities, can be differentiated from the alloSCT donor stem cell grafts. These HSPC-derived DC subsets
are highly potent stimulators of tumor-reactive T cells and NK cells. Together, these findings indicate that vaccination with HSPC-DCs may be an attractive adjuvant therapy post alloSCT to boost GVT immunity.

**Acknowledgements**

We thank Rob Woestenenk, Frans Maas and Tim Hutten for their technical assistance. Furthermore, we thank Johan Van den Bergh from the Laboratory of Experimental Hematology at the University of Antwerp for his technical input on DC-mediated activation of NK cells.
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Supplementary Figure 1. Addition of IL-6 during ex vivo culture of CD34+ HSPCs inhibits DC differentiation, while supplementation with HS and AA results in improved generation of HSPC-DCs.

(A-C) G-CSF-mobilized CD34+ HSPCs were cultured in Stemspan ACF medium (Stemcell Technologies, catalog# 09855) supplemented with Flt3L, SCF and TPO (FST) and SR1 in the absence or presence of 1, 10 or 100 ng/mL IL-6 (Immunotools, catalog# 11340066). After three weeks culture, the frequency of BDCA1+ mDCs and pDCs was determined by flow cytometry. (A) Representative plots showing percentage of BDCA1+ mDC and pDC with or without 100 ng/mL IL-6 (n=4). DC are gated from CD14 - HLA-DR+ cells, but numbers in plots show percentage DC within total cultured cells. (B) Frequency within total cultured cells and (C) total yield from 10⁶ CD34+ cells of BDCA1+ mDC and pDC. (B-C) Data are depicted as mean ± SEM of three independent donors. (D-E) G-CSF-mobilized CD34+ HSPCs were cultured for two weeks in GMP-compliant Cellgro DC medium supplemented with Flt3L, SCF and TPO (FST) and SR1 in the absence or presence of 2% HS and 50 µg/mL AA. Fresh medium containing SR1, FST, HS and AA was added every 2-3 days. At every medium-refreshment, AA was added to a final concentration of 50 µg/mL. After two weeks, the frequency of BDCA1+ mDC and pDC was determined by flow cytometry, followed by overnight TLR maturation. (D) Frequency within total cultured cells and (E) total yield from 10⁶ CD34+ cells of BDCA1+ mDC and pDC. (D-E) Each dot represents an independent donor tested, lines indicate the mean value (n=3). Statistical analysis was performed by using one-way ANOVA, followed by Bonferroni’s multiple comparison test. (A-C) Each condition supplemented with IL-6 was compared to FST alone (0 ng/mL IL-6). *p<0.05, ** p<0.01, ***p<0.001
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**Supplementary Figure 3. Performance of freshly isolated CD34+ HSPCs versus thawed CD34+ HSPCs.** HSPC-DCs were generated as described in Figure 1 from either freshly isolated CD34+ HSPCs or CD34+ HSPCs that were cryopreserved and thawed for later use. Comparison of (A) total fold expansion and (B-C) frequency of (B) BDCA1+ mDCs and (C) pDCs. Each dot represents an independent donor, lines indicate the mean value ± SEM (n=3-6).

**Supplementary Figure 2 (on the left). Gating strategy and phenotype of generated HSPC-DCs.** HSPC-DCs were generated as described in Figure 1 and subsequently analyzed by flow cytometry. Density plots from a representative FST culture, where HSPC-DCs were first gated as (A) live (Sytox blue, FS versus SS), singlets (FS/SS TOF versus FS/SS INT) and autofluorescent negative (FL4-) cells. The frequencies of the different DC subsets were defined as follows: (B) BDCA1+ mDCs were defined as BDCA1+HLA-DR+ cells within autofluorescent negative cells. (C) BDCA3+ mDCs were defined as HLA-DR’BDCA3’/DNGR1+ cells within autofluorescent negative cells. (D) pDCs were defined as CD14 HLA-DR’CD123’BDCA2’ cells within autofluorescent negative cells with a low FS/SS scatter. Supplementary Figure 2 further depicts the expression of other DC-related markers on the different DC subsets.
Supplementary Figure 4. Phenotypical maturation and cytokine secretion by sorted CD34-derived DC subsets. (A-B) HSPC-DCs were generated as described in Figure 1, and subsequently BDCA1+ mDCs were sorted from G4 culture and pDCs from FST culture. Next, BDCA1+ mDCs were cultured with GM-CSF and IL-4 (G4, black lines) with or without R848 and Poly I:C (RPI:C, green lines), while pDCs were cultured with IL-3 (black lines) with or without CpG-A (blue lines) or R848 (green lines). After overnight maturation, phenotypical maturation was assessed by flow cytometry and cytokine secretion by ELISA. (A) Secretion of IL12p70, TNF-α and IFN-α. Lines indicate median values (n=3-9). (B) Histograms of one representative donor show the expression of CD80, CD86, CD83, PD-L1, HLA-DR and HLA-ABC compared to isotype control (grey lines). (C) HSPC-DCs were generated as described in Figure 1. Next, total cultured cells were stimulated overnight with TLR ligands at 5 x 10^5 cells/mL. Secretion of IL-12p70 and IFN-α was evaluated by ELISA. Lines indicate median values (n=6-8).
Supplementary Figure 5. HSPC-derived BDCA1+ mDCs efficiently prime naïve MiHA-specific T cells. HSPC-DCs were generated as described in Figure 1, and subsequently BDCA1+ mDCs were sorted from G4 culture. Next, BDCA1+ mDCs were stimulated with R848 and Poly I:C (RPI:C) in the presence of GM-CSF and IL-4. Purified CD8+ T cells from HA8-negative HLA-A2+ donor were cultured for one week with autologous HA8 peptide-loaded TLR-matured BDCA1+ mDCs. Cells were screened for the presence of HA8-specific CD8+ T cells using flow cytometry on day 7. The number in the dot plot represents the percentage of tetramer-positive cells within CD3+CD8+ T cells.
Supplementary Figure 6. CD8⁺ T cell responses. (A) HSPC-DCs were generated as described in Figure 1, and subsequently BDCA1⁺ mDCs were sorted from G4 culture and pDCs from FST culture. Next, the different DC subsets and autologous MoDCs were activated with respective TLR-ligands and cytokines (GM-CSF and IL-4 (MoDCs and BDCA1⁺ mDCs) or IL-3 (pDCs)) as indicated in the figure. Patient PBMCs containing low frequencies of HA1-specific CD8⁺ T cells were subsequently stimulated with TLR-matured non-peptide loaded DCs at 1:0.1 ratio. After one week stimulation, cells were screened for the presence of HA1-tetramer positive CD8⁺ T cells using flow cytometry. Density plots show tetramer stainings of cultures with PBMCs from Pt. 3 and 4 stimulated with non-peptide loaded DC (the relevant controls for tetramer stains depicted in Figure 3). The frequency of HA1-specific CD8⁺ T cells at day 0 was 0.01% and 0.61% in Pt. 3 and 4, respectively. The numbers in the dot plots represent the percentage of positive cells within the CD3⁺CD8⁺ T cells. Pt. = patient. (B) HSPC-DCs were generated as described in Figure 1. Next, total cultured cells were stimulated overnight with TLR ligands and cytokines: G4 culture was stimulated with G4+RPI:C, while FST culture with IL-3+CpG-A. TLR-matured total cultured HSPC-DCs were seeded in triplicate and loaded for 1 hour with 1 µM HA1 or CMV peptide at 37°C. Next, without washing, CTLs specific for HA1 or CMV were added at 1:1 ratio and co-cultured for 24 hours. Concentrations of IFN-γ were determined by ELISA. Results are depicted as mean ± SD.
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Supplementary Figure 7. Flow cytometric analysis of HSPC-DC cultures. HSPC-DCs were generated as described in Figure 1 and subsequently analyzed by flow cytometry for HLA-DR, CD14, CD11b, CD15 and CD34 expression. (A) Density plots from one representative donor showing the frequencies of HLA-DR⁺ and HLA-DR⁻ cells within total FST- and G4-cultured cells. (B) Frequencies of HLA-DR⁺ and HLA-DR⁻ cells within total FST- and G4-cultured cells of 7 different donors. Lines indicate mean values. (C) Density plots from one representative donor depicting expression of CD14, CD11b, CD15 and CD34 within HLA-DR⁺ cells. Numbers in plots indicate percentage positive cells within gated cells (gates are indicated within brackets above FACS plots). (D) Density plots from one representative donor showing CD15 and CD11b expression within HLA-DR⁻ cells. Numbers in plots indicate the percentage of positive cells within the HLA-DR⁻ population. (E) Frequency of HLA-DR⁻ cells expressing CD15 and CD11b out of total cultured cells. Lines indicate mean values of 7 independent donors.
CLEC12A-mediated antigen uptake and cross-presentation by human dendritic cell subsets efficiently boosts tumor-reactive T cell responses

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*These authors contributed equally.

Abstract

Potent immunotherapies are urgently needed to boost anti-tumor immunity and control disease in cancer patients. As dendritic cells (DCs) are the most powerful antigen-presenting cells, they are attractive means to reinvigorate T cell responses. An appealing strategy to employ the effective antigen processing and presentation machinery, T cell stimulation and cross-talk capacity of natural DC subsets is in vivo tumor antigen delivery. In this context, endocytic C-type lectin receptors are attractive targeting molecules. Here, we investigated whether CLEC12A efficiently delivers tumor antigens into human DC subsets, facilitating effective induction of CD4+ and CD8+ T cell responses. We confirmed that CLEC12A is selectively expressed by myeloid cells, including myeloid and plasmacytoid DC subsets (mDCs and pDCs). Moreover, we demonstrated that these DC subsets efficiently internalize CLEC12A, whereupon it quickly translocates to the early endosomes and subsequently routes to the lysosomes. Notably, CLEC12A antibody targeting did not negatively affect DC maturation nor function. Furthermore, CLEC12A-mediated delivery of keyhole limpet hemocyanin (KLH) resulted in enhanced proliferation and cytokine secretion by KLH-experienced CD4+ T cells. Most importantly, CLEC12A-targeted delivery of HA-1 long peptide resulted in efficient antigen cross-presentation by mDCs and pDCs, leading to strong ex vivo activation of HA-1-specific CD8+ T cells of patients after allogeneic stem cell transplantation. Collectively, these data indicate that CLEC12A is an effective new candidate with great potential for in vivo antigen delivery into mDCs and pDCs, thereby employing the specialized functions and cross-talk capacity of these DC subsets to boost tumor-reactive T cell immunity in cancer patients.
Introduction

The prominent role of dendritic cells (DCs) in orchestrating immune responses has provided the rationale for the development of DC-based strategies to boost anti-tumor immune responses in cancer patients.\(^1\) DCs are the most powerful antigen-presenting cells and efficiently initiate and reactivate CD4\(^+\) and CD8\(^+\) T cell responses.\(^2\) Importantly, they have the unique capacity to cross-present extracellular antigens, including tumor antigens, in major histocompatibility complex (MHC) class I molecules to CD8\(^+\) T cells.\(^3,4\) Upon tumor antigen presentation and co-stimulation by the DCs, tumor-reactive T cells become activated, expand and attack tumor cells. Moreover, long-lasting memory against recurrent disease is formed. Importantly, tumor regression has been observed following DC-based vaccination in patients with hematological cancers.\(^5-7\) In addition, productive T cell responses could be boosted by DC vaccination of patients treated with allogeneic stem cell transplantation (alloSCT).\(^8-11\) In this context, T cells could be directed against tumor antigens or recipient-specific allo-antigens restricted to hematological tumor cells, known as minor histocompatibility antigen (MiHA).\(^12,13\)

Nevertheless, we and others have observed that often the induction and/or reactivation of anti-tumor immune responses is inadequate, contributing to disease progression or relapse.\(^14\) This illustrates the urgency to develop alternative DC-based strategies to more effectively boost anti-tumor immunity. One such strategy involves the exploitation of the powerful antigen presentation capacity of natural DC subsets by in vivo tumor antigen delivery. Two key populations of human DCs are myeloid DCs (mDCs) and plasmacytoid DCs (pDCs).\(^15\) mDCs are further subdivided based on surface expression of BDCA1\(^+\) (CD1c) and BDCA3\(^+\) (CD141). Each DC subset has its specialized function in the induction and maintenance of immune responses.\(^16\) Notably, pre-clinical studies indicate that simultaneous engagement and cross-talk of multiple DC subsets is important for generating more potent and broader anti-tumor responses.\(^17,19\)

Attractive cell surface molecules for antibody-mediated in vivo tumor antigen delivery into DC subsets are the endocytic C-type lectin receptors (CLRs).\(^20-25\) CLRs are pattern-recognition receptors that mediate recognition and uptake of pathogens, as well as antigens exposed or released upon cell death. Furthermore, their ligation can induce intracellular signalling pathways regulating DC function. In this study, we investigated the potential of a relatively unexplored CLR, C-type lectin domain family 12 member A (CLEC12A), as antigen delivery receptor. CLEC12A is also known as myeloid inhibitory C-type lectin-like receptor (MICL), C-type lectin-like molecule-1 (CLL-1), dendritic-cell-associated C-type lectin 2 (DCAL-2) and CD371. Notably, CLEC12A is an attractive candidate, as it is broadly expressed by all human DC subsets.\(^26\) The function of CLEC12A has not been fully elucidated, though recently Neumann et al. found CLEC12A to be involved in the control of sterile inflammation by neutrophils.\(^27\) They identified that CLEC12A binding to uric acid crystals, released by dying cells, inhibits neutrophil-derived reactive oxidant species production, thereby reducing infiltration of immune cells to the site of damage.
More recently, CLEC12A was reported to be involved in microbial defense in myeloid cells, in particular in bacterial autophagy.\textsuperscript{28} It was postulated that CLEC12A is involved early during bacterial autophagy at the level of pathogen recognition initiated upon membrane damage. Notably, Chen\textit{ et al.} demonstrated that targeting of human monocyte-derived DCs (MoDCs) with αCLEC12A antibody modulated Toll-like receptor (TLR)-mediated maturation and cytokine secretion.\textsuperscript{29} In addition, Lahoud\textit{ et al.} have proposed CLEC12A as a potential target for specific antigen delivery.\textsuperscript{30,31} They showed \textit{in vivo} induction of OVA-specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell responses after administration of OVA-conjugated αCLEC12A antibody in mouse models. Yet, still little is known about CLEC12A’s internalization mechanism, intracellular routing and fate, and most importantly whether CLEC12A can efficiently facilitate antigen cross-presentation by human DC subsets. Our aim was to acquire insight into these mechanisms and to explore CLEC12A’s potential for targeted antigen delivery into human DCs to promote CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell responses. Here, we validated the discriminative expression pattern of CLEC12A, and showed that it is efficiently internalized by human BDCA1\textsuperscript{+} mDCs, BDCA3\textsuperscript{+} mDCs and pDCs upon targeting with specific antibodies. We observed that CLEC12A initially translocates to the endosomes, whereupon it quickly routes to the lysosomes. Notably, CLEC12A antibody binding did not negatively affect DC phenotype nor allogeneic T cell stimulation capacity. Importantly, we showed that antigens delivered via CLEC12A are efficiently processed by DCs and (cross-)presented to antigen-experienced T cells. These findings demonstrate that CLEC12A is an appealing targeting receptor for selective \textit{in vivo} delivery of tumor antigens into mDC and pDC subsets to boost tumor-reactive immune responses in cancer patients.

**Materials and Methods**

**Patient and donor material**

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats (Sanquin, Nijmegen, the Netherlands) or from leukapheresis products using Ficoll-Hypaque density centrifugation. PBMCs containing KLH-specific T cells were obtained from multiple myeloma patients following vaccination with KLH-pulsed tumor antigen-presenting DCs. PBMCs containing HA-1 specific T cells were obtained from patients after alloSCT.\textsuperscript{9,10} Pre-vaccination leukapheresis material was used to isolate BDCA1\textsuperscript{+} mDCs, BDCA3\textsuperscript{+} mDCs and pDCs, and culture monocyte-derived DCs (MoDCs). All material of patients and healthy donors was obtained after written informed consent, according to institutional guidelines.

**DC isolation and culture**

To generate MoDCs, monocytes were isolated from PBMCs via plastic adherence in tissue culture flasks (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) and cultured in X-VIVO 15 medium (Lonza, Verviers, Belgium) supplemented with 2% human serum.
CLEC12A-mediated antigen cross-presentation by DCs

(HS; PAA laboratories, Pasching, Austria), 500 IU/mL interleukin (IL)-4 and 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF; both Immunotools, Friesoythe, Germany). At day 3, immature MoDCs were harvested and directly used in experiments or routinely matured for 48 hours with 500 IU/mL IL-4, 800 IU/mL GM-CSF, 5 ng/mL IL-1β, 15 ng/mL IL-6, 20 ng/mL tumor necrosis factor (TNF)-α (all Immunotools), and 2.5 µg/mL PGE2 (prostaglandin E2, Pfizer; conventional cytokines).

PBMCs containing naturally occurring BDCA1+ mDCs, BDCA3+ mDCs and pDCs were labeled with fluorophore-conjugated αCD11c, αBDCA1, αBDCA2, αBDCA3 and αCD123 (all Biolegend, CA, USA). Subsequently, CD11c+BDCA1+ mDCs, CD11c+BDCA3+ mDCs and CD123+BDCA2+ pDCs (Supplementary Figure 1A) were sorted using the ARIA SORP (Becton Dickinson, Franklin Lakes, NJ, USA) with >95% purity. BDCA1+ mDCs and BDCA3+ mDCs were matured with 800 IU/mL GM-CSF, 10 µg/mL Poly I:C (Polyinosinic:polycytidylic acid, Sigma Aldrich) and 5 µg/mL R848 (Resiquimod, Enzo Life Sciences, Raamsdonkveer, the Netherlands), and pDCs with 10 ng/mL IL-3 (Immunotools) and 5 µg/mL R848.

Expression and internalization

CLEC12A and DEC205 expression and their internalization capacity was analyzed by flow cytometry. Healthy donor PBMCs or day 3 MoDCs were incubated for 30 minutes with 10 µg/mL αCLEC12A (clone 687317, R&D systems Minneapolis MN, USA) or mIgG2b isotype control (Biolegend) at 4°C. Subsequently, cells were incubated for 0-90 minutes at 37°C to allow endocytosis. Then, goat-anti-mouse Alexa Fluor-647 (Life Technologies, Carlsbad, CA, USA) was added. To inhibit clathrin-mediated endocytosis, cells were incubated in hypertonic 450mM sucrose solution (Boom, Meppel, the Netherlands). Blockade of clathrin-mediated uptake was reversed by sucrose washout and resuspension in PBS. Subsequently, cells were labeled with fluorophore-conjugated αBDCA1, αBDCA2, αBDCA3, αCD123, αCD11c (Miltenyi Biotec, Bergisch Gladbach, Germany; BD or Biolegend). DCs were analyzed on a CyAn ADP or a Gallios flow cytometer (both Beckman Coulter) and gated as CD11c+BDCA1+ mDCs, CD11c+ BDCA3+ mDCs and BDCA2+CD123+ pDCs (Supplementary Figure 1B). Data was analyzed with Kaluza V1.3 (Beckman Coulter).

Intracellular routing

To study CLEC12A internalization and investigate its intracellular routing, day 3 MoDCs were adhered to RetroNectin-coated (Takara, Otsu, Japan) coverslips. After blocking Fc-receptors with total human IgG (hIgG; Sanquin) and goat serum (CDL, Nijmegen, the Netherlands), MoDCs were incubated for 30 minutes with 10 µg/mL αCLEC12A (mIgG2b, clone 687317, R&D systems) or αDEC205 (mIgG2b, clone MG38, eBioscience) at 4°C. Subsequently, cells were labeled with Alexa Fluor-488-conjugated goat-anti-mouse IgG2b (Life Technologies, Carlsbad, CA, USA) and DCs were incubated for 0-60 minutes at
37°C. Subsequently, cells were fixed with 4% paraformaldehyde and incubated with αHLA-DR/DP (mIgG2a, clone Q5/13). Thereafter, HLA-DR/DP staining was visualized using Alexa Fluor-564-conjugated goat-anti-mouse IgG2a (Life Technologies). Then, after permeabilization, αEEA-1 (rabbit IgG, Abcam, Cambridge, UK) or αLAMP-1 (rabbit IgG, Sigma Aldrich, St. Louis, MO, USA) was added for 30 minutes, followed by staining with Alexa Fluor-647-conjugated goat-anti-rabbit IgG (polyclonal, Life Technologies). Coverslips were mounted on slides with Mowiol (Sigma Aldrich). Images were acquired with an Olympus FV1000 Confocal laser scanning microscope (Olympus America Inc., Melville, NY, USA) or a LSM 510 META confocal microscope (Zeiss, Jena, Germany). Isotype controls mIgG2b, mIgG2a and rabbit IgG (Biolegend, Miltenyi Biotec, Jackson ImmunoResearch Laboratories, respectively) did not show fluorescent staining. Pictures were analyzed with Image J V1.46R and quantified with JACoP plugin V2.1.1.

**DC phenotype and function upon CLEC12A antibody binding**

Day 3 MoDCs were pre-incubated with 10 µg/mL αCLEC12A (hlgG1) or hlgG1 isotype control (Merus B.V., Utrecht, the Netherlands) and matured with conventional cytokines; 1 µg/mL LPS (Lipopolysaccharide, Sigma Aldrich); 10 µg/mL Poly(I:C) or 5 µg/mL R848. After 48 hours, IL-6, IL-10 and TNF-α concentrations were quantified by ELISA according to manufacturer’s instructions (Human IL-6 and IL-10 ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA, USA); PeliPair human TNF-α ELISA reagent set (Sanquin)). MoDC phenotype was analyzed by flow cytometry using: αCD14, αCCR7, αCD80, αCD86, αHLA-ABC (all Biolegend), αHLA-DR (Beckman Coulter), αCD83 (BD) and appropriate isotype controls (all Biolegend). Cells were analyzed using a FC500 flow cytometer (Beckman Coulter). For allogeneic mixed lymphocyte reaction (alloMLR), T cells were prepared by depleting healthy donor PBMCs of CD14+ and CD19+ cells with magnetic beads (BD), and subsequently labeled with 1.25µM CFSE (carboxyfluorescein diacetate succinimidyl ester, Life technologies)\(^3\). Next, 1 x 10^5 T cell-enriched leukocytes and 1 x 10^4 mature MoDCs were seeded in 200 µL IMDM (Iscove-modified Dulbecco medium, Life technologies) supplemented with 10% HS in round-bottom 96-well plates (Corning Costar) in triplicate or sixplicate. After 5 days of co-culture, supernatant was collected for interferon (IFN)-γ ELISA (Pierce Endogen, Rockford, IL, USA). Furthermore, cells were harvested, stained with αCD4, αCD3 (both Biolegend) and αCD8 (Life technologies), and subsequently analyzed for CFSE dilution within CD3+CD4+ and CD3+CD8+ T cells using a Gallios flow cytometer.

**KLH-specific T cell activation**

Day 3 MoDCs were generated, and BDCA1+ mDCs and pDCs were isolated from leukapheresis material. DCs were incubated for 30 minutes at 4°C with total hlgG (Sanquin) in the presence of 10 µg/mL αCLEC12A (mIgG2b or biotinylated hlgG1) or isotype control antibody. Then, in case of mouse antibodies, secondary labeling with 10
µg/mL biotinylated goat-anti-mouse IgG (Life technologies) was performed for 30 minutes at 4°C. Subsequently, DCs were washed and 2 µg/mL KLH-conjugated abiotin antibody was added for 30 minutes at 4°C. Finally, cells were washed and MoDCs were matured for 48 hours, mDCs and pDCs for 24 hours. Then, patient PBMCs containing KLH-specific T cells were thawed, depleted from monocytes using CD14 IMag beads (BD), labeled with 1.25µM CFSE and resuspended in IMDM/10% HS to a concentration of 0.5 x 10^6 cells/mL. These were added to the DCs at a peripheral blood lymphocytes (PBLs):DCs ratio of 1:0.1 in 96-well round-bottom plates (in triplicate). Cells were co-cultured in a volume of 200 µL at 37°C. After 4 days, supernatant was harvested and pooled for analysis of IFN-γ production by cytokine bead array (CBA; BD) and CD4+ T cell proliferation was evaluated, as described before.

**HA-1 cross-presentation assay**

To evaluate receptor-mediated antigen cross-presentation, synthetic long peptide for the MiHA HA-1 (azido propionic acid-KLKECVLHDDLLEARRPRAHE-biotin; Leiden University Medical Center (LUMC), the Netherlands) was conjugated to αCLEC12A (hIgG1), αDEC205 (IgG2b, clone MG38, BD) or respective isotype controls (hIgG1 and mIgG2b), using the copper-free click reaction. In short, a highly reactive succinimidyl-cyclooctyne variant, 2,5-dioxopyrrolidin-1-yl4-(((1R,8S,9s)-bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)amino) butanoate (BCN, Synaffix, Oss, the Netherlands), was incubated with the corresponding antibody at a 15 molar excess, following manufacturer's instructions. Thereafter, the BCN-conjugated antibody was incubated overnight with a 3 molar excess of HA-1 long peptide and purified using a 10kDa dialysis cassette (Thermo scientific). Conjugation efficiency was determined with ELISA. Furthermore, binding capacity and specificity of the HA-1 conjugated αCLEC12a and αDEC205 antibodies, as compared to the isotype controls, was validated by flow cytometry by staining the biotin-tag on the HA-1 long peptide with αbiotin-FITC. DC subsets, isolated from leukapheresis material of HA-1 negative healthy donors, were pre-incubated for 30 minutes at 4°C with 1 mg/mL total hIgG (Sanquin) to block Fc-receptors, followed by 30 minutes incubation at 4°C with 10 µg/mL αCLEC12A/HA-1, αDEC205/HA-1 or corresponding isotype/HA-1 conjugates. Furthermore, as an additional control, DCs were labeled with αCLEC12A/HA-1 or αDEC205/HA-1 conjugate in the presence of 100 µg/mL excess unconjugated αCLEC12A or αDEC205 antibody to demonstrate receptor-specific uptake. As the peptide conjugation efficiency slightly differed with the corresponding isotype control antibodies, the αCLEC12A or αDEC205 block with excess unconjugated antibody was used in all cross-presentation assays. Subsequently, DCs were washed and incubated at a concentration of 0.1 x 10^6 cells/mL in IMDM in 96-well round-bottom plates at 37°C (in triplicate). As positive control, DCs were pulsed with 1µM HA-1 short peptide (VLHDDILLEA). After 3 hours of incubation, maturation stimuli and 10% HS were added and DCs were incubated overnight. Thereafter, DCs were co-cultured overnight with HA-1
T cell receptor (TCR) transduced T cells at a T:DC ratio of 1:1 in a volume of 200 µL in the presence of αCD107a (BD). Then, supernatant was harvested for IFN-γ ELISA, and T cells were stained with αCD8 (Beckman Coulter) and αCD137 (Biolegend) and analyzed for activation using a Gallios flow cytometer.

In the antigen recall assay using primary patient T cells, BDCA1+ mDCs and pDCs were labeled with the αCLEC12A/HA-1 conjugate, as described before, and plated at a concentration of 0.8 x 10^6 cells/mL in IMDM in a 24-well plate. Due to limited availability of patient material these experiments were only performed with the αCLEC12A/HA-1 conjugate in the presence/absence of excess unjugated antibody as negative control. After 3 hours of incubation at 37°C, maturation stimuli and 10% HS were added and DCs were cultured overnight. Thereafter, DCs were co-cultured for 7 days with CD14-depleted PBMCs containing HA-1 specific memory T cells obtained from HA-1 mismatched patients after alloSCT, at a ratio of PBL:DC of 1:0.2 in a total volume of 1 ml. After 4 days, 1 ml IMDM supplemented with 10% HS, 100 IU/mL IL-2 and 10 ng/mL IL-15 (Immunotools) was added. At day 7, cells were harvested, counted and the percentage of HA-1-specific CD8+ T cells was determined by staining with αCD3 (biolegend), αCD8 (Beckman Coulter), HA-1 PE and APC tetramers (both from LUMC). Cells were analyzed on a Gallios flow cytometer.

**Statistical analysis**

Data were analyzed using one-way ANOVA followed by a Bonferroni post-hoc test. P-values <0.05 were considered significant.

**Results**

**CLEC12A is broadly expressed and efficiently internalized by human DC subsets**

To study the suitability of CLEC12A as a targeting receptor for antigen delivery, we first validated its expression on human DC subsets using flow cytometry. We confirmed that MoDCs and naturally occurring BDCA1+ mDCs, BDCA3+ mDCs and pDCs highly expressed CLEC12A (Figure 1A and Supplementary Figure 2A). Since expression of CLEC12A was progressively lost during differentiation of MoDCs (Supplementary Figure 2B), we selected day 3 MoDCs for further experiments.

Next, we investigated the CLEC12A internalization potential of day 3 MoDCs, BDCA1+ mDCs, BDCA3+ mDCs and pDCs using flow cytometry. Following incubation at 37°C, CLEC12A was quickly and efficiently internalized by all DC types (Figure 1B). Although, CLEC12A expression was lower on pDCs than on mDCs, their CLEC12A internalization efficacy was similar. These findings were confirmed by confocal microscopy. At start, CLEC12A was located on the plasma membrane and co-stained with MHC-II molecules (Figure 1C). Notably, after 1 hour incubation at 37°C, all DC types showed clear clusters of CLEC12A in intracellular compartments with hardly any CLEC12A expression on the
Figure 1. CLEC12A is highly expressed and efficiently internalized by human DC subsets. (A) CLEC12A expression on day 3 MoDCs and BDCA1+ mDCs, BDCA3+ mDCs and pDCs was analyzed by flow cytometry. Open black histograms correspond to cells stained with CLEC12A, while filled gray histograms represent the isotype control. Numbers in the plots represent the mean fluorescence intensity. Data of one representative donor out of 3 is shown. (B) Day 3 MoDCs and mDCs and pDCs were labeled with αCLEC12A, followed by 0-90 minutes incubation at 37°C. Surface expression of bound αCLEC12A was analyzed by flow cytometry following labeling with goat-anti-mouse IgG. CLEC12A internalization is calculated relative to the surface expression at start based on the mean fluorescence intensity (MFI). Data are shown as mean ± SEM of 3 independent experiments. (C) Confocal analysis of CLEC12A internalization in day 3 MoDCs and FACSSorted BDCA1+ mDCs and pDCs. DCs were stained with mouse αCLEC12A, followed by labeling with Alexa Fluor-488-conjugated goat-anti-mouse IgG2b (green). Then, internalization was allowed for 1 hour at 37°C, with subsequent staining for extracellular MHC class II with mouse-anti-human HLA-DR/DP, followed by Alexa Fluor-564-conjugated goat-anti-mouse IgG2a (red). Scale bar length is shown in the figure.
cell surface. These data demonstrate that human BDCA1⁺ mDCs, BDCA3⁺ mDCs, pDCs and day 3 MoDCs highly express CLEC12A and efficiently internalize this CLR following antibody targeting.
Clathrin-independent CLEC12A translocation to the endosomal/lysosomal compartments

Intracellular fate and antigen release are essential parameters determining the efficacy of antigen processing and presentation by DCs. Therefore, we investigated the internalization mechanism and intracellular routing of CLEC12A. The classical pathway for receptor-mediated endocytosis is dependent on clathrin-coated pits.36 The CLR DEC205 is known to internalize via this classical route. To evaluate whether the same endocytosis mechanism applies to CLEC12A, we disrupted clathrin-coated pits using hypertonic solution.32 Interference with clathrin-mediated uptake almost completely blocked DEC205 internalization by day 3 MoDCs, BDCA1+ mDCs and pDCs (Figure 2A), while CLEC12A endocytosis was not affected. By means of sucrose washout, we could restore DEC205 internalization, indicating that the observed effects are the result of the hypertonic milieu.

Next, we studied the intracellular routing of CLEC12A, in comparison to DEC205, in time on day 3 MoDCs by analyzing their co-localization with EEA-1, an early endosome marker, or LAMP-1, a marker of the late endosomal/lysosomal compartment. At start, both DEC205 and CLEC12A were localized on the cell membrane, yet already after 5 minutes of endocytosis co-localization was observed with the early endosomes (Figure 2B and Supplementary Figure 3). Notably, while DEC205 co-localization with the early endosomes was lost after 15 minutes, CLEC12A still co-localized with EEA-1. Gradually, both receptors routed to the lysosomes with clear co-localization at 60 minutes after internalization. These co-localization patterns were confirmed with the Manders coefficient (Figure 2C-F).

Together, these data demonstrate that, in contrast to DEC205, CLEC12A is not internalized via the classical clathrin-coated pits, but follows a distinct endocytosis pathway. Yet, the intracellular routing of both receptors appears highly similar, as both...
co-localize with the early endosome after internalization and subsequently route to the lysosomes.

**CLEC12A targeting does not affect DC maturation and T cell stimulatory capacity**

As CLEC12A contains an ITIM motif in its cytoplasmic tail, it might function as an inhibitory receptor upon triggering. Therefore, we investigated whether CLEC12A antibody binding, alone or in combination with different maturation stimuli, modulated DC phenotype or function. CLEC12A antibody binding by itself did not affect expression levels of maturation marker CD83, co-stimulatory molecules CD80/CD86, co-inhibitory ligand PD-L1 nor HLA-DR (Figure 3A). Furthermore, 48 hours incubation of αCLEC12A labeled MoDCs in the presence of conventional cytokines, LPS, Poly(I:C) or R848 did not influence expression levels of DC maturation markers (Figure 3B), as compared to isotype control treated DCs. Additionally, CLEC12A antibody binding did not affect production of the immunoregulatory cytokine IL-10 nor the pro-inflammatory cytokine TNF-α (Figure 3C). In contrast, we observed an almost 2-fold increase in IL-6 secretion by LPS- and cytokine-matured MoDCs following CLEC12A targeting, as compared to isotype control treatment. Importantly, the capacity of mature MoDCs to promote allogeneic T cell proliferation and IFN-γ secretion was not affected by CLEC12A targeting (Figure 3D,F). These data indicate that CLEC12A antibody targeting does not negatively affect DC maturation, phenotype, nor T cell stimulatory potential.

**CLEC12A efficiently delivers KLH into DCs, resulting in potent CD4+ T cell recall responses**

As CLEC12A is efficiently internalized and antibody binding does not affect DC function, we next investigated whether CLEC12A could be used for antigen delivery. Hereeto, DC subsets were labeled with CLEC12A-biotin and abiotin/KLH conjugates, matured and then co-cultured with patient PBMCs containing KLH-experienced CD4+ T cells. CLEC12A-mediated KLH delivery into day 3 MoDCs resulted in a 2-fold increase of CD4+ T cell proliferation (Figure 4A) and enhanced IFN-γ production (Figure 4B) as compared to isotype control treatment. Importantly, CLEC12A-mediated KLH uptake and release into BDCA1+ mDCs showed superior induction of KLH-specific T cell proliferation (Figure 4C). Moreover, these T cells secreted high levels of IFN-γ (Figure 4D). Interestingly, despite lower CLEC12A expression, CLEC12A-mediated KLH delivery in pDCs efficiently promoted proliferation and IFN-γ production of KLH-experienced T cells (Figure 4E,F). Although, T cell proliferation was lower in pDC co-cultures as compared to mDC co-cultures, IFN-γ levels were equal in both co-cultures, indicating that pDC stimulation promotes higher IFN-γ secretion per T cell. These data demonstrate that CLEC12A efficiently enables KLH uptake, and its subsequent processing and presentation in MHC-II molecules, eliciting productive CD4+ T cell activation and proliferation.
Figure 3. CLEC12A ligation does not negatively affect DC function. Day 3 MoDCs were incubated for 48 hours with αCLEC12A or hlgG1 isotype control in the absence or presence of different maturation stimuli. (A) Expression of CD83, CD80, CD86, PD-L1 and HLA-DR on non-matured CLEC12A antibody-treated (black line) or isotype-treated (dark grey line) MoDCs was determined by flow cytometry. Isotype controls for the respective maturation markers are depicted in light grey. Data of one representative donor out of 4 independent experiments is shown. (B) Relative expression of the DC maturation markers CD83, CD80, CD86 and CCR7 and (C) relative secretion of IL-6, IL-10 and TNF-α for antibody-treated MoDCs cultured with various maturation stimuli. Data are calculated for αCLEC12A-treated cells relative to cells incubated with hlgG1 (αCLEC12A/IgG). Graphs show the median with range of 7 independent experiments. (D-F) Mature αCLEC12A (black lines/circles) or isotype-treated (grey lines/white circles) MoDCs were co-cultured with allogeneic CFSE-labeled T cells at a ratio of 1:10 for 5 days. Subsequently, T cell proliferation was determined by flow cytometry and IFN-γ secretion by ELISA. (D) Representative histograms show CSFE dilution of CD4+ and CD8+ T cells of one T cell donor out of 4 donors. (E) CD3+CD4+ and CD3+CD8+ T cell proliferation and (F) IFN-γ secretion of 4 different T cell donors tested. Data were analyzed using one-way ANOVA followed by a Bonferroni post-hoc test, *P <0.05.
CLEC12A-targeted long peptide is efficiently cross-presented by natural DC subsets to CD8+ T cells

For effective boosting of anti-tumor immunity, cross-presentation of internalized tumor antigens to cytotoxic CD8+ T cells is essential. Therefore, we investigated whether CLEC12A-mediated delivery of MiHA long peptide leads to effective cross-presentation by BDCA1+ mDCs, BDCA3+ mDCs and pDCs. For this, DC subsets were labeled with aCLEC12A/Ha-1 or aDEC205/Ha-1 conjugates. Subsequently, DCs were matured and co-
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Figure 5. CLEC12A-delivered HA-1 long peptide is efficiently cross-presented by BDCA1+ mDCs, BDCA3+ mDCs and pDCs. FACS sorted BDCA1+ mDCs, BDCA3+ mDCs and pDCs were incubated with long HA-1 peptide chemically conjugated to αCLEC12A (αCLEC12A/HA-1) or αDEC205 (αDEC205/HA-1). As negative controls, DCs were not labeled (DC-), labeled with isotype/HA-1 conjugates, or the specific conjugate in the presence of excess unconjugated antibody (blocked αCLEC12A/HA1 or αDEC205/HA1). As positive control, DCs were pulsed with 1µM short HA-1 peptide. BDCA1+ mDCs, BDCA3+ mDCs and pDCs were subsequently matured overnight, followed by 24 hour co-culture with HA-1-TCR transduced T cells (between 40% and 66% HA-1-tetramer positive, data not shown) at a ratio of 1:1. T cell activation was assessed by determining the percentage of CD107a positive CD8+ T cells using flow cytometry, and by analysis of IFN-γ secretion using ELISA. (A) Dot plots show CD107a expression after co-culture with αCLEC12A/HA-1 targeted or non-targeted BDCA1+ mDCs of one representative donor. (B-D) Percentage of CD107a+CD8+ T cells and IFN-γ production after co-culture with (B) BDCA1+ mDCs, (C) pDCs targeted with αCLEC12A/HA-1 or αDEC205/HA-1 or (D) BDCA3+ mDCs targeted with αCLEC12A/HA-1. Data are depicted as the mean ± SD of triplo measurements of one representative donor. For the αCLEC12A/HA-1 conjugate n=6 (BDCA1+ mDC), n=5 (pDC) or n=1 (BDCA3+ mDC) independent experiments were performed. For αDEC205/HA-1 n=2 independent experiments were performed. Data were analyzed using one-way ANOVA followed by a Bonferroni post-hoc test, ***P<0.001.
cultured with HA-1 TCR transduced CD8+ T cells. Notably, we confirmed specific binding of the αCLEC12A/HA-1 and αDEC205/HA-1 conjugates to BDCA1+ mDCs, BDCA3+ mDCs and pDCs using flow cytometry by staining the biotin-tag on the HA-1 long peptide with avidin-FITC (Supplementary Figure 4B). DCs labeled with either αCLEC12A/HA-1 or αDEC205/HA-1 conjugate in the presence of excess unconjugated specific antibody or isotype/HA-1 conjugates were incapable of activating HA-1 TCR-transduced CD8+ T cells (Figure 5A-D and Supplementary Figure 4C). Importantly, CLEC12A- and DEC205-mediated HA-1 delivery and cross-presentation by BDCA1+ mDCs, BDCA3+ mDCs and pDCs resulted in significant activation of HA-1 TCR-transduced CD8+ T cells, as demonstrated by significant up-regulation of the degranulation marker CD107a (Figure 5A-D) and activation marker CD137 (Supplementary Figure 4C). Furthermore, DC subsets targeted with αCLEC12A/HA-1 or αDEC205/HA-1 conjugates efficiently promoted IFN-γ secretion, as compared to the controls (Figure 5B-D). Interestingly, CLEC12A and DEC205 showed similar capacity to deliver antigens for cross-presentation and activation of HA-1-specific CD8+ T cells.

To evaluate the efficacy of CLEC12A as an attractive target for facilitating antigen cross-presentation to patient-derived T cells, we stimulated PBLs of alloSCT patients containing HA-1-reactive CD8+ memory T cells with BDCA1+ mDCs (Figure 6A) or pDCs (Figure 6B) loaded with the αCLEC12A/HA-1 conjugates or short HA-1 peptide (i.e. positive control). After 7 days of co-culture, increased percentages of HA-1-specific CD8+ memory T cells were observed as compared to stimulation with non-peptide loaded DCs. Moreover, when DCs were labeled with the αCLEC12A/HA-1 conjugate in the presence of excess unconjugated αCLEC12A, no increase in the percentage of HA-1-specific CD8+ T cell was observed. Furthermore, also augmented absolute numbers of HA-1-specific CD8+ T cells were detected upon stimulation with BDCA1+ mDCs (Figure 6C) or pDCs (Figure 6D) labeled with the αCLEC12A/HA-1 conjugates or short HA-1 peptide. Collectively, these findings demonstrate that CLEC12A effectively mediates HA-1 long peptide delivery and cross-presentation by BDCA1+ mDCs and pDCs, resulting in strong activation of HA-1-reactive CD8+ T cells of patients after alloSCT.

**Discussion**

Although the immune system is capable of eliciting tumor-reactive immune responses, the magnitude and functionality of these responses is often inadequate. Therefore, powerful strategies are needed to boost anti-tumor immunity in order to prevent disease progression. As DCs are the most powerful antigen-presenting cells, they are highly attractive vehicles for boosting T cell responses. An appealing strategy to employ the specialized functions and cross-talk of natural DC subsets and exploit their potent antigen processing and presentation machinery is targeted delivery of tumor antigens in vivo via endocytic receptors. In this study, we evaluated the potential of CLEC12A, an
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endocytic CLR, as a new targeting candidate for simultaneous antigen delivery into all human DC subsets.

We confirmed that the expression pattern of CLEC12A is restricted to hematopoietic cells of myeloid origin, including monocytes, granulocytes, BDCA1+ mDCs, BDCA3+ mDCs and pDCs, as has been reported by others.26,43 Next, we demonstrated that CLEC12A is efficiently internalized by all human DC subsets. Most CLRs, such as DCIR44, DC-Sign45 and DEC20551 endocytose via the classical clathrin pathway. Interestingly, CLEC12A endocytosis was not affected in hypertonic conditions, indicating that its mechanism of internalization does not involve clathrin-coated pits. Alternatively, similar to Langerin (CD207),46 CLEC12A might be endocytosed via the caveolin pathway. However, so far we were not able to demonstrate caveolin involvement in CLEC12A internalization (data
not shown). Next to the capacity to internalize, the intracellular fate and cargo release are key parameters determining the suitability and efficacy of CLEC12A for targeted antigen delivery and presentation via MHC-I and MHC-II molecules. In general, receptor/antigen trafficking to the lysosomes is considered to result in loading onto MHC-II molecules and subsequent CD4+ T cell activation.\textsuperscript{47} Whereas pre-lysosomal localization has been postulated to be more favourable for antigen processing and cross-presentation via MHC-I molecules to CD8+ T cells.\textsuperscript{48-52} Nevertheless, DEC205, which quickly routes to the lysosomes, can efficiently facilitate antigen cross-presentation.\textsuperscript{52-55}

To elucidate the intracellular fate of CLEC12A we performed co-localization studies. CLEC12A and DEC205 showed similar intracellular routing: initial co-localization with the early endosomes, followed by further trafficking to the lysosomes where they were retained. However, CLEC12A co-localization with the early endosomes lasted longer. In accordance, Begun et al. recently reported CLEC12A co-localization with Rab5 another early endosome marker.\textsuperscript{28} These findings suggest that CLEC12A might provide a good window for endosomal escape of targeted antigen, thereby facilitating effective cross-presentation.

CLR\textsubscript{s} are known to shape immune responses via the signaling motifs in their cytoplasmic tails or via the association with adaptor molecules that elicit downstream signaling cascades. CLEC12A contains an inhibitory ITIM motif in its cytoplasmic tail. Recently, Neumann \textit{et al.} identified that CLEC12A expressed on neutrophils is involved in controlling sterile inflammation.\textsuperscript{27} They discovered that CLEC12A, upon triggering by uric acid crystals, inhibits Syk-dependent reactive oxygen species production, thereby limiting infiltration of immune cells to the site of damage. In contrast, Chen \textit{et al.} observed positive as well as negative effects on DC phenotype and function upon αCLEC12A binding, dependent on the type of TLR activation.\textsuperscript{29} In this report, we showed that binding of our αCLEC12A did not promote DC maturation. Moreover, no effect on CD86, CD80, CD83 or CCR7 expression was observed following αCLEC12A binding in combination with different maturation stimuli. In contrast, Chen \textit{et al.} observed increased expression of CCR7 following αCLEC12A ligation in combination with TLR4 stimulation.\textsuperscript{29} These opposite findings might be attributed to differences in epitope recognition of the used antibodies or isotype-related effects. Surprisingly, we observed augmented IL-6 secretion upon CLEC12A targeting in the presence of conventional cytokine maturation or LPS-mediated TLR-4 stimulation. Despite this effect on IL-6 secretion, no difference in allogeneic T cell stimulatory capacity was observed for αCLEC12A versus isotype control-treated MoDC\textsubscript{s}.

Because of its efficient internalization capacity and the lack of negative effects following antibody binding, we consider CLEC12A to be an interesting candidate for targeted antigen delivery into DC\textsubscript{s}. Importantly, we demonstrated that targeted delivery via CLEC12A enabled KLH presentation onto MHC-II molecules of human BDCA1+ mDC\textsubscript{s}, pDC\textsubscript{s} and MoDC\textsubscript{s}, thereby promoting profound proliferation and IFN-γ secretion by KLH-
experienced CD4+ T cells. Furthermore, we provided the first mechanistic evidence that human mDCs and pDCs are capable of cross-presenting natural tumor antigens delivered via CLEC12A, facilitating strong activation of tumor-reactive CD8+ T cells of cancer patients. Although data on the BDCA3+ mDC are merely indicative as experiments were only performed for one donor. Previously, Lahoud et al. reported induction of humoral and cellular immune responses after CLEC12A-mediated ovalbumin delivery into mouse antigen presenting cells in vivo.30,31 Yet, they demonstrated CLEC9A and DEC205 to be more effective targets than CLEC12A. In contrast, in our studies CLEC12A performed equally good as DEC205 in antigen cross-presentation assays by human DC subsets. These opposing findings could possibly be attributed to differences in epitope recognition of the used antibodies and functionality after cargo conjugation, conjugation efficiency, or related to differences in the murine and human model systems.

In addition to other CLRs for which antibodies are currently in phase I/II clinical investigation, CLEC12A holds great promise for future cargo delivery applications. Using nanoparticles combined delivery of tumor antigens and immunomodulatory agents, such as TLR ligands or siRNA against co-inhibitory molecules, could be realized to further improve DC immunogenicity in vivo. Previously, we showed that silencing the co-inhibitory molecules PD-L1/L2 on DCs resulted in superior T cell stimulatory potential.56,57 Another interesting application for CLEC12A targeted cargo delivery is in the setting of acute myeloid leukemia and myeloid dysplastic syndrome. CLEC12A is differentially expressed by myeloid leukemia stem cells and blasts, but not by healthy hematopoietic stem cells.43,58 By favourably modulating the immunogenicity of leukemic stem cells and blasts, more powerful tumor-reactive T cell responses could be elicited.

In conclusion, we showed that human BDCA1+ mDCs, BDCA3+ mDCs, pDCs and ex vivo generated MoDCs highly express CLEC12A. CLEC12A is efficiently internalized via a clathrin-independent mechanism and is quickly translocated from the early endosomes to the lysosomes. Notably, we did not observe negative effects of CLEC12A antibody binding on DC phenotype or functionality. Most importantly, we demonstrated that CLEC12A targeted antigen delivery results in potent activation of KLH-reactive CD4+ T cell responses and highly effective cross-presentation to HA-1-reactive CD8+ T cells of alloSCT patients. These data indicate that CLEC12A is an attractive candidate for in vivo targeting of human DC subsets with tumor antigens, thereby employing the specialized functions and cross-talk of these DCs to boost tumor-reactive T cell immunity in cancer patients.

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References

CLEC12A-mediated antigen cross-presentation by DCs


CLEC12A-mediated antigen cross-presentation by DCs


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Supplementary Figure 1. Flow cytometry gating strategies for human DC subsets. (A) Gating strategy for flow-assisted cell sorting of BDCA1+ mDCs, BDCA3+ mDCs and pDCs from leukapheresis products. First, single cell leukocytes were gated using forward- and sideward-scatter characteristics. Then, CD14- and CD19-negative cells were selected. Finally, BDCA1+ mDCs were defined as CD11c+BDCA1+ cells, and BDCA3+ mDCs as CD11c+BDCA3+ cells. pDCs were characterized based on BDCA2+ and CD123+. Density dot plots of one representative donor are shown. (B) For phenotypical analyses and functional experiments, DC subsets were gated as follows: leukocytes were defined by forward-and sideward-scatter characteristics, and single cells were gated using time of flight (TOF). Next, dead cells were excluded using sytox blue staining. BDCA1+ mDCs were defined as CD11c+BDCA1+ cells, and BDCA3+ mDCs as CD11c+BDCA3+ cells. pDCs were characterized as BDCA2+CD123+. Density dot plots of one representative donor are shown.

Supplementary Figure 2. Expression of CLEC12A is limited to cells of myeloid origin and immature MoDC. (A) Expression of CLEC12A on CD8+ T cells, CD4+ T cells, B cells, NK cells, monocytes, BDCA1+ mDCs, pDCs and granulocytes was analyzed using flow cytometry. Data are expressed as ΔMFI, N=5 healthy donors. Lines indicate the mean. (B) Expression of DEC205 and CLEC12A during MoDC differentiation was analyzed by flow cytometry. MoDCs were analyzed at the immature stage (day 3 and day 7), and after maturation with conventional cytokines (day 9). Expression of DEC205 and CLEC12A is shown in black lines, and the corresponding isotype controls are depicted in light grey. Numbers in the plots represent the mean fluorescence intensity. Data of one representative donor out of 3 independent experiments is shown.
Supplementary Figure 3. CLEC12A co-localization with the endosomal/lysosomal compartments. Confocal laser scanning microscopy analyses of DEC205 (A,C) and CLEC12A (B,D) internalization in day 3 MoDCs and co-localization with early endosomes (A,B) and lysosomes (C,D) in time. MoDCs were incubated with αDEC205 or αCLEC12A, followed by staining with Alexa Fluor-488-conjugated goat-αmouse IgG2b (green). Receptors were allowed to internalize for 0-60 minutes at 37°C. Then, MoDCs were stained for membrane MHC-II molecules using anti-HLA-DR/DP, which was visualized with Alexa Fluor-564-conjugated goat-αmouse IgG2a (blue). Finally, MoDCs were permeabilized, stained for early endosomes (EEA-1) or late endosomes (LAMP-1), whereupon visualization with Alexa Fluor-647-conjugated goat-αrabbit IgG was performed (red). Images were acquired on a Olympus FV1000 Confocal laser scanning microscope, 60x oil objective (Numerical Aperture, 1.47) and were analyzed using Image J V1.46R software. Data of one representative donor out of 3 independent experiments is shown. The size of the scale bar is 5μm.
Supplementary Figure 4. αCLEC12A/HA-1 and αDEC205/HA-1 long peptide conjugates are efficiently generated, and specifically deliver antigen for cross-presented by human mDCs and pDCs. (A) To compare conjugation efficiency of HA-1 long peptide to αCLEC12A, αDEC205, hIgG1 and mIgG2b isotype controls, a direct ELISA was performed. For this, titrated concentrations of conjugated antibody/HA-1 constructs were allowed to adhere overnight to Nunc high immuno absorbance plates (Thermo scientific, Roskilde, Denmark). As the HA-1 long peptide has a biotin tag, conjugation efficiency was subsequently determined by incubation with streptavidin–HRP (Sanquin), followed by a color reaction with 3,3',5,5'-Tetramethylbenzidine (TMB) peroxidase substrate solution (KPL, Gaithersburg, MD, USA). This enzymatic reaction was stopped with 1M H₃PO₄ (Merck) and absorbance was measured at 450 nm. Graphs show αCLEC12A and αDEC205 conjugates (closed symbols), or isotype control conjugates (open symbols). (B) Binding efficacy of the antibody/HA-1 conjugates was determined by flow cytometry. PBMCs of healthy donors were incubated with 1 mg/mL total human IgG (Sanquin) to block Fc-receptors, followed by staining with 10 µg/mL αCLEC12A/HA-1 or αDEC205/HA-1 (green lines) or the corresponding isotype/HA-1 (black line) conjugates. As a control for specificity, 10x excess unconjugated αCLEC12A or αDEC205 (red line) was added to block specific binding prior to incubation with antibody/HA-1 conjugates. Finally, binding of antibody/HA-1 construct was visualized by
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addition of biotin-FITC. Light grey lines in histograms depict staining with biotin-FITC alone. (C) BDCA1+ mDCs and pDCs were incubated αCLEC12A/HA-1 or αDEC205/HA-1 long peptide conjugates. As negative controls, DCs were not labeled (DC-), labeled with corresponding isotype control/HA-1 conjugates, or labeled with the antibody/HA-1 conjugate in the presence of excess unconjugated αCLEC12A or αDEC205 (blocked αCLEC12A/HA1 or αDEC205/HA1). As positive control, DCs were pulsed with 1µM short HA-1 peptide. BDCA1+ mDCs, and pDCs were subsequently matured overnight, followed by 24 hour co-culture with HA-1 TCR transduced CD8+ T cells (between 40% and 66% HA-1-tetramer positive, data not shown) at a ratio of 1:1. T cell activation was assessed by determining the percentage of CD107a- and CD137-positive CD8+ T cells using flow cytometry, and by analysis of IFN-γ secretion using ELISA. Data are depicted as the mean ± SD of triplo measurements of one representative donor. For the αCLEC12A/HA-1 conjugate n=6 (BDCA1+ mDC) or n=5 (pDC) independent experiments were performed. For αDEC205/HA-1 n=2 independent experiments were performed.
The aryl hydrocarbon receptor antagonist StemRegenin 1 improves in vitro generation of highly functional NK cells from CD34+ hematopoietic stem and progenitor cells

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Abstract

Early natural killer (NK) cell repopulation after allogeneic stem cell transplantation (alloSCT) has been associated with reduced relapse rates without an increased risk of graft-versus-host disease (GVHD), indicating that donor NK cells have specific anti-leukemic activity. Therefore, adoptive transfer of donor NK cells is an attractive strategy to reduce relapse rates after alloSCT. Since NK cells of donor origin will not be rejected, multiple NK cell infusions could be administered in this setting. However, isolation of high numbers of functional NK cells from transplant donors is challenging. Hence, we developed a cytokine-based \textit{ex vivo} culture protocol to generate high numbers of functional NK cells from granulocyte colony-stimulating factor (G-CSF)-mobilized CD34$^+$ hematopoietic stem and progenitor cells (HSPCs). In this study, we demonstrate that addition of aryl hydrocarbon receptor antagonist StemRegenin 1 (SR1) to our culture protocol potently enhances expansion of CD34$^+$ HSPCs, and induces expression of NK cell-associated transcription factors promoting NK cell differentiation. As a result, high numbers of NK cells with an active phenotype can be generated using this culture protocol. These SR1-generated NK cells exert efficient cytolytic activity and IFN-γ production towards acute myeloid leukemia and multiple myeloma cells. Importantly, we observed that NK cell proliferation and function is not inhibited by cyclosporin A, an immunosuppressive drug often used after alloSCT. These findings demonstrate that SR1 can be exploited to generate high numbers of functional NK cells from G-CSF mobilized CD34$^+$ HSPCs, providing great promise for effective NK cell based immunotherapy after alloSCT.
**Introduction**

Natural killer cells (NK cells) are CD3 CD56+ lymphocytes, which are part of the innate immune system and play an important role in the defense against virus-infected and transformed cells. NK cell activation and subsequent killing of target cells is regulated by a balance in their expression levels of inhibitory receptors, including the killer-immunoglobulin like receptors (KIRs) and CD94/NKG2A heterodimer, versus activating receptors, such as DNAX accessory molecule-1 (DNAM-1), natural cytotoxicity receptors (NCRs) and NKG2D. In homeostasis, NK cells are inhibited by their inhibitory receptors recognizing self human leukocyte antigen (HLA) class I molecules and/or HLA-E molecules presenting conserved HLA class I leader sequences. However, an NK cell-mediated anti-tumor effect can be induced by upregulation of activating ligands or downregulation of HLA class I molecules on tumor cells. In addition, in the setting of haploidentical allogeneic stem cell transplantation (alloSCT), donor NK cells may lack expression of inhibitory KIRs for recipient HLA class I molecules and hence be activated. This phenomenon is called missing-self recognition and can contribute to the curative graft-versus-tumor (GVT) effect.1

Because of their ability to kill tumor cells, NK cells are considered potent effectors for adoptive immunotherapy against cancer. So far, promising results have been obtained by infusion of haploidentical NK cells after immunosuppressive chemotherapy in adult and childhood acute myeloid leukemia (AML).2-4 However, a limitation in these studies is the relatively low NK cell numbers that can be enriched from aphaeresis products for multiple infusions. Furthermore, contaminating allo-reactive T cells risk the induction of graft-versus-host disease (GVHD), especially when interleukin (IL)-2 or IL-15 are co-administrated to boost NK cell survival and expansion. In order to generate high numbers of allogeneic NK cells completely devoid of T cell contamination, a Good Manufacturing Practice (GMP)-compliant, cytokine-based *ex vivo* culture protocol has been developed by our group.5,6 Using this procedure, CD34+ hematopoietic stem and progenitor cells (HSPCs) isolated from umbilical cord blood (UCB) can be expanded over 2000-fold in large-scale bioreactors into a mixture of immature and mature NK cells with a purity > 80%. Pre-clinical studies conducted in NOD/SCID-IL2Rnull mice demonstrated that these HSPC-NK cells have BM homing capacity, display IL-15-driven *in vivo* expansion, and prolong survival of leukemia-bearing mice.7 Currently, administration of this HSPC-NK cell product following immunosuppressive chemotherapy is being investigated in a phase I clinical trial in older AML patients who are not eligible for alloSCT (see www.trialregister.nl and search for 2818).

In HLA-matched non-myeloablative and T cell-depleted alloSCT, early NK cell repopulation has been associated with decreased relapse rates, without increasing GVHD incidence.8,9 Moreover, high NK cell numbers in stem cell grafts have been associated with a decreased incidence of GVHD.10 In addition, transplants from donors with KIR-B haplotypes, containing several activating KIRs, led to lower rates of relapse and
improved survival. For these reasons, it would be highly valuable to exploit HSPC-NK cell products for adoptive immunotherapy after alloSCT. Since NK cells of donor origin will not be rejected, multiple NK cell infusions without the need for immunosuppressive chemotherapy to prevent rejection, could be administered after alloSCT. Consequently, these cells may potentially induce long-term GVT effects. However, to obtain large numbers of NK cells from donor origin, peripheral blood (PB) or bone marrow (BM)-derived CD34+ HSPCs, which have a lower expansion potential compared to UCB-derived CD34+ HSPCs, should be expanded and differentiated into NK cells.

Recently, it was described that expansion of CD34+ HSPCs can be enhanced by inhibition of the aryl hydrocarbon receptor (AhR) using the antagonist StemRegenin 1 (SR1). AhR is a ligand-inducible transcription factor, which plays an important role in biological responses towards xenobiotic agents such as digoxin. Yet, it has become clear that AhR also has multiple naturally occurring ligands, like tryptophan metabolites and dietary compounds. Furthermore, AhR turned out to regulate differentiation of multiple immune cells including dendritic cells, regulatory T cells, γδ T cells, Th17 cells and notably NK cells. Based on these findings, we hypothesized that addition of SR1 to our culture system might improve expansion of CD34+ HSPCs, and differentiation of these cells into NK cells. We found that SR1 not only enhances expansion of CD34+ HSPCs, but also upregulates the expression of early and late NK cell specific transcription factors, thereby potentiating differentiation of SR1-expanded CD34+ cells into NK cells. These SR1-induced NK cells have a high purity, express high levels of activating receptors and efficiently target AML and multiple myeloma (MM) cells. Importantly, proliferation and cytolytic functions of SR1-induced HSPC-NK cells are not inhibited by cyclosporin A (CsA), in contrast to mycophenolic acid (MPA), which is used only shortly after alloSCT, facilitating multiple infusions relatively shortly after alloSCT. Therefore, our SR1 culture system holds great promise for future donor HSPC-NK cell adoptive immunotherapy after alloSCT to boost anti-tumor and anti-viral immunity, leading to prolonged relapse-free survival.

**Materials and Methods**

**Cell lines**

Cell lines (K562, THP1, HL-60, U266, UM9, RPMI8226) were cultured in Iscove-modified Dulbecco medium (IMDM; Invitrogen, Carlsbad, CA, USA) containing 50 U/mL penicillin, 50 µg/mL streptomycin and 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands).

**Isolation of CD34+ stem and progenitor cells**

Bone marrow samples were collected from healthy donors after written informed consent. Bone marrow-derived mononuclear cells were isolated by Ficoll-hypaque (1.077 g/mL; GE
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Healthcare, Uppsala, Sweden) density gradient centrifugation. Peripheral blood-derived mononuclear cells were obtained from apheresis material from stem cell donors who were treated with granulocyte colony-stimulating factor (G-CSF; Neupogen®) 10 µg/kg/day subcutaneously for 5 to 6 days, after written informed consent. CD34+ HSPCs were isolated using anti-CD34 immunomagnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer’s instructions. CD34+ HSPCs were directly used for NK cell generation.

**Ex vivo expansion of CD34+ HSPCs, and differentiation into NK cells**

CD34+ HSPCs were plated into 24-well or 6-well tissue culture plates (Corning Incorporated, Corning, NY, USA). Cells were expanded during 9 or 10 days with a high-dose cytokine cocktail (Expansion cocktail I) consisting of 25 ng/mL IL-7 (Immunotools), 25 ng/mL SCF (stem cell factor, Immunotools), 25 ng/mL TPO (thrombopoietin, Cellgenix) and 25 ng/mL Flt3L (FMS-like tyrosine kinase 3 ligand, Immunotools). From day 9 or 10 until day 14 or 15, TPO was replaced by 20 ng/mL IL-15 (Miltenyi). After day 14 or 15, cell differentiation was initiated by replacing Expansion cocktail I by a new high-dose cytokine cocktail (Differentiation cocktail) consisting of 20 ng/mL IL-7, 20 ng/mL SCF, 20 ng/mL IL-15 and 1000 U/mL IL-2 (Proleukin®, Chiron, München, Germany). Where mentioned, 2 µM SR1 (Cellagen Technology, San Diego, CA, USA) was added to the culture medium (see Figure 1A). Cells were cultured in Cellgro GMP DC medium (Cellgenix, Freiburg, Germany) supplemented with 10% human serum (HS; Sanquin Bloodbank, Nijmegen, The Netherlands) and a low dose cytokine cocktail consisting of 250 pg/mL G-CSF (Filgrastim, “Neupogen” - Amgen Corp. USA), 10 pg/mL GM-CSF (granulocyte-macrophage colony-stimulating factor) and 50 pg/mL IL-6 (both Immunotools, Friesoythe, Germany). During the first 14 or 15 days of culture, low molecular weight heparin (Clivarin®; Abbott, Wiesbaden, Germany) was added to the medium in a final concentration of 20 µg/mL. Freshly isolated CD34+ cells were plated at a concentration of 1 – 4 x 10⁵/mL. After 3 days of culture, cells were transferred to a new plate in order to deplete for stromal cells. From day 14 or 15 onward, cell counts were kept above 2 x 10⁶ cells/mL. Cell cultures were refreshed with at least 30% new medium every 2 to 3 days. Cultures were maintained at 37°C, in 95% humidity, and 5% CO₂. NK cells were used in experiments after 5 weeks of culture.

**RNA isolation and real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)**

Total RNA from 0.5 - 2 x 10⁶ cells, collected weekly from HSPC-NK cell cultures, was isolated using the Quick-RNA™ MicroPrep Kit (Zymo Research, CA, USA). Next, cDNA was synthesized using M-MLV-reverse transcriptase (Invitrogen) in a standard reaction as described earlier, after which real-time PCR was performed using the following Taqman Gene expression assays (Applied Biosystems, Forster City, CA, USA): AhRR (Hs01005075...
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m1); TOX (Hs01055573_m1); ID2 (Hs04187239_m1); EOMES (Hs00172872_m1); GATA3 (Hs00231122_m1); SH2D1B (Hs01592483_m1); IFNG (Hs00989291_m1); GZMB (Hs01554355_m1); PRF1 (Hs99999108_m1). For all genes, Ct values were normalized to GAPDH (Hs02758991_g1) by calculating \( \Delta \text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}} \) per sample. Finally, gene expression levels were quantified relative to GAPDH as follows: \( 2^{-\Delta \text{Ct}} \).

**Flow cytometry (FCM)**

Cell numbers and expression of cell-surface markers were determined by FCM. Anti-human CD45-ECD (J.33, Beckman Coulter, Woerden, The Netherlands) and anti-CD56-PC7 (HCD56, Biolegend, San Diego, CA, USA) antibodies were used to follow cell number and NK cell differentiation during culture using the Coulter FC500 flow cytometer (Beckman Coulter). The population of viable CD45+ cells was determined by exclusion of 7-AAD (Sigma, St Louis, MO, USA) positive cells. For phenotypical analysis, cells were incubated with antibodies in FCM buffer (PBS/0.5% bovine serum albumin (BSA; Sigma) for 30 minutes at 4°C. After washing, cells were resuspended in FCM buffer and analyzed. The following conjugated monoclonal antibodies were used for NK cell phenotyping: anti-NKG2A-PE (Z199; Beckman Coulter), anti-DNAM-1-FITC (DX11; BD Biosciences Pharmingen, Breda, The Netherlands), anti-CD16-FITC (3G8), anti-CD3-FITC (UCHT1), anti-NKG2D-PE (1D11), anti-NKp30-PE (P30-15), anti-NKp44-PE (P44-8), anti-NKp46-PD (9E5), anti-CD158b-PE (Dx27), anti-CD158e-PE (Dx9), anti-CD158a/h-PE (HP-MA4), anti-CD62L-PE (DREG56), anti-CD253-PE (RIK-2), anti-CXCR3-PE (G025H7), anti-CXCR4-PE (12G5), anti-IgG1-PE (MOPC-21), anti-IgG2a-PE (MOPC-173), anti-IgG2b-PE (MCP-11), anti-IgG1-FITC (MOPC-21; all from Biolegend).

**Fluorescence-activated cell sorting (FACS) of HSCP-NK cells**

After 5 weeks of culture, CD56+ cells were isolated from the total cultured cells. For this purpose cells were stained for 15 minutes at 4°C using the appropriate concentration of anti-CD56-PECy7 (HCD56, Biolegend). Cells were washed and resuspended in FACS buffer at a concentration of 1.5 x 10^6/mL, and subsequently sorted at the FACS Aria Cell Sorter (BD Biosciences).

**FCM-based cytotoxicity assays**

Cell lines were labeled with 1 µM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Invitrogen, Eugene, OR, USA) and primary AML blasts were labeled with 1.5 µM CFSE, both in a concentration of 1 x 10^7/mL for 10 minutes at 37°C. The reaction was terminated by adding an equal volume of FCS. After washing, cells were resuspended in IMDM/10% FCS to a final concentration of 3 x 10^5/mL. Target cells (3 x 10^4) were co-cultured in triplicate with effector cells at different effector:target (E:T) ratios in a total volume of 200 µL IMDM/10% FCS in 96-wells round-bottom plates (Corning Incorporated). Effector cells and target cells alone were plated out in triplicate as controls.
AhR inhibition facilitates NK cell generation

In experiments with primary AML blasts, AML blasts were derived from bone marrow samples from 5 patients at the time of diagnosis and were supplemented with IL-3 (50 ng/mL; Cellgenix), SCF (25 ng/mL), Flt3L (20 ng/mL), GM-CSF (100 ng/mL), G-CSF (100 ng/mL) and IL-15 (5 ng/mL). To measure degranulation of NK cells, anti-CD107a-PC7
(H4A3, Biolegend) was added to the co-culture. After overnight co-culture at 37°C, 50 µL supernatant was discarded and 50 µL Coulter® Isoton® II Diluent containing 0.2 µL 7-AAD was added instead. Cells were harvested and the number of viable target cells was quantified by FCM by gating on forward scatter and side scatter and exclusion of 7-AAD positive cells. Target cell survival was calculated as follows: % survival = ([absolute number of viable CFSE+ target cells co-cultured with NK cells]/[absolute number of viable CFSE+ target cells cultured in medium]) x 100%. The percentage specific lysis was calculated as follows: % lysis = (100-[% survival]), as described earlier by Jedema et al.\textsuperscript{25,26} Degranulation of NK cells during overnight co-culture was determined as the percentage of CD107a expressing cells measured by FCM.

**Enzyme-linked immunosorbent assays (ELISA)**

The production capacity of interferon (IFN)-γ and Granzyme B by NK cells were evaluated by ELISA according to manufacturer instructions (IFN-γ: Pierce Endogen, Rockfore, IL, USA; Granzyme B; Mabtech, Sweden). To this end, NK cells (1 x 10^5) were co-cultured in triplicate with target cells (1 x 10^5) in a total volume of 200 µL IMDM/10% FCS in 96-wells round-bottom plates (Corning Incorporated). NK cells alone were plated out in triplicate as controls. After incubation overnight at 37°C, 150 µL supernatant was collected and stored at -20°C until use.

**Proliferation assays**

HSPC-NK cells were cultured according to our culture protocol for 35 days. After 35 days, NK cells were labeled with 1 µM CFSE as described previously. After staining, cells were resuspended in differentiation medium at a concentration of 2 x 10^6/mL and plated in duplicate in a 96-well plate. CsA (Biovision Incorporated, Milpitas, CA, USA) or MPA (Sigma-Aldrich, Zwijndrecht, The Netherlands) were added to final concentrations of 0.01 to 1 µg/mL (CsA) or 0.1 to 10 µg/mL (MPA). Half of the medium containing MPA or CsA in the final concentration was refreshed every 2-3 days. After 7 days, cells were harvested. The number of viable target cells was quantified by FCM by gating on forward scatter and side scatter and exclusion of 7-AAD positive cells. Proliferation was analyzed by determining the CFSE dilution within CD56+ cells.

**Statistical analysis**

Results from different experiments are described as mean ± standard error of the mean (SEM). Statistical analysis was performed using a one-tailed paired student’s t-test, a two-tailed unpaired student’s t-test or one-way ANOVA if values had a normal distribution (normality was determined using the Kolmogorov-Smirnov test). For values without normal distribution we used the Wilcoxon matched-pairs signed rank test. Differences were considered to be significant for p values <0.05.
Results
SR1 enhances expansion of PB- and BM-derived CD34+ HSPCs and improves NK cell differentiation

In this study, we investigated whether NK cells could be generated in vitro from PB or BM-derived CD34+ HSPCs. We used the feeder-free culture protocol described in Figure 1A, which was reported previously to generate high numbers of functional NK cells from UCB-derived CD34+ HSPCs. However, in our initial cultures using PB- or BM-derived HSPCs, expansion and differentiation were low, which was associated with high numbers of stroma-like cells observed in culture plates (data not shown). Therefore, we investigated transfer of non-adherent cells to a new culture plate after 3 days of culture. Although this resulted in much lower outgrowth of stromal cell layers in the culture plates, expansion and NK cell differentiation were still low. For that reason, we investigated whether addition of the AhR antagonist SR1 (2 µM), which is known to improve expansion of CD34+ HSPCs and to influence NK cell differentiation, could improve NK cell generation from HSPCs. Importantly, addition of SR1 strongly enhanced expansion of CD34+ cells from both PB-derived HSPCs (Figure 1), as well as BM-derived HSPCs (Supplementary Figure 1A,B). Mean NK cell yield after 5 weeks of culture was 235 fold (range 115 – 904 fold; n = 10, Figure 1D) for G-CSF mobilized CD34+ cells, and 129 fold (range 33 – 301; n=4, Supplementary Figure 1B) for BM-derived CD34+ HSPCs. Interestingly, differentiation also improved by addition of SR1 to our cultures, resulting in a purity of 83% ± 9% for G-CSF-mobilized CD34+ HSPCs, and 84% ± 18% for BM-derived CD34+ cells. The remaining non-NK cells in the cultures represented mainly CD14+ and/or CD15+ mature monocytic and myelocytic cells (11% ± 6%). A small frequency of CD34+ cells could also be detected at the end of the culture process (0.7% ± 0.4%). Most importantly, the amount of CD3+ T cells in the SR1-based NK cell cultures investigated was very low (0.1% ± 0.1%). Altogether, these data demonstrate that the AhR pathway negatively influences NK cell development, and that by combining SR1 with cytokine mixtures, high numbers of CD56+ NK cells can be generated from G-CSF-mobilized and BM-derived CD34+ HSPCs ex vivo. Since G-CSF-mobilized CD34+ HSPCs are the main stem cell source for alloSCT, we concentrated on these HSPC-NK cells for further investigations.

SR1 influences expression of transcription factors important for NK cell differentiation and maturation

To gain insight into the molecular processes behind the SR1-enhanced differentiation of CD34+ HSPCs into NK cells, we analyzed the gene expression profile of several transcription factors that are described to be important for NK cell differentiation and maturation. To analyze the culture composition, we concomitantly determined the percentage of CD56+ cells in our cultures at different time points (Figure 2A). Next, we compared expression levels of several transcription factors in the presence or absence of SR1 in total cells at these time points in our ex vivo culture system (Figure 2B). We
observed efficient down regulation of AHR repressor (AhRR, i.e. direct target gene of AhR signaling\textsuperscript{14}) in the presence of SR1, indicating that a concentration of 2 µM SR1 is sufficient for AhR inhibition. Interestingly, we observed higher expression of thymocyte selection-associated HMG box factor (TOX), which is important in early NK cell differentiation,\textsuperscript{27} from day 7 onward (Figure 2B,C). This suggests that SR1 increased the number of NK cell precursors, even before the induction of NK cell differentiation was initiated by addition to IL-15 to the culture, and before CD56 acquisition. Expression of ID2, which is important for NK cell maturation,\textsuperscript{28,29} was increased from day 14 onwards, after addition of IL-15 to the culture (Figure 2B). Eomesodermin (EOMES), another factor important for NK cell maturation, was upregulated from week three onward. Finally, expression of GATA-3 and

![Figure 2. SR1 reduces expression of AhRR and increases expression of several transcription factors important for NK cell differentiation and NK cell effector functions.](image)

Cells generated from CD34\textsuperscript{+} HSPCs in the presence or absence of SR1 were collected at different time points. (A) Kinetics of CD56 expression in the cultures used for weekly mRNA isolation were determined using FCM. Means ± SEM of 4 - 6 different donors. (B) Expression levels of AhRR and transcription factors relative to GAPDH at different time points during culture were determined using qRT-PCR. Means ± SEM of 4 - 6 different donors. (C) Expression levels of AhRR and TOX relative to GAPDH after 7 days of culture were determined using qRT-PCR. Means ± SEM of 6 different donors. (D) Expression levels of AhRR and transcription factors in CD56\textsuperscript{+} cells which were FACS sorted from cultures after 5 weeks of culture were determined using qRT-PCR. Means ± SEM of 4 different donors. *p<0.05. Paired one-tailed student’s t-test.
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Ewing’s sarcoma-associated transcript 2 (EAT-2; SH2D1B), required to develop cytotoxic functions, was increased in cells cultured in the presence of SR1 (Figure 2B).

To investigate whether the increased expression levels of these transcription factors reflected a difference between NK cells generated in the presence or absence of SR1, or resulted from the different composition of the cultures, CD56+ NK cells, generated in the presence or absence of SR1, were sorted by FACS from HSPC-NK cultures after 5 weeks. Subsequently, gene expression levels of the previously mentioned transcription factors were determined. We did not observe increased expression of the NK cell differentiation factors TOX, ID2 and EOMES suggesting that SR1 increases the number of NK cell progenitors resulting in more NK cells, but it does not intrinsically change HSPC-NK cells (Figure 2D). In addition, expression levels of GATA-3 and EAT-2 were similar in CD56+ NK cells cultured in the presence of SR1. Expression of GZMB and PERF was slightly higher, but not significantly increased. Furthermore, IFN-γ was not increased in NK cells cultured in the presence of SR1, but these cells were not exposed to target cells prior to mRNA analysis.

Collectively, these data demonstrate that addition of SR1 to our ex vivo culture system blocks function of AhR, resulting in the upregulation of several transcription factors that are required for NK cell differentiation and maturation.

SR1-generated HSPC-NK cells have an activated and mature phenotype

To further elucidate the effect of SR1 on NK cell activation status and function, we investigated the influence of SR1 on the phenotype of our ex vivo-generated CD56+ NK cells. After 35 days of culture in the presence of SR1, NK cell phenotype was analyzed using FCM (Figure 3A-D and Supplementary Figure 1C). SR1-generated NK cells expressed high levels of NKG2A, which indicates transition between stage 3 and stage 4 NK cell progenitors. CD16, important for antibody-dependent cell mediated cytotoxicity (ADCC), was expressed on 22.7 ± 2.6% of the CD56+ cells. Furthermore, we found high expression levels of the activating markers DNAM-1, NKG2D, NCRs, and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). Expression of KIRs was observed in a low percentage of CD56+ cells. However, expression levels were similar as observed for UCB-NK cells generated in our culture system. Importantly, our NK cells also expressed high levels of CD62L and chemokine (C-X-C motif) receptor 3 (CXCR3), which are involved in homing to the lymphoid organs and trafficking of NK cells towards inflammation in vivo. Interestingly, presence of CD62L, CXCR3, DNAM-1 and TRAIL were significantly higher in CD56+ cells generated in the presence of SR1 (Figure 3C,D). Increased expression of DNAM-1 and TRAIL suggests that NK cells generated in the presence of SR1 are more active as compared to NK cells generated in the absence of SR1. We did not find significant differences in expression of the other molecules in the cultures with SR1 compared to the cultures without SR1 (data not shown). Altogether, these results indicate that SR1-generated HSPC-NK cells consist of a mixture of immature and mature...
Figure 3. NK cells generated in the presence of SR1 have an active and mature phenotype. NK cells were generated from CD34+ progenitor cells in the presence or absence of SR1. After 5 weeks a phenotypical analysis was performed by FCM. (A) Phenotype of a representative NK cell product. Density plot of live cells gated on forward scatter/ side scatter. (B) Expression level of several NK cell specific surface antigens on live CD56+ NK cells from 3 - 8 different donors (Mean ± SEM) are shown. (C) Expression levels of NK cell-specific antigens on CD56+ NK cells generated in the presence (grey histograms) or absence (black histogram) of SR1 from one representative donor as compared to isotype controls (white histogram). Numbers represent % positive cells. (D) Expression levels of NK cell-specific antigens on CD56+ NK cells generated in the presence or absence of SR1 from 3 - 7 different donors (Mean ± SEM) are shown. *p<0.05, **p<0.01, Paired one-tailed student’s t-test.
NK cells expressing various activating receptors, similarly to our previously described UCB-derived NK cells.\textsuperscript{6}

**SR1-generated HSPC-NK cells have efficient IFN-γ production capacity and cytolytic activity against AML and MM cells**

Next, we investigated the functional activity of SR1-generated NK cells, and compared them with NK cells generated in the absence of SR1. After 5 weeks of culture, NK cell products were harvested and co-cultured overnight with AML or MM tumor cell lines. Target cell induced IFN-γ production was determined. We found that our SR1-generated HSPC-NK cells have a good IFN-γ producing capacity, which is improved in cells generated in the presence of SR1 (Figure 4A). Subsequently, we compared the killing capacity of NK cells generated in the presence of absence of SR1. Therefore, $1 \times 10^4$ CD56\textsuperscript{+} NK cells were co-cultured overnight with different AML cell lines. We observed significantly improved killing of K562 and HL-60 cells, and also a trend towards improved killing of THP-1 cells by SR1-generated NK cells (Figure 4B). To rule out an effect of non-NK cells in the cultures, we repeated the killing experiments using sorted NK cells. Therefore, CD56\textsuperscript{+} NK cells were sorted from the NK cell products after 5 weeks of culture. Next, NK cells were co-cultured overnight with AML or MM tumor cell lines as described earlier. Importantly, we also observed higher killing of K562, HL-60 and RPMI8226 cells by sorted SR1-generated NK cells (Figure 4C). In addition, granzyme B production was enhanced in SR1-generated HSPC-NK cells as compared to NK cells generated in the absence of SR1 (Figure 4D). These results indicate that addition of SR1 to our culture system not only increases the number of CD56\textsuperscript{+} cells we can generate, but also enhances the functional activity of the CD56\textsuperscript{+} NK cells.

To further confirm the functional activity of SR1-generated NK cells, we investigated degranulation of these cells after overnight co-culture with AML or MM tumor cell lines. We found marked degranulation of our SR1-generated NK cells upon co-culture with the different cell lines (Figure 4E,F). Most efficient degranulation was observed against major histocompatibility complex (MHC)\textsuperscript{neg} K562 cells as well as the MHC-expressing AML cell line HL-60. We also observed marked degranulation upon co-culture with the MM cell lines U266 and RPMI8226. The MM cell line UM9 was the least potent cell line to induce degranulation.

Next, we performed cytotoxicity experiments using SR1-generated HSPC-NK cells harvested after 5 weeks of culture. For this, NK cells were co-cultured overnight with different CFSE-labeled AML (K562, HL-60 and THP-1) and MM (UM9, U266 and RPMI8226) cell lines. The next day, specific killing was determined by FCM. We observed very efficient killing of most AML and MM cell lines, even at an E:T ratio of 1:1 (Figure 4G). Subsequently, we investigated whether patient-derived primary AML blasts were susceptible to killing by SR1-induced NK cells. Therefore, we performed cytotoxicity assays with AML blasts from 5 different patients. Importantly, AML blasts could be
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potently killed within 3 days of co-culture (Figure 4H). We observed some variance in susceptibility between the different patients, but variance between HSPC-NK cell donors was small, indicating that the quality of the SR1-induced NK cell products is consistent.

Taken together, these data demonstrate that SR1-induced HSPC-NK cells, generated from G-CSF-mobilized CD34+ cells, mediate efficient IFN-γ production, degranulation and cytolytic activity against hematological tumor cells. Furthermore, as expected by increased expression of activating markers, SR1 augments IFN-γ production and cytotoxic activity of ex vivo-generated HSPC-NK cells.

**HSPC-NK cell proliferation, viability and function is inhibited by MPA but not by CsA**

Increased NK cell numbers after non-myeloablative alloSCT have been associated with decreased relapse rates, without an increased risk for GVHD.8,9 Hence, adoptive transfer of ex vivo-generated NK cells shortly after alloSCT is an attractive approach to improve patient outcome. Our therapeutic strategy is to apply HSPC-NK cell adoptive transfer for high-risk patients treated with non-myeloablative alloSCT (Figure 5A). For this purpose, we want to exploit 15-30 x 10⁶ CD34+ cells from the G-CSF-mobilized donor stem cell graft, which represents approximately 5-10% of the total graft, for ex vivo NK cell generation. These HSPC-NK cells can be infused as a single infusion, or multiple infusions after alloSCT, to improve the GVT effect. However, these alloSCT patients are treated with immunosuppressive drugs to prevent GVHD, so the effect of these drugs on HSPC-NK cell proliferation and viability should be known. For that reason, we investigated the effect of CsA and MPA, which are often used after non-myeloablative alloSCT, on SR1-induced NK cells. For this purpose, week 5 HSPC-NK cells were cultured for 7 additional days in the presence of absence of therapeutic concentrations of MPA or CsA (Figure 6).

**Figure 4 (on the left). NK cells generated in the presence of SR1 are functionally active.** NK cells were generated from CD34+ HSPCs in the presence or absence of SR1. After 5 weeks the functional activity of the cells was investigated. (A-G) NK cells were co-cultured overnight with the leukemia cell lines K562, HL-60 or THP-1, or the MM cell lines RPMI8226, U266 or UM9 at an E:T ratio 0.3:1, 1:1 or 3:1. (A) IFN-γ levels were determined by ELISA in the co-culture supernatants of 1 x 10⁶ CD56+ NK cells and 1 x 10⁵ target cells. Means ± SEM of 3 - 4 donors generated in the presence or absence of SR1 are shown. (B) Specific killing of leukemia cell lines at an E:T ratio 0.3:1 was determined in a FCM-based cytotoxicity assay. Means ± SEM of 3 donors generated in the presence or absence of SR1 are shown. (C-D) After 5 weeks of culture, CD56+ NK cells were FACS sorted from the cultures, and co-cultured with leukemia cell lines or the MM cell line RPMI8226 at an E:T ratio 1:1. Cultures were supplemented with IL-15 (5 ng/mL). (C) Specific killing of the cell lines was determined in a FCM-based cytotoxicity assay. Data are depicted as mean ± SD. (D) Granzyme B production by the CD56+ NK cells was measured by ELISA. Data are depicted as mean ± SD. (E-F) Degranulation of SR1-generated CD56+ cells after co-culture at an E:T ratio 1:1, determined by FCM as the percentage of CD107a expressing cells. One representative donor (E) and means ± SEM of 4 - 7 donors (F) are shown. (G) Specific killing of leukemia or MM cell lines by SR1-generated NK cells at different E:T ratios was determined in a FCM-based cytotoxicity assay. Means ± SEM of 3 - 7 donors are shown. (H) Specific killing of primary AML cells by SR1-generated NK cells from 5 different patients (#1 AML-M2, #2 AML-M2, #3 AML-M4, #4 AML-M5, #5 AML-M0) was determined for 5 different HSPC donors. Specific killing was determined after 1, 2 and 3 days of co-culture in a FCM-based cytotoxicity assay at an E:T ratio of 3:1. Data are displayed as mean ± SD of triplicate samples. *p<0.05, ** p<0.01, *** p<0.001. Paired one-tailed (A, B, F) or an unpaired two-tailed (C-D) student’s t-test.
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After 7 days, we analyzed NK cell proliferation and viability using FCM (Figure 5B-D). We found that proliferation of HSPC-NK cells was already inhibited by MPA in a therapeutic concentration of 0.1 µg/mL. In contrast, CsA, even at a concentration of 1 µg/mL, did not inhibit HSPC-NK cell proliferation (Figure 5B,C). In addition, NK cell viability was not affected by CsA, while MPA induced dose-dependent cell death (Figure 5D). Next, we investigated the effect of MPA and CsA on HSPC-NK cell function. For this, CD56+ NK cells cultured in the presence of MPA or CsA were co-cultured overnight with K562 cells at an E:T ratio of 1:1. As a control, week 6 CD56+ NK cells were used. We observed impaired killing by NK cells cultured in the presence of MPA; however, CsA did not inhibit NK cell-mediated killing (Figure 5E). In addition, we observed impaired IFN-γ production after incubation with MPA, while incubation with CsA even enhanced IFN-γ production (Figure 5F). These data indicate that adoptive transfer of SR1-generated HSPC-NK cells can be applied in patients treated with CsA, but infusion should be postponed until cessation of MPA therapy.

Discussion

NK cells are the first lymphocyte population recovering after alloSCT, and have several important functions shortly after alloSCT.36 First of all, they are involved in defence against viral infections such as cytomegalovirus (CMV) infections, which can cause high morbidity shortly after alloSCT. Furthermore, high NK cell numbers shortly after non-myeloablative and T cell-depleted alloSCT have been associated with reduced relapse rates without an increased risk of GVHD, indicating that allogeneic donor NK cells have specific antitumor activity. Nevertheless, it was shown that early engrafting NK cells have decreased cytokine producing capacity.39 So, in order to further exploit the

Figure 5 (on the left). NK cell viability, proliferation and function is inhibited by MPA but not by CsA. (A) Suggested strategy for adoptive transfer of ex vivo generated SR1-NK cells after non-myeloablative alloSCT. A patient is conditioned with non-myeloablative conditioning; subsequently, the patient receives a T cell replete stem cell graft. Ten percent of CD34+ cells of the donor graft are used for NK cell generation. To prevent GVHD, patients are treated with MPA and CsA. After cessation of MPA (around day 28 after transplantation), NK cells can be infused as a single infusion or multiple infusions. (B-F) The effect of immunosuppressive drugs used after non-myeloablative alloSCT was investigated. NK cells were generated from CD34+ HSPCs in the presence of SR1. After 5 weeks, NK cells were cultured for 7 more days in the presence or absence of different dosages of MPA or CsA. (B) Proliferation of CFSE-labelled NK cells cultured in the presence of absence of drugs measured by CFSE dilution in a representative donor. (C) Proliferation of NK cells in the presence or absence of drugs measured as CFSE dilution. Mean MFI relative to MFI of control cells ± SEM of 4 different donors is shown. (D) The number of living cells determined by forward scatter/side scatter and exclusion of 7-AAD positive cells. Percentage of living NK cells cultured for 7 days in the presence or absence of drugs. Mean viability relative to control cells ± SEM of 4 different donors is shown. (E-F) 1 x 10⁵ CD56+ NK cells cultured in the presence or absence of MPA or CsA were co-cultured overnight with K562 cells in the presence of IL-15 (5 ng/mL), at an E:T ratio of 1:1. (E) Specific killing of K562 cells was determined in a FCM-based cytotoxicity assay. Data are depicted as mean ± SD and are representative of 2 different donors. (F) IFN-γ levels were measured in the supernatant after overnight co-culture using ELISA. Data are depicted as mean ± SD and are representative of 2 different donors. ** p<0.01, *** p<0.001, One-way ANOVA.
beneficial effects of NK cells after alloSCT, adoptive transfer of functional and rapidly maturing NK cells would be an attractive immunotherapeutic strategy. Since donor-derived NK cells will not be rejected post-transplant, multiple NK cells infusions without the need for immunosuppressive chemotherapy to prevent rejection, will be feasible in this setting. However, high numbers of functional NK cells of donor origin are needed to apply this strategy. Since isolation of sufficient numbers of NK cells from donors, without contaminating allo-reactive T cells, is challenging; we investigated if high numbers of functional NK cells could be generated from G-CSF mobilized CD34+ HSPCs, using an ex vivo cytokine-based culture protocol. This protocol was developed by our group earlier, and high numbers of pure and functional NK cells with in vivo maturation capacity, can be generated from UCB-derived CD34+ HSPCs using this GMP-compliant protocol.5-7

We found that NK cell expansion and differentiation from G-CSF-mobilized and BM-derived CD34+ cells in the absence of SR1 was very limited, as compared to NK cell expansion and differentiation of UCB-derived CD34+ HSPCs observed in our previously developed culture protocol. Interestingly, we found that addition of the AhR antagonist SR1 to our cultures greatly enhanced NK cell expansion and differentiation. As a result, we can expand G-CSF-mobilized CD34+ cells on average 268-fold using our newly developed SR1-based protocol. In addition, SR1-generated NK cell products are 83% ± 9% pure. The remaining non-NK cells in the cultures represented mainly CD14+ and/or CD15+ mature monocytic and myelocytic cells. Probably due to SR1 addition still some remaining low amount CD34+ cells could be detected. However, contaminating CD3+ T cells were either not detectable or at very low frequency (0.1% ± 0.1%). Furthermore, we found that SR1-NK cells have a similar phenotype as our NK cells previously generated from UCB-derived CD34+ HSPCs, which is a highly active phenotype, characterized by expression of high levels of activating NK cell receptors.5-7 Interestingly, expression levels of CD62L, CXCR3, DNAM-1 and TRAIL were even higher on NK cells generated in the presence of SR1. CD62L and CXCR3 are involved in homing to lymphoid organs and trafficking towards inflammation in vivo, so high expression of these markers can contribute to trafficking of NK cells toward hematological tumor cells in the lymphoid organs.7,34,35 In addition, it was described that CD62L indicates an unique subset of polyfunctional NK cells combining the ability to produce IFN-γ with cytotoxic properties.40 DNAM-1 and TRAIL are activation markers, increased expression levels of these markers suggests that SR1-generated NK cells are more active as compared to NK cells generated in the absence of SR1. This was indeed confirmed in functional studies showing that SR1-induced NK cells have increased INF-γ production capacity and an increased capability to kill AML cell lines, as compared to NK cells generated in the absence of SR1. Furthermore, SR1-generated NK cells show efficient degranulation against AML and MM cell lines. Besides, even at low E:T ratios, these cells efficiently kill hematological tumor cell lines, and most importantly, patient-derived primary AML blasts. To further investigate the applicability of our SR1-NK cell product after alloSCT, we investigated the effect of the immunosuppressive drugs MPA.
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en CsA, which are commonly used after non-myeloablative alloSCT, on our SR1-NK cell product. Importantly, we found that therapeutic CsA concentrations do not affect HSPC-NK cell viability, proliferation or function, while exposure to therapeutic levels of MPA did have a negative effect. Exposure to CsA did even enhance IFN-γ production by the HSPC-NK cells. This is in accordance with the effect of CsA and MPA on naturally occurring NK cells described in literature. These results provide strong rational to infuse SR1-generated HSPC-NK cells relatively short after alloSCT, since MPA treatment is generally stopped at day 28 after alloSCT. CsA, which is usually prescribed for at least six months, will most likely not affect NK cell viability, proliferation or function in vivo upon transfer.

Several methods to generate NK cell products from donor origin for immunotherapy have been reported. In most studies, NK cells are isolated from aphaeresis products using magnetic cell sorting systems. However, poor recovery and viability is a common problem in these procedures, therefore NK cells require cytokine stimulation and expansion before infusion. NK cell numbers up to 7.6 x 10^9 have been reported using this method. Nevertheless, large-scale aphaeresis procedures are necessary to obtain this amount of NK cells, and contaminating T cells can potentially cause GVHD. To circumvent these problems, NK cell generation from CD34+ HSPCs is an attractive option. Yoon et al. recently reported safe infusion of NK cells generated using a feeder-free 6-week culture procedure after HLA-mismatched alloSCT. Using this procedure, an average number of 9.28 x 10^6 NK cells/kg was generated from 2.22 x 10^6 donor CD34+ HSPCs/kg. In our system, the average NK cell expansion from CD34+ HSPCs was 235 fold (range 115 – 904 fold). Therefore, if we use 10% of the G-CSF-mobilized CD34+ HSPCs isolated from donors for alloSCT, we will have on average 0.5 x 10^6 NK cells/kg. In case of a 70 kg donor and patient, we will get on average 8 x 10^9 NK cells (> 1 x 10^8 NK cells/kg). So we will be able to infuse large numbers of NK cells from donor origin using our SR1-based NK cell generation protocol.

In the present study, we showed that the AhR antagonist SR1 greatly enhances NK cell expansion and differentiation. AhR is a ligand-inducible transcription factor which has extensively been studied in the context of its activation by environmental pollutants, such as dioxins and polycyclic aromatic hydrocarbons. Until recently, little was known about the physiological role of AhR, but a growing body of evidence shows that AhR has multiple endogenous activators, and is involved in several physiological processes. Examples of endogenous ligands are metabolites of dietary substances and tryptophan metabolites like cinnabarinic acid, 6-formylindolo[3,2-b]carbazole (FICZ), which can be produced in the skin upon light exposure, and the indoleamine 2,3-dioxygenase 1 (IDO1)-metabolite kynurenin. AhR also regulates differentiation of various immune cells like T helper 17 (Th17) cells, dendritic cells, regulatory T cells, and γδ T cells. Recently, Hughes et al. reported that antagonism of AhR promotes differentiation of immature innate lymphoid cells into NK cells expressing EOMES and TBET. In our SR1-
based HSPC-derived NK cell culture, we also observed upregulation of EOMES, which is involved in NK cell maturation, and additionally we found upregulated expression of TOX, ID2, GATA-3 and EAT-2 after AhR inhibition. TOX is important for early NK cell development, ID2 is involved in NK cell maturation and GATA-3 and EAT-2 are important for NK cell effector functions. Interestingly, expression of TOX markedly decreased in the first week of culture in our culture system in the absence of SR1. In the presence of SR1, TOX expression is more preserved, resulting in significantly higher TOX expression after one week. This suggests that before induction of differentiation by addition of IL-15, early NK cell progenitors are expanded in the presence of SR1, explaining increased NK cell numbers generated in the presence of SR1, IL-15 and IL-2. To our knowledge, this is the first report of an applying AhR blocking in an in vitro system to generate high numbers of functional NK cells.

In conclusion, we developed a cytokine-based ex vivo culture system, which enables us to generate high numbers of very potent NK cells from G-CSF mobilized CD34+ HSPCs. Addition of SR1 to the culture system induced upregulation of multiple NK cell-related transcription factors, and resulted in generation of NK cell products with very high cell numbers and purity. These NK cells have an active phenotype and are highly functional in vitro, therefore they hold great promise for future adoptive HSPC-NK cell therapy after alloSCT.

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References

Chapter 6


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Supplementary Figure 1. SR1 enhances expansion and NK cell differentiation of bone marrow (BM)-derived CD34+ HSPCs, thereby NK cells with a mature and active phenotype can be generated. CD34+ HSPCs were isolated from BM samples. The cells were cultured for 5 weeks in the ex vivo cytokine-based culture protocol described in the Materials and Method section and Figure 1A. (A) Kinetics of differentiation and expansion of CD56+ cells in the presence or absence of 2 µM SR1 from one representative donor. (B) Summary of total expansion, NK cell yield (calculated by [total expansion x percentage of CD56+ cells]/100), and NK cell differentiation after 5 weeks of culture in our ex vivo culture protocol in the presence or absence of 2 µM SR1 in 4 donors. (C) After 5 weeks of culture, expression of NK cell markers was analyzed using FCM. Expression levels and MFI of several NK cell specific surface antigens on living cells. Mean ± SEM of 2 - 3 different donors are shown. Paired one-tailed student’s t-test.
7

English summary
Chapter 7

**Thesis objective**

Though the cure rate for patients with hematological malignancies has significantly increased in the last decades with optimized therapeutic strategies, many patients still succumb to the disease. They are either non-responsive or develop resistance to current therapies, or minimal residual disease remains which can eventually lead to relapse. To date, the most effective and the only curative treatment option available for many aggressive hematological cancers, is allogeneic stem cell transplantation (alloSCT) followed by donor lymphocyte infusion (DLI). AlloSCT has the potential to be curative due to induction of a potent and long-lasting anti-tumor immune response, called the graft-versus-tumor (GVT) response. In the GVT response, it has been shown that donor-derived natural killer (NK) cells and cytotoxic CD8+ T cells specific for minor histocompatibility antigens (MiHAs) and tumor-associated antigens (TAAs) are the prime effector cells that are responsible for elimination of the residual malignant cells. Furthermore, tumor-specific memory T cells are formed after the initial anti-tumor response, which have the potential to convey life-long protection against tumor cell recurrence. However, relapses do frequently occur in transplanted patients, which is the major cause of treatment failure and mortality.

There are various reasons why patients relapse. Patients can relapse early after transplant if the malignant clone grows out before GVT-effector cells are at sufficient levels to control the disease. In this regard the efficacy of the conditioning regimen prior to transplant plays an important role; reduced-intensity and non-myeloablative conditioning regimens that have been developed to make alloSCT feasible for older and medically less fit patients, are associated with higher relapse rates due to less effective reduction of tumor load, thereby hampering productive GVT immunity. Relapse can also occur due to failure of donor-derived lymphocytes to launch anti-tumor responses (*e.g.* minimal/unfavorable MiHA mismatch between patient and donor, insufficient antigen presentation or limited NK cell activation). Additionally, relapse can occur after an initial period of effective GVT immunity, if no long-term memory against the malignant disease is formed, the immune system is weakened or the immune effector cells become tolerant to the residual disease. Furthermore, the malignant cells in some cases employ various mechanisms and establish an immunosuppressive microenvironment, to escape immune recognition and elimination. Besides relapse, other transplant-related complications can arise, such as graft-versus-host disease (GVHD) and opportunistic infections. As alloSCT, despite these limitations, has the potency to cure patients, great efforts are being made to tailor this treatment strategy for improved outcome. These improvements focus on reducing the incidence and severity of GVHD and infectious complications, while boosting GVT immunity to increase the remission rate and duration of remission.

In this thesis, we focused on the pre-clinical development of immunotherapeutic strategies that have the potential to specifically boost the GVT effect, without increasing the risk of GVHD. We describe protocols for *ex vivo* generation of different dendritic
cell (DC) subsets and NK cells from CD34+ hematopoietic stem and progenitor cells (HSPCs), that could potentially be used for DC vaccination and adoptive NK cell transfer, respectively. Furthermore, we evaluated the potential of CLEC12A, an endocytic receptor expressed on the surface of DCs, as a new targeting candidate for in vivo delivery of MiHAs and TAAs.

**DC vaccination using ex vivo-loaded HSPC-derived DC subsets**

DC-based cancer vaccination has been widely investigated for the past two decades due to the central role of DCs in orchestrating adaptive and innate immunity. The basic concept is to use tumor antigen-loaded DCs to induce tumor-specific T cell responses that are capable of lysing malignant cells that express the respective antigen. In this respect, multiple clinical trials using monocyte-derived DCs (MoDCs) have been performed in a wide range of different types of cancer. These trials have demonstrated that DC vaccination is feasible, safe and capable of promoting in vivo tumor-specific immune responses. However, only a minority of patients experiences objective clinical responses, which has impeded routine implementation of DC vaccination in standard clinical practice. Hence, efforts to improve and optimize this personalized immunotherapy are ongoing. One strategy is to optimize the efficacy of the commonly used MoDCs, and is the focus of extensive pre-clinical and clinical research. In this regard, our group is investigating whether the immunogenicity of MoDCs can be enhanced by silencing their expression of co-inhibitory molecules such as PD-L1 and PD-L2.1-3 Furthermore, van den Bergh et al. presented a strategy for enhancing the capacity of MoDCs to engage NK cells in the anti-tumor response, by engineering the DCs to trans-present and secrete interleukin (IL)-15.4,5 Another promising strategy was developed by Prof. Kris Thielemans and his colleagues, where MoDCs are electroporated with mRNA encoding CD40 ligand (CD40L), constitutive active form of TLR4, and CD70 (referred to as TriMix-DCs). The major advantage of this vaccine formula is that the DCs can be infused within few hours after electroporation, which means that they will mature and secrete most of their stimulatory cytokines in vivo.6

Besides increasing the immunogenicity of MoDCs, it is promising to use different DC subsets for vaccination such as plasmacytoid DCs (pDCs), BDCA1+ myeloid DCs (mDCs) and BDCA3+ mDCs. Each of these DC subsets have a distinct role in the immune system and a different cytokine secretion profile. For example pDCs secrete high levels of type I interferons (IFN-α/β), which have pleiotropic effects on multiple cell types and is particularly important for NK cell functions and anti-viral immunity. On the other hand, the mDC subsets secrete interleukin (IL)-12, which together with their high expression levels of major histocompatibility complex (MHC) and co-stimulatory molecules, makes them particularly efficient in generating CD4+ T-helper 1 (Th1) T cell responses and priming naïve CD8+ T cells. Moreover, studies indicate that the different DC subsets act...
synergistically via cross-talk, thereby potentiating each other’s stimulatory capacity. Thus simultaneous vaccination with multiple different subsets might result in a broader and more potent immune response (i.e. due to DC cross-talk and engagement of both T cells and NK cells) and thereby better therapeutic efficacy than “standard” MoDC vaccines. In chapters 2-4, we therefore explored the possibility of generating different DC subsets from HSPCs ex vivo.

In chapter 2, we optimized a protocol for generation of myeloid-like DC subsets from umbilical cord blood (UCB) CD34+ HSPCs. HSPCs were expanded for 11 days in medium supplemented with Flt3L (FMS-like tyrosine kinase 3 ligand), SCF (stem cell factor), IL-3, IL-6 and TPO (thrombopoietin). After 11 days culture, the expanded precursor cells were washed, reseeded and continued in culture in medium containing GM-CSF (granulocyte-macrophage colony-stimulating factor) and IL-4 with or without transforming growth factor (TGF)-β1, thereby inducing their differentiation into DCs. Within this culture system, >60% of the cells co-expressed CD11c and HLA-DR, markers that are characteristic for monocytes and DCs. Furthermore, DCs with a phenotype similar to interstitial/dermal DCs and Langerhans cells (LCs) could be identified, based on the expression of CD1a and CD207 (langerin), respectively. The proportion of langerin-expressing cells was higher in cultures supplemented with TGF-β1, which is in line with findings by others.7,8 These UCB-derived DCs efficiently activated and induced proliferation of both viral antigen-specific and MiHA-specific human CD8+ T cells in vitro, as well as in vivo in immunodeficient mice. However, within this culture system, DCs with a phenotype of genuine blood DC subsets, such as pDCs, BDCA1+ mDCs and BDCA3+ mDCs were not identified. In chapter 3, we therefore explored other means to generate these subsets from CD34+ HSPCs. Here, we discovered that inhibition of the aryl hydrocarbon receptor (AhR), using the antagonist StemRegenin 1 (SR1), significantly promoted the development of pDCs and mDCs from HSPCs in vitro. For this, CD34+ HSPCs were expanded in medium containing Flt3L, SCF, TPO and IL-6 with or without SR1 for three weeks. In SR1-supplemented cultures, the different DC subsets (pDCs, BDCA1+ mDCs and BDCA3+ mDCs) could be identified, while their frequency in cultures without SR1 was negligible. Furthermore, we confirmed that the SR1-induced DC differentiation was AhR-dependent, by addition of the agonist VAF347, which dose-dependently blocked the observed SR1-induced DC development. We further characterized the SR1-generated DC subsets, and found them to be phenotypically and functionally comparable to their peripheral blood counterparts. After Toll-like receptor (TLR) stimulation, they secreted high levels of subset-specific inflammatory cytokines, upregulated co-stimulatory molecules and maturation markers, and were capable of activating T cells.

In chapter 4, we set out to optimize this SR1-based culture protocol further for eventual clinical application, by establishing culture conditions translatable to good manufacturing practice (GMP) which would result in sufficient numbers of functional DC subsets for vaccination. In this study, we focused on the generation of DCs from
CD34+ HSPCs obtained from granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood, as this stem cell source is most commonly used for transplantation of patients with hematological cancers. Hereby, HSPC-derived DC subset vaccines could be generated from a portion of the donor graft to boost GVT immunity post-transplantation. The established GMP-compliant protocol combined aspects from the two different culture systems presented in chapters 2 and 3, with some additional modifications: in brief, HSPCs were expanded for 7-13 days using Flt3L, SCF, TPO (abbreviated as FST) and SR1, where after the culture was split into two differentiation protocols. One part of the expanded cells was continued in culture for one week in FST and SR1-supplemented medium (FST culture) which favoured generation of IFN-α-producing pDCs, though both BDCA1+ mDCs and BDCA3+ mDCs were also present. The other part of the expanded cells was provided with a myeloid differentiation boost by addition of GM-CSF and IL-4 (called G4 culture) which resulted in high numbers of IL-12-producing BDCA1+ mDCs. To the best of our knowledge, this protocol resulted in the highest reported numbers of pDCs, BDCA1+ mDCs and BDCA3+ mDCs that can be simultaneously generated ex vivo from HSPCs using GMP-compliant components, and additionally exceeds the numbers that can be isolated from peripheral blood.

In chapter 4, we next assessed the potential of HSPC-derived pDCs (enriched from the FST culture) and HSPC-derived BDCA1+ mDCs (enriched from the G4 culture) to induce anti-tumor T cell and NK cell responses ex vivo. To this end, BDCA1+ mDCs were superior in priming naïve T cells and expanding patient-derived MiHA-specific CD8+ T cells. These T cells were highly functional, as determined by IFN-γ and degranulation responses upon peptide restimulation. On the other hand, HSPC-derived pDCs were superior in inducing IFN-γ secretion by effector memory T (Tem) cells and cytolytic activity of NK cells, which can likely be attributed to their high secretion of IFN-α. pDC-priming of NK cells resulted in augmented TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) expression and significantly increased lysis of tumor cells. Collectively, these results show that the different HSPC-DCs are potent stimulators of anti-tumor effector cells and display subset-specific characteristics in line with the literature, i.e. the mDCs were better T cell stimulators, while the pDCs were superior in induction of NK cell responses. These results encourage us to believe that a vaccine composed of the different HSPC-derived DC subsets would induce a broader immune response by engaging both adaptive and innate immune effector cells (i.e. T cells and NK cells, respectively), which hopefully would result in superior therapeutic efficacy than has been obtained with previous MoDC-based vaccination trials.

**In vivo-targeting of DCs**

An alternative to using ex vivo-generated and tumor antigen-loaded DCs for immunotherapy, is to target the different DC subsets in vivo. The concept of this approach
is to deliver tumor antigens to the DCs, via antibodies directed against specific antigen-uptake receptors, and simultaneously deliver adjuvants (such as TLR ligands) to promote maturation of the targeted DCs to facilitate antigen (cross-)presentation and activation of cellular immune responses. Hereby, extensive, labour intensive and costly \textit{ex vivo} culturing and handling of DCs can be avoided. Furthermore, \textit{in vivo} targeting has the potential to be more broadly applicable, since the vaccine does not need to be tailor-made for each individual patient. However, this strategy is not yet as far developed as \textit{ex vivo}-loaded DC vaccination and only few clinical trials have been performed using this approach. For implementation, multiple parameters need to be further researched, including a thorough characterization of antigen-uptake receptors.

A potential targeting receptor needs to facilitate efficient delivery of the antigen into intracellular compartments for processing and loading onto MHC. To this end, we have investigated the potential of CLEC12A, an endocytic C-type lectin receptor, for targeting of human DC subsets in \textit{chapter 5}. In this study, we validated the discriminative expression pattern of CLEC12A and confirmed that it is selectively expressed by myeloid cells, including monocytes and granulocytes, and the different DC subsets: BDCA1\(^+\) mDCs, BDCA3\(^+\) mDCs and pDCs. Furthermore, we demonstrated that upon binding by CLEC12A antibodies, CLEC12A is efficiently internalized by all the different DC subsets and traffics to intracellular compartments involved in the antigen-presentation process. Besides facilitating endocytosis, C-type lectin receptors like CLEC12A can also shape immune responses by involvement of their intracellular signaling domains. CLEC12A contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail, and might therefore impair DC maturation and function upon triggering. However, we did not observe negative effects after antibody targeting of CLEC12A on TLR-induced expression of DC maturation markers or cytokine secretion, nor on the capacity of the targeted DCs to induce activation of allogeneic T cells. With regards to antigen delivery, we demonstrated that CLEC12A-dependent antigen uptake lead to efficient antigen presentation to both CD4\(^+\) and CD8\(^+\) T cells by the different DC subsets. We showed that delivery of keyhole limpet hemocyanin (KLH) resulted in expansion of and IFN-γ-secretion by KLH-experienced CD4\(^+\) T cells. Importantly, all primary DC subsets, BDCA1\(^+\) mDCs, BDCA3\(^+\) mDCs and pDCs, were capable of cross-presenting a long MiHA peptide delivered via CLEC12A, resulting in strong \textit{ex vivo} activation of patient-derived MiHA-specific CD8\(^+\) T cells. Cumulatively, these data indicate that CLEC12A is an interesting candidate for targeted antigen delivery into DCs.

**Adoptive transfer of NK cells**

Besides T cells, allo-reactive NK cells are important players in the GVT response and have been associated with reduced relapse rates and prolonged disease-free survival. The beneficial effect is attributed to NK cell activation because of selective (stress-induced)
upregulation of NK-activating ligands and/or downregulation of MHC class I molecules (NK-inhibitory ligands) on the targeted tumor cells. Of importance, allo-reactive NK cells do not cause GVHD, and may even suppress the induction and/or severity of GVHD. These observations have sparked the interest of many research groups to apply adoptive transfer of NK cells as adjuvant therapy post-transplantation, with the goal to eliminate residual or refractory disease without induction of GVHD. Furthermore, NK cells participate in the defence against viral and fungal infections post-transplantation, which additionally makes them attractive for adoptive transfer. Despite reports of clinical efficacy using NK cells isolated from peripheral blood, their use as immunotherapy for cancer has been limited by several factors, including low cell numbers, relatively low activation status and contaminating allogeneic T cells that can cause GVHD. To surpass these limitations, our group has developed a novel GMP-compliant NK cell product by ex vivo differentiation of NK cells from UCB-derived CD34+ HSPCs using a cytokine-based culture protocol. With this approach, high yield of cytotoxic NK cells, devoid of contaminating T and B cells, can be achieved.9,10 These UCB-derived NK cells have recently been examined for safety, toxicity and biological properties in a phase I clinical study. Though the trial was not designed to evaluate clinical benefit, a temporary decrease in residual disease was observed in the bone marrow of some patients after cyclophosphamide/fludarabine conditioning and UCB-NK cell infusion.11 These results are promising for further development of adoptive cell therapy using HSPC-derived NK cells.

In chapter 6, we explored the feasibility of generating NK cells from other HSPC sources than UCB, namely G-CSF-mobilized peripheral blood and bone marrow, for the same reasons as listed above for HSPC-DCs: to facilitate the generation of adjuvant cellular therapeutic products from the stem cell donor. However, adult CD34+ HSPCs have a lower expansion potential and NK cell differentiation capacity than young, UCB-derived CD34+ HSPCs. This illustrates that modifications of the ex vivo NK cell generation protocol were necessary. Therefore, based on findings described in the literature and our own experience with SR1 in the HSPC-DC culture system, we explored whether exposure of CD34+ HSPCs to SR1 would augment their expansion rate and potentiate NK cell differentiation. Indeed, addition of SR1 to our cytokine-based ex vivo NK cell culture protocol significantly enhanced the expansion of adult CD34+ HSPCs. Furthermore, SR1-exposure induced expression of transcription factors important in NK cell differentiation, such as TOX (thymocyte selection-associated HMG box factor), thereby promoting differentiation of the SR1-expanded HSPCs into NK cells. As a result, SR1-addition enabled routine generation of high numbers of CD56+CD3- NK cells from adult HSPC with 83% purity in average. The generated NK cells uniformly expressed high levels of activating NKG2D and natural cytotoxicity receptors (NCRs) and efficiently lysed myeloid leukemia and multiple myeloma cell lines, as well as patient-derived acute myeloid leukemia (AML) blasts. Interestingly, we observed that SR1-generated NK cells showed enhanced functional activity compared to NK cells generated in the absence of SR1: they had elevated expression
of DNAM-1 and TRAIL, secreted higher levels of IFN-γ and granzyme B, and exhibited increased cytolytic potential. Furthermore, SR1 augmented their expression of the homing receptors CD62L and CXCR3, which could promote trafficking of HSPC-NK cells towards hematological tumor cells in lymphoid organs.

We are interested in exploring the application of these SR1-generated NK cells as adjuvant therapy shortly after alloSCT, however patients then frequently receive the immunosuppressive drugs cyclosporin A (CsA) and mycophenolic acid (MPA) for suppression of allo-reactive T cells and prevention of GVHD. Therefore, we studied the effect of CsA and MPA on the viability, proliferation and functionality of the generated NK cell product in vitro. Therapeutic concentrations of CsA did not negatively affect any of these parameters, which indicates that HSPC-NK cell therapy could be given parallel to CsA administration (which usually lasts at least 6 months post-transplantation). In contrast, MPA inhibited the proliferation, viability, cytolytic potential and IFN-γ secretion of the NK cells. MPA treatment is generally only applied in the first month post-transplant, thus indicating that NK cell adoptive transfer could be realised from the 2nd month onwards.

Conclusion

In conclusion, this thesis describes the pre-clinical development of various immunotherapeutic strategies that could potentially be applied as adjuvant therapies post-alloSCT to improve its therapeutic efficacy. The HSPC-derived DC subsets, presenting hematopoietic-restricted MiHAs or TAAs, could potentially be used as vaccine post-alloSCT for selective induction of GVT immunity. Alternatively, targeted nanovaccines, containing MiHAs and/or TAAs, could be directly delivered to multiple DC subsets in vivo via the C-type lectin receptor CLEC12A. Finally, HSPC-NK cells could be applicable for adoptive transfer early after alloSCT, to mediate tumor clearance and anti-viral defense/control. Besides the potential therapeutic implication of these strategies, the DC- and NK-generation protocols described in this thesis may provide a tool for mechanistic studies of human DC and NK cell development and biology ex vivo. Further discussion on the findings described in this thesis, and their potential implication in future treatment schemes is presented in chapter 8.
References


General discussion and future perspectives
Aryl hydrocarbon receptor antagonism for generation of immunocellular therapeutic products

In chapters 3 and 6, we describe how the synthetic small-molecule compound StemRegenin 1 (SR1) greatly improves ex vivo generation of dendritic cells (DCs) and natural killer (NK) cells from CD34+ hematopoietic stem and progenitor cells (HSPCs).1,2 SR1 was identified by Boitano et al. in 2010, when they performed a library screening for a compound that could promote ex vivo expansion of CD34+ HSPCs.3 In their study, SR1 induced a 17-fold increase in the number of cells with engraftment capacity in immunodeficient mice. The therapeutic potential of SR1 was tested in the allogeneic stem cell transplantation (alloSCT) setting, where transplantation with SR1-expanded umbilical cord blood (UCB)-HSPCs resulted in significantly faster engraftment in patients as compared to unmanipulated UCB units.4 The discovery of SR1 has improved the potential of UCB units as HSPC source for alloSCT in the future, which so far was limited due to the low number of cells in UCBS. This facilitates the selection of suitable donor for adult patients in need of SCT.

Boitano et al. demonstrated that SR1 is an antagonist of the aryl hydrocarbon receptor (AhR).3 The AhR is a ligand-inducible transcription factor belonging to the basic helix-loop-helix transcription factor family.5 AhR regulates the transcription of a number of genes, involved in an array of physiological and pathological pathways. It is best known for its dominant role in xenobiotic-induced toxicity and carcinogenesis, as it mediates transcriptional responses to environmental toxins such as TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) and polycyclic hydrocarbons. These toxins are the prototypic ligands for AhR, and have been extensively studied. However, once AhR knockout mice were investigated, the additional role of AhR in normal developmental and physiological pathways became evident. These mice showed developmental defects in the reproductive, hepatic, hematopoietic and cardiovascular system. Furthermore, in vitro and in vivo studies demonstrated an important role of AhR within the immune system, including regulation of the acute phase response, Th17 (T-helper (Th) type 17) immune cell differentiation and modulation of NF-κB activity and cytokine signaling.6-9 These findings supported the existence of endogenous AhR ligands.10 The majority of the natural/endogenous AhR ligands that have been identified so far are either heme-, arachidonic acid-, or tryptophan-derived metabolites.11 Heme-derived metabolites include bilirubin and biliverdin, while examples of arachidonic acid metabolites are prostaglandins, such as PGG2 (prostaglandin G2). However, the most prominent endogenous AhR ligands are derived from tryptophan metabolism. These include compounds derived from 1) indoleamine 2,3-dioxygenase (IDO) or tryptophan-2,3-dioxygenase activity (e.g. kynurenine, kynurenic acid and xanthurenic acid) 2) serotonin pathway (e.g. 5-hydroxytryptophan) 3) photometabolism, i.e. metabolism in response to UV-light exposure (e.g. 6-formylindolo[3,2-b]carbazole (FICZ)), and 4) metabolism by commensal gut microflora of dietary substances (e.g. indirubin, indigo and indole-3-carbinol). Given the versatility
of ligands, they probably act in a tissue- and context-specific manner. Furthermore, some
bind with high affinity, while others are only weak agonists or found at very low levels that
are likely insufficient to activate the AhR. It could also be that endogenous ligands exist
that have an antagonistic effect on AhR activity. However, these have not been described
so far.

Upon activation, the prototypic way of AhR-mediated gene transcription is as follows\(^5\).
In its non-activated/unligated state, AhR resides in the cytoplasm, but upon ligation
it translocates to the nucleus where it forms a heterodimer with ARNT (AhR nuclear
translocator). This heterodimer binds to so called xenobiotic responsive elements (also
termed dioxin responsive elements (DREs)) located in the upstream regulatory region of
target genes, thereby initiating transcription. AhR signaling is fine-tuned and controlled
by an elegant negative feedback loop, where the AhR induces expression of the AhR
repressor (AhRR), which then competes with AhR for heterodimerization with ARNT.
As AhRR-ARNT heterodimers are transcriptionally inactive, this abolishes AhR-induced
gene expression. AhR-mediated gene regulation is though more complex than this, as AhR
does not only induce transcription of genes, but can also repress gene expression, even
in a DRE-independent manner. It has for example been reported that AhR regulates the
proteasome-dependent degradation of specific transcription factors, such as the estrogen
receptor \(\alpha\) (ER\(\alpha\)), by acting as a ligand-dependent E3 ubiquitin ligase.\(^12\) AhR activation can
also facilitate gene transcription by indirectly promoting binding of other transcription
factors to the genome. One mechanism through which this occurs is that AhR binds to
DREs upstream of the transcriptional start site, where it dismisses histone deacetylase
(HDAC)-containing co-repressor complexes, thereby lifting the repressive state of the
promoter. This allows other transcription factors and RNA (ribonucleic acid) polymerase
to bind to the promoter and initiate transcription.\(^13,14\) Thus, the AhR may either stabilize or
promote the binding of other transcription factors to the genome, or inhibit the functions
of those proteins by promoting their degradation. These examples illustrate that AhR
signaling and its regulatory activity is very complex to study.

Because of the findings of Boitano \emph{et al.}, we tested the addition of SR1 to improve
expansion of HSPCs when we were developing a culture protocol for \emph{ex vivo} generation of
plasmacytoid and myeloid DCs (pDCs and mDCs, respectively) from HSPCs. By that time,
there were no publications describing that AhR was involved in human DC development,
besides one publication implicating AhR in Langerhans cell (LC) differentiation.\(^15\) It
was thus an exciting finding when we noticed that SR1 addition strongly promoted
differentiation of pDCs, BDCA1\(^+\) mDCs and BDCA3\(^+\) mDCs in our cultures, even before
we initiated specific DC differentiation protocols. This was probably one of the more
memorable days of mine in the lab during the PhD track. During the revision of our
manuscript, Liu \emph{et al.} corroborated our findings by showing that AhR knockout mice have
a higher frequency and number of pDCs in lymphoid organs compared to wild-type mice,
and that \emph{ex vivo} FMS-like tyrosine kinase 3 ligand (Flt3L)-induced pDC differentiation
from mouse bone marrow HSPCs is promoted by AhR antagonism (using CH223191 compound) and inhibited by AhR activation (using TCDD).\textsuperscript{16} However, in their models, mDC development was not affected, illustrating that there might be intrinsic differences in AhR involvement in human and murine DC development. We focused on the direct clinical utilization of our discovery (\textit{i.e.} generation of HSPC-pDC/mDC vaccines) and did not look further into the mechanism underlying SR1-induced DC differentiation, though of course this is highly interesting. DC development is governed by complex transcriptional control, where different transcription factors are involved in the (later stages of) differentiation of each subset.\textsuperscript{17} The E2-2/TCF4 transcription factor is a central regulator of pDC differentiation, BATF3 regulates BDCA3\textsuperscript{+} mDC differentiation and IRF4 has been implicated as the transcription factor regulating BDCA1\textsuperscript{+} mDC development. Other transcription factors that have been implicated to play a pivotal role in DC development, but are not specific for one subset, include GATA2, ID2, IRF8, PU.1 and members of the NF-κB pathway. AhR could influence DC development by either directly or indirectly regulating the expression or activity of one or more of these transcription factors. It is interesting that AhR inhibition promoted the differentiation of all the different DC subsets, pDCs as well as mDCs, indicating that AhR-mediated regulation is involved somewhere early in the DC developmental pathway, before pDC- and mDC-specific transcription factors become active. On the other hand, it could be that AhR influences pathways common to later stages of differentiation. A publication by Platzer \textit{et al.}, implies that AhR controls human monocyte and LC differentiation by modulation of PU.1 expression.\textsuperscript{15} They demonstrate that AhR activation (using the agonist VAF347) inhibits PU.1 induction, and that the lack of PU.1 results in the arrest of monocyte and LC development at a precursor cell stage. Interestingly, Angelos \textit{et al.} recently demonstrated that SR1 inhibition results in upregulation of PU.1 (and also GATA2) during \textit{ex vivo} differentiation of CD34\textsuperscript{+} HSPCs from human embryonic stem cells.\textsuperscript{18} Studies in murine models have shown that deletion of PU.1 hampers pDC and mDC development.\textsuperscript{19,20} Thus it could be that SR1-induced DC differentiation might be related to AhR-mediated control of PU.1 transcriptional activity in HSPCs. AhR-mediated regulation of DC differentiation could also be on the cytokine level, such as by maintaining interleukin (IL)-6 secretion. We and others have demonstrated that IL-6 hampers DC differentiation (chapter 4).\textsuperscript{21-23} Multiple studies have shown that natural AhR ligands are present at biologically active concentrations in culture medium, which can contribute to basal activation of the AhR during cell culture.\textsuperscript{24,25} These ligands are primarily tryptophan-derived metabolites, that form due to light exposure or IDO activity of the cultured cells.\textsuperscript{25,26} Studies have shown that \textit{in vitro} AhR activation (by \textit{e.g.} kynurenic acid) synergistically enhances NF-κB-induced IL-1β and IL-6 secretion (by the before-mentioned mechanism where ligated AhR lifts the repressive state of promoters, allowing binding of other transcription factors, in this case NF-κB).\textsuperscript{7,13,14,27-29} Furthermore, DiNatale \textit{et al.} showed that IL-6 signaling can be abrogated in tumor cell lines that depend on autocrine IL-6 for their growth by
applying AhR antagonists.\textsuperscript{27} As we observed that IL-6 strongly hampers SR1-induced DC differentiation (chapter 4), it is tempting to speculate whether SR1 improves DC differentiation by blocking basal levels of AhR-induced IL-6 secretion. Why and how IL-6 inhibits DC differentiation needs to be further elucidated, just like AhR involvement itself. Furthermore, it also remains to be demonstrated to which extend these pathways are involved in natural DC development \textit{in vivo}.

The role of AhR inhibition in NK cell development is more extensively researched than in DC development. In our \textit{ex vivo} NK-generation culture protocol, addition of SR1 enhanced proliferation of HSPCs and promoted NK cell differentiation, resulting in high numbers of functional NK cells.\textsuperscript{1} Beside our study, three other studies have shown that \textit{ex vivo} AhR inhibition, using for example SR1, promotes differentiation of human NK cell precursors into interferon (IFN)-\textgamma producing, cytolytic mature NK cells.\textsuperscript{18,30,31} NK cells belong to the group of innate lymphoid cells (ILCs), and differentiate (at least partially) from bone marrow CD34\textsuperscript{+} HSPCs via the same pathway as IL-22- and IL-17-producing ILCs.\textsuperscript{32} Hughes \textit{et al.} identified one potential NK cell differentiation pathway, where NK cells differentiate from ILCs that secrete IL-22 and highly express IL-1 receptor type 1 (called ILC3s).\textsuperscript{30} They demonstrated that this transition, from ILC3 to NK cell, is controlled by AhR activity. They show that ILC3s have high expression of AhR, and that silencing or blocking AhR reduces RORC expression (\textit{i.e.} the main transcription factor used for characterizing ILC3s), while it promotes expression of the transcription factors TBET and EOMES. EOMES in particular, is indispensable for NK cell differentiation. During our SR1-induced NK cell differentiation from CD34\textsuperscript{+} HSCPs, we also saw EOMES gradually increase during the culture. It would be interesting to investigate whether we can identify the ILC3-NK cell transition stage described by Hughes \textit{et al.} as an intermediate stage in the differentiation of NK cells from CD34\textsuperscript{+} HSPCs in our culture system, and whether increased SR1-mediated transition through this stage is responsible for the increased NK cell differentiation we see. It could also be that SR1 blockade enhances NK cell differentiation because of AhR-mediated control at other stages of NK cell development. Interestingly, we observed a significant SR1-mediated increase in TOX expression after 1 week expansion of CD34\textsuperscript{+} HSPCs, before NK differentiation was induced by addition of IL-15.\textsuperscript{1} TOX is a multifunctional transcription factor, that has been demonstrated to be pivotal for development of ILCs, including NK cells.\textsuperscript{33-35} Seehus \textit{et al.} demonstrated that TOX deficiency resulted in early defects in the survival or proliferation of ILC progenitor cells in mice, and also affected ILC differentiation at a later stage.\textsuperscript{34} Thus it might be that AhR is involved in different developmental phases of NK cells in humans, but that, as well as the molecular mechanism behind AhR-mediated control of NK cell development, needs to be further elucidated.

Cumulatively, this overview shows that AhR regulation of immune cell differentiation and functionality is very complex and not yet well understood. But the identification of SR1 has provided means of exploiting this pathway for more efficient generation of
cellular immunotherapeutic products for clinical application. It would be interesting to see if addition of SR1, or other AhR-modulating agents, would be suitable for enhancing the efficiency and potency of other cellular products that are generated ex vivo.

**Primary DC subset vaccinations and synergistic cross-talk**

It is postulated that by using primary DC subsets, such as pDCs and mDCs, better efficacy of DC vaccination may be reached than by using monocyte-derived DCs (MoDCs).\(^{36}\) However, the low frequency of primary DC subsets in blood has limited their use in DC vaccination trials so far. Nevertheless, three trials have recently been performed where pDCs or BDCA1\(^+\) mDCs isolated from peripheral blood of patients were used. Prof. de Vries and colleagues performed the first clinical study utilizing activated pDCs,\(^{37}\) and also carried out a trial with BDCA1\(^+\) mDCs.\(^{38}\) Both these trials were performed in end-stage melanoma patients. To allow maximal efficacy of low DC numbers, vaccines were directly administered to the lymph nodes. Although these trials were designed as safety and feasibility studies, promising immunological and clinical results were obtained. Patients treated with activated and antigen-loaded pDCs showed an enhanced systemic expression of IFN-α-induced genes, indicating systemic secretion of type I IFNs, and a portion of the patients increased their number of circulating tumor-specific CD4\(^+\) and CD8\(^+\) T cells. Remarkably, pDC vaccinated patients had significantly improved overall survival compared to historical control patients treated with standard chemotherapy. However, their progression free survival was similar to these controls. Unfortunately, NK cell responses were not reported for these patients, which would have been very interesting to observe in the light of the enhanced IFN-α signature. Out of the 14 melanoma patients treated with BDCA1\(^+\) mDCs, three patients had detectable multifunctional tumor-specific CD8\(^+\) T cells in their circulation following vaccination. Notably, the presence of these T cell responses coincided with significantly improved progression-free and overall survival. The third clinical trial using primary DC subset was reported by Prue et al.,\(^{39}\) where metastatic prostate cancer patients were vaccinated with immature, tumor peptide-loaded BDCA1\(^+\) mDCs.\(^{39}\) In this trial, no tumor-specific immune responses were detected. In the melanoma trial, GM-CSF (granulocyte-macrophage colony-stimulating factor) was used for maturation of the BDCA1\(^+\) mDCs, while immature DCs were used in the trial of Prue et al. For both trials, one can hypothesise that a stronger maturation signal, e.g. with TLR (Toll-like receptor) ligands, might have induced a more robust immune response than was currently achieved, as non-activated or improperly activated DCs can cause T cell tolerance rather than productive T cell immunity. Importantly, these three trials demonstrated the feasibility of isolating primary DC subsets from blood and using them for vaccination studies, and the results are promising for future exploration of their capacity to induce productive anti-tumor immunity.
These three trials investigated the potency of each subset separately, however it would be very interesting to combine the different subsets in one vaccine for two reasons. First, a combined vaccine, where both pDCs and mDCs are present, might induce a broader immune response (e.g. potent T cell priming as well as NK cell stimulation) because the specialized function of each subset is harnessed. Second, there are reports that the different DC subsets interact, and that bi-directional crosstalk between them can potentiate the function of each subset, resulting in enhanced anti-tumor responses (reviewed by Bakdash et al.\textsuperscript{40}). Murine tumor models have demonstrated that vaccines that either are composed of both pDCs and mDCs,\textsuperscript{41} or mDCs that were activated in the presence of pDCs,\textsuperscript{42} induce enhanced T cell responses against the tumor and generate long-term anti-tumor immunity. The observed synergistic efficacy can be attributed to pDC-derived IFN-α and pDC-mDC cell contact, which enhances the differentiation, maturation and cross-priming capacity of mDC subsets.\textsuperscript{43,44} In vivo administration of TLR agonists has also been shown to result in synergistic activation of DCs. For example, injection of pDC-activating TLR ligands results in tumor clearance and protective anti-tumor immunity by activating pDCs which subsequently results in activation and engagement of residential mDCs to present tumor-derived antigens.\textsuperscript{42,45} This effect is not only mediated by direct cross-talk between the different DC subsets, but also involves other innate immune cells, such as NK cells and NKT cells.\textsuperscript{45-48} Liu et al. demonstrated that adoptively transferred TLR-activated pDCs enhanced the recruitment and activation of NK cells to the tumor site, and their data indicate that the NK cells, through their lysis of tumor cells and IFN-γ secretion, provided mDCs with antigens and additional maturation signals and T cell cross-priming capacities.\textsuperscript{45} CpG-activated pDCs have also been shown to license NKT cells to interact with mDCs, which then can activate NKT cells through CD1d-presented lipids.\textsuperscript{47,48} NKT cells do not have a strong cytolytic capacity by themselves, but they provide IFN-γ and IL-2 which is important for the activation of cytotoxic NK cells and T cells.\textsuperscript{49,50} In addition, NKT cells can also help with further maturation and enhanced cross-priming capacity of the DCs.\textsuperscript{46} Most of these data are derived from mouse experiments, but DC cross-talk has also been demonstrated in the human setting.\textsuperscript{44,51-53} For example a study by Beek et al. demonstrated that human BDCA\textsubscript{1} mDCs and pDCs cross-activate each other when co-cultured in vitro, enhancing DC-induced NK cell-mediated cytotoxicity.\textsuperscript{53} Cumulatively, these data demonstrate that both pDCs and mDCs are important for the formation of long-lasting anti-tumor immunity, and that vaccination using multiple DC subsets is therefore a very promising strategy (Figure 1). The first clinical studies using a combination of pDCs and BDCA\textsubscript{1} mDCs for vaccination are ongoing in patients with melanoma\textsuperscript{54} (the MIND-DC (Melanoma patients Immunized with Natural Dendritic Cells) study) and prostate cancer\textsuperscript{55} at the Radboudumc lead by Prof. de Vries. The results of these studies are eagerly awaited.
Chapter 8

Figure 1. The ultimate aim with DC subset vaccination strategies. Each DC subset has unique stimulatory capacity and features which makes them attractive for vaccination, but they are also capable of potentiating each others’ stimulatory capacity via cross-talk. DC subset vaccination is therefore a promising approach for generating a broad and potent anti-tumor immune response. Cancer patients could be vaccinated either with TLR-activated *ex vivo*-loaded (HSPC)-DC subsets or with *in vivo*-targeting nanovaccines containing TLR ligands and tumor antigens. After vaccination, the activated DCs interact with immune effector cells *in vivo*, via cell surface molecules (e.g. MHC-TCR interaction, and co-stimulatory molecules) and cytokine secretion (e.g. IL-12 and IFN-α). Subsequently, these cells become activated and acquire effector functions (e.g. IFN-γ, IL-2 and granzyme secretion). Interaction of DCs with CD4+ T cells promotes pro-inflammatory Th1 responses, which provides additional help to CD8+ CTLs and NK cells for strong and sustained effector functions. Eventually, the activated CTLs and NK cells kill the tumor cells. The activated tumor-specific CD4+ and CD8+ T cells can thereafter become long-lived memory cells, important for long-term remission and tumor control in patients. pDC, plasmacytoid dendritic cell; mDC, myeloid DC; HSPC, hematopoietic stem and progenitor cell; MHC, major histocompatibility complex; CSM, co-stimulatory molecule; IFN, interferon; IL-12, interleukin-12; NK cell, natural killer cell; Th1, T helper type 1; CTL, cytotoxic T lymphocyte; TCR, T cell receptor; TLR, Toll-like receptor.

Clinical implementation of HSPC-DC vaccines

Combined vaccination with pDCs and mDCs is also an interesting approach in the alloSCT setting, as an adjuvant therapy for boosting GVT immunity to prevent relapse of haematological malignancies. GVT immunity is primarily mediated by donor-derived allo-reactive NK cells, as well as CD8+ cytotoxic T lymphocytes (CTLs) specific for minor histocompatibility antigens (MiHAs) or tumor-associated antigens (TAAs). These tumor-reactive lymphocytes are either derived from the stem cell graft or from a donor lymphocyte infusion (DLI) administered post-transplantation after stopping immunosuppression. DLI is one of the current adjuvant therapies applied for treatment and prevention of relapse. Despite the induction of tumor-reactive responses in patients
treated with DLI, the numbers and/or functionality of the immune effector cells are often inadequate for keeping the tumor growth under control. Thus, for boosting the efficacy of the alloSCT and DLI, few clinical trials performed by us and others have investigated the accompanying infusion of MiHA- or TAA-loaded MoDCs. These studies demonstrated that DC vaccination in this setting is safe and does not induce or exacerbate graft-versus-host disease (GVHD). However, similar to MoDC vaccinations in solid cancers, objective clinical responses have been limited. We envision that by using pDC and mDC vaccines instead of MoDCs, better clinical efficacy might be reached because of the simultaneous induction of both donor-derived tumor-specific T cells and cytotoxic NK cells, and the potential of the DC subsets to synergistically enhance each other’s stimulatory capacity. Instead of isolating the primary DC subsets directly from peripheral blood of the donors, chapters 3 and 4 of this thesis describe the development of a protocol to generate genuine DC subsets from CD34+ HSPCs of the donor stem cell graft. By generating the DCs from HSPCs, higher numbers of DCs for multiple vaccination rounds can be achieved than would be possible by isolation from peripheral blood. Furthermore, direct generation of DC subset vaccines from a small part of the stem cell graft would limit the burden for the donor, as a second apheresis would not be needed. We demonstrated that the HSPC-derived DC subsets are highly potent inducers of ex vivo tumor-reactive T cell and NK cell responses, with the generated BDCA1+ mDCs being better T cell stimulators, while the pDCs were superior NK cell stimulators. Their potential synergistic interplay remains to be further explored, as well as their in vivo efficacy.

We would like to further explore the pre-clinical development of HSPC-derived DC subsets and eventually translate it into a clinical study, where patients are vaccinated with MiHA- and/or TAA-loaded HSPC-DCs post-transplantation. With careful selection of MiHA that have a hematopoietic-restricted expression, and/or TAAs that are highly expressed by the malignant cells, a specific GVT immunity could be induced, with minimal risk of inducing GVHD. But before HSPC-DCs can be tested in the clinic for assessing feasibility, safety and efficacy, multiple aspects need to be further investigated. We need to develop and validate a (closed) culture system for large-scale production of HSPC-DCs under GMP (good manufacturing practice) conditions, and, if needed, optimize the culture conditions accordingly to obtain high yields of the different DC subsets. Furthermore, we need to investigate whether isolation or enrichment of the different DC subsets will be needed, or whether the whole bulk of cultured cells can be used as vaccine. To be able to use the whole bulk as a vaccine, we need to exclude that the non-DCs in the culture (which are mainly monocytic, myelocytic and other hematopoietic progenitor cells) exert suppressive effects on the activation and function of tumor-reactive effector cells. Additionally, we need to determine which setting would be most optimal for the maturation of the DCs. For example, is it best to simultaneously activate the pDCs and mDCs during co-culture or to stimulate them separately? Here we need to take into account whether isolated DCs or whole bulk would be used. Related to this,
other questions need to be resolved, such as which maturation cocktail/agent should be used and what is the ideal duration of stimulation? And dependent on whether DCs are co-activated or not; should they be injected separately or together? If together, at which ratio? If separately, should they be infused simultaneously (e.g. at the same or different location) or consecutively (e.g. on different days)?

The protocol chosen for maturation of DCs for vaccination should ideally generate DCs that secrete pro-inflammatory and Th1-skewing cytokines/chemokines and highly express co-stimulatory receptors, antigen-presenting molecules and lymph node homing receptors such as CCR7. Pre-clinical studies have demonstrated that the most optimal maturation can be achieved by using TLR agonists, with or without pro-inflammatory cytokines. TLR agonists are for example essential for proper secretion of the Th1-skewing and CTL-stimulatory cytokine IL-12 by BDCA1+ mDCs and BDCA3+ mDCs. Moreover, numerous studies have shown that combination of two or more TLR agonists, for example R848 (Resiquimod; TLR7/8 agonist) with Poly I:C (Polyinosinic:polycytidylic acid; TLR3 agonist) or LPS (Lipopolysaccharide; TLR4 agonist), induces significantly higher IL-12 secretion than stimulation with only one TLR agonist.60-63 Gautier et al. report a role for secreted IFN-α that works in an autocrine loop to induce the observed synergism. 60 These results were corroborated by Kreutz et al., who additionally demonstrated how IFN-α production, initiated upon ligation of one TLR, induces expression of other TLRs, even TLRs that have been reported to be absent in the steady state.64 For example, poly I:C-induced IFN-α secretion resulted in upregulation of TLR7 in BDCA1+ mDCs, which thereby rendered them more responsive to TLR7 agonists, facilitating amplified cytokine production upon dual stimulation with Poly I:C and R848.44 This same mechanism likely plays a role in the synergistic cross-talk between the different DCs subsets that is discussed above, i.e. pDC-derived IFN-α may directly modulate the TLR levels of mDCs, making them more responsive to danger signals in the local environment. Because of the synergistic induction of IL-12 production upon dual TLR stimulation, we chose to use a combination of R848 and Poly I:C for activating HSPC-derived mDCs in our studies (chapter 3 and 4). In line with literature, this maturation regimen resulted in IL-12 secretion, and the DCs could efficiently activate both anti-tumor T cell and NK cell responses.2,22 In general, these findings provide a rational for combining multiple TLR agonists for maturation of the primary DC subsets in the DC vaccination setting. Care needs to be taken though, that for simultaneous maturation of co-cultured mDCs and pDCs, a maturation mix/agent would be needed that optimally activates all DC subsets, without interfering with the maturation of the other subset(s). The TLR repertoire of pDCs is more limited than of mDCs, as they primarily express TLR7 and TLR9, which can be activated using synthetic agonists such as R848 and CpG oligodeoxynucleotides (CpG ODNs), respectively. In our studies, we stimulated the HSPC-derived pDCs with CpG-A which induces high secretion of type 1 IFNs. We also used R848 which induces lower IFN levels than CpG-A, but stimulates better phenotypical maturation (i.e. upregulation of
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Combining TLR7 or TLR9 agonists with the agents that are most favourable for mDCs, for simultaneous maturation of pDCs and mDCs, is not straightforward. For example, studies show that CpG ODNs can have an inhibitory effect on poly I:C-induced maturation of mDCs.\textsuperscript{53,65} Moreover, TLR7 agonists have been demonstrated to inhibit CpG-induced IFN-α production.\textsuperscript{66-68} These findings likely restrict the selection of maturation agent for both subsets to just one TLR agonist. Even though pDCs and mDCs do not have an overlapping TLR repertoire, there is one type of danger signal that both subsets can respond to, which is single stranded RNA (ssRNA). ssRNA and synthetic analogs such as R848 are recognized by TLR7 (by pDCs) and TLR8 (by mDCs), and are therefore possible candidates to use for simultaneous pDC and mDC maturation. Besides the issues with distinct TLR responsiveness of the different DC subsets, the choice and combinations of stimulants are also limited due to the scarce availability of clinical-grade TLR agonists. In this regard, Skold et al. recently developed a formula based on clinically applicable reagents, where RNA is stabilized with protamine to use as universal DC stimulation agent.\textsuperscript{69} These protamine-RNA complexes induced a mature phenotype of both pDCs and BDCA1⁺ mDCs, and their respective secretion of IFN-α and IL-12 \textit{in vitro}. In the ongoing clinical trial with primary pDCs and mDCs in patients with prostate and melanoma cancers mentioned above, protamine-RNA is used as the maturation agent.\textsuperscript{54,55} It would be interesting to evaluate whether protamine-RNA or other TLR7/8 agonists could be utilized in the clinical implementation of HSPC-DC vaccines.

Besides finding the optimal maturation agent, other strategies could be applied to enhance the immunogenicity of HSPC-DC vaccines. As the culture protocol for generation of the DC subsets from CD34⁺ HSPCs takes 2-3 weeks, there is room to perform additional \textit{in vitro} manipulations. Mature DCs do not only express T cell co-stimulatory molecules on their surface, but also co-inhibitory molecules (CIMs), such as PD-L1 and PD-L2 (programmed death-1 (PD-1) ligand 1 and 2, respectively). Multiple studies demonstrated how interference with the PD-1/PD-L pathway augments the activation and expansion of tumor-reactive T cells. In our group we have developed a strategy to silence PD-L molecules on the surface of MoDCs, by PD-L siRNAs (small interfering RNAs) transfection technology. These PD-L-silenced MoDCs have superior efficacy in boosting MiHA-specific T cell responses, both \textit{in vitro} and in murine models,\textsuperscript{70-73} and are currently being investigated for safety, toxicity and immunostimulatory capacity in a phase I study. Preliminary data of the first four patients show promising results, where three of them show a clear increase in the frequency of MiHA-specific T cells post-vaccination (personal communication W.Hobo). It would therefore be interesting to silence these molecules during generation of HSPC-DCs, for enhanced vaccine efficacy. Next to the PD-1/PD-L1 pathway, there are other inhibitory molecules expressed by T cells that can interfere with induction of anti-tumor immunity, such as LAG-3 (lymphocyte-activating gene 3), TIM-3 (T cell immunoglobulin and mucin-domain containing 3) and
TIGIT (T cell immunoreceptor with Ig and ITIM domains).\textsuperscript{74,75} Interestingly, recent studies demonstrated that also NK cells express PD-L1, TIM-3 and TIGIT, and indicated that these molecules are involved in inhibiting NK cell function following ligation with cognate ligands on DCs, tumor cells and other cells in the tumor microenvironment.\textsuperscript{49,76-79} Further research is needed to elucidate the specialized function of these molecules and whether modulation of these pathways, in T cells and NK cells, has therapeutic implications. It would be interesting to map the expression of TIM-3 and TIGIT ligands on HSPC-DCs and to investigate whether silencing their expression level (in addition to silencing PD-1 ligands) can improve DC-mediated activation of NK cells and T cells.

**In vivo-targeted DC vaccination**

The powerful antigen-presenting capacity of DCs, the specialized features of the different DC subsets and their potential synergistic cross-talk can also be utilized for induction of anti-tumor immunity by direct antigen-targeting \textit{in vivo} (Figure 1).\textsuperscript{80,81} In this setting, besides the delivery of an antigen to the DCs, it is pivotal to simultaneously deliver a maturation agent (e.g. TLR agonists), for induction of productive immune response rather than tolerance. Moreover, studies demonstrated that delivery of antigens and TLR agonists to the same cellular compartment is essential for effective antigen presentation.\textsuperscript{82-85} The most promising approach for achieving such co-delivery is encapsulating the antigens and maturation agent(s) in a polymer-based or lipid-based nanoparticles.\textsuperscript{80} Furthermore, linking these nanoparticles to antibodies directed against endocytic receptors expressed on the surface of the DCs is an elegant strategy for specific delivery of the cargo into DCs and induction of anti-tumor immune responses. Multiple endocytic receptors have been studied for this purpose.\textsuperscript{86} These receptors have varying cell type specificity, some are specifically expressed by only one DC subset, while others are more broadly expressed. Thus the choice of targeting receptor will determine to which cell the antigen is delivered. As activation of tumor-specific CD8\(^+\) CTLs is instrumental for productive anti-tumor immunity, DC subsets that are capable of cross-presentation are considered the most interesting targets. Interestingly, a study by Idoyaga \textit{et al.} implies that the targeted DC subset and not the antigen-capturing receptor is a dominant force that determines the magnitude of T cell responses following directed antigen delivery.\textsuperscript{87} They demonstrated how antigen targeting to murine CD8α\(^+\) mDC subsets (the superior cross-presenting DC subset in mice) induced a stronger immune response than via CD8α\(^-\) mDCs, no matter whether the antigen was delivered via DEC-205, CLEC9A or Langerin.\textsuperscript{87} This notion may not necessarily apply to the human setting, because the functional division between the two human mDC subsets is not as straightforward as in mice. Based on their (gene) expression profile and excellent cross-presentation capacity, human BDCA3\(^+\) mDCs have been described as the human equivalent of mouse CD8α\(^+\) mDCs.\textsuperscript{88} However, we (chapter 5) and others demonstrated that BDCA1\(^+\) mDCs are also highly capable of priming
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CD8+ T cells, and are in many settings equally efficient at cross-presentation as BDCA3+ mDCs.89-94 Human pDCs have also been shown to be very good cross-presenters.89,90,94 However, studies have shown that pDCs have a limited ability to phagocytose large particles and dead cells, making them less efficient at cross-presenting particulate or necrotic cell-derived antigens.89,94 Nevertheless, they process and (cross-)present small exogenous antigens, and antigens that are delivered via endocytic receptors with equal efficiency as the mDC subsets.89,94 Thus, in the human setting, it is most interesting to assess the efficacy of targeting multiple DC subsets at once, to maximize the potency of in vivo-targeted vaccines. There are few candidate receptors that are universally expressed by all the primary human DC subsets. These include DEC-205, CD40, CD32 and CLEC12A.86,89 Importantly, it has been demonstrated for CLEC12A (chapter 5) and the other three receptors that they can facilitate in vitro targeted delivery of antigens to pDCs, BDCA1+ mDCs and BDCA3+ mDCs, leading to uptake, processing and (cross)-presentation of antigens to both CD4+ and CD8+ T cells.89,91,93,95,96 The different studies are not yet conclusive whether one receptor is superior for cargo delivery over another, as the different receptors have not been compared head-to-head for all the different DC subsets. Of note, when performing such comparisons, the targeting compounds need to have similar characteristics, such as similar amount of coupled antigen and antibodies of similar affinity. As this can be technically challenging to obtain, no proper head-to-head comparisons have been made so far.

A DEC-205 targeting antibody is most advanced in clinical development. Currently a number of clinical trials are ongoing using DEC-205 antibody fused to an antigen, including the NY-ESO-1 tumor antigen (CDX-1401) or HIV gag p24 protein (DCvax-001)101. These anti-DEC-205 drug conjugates are being investigated together with subcutaneous (sc) PolyICLC (Hiltonol), topical/sc R848 (resiquimod) and/or sc recombinant Flt3L (CDX-301) for providing enhanced DC responses. The results of the first trial have been published by Dhodapkar et al., where CDX-1401 together with TLR agonists was well tolerated and induced humoral and cellular NY-ESO-1-specific immune responses in most of the patients.102 It will be interesting to see whether this treatment can induce durable clinical responses in cancer patients in other (randomized follow-up) trials. It will also be interesting to observe whether the separate administration of TLR is sufficient, or whether use of nanoparticle carriers, that co-deliver antigens and TLR ligands to the same cell, results in most optimal anti-tumor responses.

As mentioned above, co-administration of maturation agents is important for efficient activation and function of the DCs. Like for ex vivo-loaded primary DC vaccines, the choice of maturation agent would depend on the DC subset targeted. Thus, some type of a TLR7/8 agonists would likely be the best choice for optimal maturation of all the different DC types. Notably, targeted delivery to DC surface receptors is not limited to delivery of antigens and TLRs, but could also be applied for other immunomodulatory agents, such as CIM-silencing siRNAs. By combining such siRNAs with TLR ligands and antigens in
nanocarriers, activated DCs with superior potential to stimulate tumor-specific T cell (and NK cell) responses could potentially be generated. It will be interesting to follow the development in this field in the coming years.

Potential (combination) therapies post-alloSCT

The different immunotherapeutic strategies that are described in this thesis could potentially be used as adjuvant therapies post-alloSCT to boost GVT immunity (Figure 2). It is the plan to investigate their safety and potency in vivo in the forthcoming years. In this regard, it would be interesting to evaluate whether their efficacy could be enhanced by combination with other immunotherapies or anti-cancer drugs. Hypomethylating agents (HMAs) such as decitabine and azacitidine are for example attractive drugs for combination therapies. HMAs are currently standard of care for higher-risk MDS (myelodysplastic syndrome) patients and AML (acute myeloid leukemia) patients that relapse early post-alloSCT. HMAs are also being evaluated for other haematological malignancies, both in the non-transplant and transplant setting. HMAs are well tolerated and have a direct anti-proliferative and pro-apoptotic effect on tumor cells. Besides these direct anti-tumor effect, HMAs possess immunomodulatory efficacy and can thereby promote anti-tumor immunity. It has been shown that HMAs upregulate expression levels of epigenetically-silenced TAAs and MiHAs (including MAGE, NY-ESO-1, PRAME, WT1 and HA1), and thereby render the tumor cells more susceptible to recognition and destruction by antigen-specific T cells.103-109 Also, the HMAs may improve the priming of antigen-presenting T cells by providing host DCs with necrotic tumor cell material (re-expressing silenced TAAs and MiHAs) as a result of their direct pro-apoptotic activity. HMAs may also further modulate immune responses by upregulating co-stimulatory molecules (CD80, CD86) and CIMs (PD-L1, PD-L2) on tumor cells and DCs.110-112 Studies also indicated that HMA treatment can enhance anti-tumor NK cell responses. With their epigenetic modulation, HMAs have been described to upregulate NK-activating molecules on tumor cells, like NKG2D and DNAM-1 ligands, and thereby sensitize tumors to NK cell-mediated killing.113-116 Furthermore, HMAs induce the expression of killer immunoglobulin-like receptors (KIR) on the surface of NK cells, which further aids their recognition of aberrant cells.116-118 Decitabine, rather than azacitidine, seems to be the favourable combination with NK cells.114,116 A new publication by Cany et al. shows that decitabine, but not azacitidine, positively modulated the in vivo anti-leukemic potential of NK cells by sensitizing leukemic cells to lysis, enhancing NK cell maturation and cytolytic function and improving NK cell trafficking and accumulation at the tumor site.116 In the alloSCT setting, HMAs have either been included as part of the conditioning regimen prior to alloSCT or as maintenance therapy post-alloSCT.119,120 In these patients, increased diversity and frequencies of tumor-specific T cell responses has been observed, which correlated with improved relapse-free survival after alloSCT.119,120 The effect of HMAs on
NK cell numbers and activity in HMA-treated alloSCT patients have not been reported yet, but one could imagine that those can be positively influenced. Taken all together, these data indicate that it would be very interesting to combine HMA treatment post-alloSCT with ex vivo antigen-loaded HSPC-DC subset vaccines, in vivo-targeting nanovaccines or adoptive transfer of HSPC-NK cells, for boosting GVT immunity.

Figure 2. HSPC-derived products for boosting GVT immunity. It is appealing to harness HSPCs for generating cellular immunotherapeutic products, such as NK cells and different DC subsets. These cells can efficiently be generated from HSPCs by blocking AhR activity using the antagonist SR1 in combination with specific cytokine mixtures. HSPC-derived NK cells could be used as therapy early after alloSCT to boost anti-tumor immunity, even while cyclosporine A therapy (an immune suppressive treatment commonly used after alloSCT to suppress allo-reactive GVHD-inducing T cell responses) is still ongoing. HSPC-derived DC subsets could be cryopreserved in multiple aliquots and given in several cycles post-alloSCT to boost tumor-reactive T cell and NK cell responses. These HSPC-derived cellular products can potentially be combined with other (immuno-)therapeutic strategies to further enhance GVT immunity. AlloSCT, allogeneic stem cell transplantation; HSPC, hematopoietic stem and progenitor cells; IS, immune suppressive therapy; DC, dendritic cell; NK cell, natural killer cell; SR1, StemRegenin 1; DLI, donor lymphocyte infusion; HMA, hypomethylating agents; KIR, killer immunoglobulin receptor; ALT-803, IL-15 superagonist complex.

As previously mentioned, one of the current adjuvant therapies applied for boosting GVT immunity and treating or preventing relapse is DLI. The DLI is a non-purified product, that has only a relatively low frequency of T cells that are capable of targeting tumor-specific MiHAs or TAAs. To improve GVT immunity, strategies have been developed to enrich and expand the tumor-specific T cells ex vivo before infusion.
However, *ex vivo* expansion protocols are often associated with expansion of effector memory T cells (T\(_{em}\)) and terminal differentiated T cells, which are quickly exhausted and less efficient in maintaining long-lasting T cell response *in vivo* than less differentiated memory T cells (e.g. stem cell memory T cells (T\(_{scm}\)) and central memory T cells (T\(_{cm}\))).\(^{121}\) Van der Waart *et al.* recently demonstrated that by inhibiting the Akt signaling pathway during *ex vivo* expansion of MiHA-specific CD8\(^+\) T cells, T cell differentiation could be limited. Thereby, highly potent early-memory MiHA-specific T cells were generated. These Akt-inhibited T cells showed superior expansion potential *in vivo*, and importantly, a superior anti-tumor efficacy in mice.\(^{122}\) Instead of combining HSPC-DC vaccination with DLI, it would therefore be interesting to combine them with *ex vivo*-expanded Akt-inhibited tumor-reactive T cells, for enhancing GVT immunity.

Even though strategies such as DC vaccination or adoptive T cell transfer can increase the number of tumor-reactive T cells, the induced T cells may become functionally impaired when they encounter residual tumor cells and bystander cells in the microenvironment that express CIMs or exploit other immunosuppressive mechanisms.\(^{123,124}\) Therapies that target these inhibitory pathways are therefore promising to combine with DC vaccination. Antibodies targeting the PD-1/PD-L pathway and the CTLA-4 pathway, cumulatively referred to as checkpoint inhibitors, have shown impressive therapeutic benefit in various solid tumors. Because of the success, these therapies are now also being evaluated in haematological malignancies, either as monotherapy or together with other drugs such as HMAs, with promising results (reviewed by Boddu *et al.*\(^{125}\)). For example, clinical benefit of checkpoint blockade has been demonstrated in patients with Hodgkin lymphoma.\(^{126}\) As with any potent cancer therapy, checkpoint inhibitors are accompanied by side effects, and often induce systemic autoimmune-related adverse events. Because of this, these antibodies have been introduced with caution in the alloSCT setting, as systemic blockade might release the brake on GVHD-inducing T cells.\(^{127,128}\) Indeed, a multicenter retrospective analysis of 31 lymphoma patients receiving anti-PD-1 antibody post-transplantation, showed that 55% of the patients developed GVHD following administration, of which half died due to this complication.\(^{127}\) Therefore, to safely boost GVT immunity post-alloSCT (in the absence or presence of DC vaccination), more specific approaches for interference with the inhibitory microenvironment are needed. One strategy to enhance tumor selectivity of checkpoint blockade would be using bispecific antibodies, where for example one arm binds to PD-L1 or another checkpoint protein and the other arm recognizes the tumor cell (e.g. via CD19 or CD20 for B cell malignancies and CD33, CD123 or CLEC12A for myeloid malignancies). This concept is in pre-clinical development for EGFR-positive cancers, where a PD-L1xEGFR bispecific antibody is under assessment for its efficacy to direct PD-1/PD-L1 inhibition to EGFR-positive cancer cells (conference abstract DTIM 2017, Koopmans *et al.*). Another strategy is to exploit nanovaccine technology, and specifically deliver nanoparticles containing siRNA against CIMs to the tumor cells. This type of strategy is not only limited to silencing checkpoint molecules like PD-L1, but
could also target other immunosuppressive factors such as IL-10, transforming growth factor (TGF)-β and IDO. In patients with AML and MDS, such siRNA-carrying nanoparticles could be delivered via CLEC12A, as CLEC12A is not only expressed by DCs, but is also highly expressed by myeloid leukemic blasts and stem cells. Similar as we have shown for DCs in chapter 5, we and others have demonstrated that AML cells efficiently internalize CLEC12A upon ligation, making it an attractive target for delivering immunomodulatory siRNAs to leukemic cells (Hutten et al. manuscript in preparation). All together, we believe that strategies that actively boost tumor-reactive immune effector cells (e.g. DC vaccination) combined with strategies that specifically interfere with the local immunosuppressive environment are promising for improving anti-tumor immunity in alloSCT patients, without inducing severe adverse systemic effects.

Multiple studies have shown that donor-derived NK cells have a potent anti-tumor effect in alloSCT patients, as early repopulation and high numbers of NK cells correlate with reduced relapse rate of transplanted patients. It is therefore an appealing strategy to adoptively transfer additional NK cells of donor origin to boost GVT immunity. In chapter 6, we describe the generation of high numbers of NK cells, with an active phenotype and high cytolytic capacity, from donor CD34+ HSPCs, that can be used for adoptive immunotherapy post-transplantation. For maximizing their tumor killing potency, combination treatment with other therapeutic agents could be considered. One strategy is combination with HMAs, as has already been described above. Another strategy is combination with anti-KIR antibodies, such as Lirilumab. Anti-KIR antibodies block inhibitory signals delivered by the tumor target cells to the NK cells, and can thereby increase the activation of NK cells and augment their killing of malignant cells. This might be particularly interesting in combination with HMAs, as they enhance KIR expression on NK cells. The pleiotropic cytokine IL-15 is a key regulator of NK cell development, expansion, maintenance and function, and is therefore a very interesting candidate to combine with NK cell therapy. For this reason, an IL-15 super-agonist ALT-803, which displays high in vivo stability and enhanced biological activity on NK cells, has been developed for therapeutic use. ALT-803 might not only potentiate NK cell responses, but could also positively influence the tumor-reactive T cells, as IL-15 is also important for T cell maintenance and proliferation. Indeed, a study in a murine transplant model demonstrated increased number of NK cells and IFN-γ-secreting CD8+ T cells following ALT-803 treatment, which resulted in enhanced anti-tumor activity without eliciting GVHD. It is therefore a promising strategy to administer ALT-803 together with (HSPC-)NK cells or (HSPC-)DC vaccines for potentiating GVT responses post-transplantation. Other candidates that are promising to combine with NK cell adoptive transfer are the immune modulatory drugs Lenalidomide and Bortezomib, that have both been shown to exert direct anti-leukemic effects, but can also potentiate NK cell expansion and functionality.
In chapter 4, we demonstrate how HSPC-derived DCs, in particular HSPC-pDCs, potently enhance NK cell activation and cytotoxicity. One could thus envision combining HSPC-DC vaccination with HSPC-NK cell adoptive transfer to enhance the therapeutic efficacy of alloSCT. However, the proportion of the donor stem cell graft that could be cryopreserved for generation of adjuvant immunotherapeutic products might not be enough to generate both HSPC-DCs and HSPC-NK cells. The benefit of asking for a second granulocyte colony-stimulating factor (G-CSF) mobilization and apheresis of the donor for obtaining additional HSPCs would need to be weighed against the associated risk and discomfort for the donor. I think therefore, that combination of HSPC-NK cells with HMAs, ALT-803, anti-KIR antibodies or even in vivo DC vaccination is a better and more realistic option for enhancing their in vivo efficacy than combination with ex vivo-loaded HSPC-DC vaccines.

In summary, there are multiple different (combination) therapies that can be considered as adjuvant therapy post transplantation (Figure 2). This calls for identification of the patients who would benefit from a certain type of (combination) treatment. DC vaccination is in particular interesting for those patients that have a MiHA mismatch with their donor, because MiHAs are very immunogenic antigens. Selecting hematopoietic-restricted MiHAs as targets, the immune response can specifically be skewed towards GVT effects, with low risk of GVHD induction. However, only a small portion of patient-donor couples have a MiHA mismatch. Therefore, exploiting vaccination with TAA-loaded DCs, particular in combination with HMAs, makes HSPC-DC vaccination a feasible approach for a larger group of patients. NK cell-based therapies are especially attractive in settings of HLA-mismatch, e.g. after haplo-identical transplant, and in a HLA-matched setting where donors have a KIR B/x genotype (genotype associated with more activating KIRs). In all cases, development and implementation of new (combination) therapies in the alloSCT setting needs to be done with great consideration and care, because of the lingering danger of inducing or exacerbating GVHD. But cumulatively, these novel therapies hold great promise for boosting GVT immunity and reducing the relapse rate, thereby improving overall survival after alloSCT.

Concluding remarks

Taken together, this thesis describes the pre-clinical development and characterization of various immunotherapeutic strategies. We demonstrate how high numbers of different DC subsets (pDCs, BDCA1+ mDCs and BDCA3+ mDCs) and cytotoxic NK cells can be efficiently generated ex vivo from CD34+ HSPCs, by blocking activity of the AhR using the antagonist SR1. This thesis mainly focused on the clinical translational utility of this finding, i.e. evaluating the ex vivo anti-tumor efficacy of the SR1-generated, HSPC-derived cellular immunotherapeutic products. Importantly, we demonstrated that the generated HSPC-DCs were comparable to their natural counterparts in blood, and strongly boosted
both T cell and NK cell responses *ex vivo*. The generated HSPC-NK cells had a highly active phenotype, and exhibited strong cytolytic capacity. In terms of clinical application of HSPC-derived DCs and NK cells, the next step will be designing clinical trials to investigate their potency, alone or in combination with other therapies, to boost GVT immunity in patients after alloSCT. Furthermore, the established SR1-dependent culture protocols will aid in new research to gain more insight into the physiological role of the AhR in DC and NK cell development. In this thesis, we also present results indicating that the C-type lectin receptor CLEC12A could be used for DC vaccination, by serving as an *in vivo* target receptor for nanovaccines. Via CLEC12A, tumor antigens could efficiently be delivered into DC subsets, which were subsequently processed and presented to CD4\(^+\) and CD8\(^+\) T cells, thereby facilitating effective induction of T cell responses. More pre-clinical research on the potential of CLEC12A as an *in vivo* DC-targeting receptor is required, for example a comparison to other targeting receptors that are currently under investigation, such as DEC-205. Overall, the results described in this thesis pave the way for the development of new and potent therapies that can boost anti-tumor immunity and improve the quality of life and overall survival of patients with haematological malignancies.
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Dutch summary / Nederlandse samenvatting
Doelstelling van de thesis

Hoewel de prognose voor patiënten met een hematologische maligniteit in de laatste decennia aanzienlijk is toegenomen door verbeterde therapieën, komt in veel patiënten de ziekte helaas terug. Deze patiënten reageren onvoldoende of ontwikkelen weerstand tegen huidige therapieën. Hierdoor kan residuale ziekte uiteindelijk leiden tot een recidief van de maligniteit. Tot op heden is allogene stamceltransplantatie (alloSCT) gevolgd door donor lymfocytininfusies (DLI) de meest effectieve behandeling voor veel agressieve hematologische kankersoorten. AlloSCT kan genezend zijn doordat krachtige en langdurige antitumor-immuunreacties geïnduceerd worden, die bekend staan als de graft-versus-tumor (GVT) reacties. Hierbij zijn donor Natural Killer (NK)-cellen met een directe cytotoxische werking en cytotoxische CD8+ T-cellen gericht tegen minor histocompatibiliteits-antigenen (MiHAs) of tumor-geassocieerde antigenen verantwoordelijk voor eliminatie van de resterende kwaadaardige tumorcellen. Daarnaast worden er na de initiële antitumor-respons tumor-reactieve geheugen T-cellen gevormd, die in staat zijn om levenslange bescherming te geven. Echter, ziekterugkomst komt ook nog vaak voor bij alloSCT patiënten, wat de belangrijkste oorzaak is van falen van de behandeling en sterfte.

Er zijn verschillende redenen waarom patiënten een terugval krijgen. Patiënten kunnen vroeg na transplantatie recidiveren als de kwaadaardige kloon uitgroeit voordat er voldoende immuun-effectorcellen van de donor gevormd zijn om de ziekte te beheersen. In dit opzicht speelt de effectiviteit van het conditioneringsregime voor transplantatie een belangrijke rol. Zo zijn er minder intensieve conditioneringsbehandelingen ontwikkeld om ook oudere en minder fitte patiënten met alloSCT te behandelen. Echter, deze mindere voorbehandelingen worden geassocieerd met hogere terugvalpercentages: als gevolg van minder effectieve tumorreductie voorafgaande aan de transplantatie wordt productieve antitumor-immuuniteit belemmerd. Terugval kan ook optreden wanneer donor T-cellen en NK-cellen onvoldoende reageren, bijvoorbeeld door minimale/ongunstige MiHA mismatches tussen patiënt en donor, onvoldoende antigeenpresentatie of beperkte NK-celetactivatie. Daarnaast kan er terugval plaatsvinden na aanvankelijk antitumor immuniteit als er geen langdurig geheugen tegen de kwaadaardige ziekte wordt gevormd, het immuunsysteem verzwakt is of als de afweercellen inactief worden voor de achtergebleven ziekte. Bovendien kunnen de tumorcellen in sommige gevallen verschillende mechanismen gebruiken om een afwerremmende omgeving te vormen waardoor ze kunnen ontsnappen aan herkenning en eliminatie door de afweercellen. Naast terugval kunnen andere alloSCT-gelateerde complicaties ontstaan, zoals omgekeerde afstotingsziekte tegen gezonde weefsels en opportunistische infecties. Aangezien alloSCT, ondanks deze beperkingen, de potentie heeft om patiënten te genezen, worden er grote inspanningen verricht om deze behandelingstrategie te verbeteren. Deze inspanningen richten zich op het verminderen van de incidentie en ernst van de afstotingsziekte en
infecties, waarbij de antitumor-immuniteit gestimuleerd wordt voor langdurige ziektevrije overleving.

In dit proefschrift hebben we ons gericht op de preklinische ontwikkeling van immuuntherapeutische strategieën die het antitumor-effect specifiek kunnen versterken, zonder het risico op afstotingsziekte te verhogen. Eén manier om dit te bewerkstelligen is door dendritische cellen (DCs) te gebruiken. DCs zijn professionele antigeen-presenterende cellen en kunnen krachtige antitumorreacties opwekken door antigenen te presenteren aan T-cellen. In dit proefschrift beschrijven we protocollen voor de *ex vivo* ontwikkeling van verschillende DC-populaties uit CD34⁺ hematopoietische stam- en voorlopercellen (HSCs), die gebruikt kunnen worden voor DC-vaccinatie. Daarnaast hebben we onderzocht of het CLEC12A eiwit, een opnamereceptor die op het oppervlak van DCs voorkomt, een goede kandidaat is voor het gericht afleveren van MiHAs en tumor-geassocieerde antigenen *in vivo*. Verder beschrijven we ook protocollen voor het kweken van grote hoeveelheden potente NK-cellen uit CD34⁺ HSCs voor aanvullende therapie.

**DC-vaccinatie met *ex vivo* beladen HSC-DC-populaties**

DC-vaccinatie voor kankerpatiënten is de laatste twee decennia uitgebreid onderzocht, vanwege de centrale rol van DCs in het dirigeren van aangeboren en verworven immuniteit. Het concept is om met tumorantigeen-beladen DCs tumorspecifieke T-celreacties te stimuleren. De tumorspecifieke T-cellen kunnen vervolgens maligne cellen lyseren die het overeenkomende antigeen op hun oppervlak presenteren. Vele klinische studies zijn uitgevoerd met DCs ontwikkeld uit monocyten (MoDCs) in een breed scala van verschillende kankersoorten. Deze studies hebben aangetoond dat DC-vaccinatie haalbaar en veilig is en in staat is om *in vivo* tumor-specifieke immuunreacties te bevorderen. Echter, slechts een minderheid van de patiënten ontwikkelt een objectieve klinische respons. Dit belemmert de implementatie van DC-vaccinatie in de standaardbehandeling. Daarom worden verschillende strategieën onderzocht om deze therapie te verbeteren en te optimaliseren. Manieren om de werkzaamheid van MoDCs te verbeteren is door: 1) liganden voor afweerremmende moleculen op T-cellen te verwijderen of 2) door constitutief actieve groeifactoren of activatiemoleculen voor T- en NK-cellen toe te voegen.

Naast het verbeteren van de immunogeniciteit van MoDCs is het zeer interessant om natuurlijke DC-populaties voor vaccinatie te gebruiken, zoals plasmacytoid DCs (pDCs), BDCA1⁺ myeloïde DCs (mDCs) en BDCA3⁺ mDCs. Elk van deze DC-populaties heeft een unieke rol in het immuunsysteem met verschillende cytokine secretieprofielen. Zo produceren pDCs hoge niveaus van type I interferonen (IFN-α/β), die pleiotrope effecten hebben op meerdere celtypen en bijzonder belangrijk zijn voor NK-celfunctie en antivirale immuniteit. Anderzijds produceren de mDC-populaties interleukine (IL)-12 dat, samen met hun hoge expressieniveaus van major histocompatibiliteitscomplex
(MHC) en co-stimulatoire moleculen, zorgt voor efficiënte inductie van CD4+ T-helper 1 (Th1) responsen en activatie van naïeve CD8+ T-cellen. Bovendien blijkt uit studies dat cross-talk tussen de verschillende DC-populaties zorgt voor synergie door versterking van elkaars afweerstimulerend vermogen. De theorie is dat gelijktijdige vaccinatie met meerdere verschillende DC-populaties kan leiden tot bredere en krachtigere afweerreacties (vanwege DC cross-talk en het activeren van zowel T-cellen als NK-cellen) en daardoor betere therapeutische werkzaamheid dan met de “standaard” MoDC-vaccins. In de hoofdstukken 2-4 hebben we daarom de mogelijkheid onderzocht om in het laboratorium verschillende DC-populaties uit CD34+ stamcellen te genereren.

In hoofdstuk 2 hebben we een protocol ontwikkeld voor het kweken van myeloïde DC-populaties uit CD34+ stamcellen afkomstig van navelstrengbloed. CD34+ stamcellen werden 11 dagen geëxpandeerd in medium met Flt3L ('FMS-like tyrosine kinase 3 ligand'), SCF ('stem cell factor'), IL-3, IL-6 en TPO (thrombopoietin). Na 11 dagen kweek werden de geëxpandeerde cellen verder gekweekt in medium met GM-CSF ('granulocyte-macrophage colony-stimulating factor') en IL-4 in zowel de aanwezigheid als de afwezigheid van 'transforming growth factor' (TGF)-β1 om differentiatie tot DCs te bewerkstelligen. Aan het einde van de kweek was >60% van de cellen positief voor CD11c en HLA-DR, markers die kenmerkend zijn voor monocyten en DCs. Bovendien konden DCs met een fenotype vergelijkbaar met interstitiële/dermale DCs en Langerhans-cellen (LC) geïdentificeerd worden, op basis van respectievelijk CD1a en CD207 (langerin) expressie. De populatie langerin-positieve cellen was groter in kweken met TGF-β1, wat overeenkomt met bevindingen van anderen. Deze HSC-DCs activeerden efficiënt de proliferatie van virale antigeen-specifieke en MiHA-specifieke humane CD8+ T-cellen in vitro en in vivo in immunodeficiënte muizen. Echter, in dit kweeksysteem konden geen DCs met het fenotype van DC-populaties in bloed, zoals pDCs, BDCA1+ mDCs en BDCA3+ mDCs geïdentificeerd worden. In hoofdstuk 3 hebben we daarom alternatieve manieren onderzocht om deze populaties uit CD34+ stamcellen te genereren. Hier ontdekten we dat remming van de ‘aryl hydrocarbon receptor’ (AhR), met behulp van de antagonist StemRegenin 1 (SR1), de in vitro ontwikkeling van pDCs en mDCs uit HSCs significant verbeterde. Hiervoor werden CD34+ stamcellen gedurende drie weken gekweekt in medium met Flt3L, SCF, TPO en IL-6 in zowel de aanwezigheid als de afwezigheid van SR1. In kweken met SR1 konden de verschillende DC-populaties (pDCs, BDCA1+ mDCs en BDCA3+ mDCs) worden geïdentificeerd, terwijl de frequenties in de kweken zonder SR1 verwaarloosbaar waren. Verder hebben we bevestigd dat de SR1-geïnduceerde DC differentiatie afhankelijk is van AhR-signalering, door de toevoeging van de AhR-agonist VAF347. Dit zorgde voor een dosisafhankelijke blokkade van de door SR1 bevorderde DC-ontwikkeling. We hebben verder aangetoond dat de SR1-gegenereerde DC-populaties fenotypisch en functioneel vergelijkbaar zijn met hun tegenhangers in het perifere bloed. Na stimulatie met ‘Toll-like receptor’ (TLR) liganden produceerden de DCs hoge niveaus van populatie-specifieke inflammatoire cytokines, verkregen ze
expressie van co-stimulatoire moleculen en maturatiemarkers en waren ze in staat om T-cellen te activeren.

In hoofdstuk 4 hebben we dit SR1-gebaseerde kweekprotocol verder doorontwikkeld voor een latere klinische toepassing door de juiste kweekomstandigheden vast te stellen. Later kunnen deze kweekomstandigheden ook vertaald worden naar ‘good manufacturing practice’ (GMP), voor het genereren van voldoende aantallen functionele DC-populaties voor vaccinatie. In deze studie hebben we ons gericht op de ontwikkeling van DCs uit CD34+ stamcellen afkomstig uit G-CSF-gemobiliseerd perifeer bloed, aangezien dit de meest gebruikte stamcelbron is voor transplantatie van patiënten met een hematologische maligniteit. Dit biedt de mogelijkheid om DC-populatievaccins te genereren uit een klein deel van het transplantaat om vervolgens GVT-immunitie te stimuleren na alloSCT. Het conform GMP-ontwikkelde protocol combineert aspecten uit de twee verschillende kweeksystemen die in hoofdstuk 2 en 3 zijn beschreven, met enkele aanvullende aanpassingen: in het kort, CD34+ stamcellen werden gedurende 7-13 dagen gekweekt met Flt3L, SCF, TPO (afgekort als FST) en SR1, waarna de kweek werd opgesplitst in twee verschillende differentiatieprotocollen. Een deel van de geëxpandeerde cellen werd gedurende nog een week gekweekt in medium met FST en SR1 (FST kweek), dat met name de ontwikkeling van IFN-α-producerende pDCs bevorderde, hoewel ook BDCA1+ mDCs en BDCA3+ mDCs werden gevormd. Het andere deel van de geëxpandeerde cellen werd voorzien van een myeloïde differentiatie boost door toevoeging van GM-CSF en IL-4 (genaamd G4 kweek), dat resulteerde in een groot aantal IL-12-producerende BDCA1+ mDCs. Voor zover wij weten levert dit kweekprotocol de hoogste aantallen BDCA1+ mDCs en BDCA3+ mDCs die gelijktijdig kunnen worden gekweekt uit HSCs onder GMP-conforme condities.

In hoofdstuk 4 hebben we het vermogen van HSC-pDCs en HSC-BDCA1+ mDCs onderzocht om ex vivo tumorreactieve T-cel en NK-cel reacties te stimuleren. Hier bleken BDCA1+ mDCs superieur in het activeren van naïeve T-cellen en het expanderen van MiHA-specifieke CD8+ T-cellen van alloSCT patiënten. Deze T-cellen waren sterk geactiveerd, zoals bleek uit efficiënte IFN-γ productie en degranulatie na herstimulatie met peptide. Aan de andere kant waren HSC-pDCs superieur in het induceren van IFN-γ secretie door ‘effectormemory’ T (Tmem) cellen en het versterken van NK-celfunctie. Dit kan zeer waarschijnlijk worden toegeschreven aan de hoge uitscheiding van IFN-α door pDCs. pDC-activatie van NK-cellen resulteerde in verhoogde TRAIL (‘tumor necrosis factor-related apoptosis-inducing ligand’) expressie en significant verbeterde lysis van tumorcellen. Tezamen tonen deze resultaten aan dat de verschillende HSC-DCs zorgen voor krachtige activatie van antitumor effectorcellen, en populatie-specifieke kenmerken vertonen die overeenkomen met beschrijvingen in de literatuur, d.w.z. dat de mDCs beter zijn in het stimuleren van T-celresponsen, terwijl de pDCs superieur zijn in het induceren van NK-celresponsen. Deze resultaten geven aan dat een vaccin samengesteld uit de verschillende HSC-DC-populaties veelbelovend is voor het opwekken
van bredere immuunresponsen door het activeren van immuuneffectortcellen van zowel het aangeboren als het verworven immuunsysteem (respectievelijk T-cellen en NK-cellen). Dit leidt hopelijk tot verbeterde therapeutische effectiviteit ten opzichte van de MoDC-gebaseerde vaccinatiestudies.

**In vivo targeting van DCs**

Een alternatief voor het gebruik van *ex vivo* gekweekte tumorantigeen-beladen DCs als immuuntherapie, is het direct activeren van de verschillende DC-populaties *in vivo*. Het concept van deze strategie is om via antilichamen die gericht zijn tegen specifieke oppervlakereceptoren gelijktijdig tumorantigenen en adjuvans (zoals TLR-liganden) af te leveren in de DCs om DC uitrijing te stimuleren en antigeen presentatie en activatie van cellulaire afweerreacties te induceren. Hierdoor kan de bewerkelijke, arbeidsintensieve en dure kweek en bewerking van DCs vermeden worden. Bovendien is *in vivo* targeting potentieel breder toepasbaar, aangezien niet voor elke individuele patiënt een uniek vaccin gemaakt hoeft te worden. Deze strategie is echter nog niet zo ver ontwikkeld als *ex vivo* beladen DC-vaccinatie en slechts enkele klinische studies hebben deze aanpak onderzocht. Voor implementatie moeten meerdere parameters verder worden bepaald, waaronder een grondige karakterisering van antigenopnamereceptoren. Deze receptoren moeten gerichte en efficiënte afgifte van het antigeen in intracellulaire compartimenten faciliteren voor het verwerken en beladen van de antigeenpeptiden in MHC moleculen.

In **hoofdstuk 5** hebben we onderzocht of het CLEC12A eiwit, een opnamereceptor, een goede kandidaat is voor het gericht afleveren van antigenen in humane DC-populaties. In deze studie bevestigden we dat CLEC12A selectief tot expressie komt op myeloïde cellen, waaronder monocyten en granulocyten, en de verschillende DC-populaties: BDCA1+ mDCs, BDCA3+ mDCs en pDCs. Verder hebben we aangetoond dat in alle DC-populaties CLEC12A na binding van het CLEC12A-antilichaam efficiënt geïnternaliseerd wordt en vervolgens naar intracellulaire compartimenten getransporteerd wordt die betrokken zijn bij de verwerking en presentatie van antigenen. Naast het opnemen van antigenen kunnen receptoren zoals CLEC12A ook immuunresponsen moduleren via hun intracellulaire signaleringsdomeinen. CLEC12A bevat een immunoreceptor tyrosine-based inhibitory motif (ITIM) in zijn cytoplasmatische deel, wat na triggering de DC uitrijing en functionaliteit zou kunnen remmen. Wij hebben echter geen negatieve effecten gevonden van CLEC12A antilichaam binding op TLR-geïnduceerde expressie van DC uitrijingmarkers of productie van cytokines, noch op de capaciteit van de DCs om allogene T-celactivatie te induceren. Daarnaast hebben we aangetoond dat CLEC12A-gemedieerde antigeenopname leidt tot efficiënte antigeenpresentatie aan zowel CD4+ als CD8+ T-cellen door de verschillende DC-populaties. We laten zien dat de aflevering van ‘keyhole limpet hemocyanin’ (KLH) resulteerde in expansie van en IFN-γ-secretie door KLH-specifieke CD4+ T-cellen. Bovendien waren alle primaire DC-populaties,
BDCA1⁺ mDCs, BDCA3⁺ mDCs en pDCs, in staat om een lang MiHA-peptide, afgeleverd via CLEC12A, te presenteren aan CD8⁺ T-cellen. Dit resulteerde in sterke ex vivo activatie van MiHA-specifieke CD8⁺ T-cellen, afkomstig van alloSCT patiënten. Samen geven deze bevindingen aan dat CLEC12A een interessante kandidaat is voor het gericht afleveren van antigenen in DCs.

**NK-cel therapie**

Naast T-cellen spelen alloreactieve NK-cellen een belangrijke rol in de antitumor-reactie. NK-celreacties na alloSCT zijn geassocieerd met lagere recidiefcijfers en langdurige ziektevrije overleving. Het gunstige effect kan worden toegeschreven aan NK-celactivatie door selectieve (stress-geïnduceerde) verhoging van de expressieniveaus van activerende NK-liganden en/of de verlaging van MHC klasse I expressie (NK remmende liganden) op de tumorcellen. Van belang is dat alloreactieve NK-cellen de eigenschap hebben dat ze geen omgekeerde afstotingsziekte veroorzaken, en mogelijk zelfs de inductie en/of de ernst van omgekeerde afstotingsreacties kunnen onderdrukken. Deze observaties hebben de aandacht van veel onderzoeksgroepen getrokken om toediening van NK-cellen te onderzoeken als adjuvante therapie na transplantatie, met als doel eventueel achtergebleven ziekte te elimineren zonder omgekeerde afstotingsziekte te induceren. Ook nemen NK-cellen deel aan de verdediging tegen virale en schimmelinfecties na transplantatie, waardoor ze nog aantrekkelijker worden als adjuvante therapie. Ondanks beschrijvingen van klinische werkzaamheid van NK-cellen geïsoleerd uit bloed, is het gebruik van dit type NK-cellen voor immuuntherapie beperkt om verschillende redenen, waaronder lage aantallen circulerende cellen, relatief lage activatiestatus en contaminatie van het product met allogene T- en B-cellen die omgekeerde afstotingsziekte kunnen veroorzaken. Om deze beperkingen te omzeilen heeft onze groep een nieuw NK-celproduct ontwikkeld. Hierbij worden NK-cellen ex vivo gekweekt uit CD34⁺ stamcellen, afkomstig uit navelstrengbloed, aan de hand van een cytokine-gebaseerd kweekprotocol. Via deze benadering kan een hoge opbrengst van cytotoxische NK-cellen, zonder contaminatie van T- en B-cellen, worden bewerkstelligd. Deze NK-cellen zijn recentelijk onderzocht voor veiligheid, toxiciteit en immunologische eigenschappen in een fase I klinische studie in oudere AML-patiënten. Hoewel het onderzoek niet was ontworpen om eventuele klinische effectiviteit te evalueren, werd in sommige patiënten een tijdelijke afname van ziekte waargenomen na de cyclophosphamide/fludarabine conditionerings en NK-celinfusie. Deze resultaten zijn veelbelovend voor verdere ontwikkeling van adoptieve celtherapie met behulp van HSC-NK-cellen.

In hoofdstuk 6 onderzochten we de haalbaarheid van het kweken van NK-cellen uit andere stamcelbronnen, namelijk G-CSF gemobiliseerd perifeer bloed en beenmerg, om dezelfde reden als hierboven beschreven voor de HSC-DCs: het aanbieden van additionele celtherapieproducten gegenereerd uit de stamcellen van de transplantatiedonor.
Echter, CD34+ stamcellen van volwassenen hebben een lagere capaciteit om zich te vermeerderen en te differentiëren tot NK-cellen dan jonge CD34+ stamcellen afkomstig uit navelstrengbloed. Dit betekent dat modificaties van ons *ex vivo* NK-cel kweekprotocol nodig waren. Uitgaande van de beschrijvingen in de literatuur en onze eigen ervaring met SR1 in het HSC-DC kweeksystem hebben we onderzocht of behandeling van CD34+ stamcellen met SR1 hun expansiesnelheid zou vergroten en NK-cel differentiatie zou bevorderen. Inderdaad bleek dat toevoeging van SR1 aan ons cytokine-gebaseerde *ex vivo* NK-cel kweekprotocol leidde tot significant verbeterde vermeerdering van volwassen CD34+ stamcellen. Daarnaast induceerde SR1 de expressie van transcriptiefactoren die belangrijk zijn voor NK-celdifferentiatie, zoals TOX (‘thymocyte selection-associated HMG box factor’), waardoor differentiatie van de SR1-gëxpandeerde HSCs tot NK-cellen bevorderd werd. De toevoeging van SR1 resulteerde vervolgens in de ontwikkeling van hoge aantallen CD56+CD3- NK cellen, met een gemiddelde zuiverheid van 83%, uit HSCs van volwassenen. Deze gekweekte NK-cellen hebben hoge expressieniveaus van de activerende NKG2D en ‘natural cytotoxicity receptors’ (NCRs) en waren effectief in het doden van myeloïde leukemie- en multiple myeloomcellijnen, alsook acute myeloïde leukemie (AML) blasten van patiënten. Een interessante bevinding die we gedaan hebben is dat de NK-cellen uit de SR1-kweken verbeterde functionele activiteit vertoonden in vergelijking met NK-cellen uit de controle kweken zonder SR1; ze vertoonden verhoogde expressie van DNAM-1 en TRAIL, produceerden hogere niveaus van IFN-γ en granzyme B en hadden betere cytotoxische capaciteit. Bovendien verhoogde SR1 ook de expressie van de homingreceptoren CD62L en CXCR3, waardoor de HSC-NK cellen mogelijk beter in staat zijn zich te bewegen naar hematologische tumorcellen in lymfoïde organen.

We zijn zeer geïnteresseerd in het onderzoek naar de toepassing van deze SR1-gegenereerde NK-cellen als adjuvante therapie kort na alloSCT, maar patiënten worden dan vaak nog behandeld met de immuunsuppressieve middelen cyclosporine A (CsA) en mycophenolic acid (MPA) om alloreactieve T-celreacties te onderdrukken en zo inductie van omgekeerde afstotingsziekte te voorkomen. Daarom hebben we *in vitro* het effect van CsA en MPA behandeling op de levensvatbaarheid, proliferatie en functionaliteit van de gegenereerde NK-cellen bestudeerd. Therapeutische concentraties van CsA hadden geen negatief effect op deze parameters, wat aangeeft dat HSC-NK celtherapie parallel aan CsA-behandeling kan plaatsvinden (welke in de meeste gevallen minstens 6 maanden duurt vanaf transplantatie). In tegenstelling hiermee remde MPA de proliferatie, levensvatbaarheid, cytolytisch vermogen en IFN-γ-secretie van de NK-cellen. MPA-behandeling wordt meestal alleen toegepast in de eerste maand na transplantatie, wat aangeeft dat de behandeling met HSC-NK-cellen vanaf de 2e maand na alloSCT mogelijk is.
Conclusie

Concluderend beschrijft dit proefschrift de preklinische ontwikkeling van diverse immuuntherapeutische strategieën die mogelijk post-alloSCT zouden kunnen worden toegepast als adjuvante therapieën om de werkzaamheid van de alloSCT te verbeteren. HSC-DC-populaties die hematopoietisch-geristriceerde MiHAs of tumor-geassocieerde antigenen presenteren zouden mogelijk post-alloSCT als vaccin kunnen worden gebruikt voor selectieve inductie van GVT-immuniteit. Alternatief kunnen gerichte nanovaccins, die MiHAs en/of tumor-geassocieerde antigenen bevatten, direct afgeleverd worden in verschillende DC-populaties in vivo via de CLEC12A receptor. Tenslotte zouden HSC-NK cellen van toepassing kunnen zijn voor adoptieve toediening vroeg na alloSCT, om eventueel residuale ziekte te elimineren en antimicrobiële verdediging/controle te bewerkstelligen. Naast de mogelijke therapeutische implicaties van deze strategieën, kunnen de DC- en NK-kweekprotocollen die in dit proefschrift zijn beschreven gebruikt worden voor ex vivo mechanistische studies naar de ontwikkeling en biologie van DC- en NK-cellen. Tezamen hebben de resultaten beschreven in dit proefschrift de weg gebaand voor de ontwikkeling van nieuwe, krachtige therapieën die de antitumor-immuniteit kunnen versterken en de kwaliteit van leven en ziektevrije overleving van patiënten met hematologische maligniteiten kunnen verbeteren.
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Acknowledgements
Curriculum Vitae
List of publications
PhD portfolio
List of abbreviations
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Dear promoters, Tim and Joop. I would also like to thank you both: Joop, thank you for your valuable and critical input on my project during discussions and presentations. I admire how you actively look at the bigger picture of every project within our laboratory, which thereby makes the research more focused and of better quality. Tim, thank you for believing in me and giving me the opportunity to start my PhD studies here. I enjoyed every meeting we had because of your endless enthusiasm and all the novel and creative ideas you brought up.

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Basav, thanks for taking care of me in the beginning of my PhD, and for teaching me all the tips and tricks on HSPC and DC cultures. This help was very valuable and laid the cornerstone for my thesis. Robbert, thank you for helping me develop my writing skills by providing me with guidance when I was writing my first scientific article. Anniek, I really appreciate having had you as officemate for almost my entire time here. I could always come to you for all type of questions, whether they were about mouse experiments, statistical calculations or T cells, you always had a good answer or suggestions about what to do. I am thankful for that. Rob, thank you for all the help you provided by sorting the different dendritic cell subsets from the HSPC-DC cultures. Mieke, I am happy I got to work together with you on the SR1-derived NK cell, it was a nice change from the dendritic cell work and expanded my knowledge.

Dear Tim and Lotte, I am honoured that you will be standing next to me as my paranymphs on April the 5th. Tim, we shared many nice moments together as we took on the PhD journey almost side by side. It was very nice to work with you on the CLEC12A targeting project and I always had a good time around you in the lab and the office. Your calmness was a very good counterbalance to my anxiety. Thank you for always being ready to listen and talk. It will be sweet to celebrate the end of our respective PhD journeys on April 5th and May 18th! Lotte, thank you for always showing interest in what I was doing, both at work and outside the office. You also gave me many good pep talks that helped me finishing writing the last chapters, even the very last sentences, this acknowledgement section! I will not be in the lab for the end of your PhD journey to cheer you over the finish line, but I am confident that you will make it. I look forward to attending your PhD defense!

I would also like to thank the remaining colleagues of the CTI lab that have been there during my journey. Jeannette, Karishma, Wieger, John, Paul, Jan, Hanny, Frans, Marij, Lisanne, Marleen, Menno, Manon, Denise, Janneke, Jesper, Jolien, Paulien and Ralph. Thank you for all the fruitful scientific discussions and for always having answers to any lab-related questions I would have. Also, thank you for general ‘gezelligheid’, both in the lab as well as outside the lab during our many teambuilding activities, congresses and ‘borrels’. Dear Marij and Lisanne, it was a great pleasure to work with you for the past two years on the collaborative project with Merus. Jesper, as you are continuing with the HSPC-DCs where I left off, I wish you all the best with the project and look forward to see the results. I think the project is in good hands and believe you will do good work.

During my PhD I had the joy to supervise three students that worked with me on my HSPC-DC project. Elif, Elja and Tim, thank you for all your hard work and for the enthusiasm your brought with you into the project. Good luck with your career.

Other members of the lab of hematology also truly deserve to be mentioned here. The bone marrow transplantation unit, thank you for reserving primary material for me so that I could perform my experiments. Colleagues of the immunophenotyping, molecular diagnostics, morphology and thrombosis and hemostasis unit, thank you for always being
super friendly and helpful when I came to you with questions. And the molecular research group: Anne, Bert, Davide, Florentine, Gorica, Jolanda, Jimmy, Maaike, Miriam, Niccolo, Pedro, Ruth, Rinske, Saskia and Tessa. Thank you for providing input on my project during presentations, for giving me advice on molecular approaches and for all the non-science related conversations during coffee breaks and borrels. Last but not least, Nelleke, thank you for keeping everything under control!

Furthermore I would like to thank you Dr. Michel Schaap for the input you gave on the projects from a clinical perspective. And prof. dr. Fred Falkenburg, thank you for providing us with tetramers for the different projects and for your critical revision of chapter 4 of this thesis.

There are also many people I would like to thank outside the lab, that supported me during this journey and helped me relax and clear my head when I was not working (truly necessary!). Irati, I am very happy to have made such a good friend that enjoys singing as much as I do. I will always cherish the time we spent together here in Nijmegen and hope we will be able to keep up the contact even though we move far away from each other. Good luck with finalizing your thesis! Farhan, I am very grateful for your first class pep talks, those really helped me to push through and finish the thesis. My expat friends, Inna, Simon, Joanna, Dimitar, Giuseppe and Chris, thank you for the support and for all the nice times we spent together. Marta, I decided to rather thank you here than as colleague, as I think we shared more non-research-related things than research-related stuff. I really enjoyed the conversations we had and the concerts we went to together. The Buena Vista Social Club concert: awesome! It was nice to connect with someone who understood how it was to move to the Netherlands and start a PhD here. Mieke, Kyra, Yvonne, Marjolijn and Tamara. I will always cherish the time I spent with you while I had Syrpa. Thank you for all the nice times in the weekends, riding together and taking care of the horses. Willibrord and the other members of the Campuskoor Veelstemmig, it was a great pleasure to sing and perform together with you. I will always keep the songs of De lastpost close to my heart. My Lindy hop buddies, you are too many to mention by name, but I want to thank you all for all the awesome dances, conversations and drinks we have had together. When I started lindy hop classes, never did I anticipate that I was entering such a welcoming, warm and social society and would make so many nice friends. I am very glad to have been part of this society and will miss you very much wherever I will be going. I hope to dance with you in the future at different workshops here and there in the world. Christine, thank you for the nice cover you designed for me!

Ann-Kathrin, Bas, Hannah, Lorenz, Matteo, Moritz and Sarah. I am so grateful for having you as friends, and so glad that we’ve been able to reunite yearly since we studied together in Sweden. I had a fantastic time at every reunion, and look forward to many
more in the future. Bas, I would also like to thank you for critically reading over the Dutch summary of my thesis. I really appreciate it.


Elsku Mamma, Pabbi, Jóna, Sunna, Siggi, Sigga Thea og Júlíus Bjarni. Ég er afar þakklát fyrir að hafa svona góða fjölskyldu á bak við mig. Þið hafir gefið mér ómetanlega stuðning í gegnum árin. Einnig er ég þakklát fyrir þá trú sem þið hafið alltaf haft á mér og minni getu. Sú trú er klárlega hvetjandi og hefur hjálpað mér að klára þetta verkefni sem þessi ritgerð er. Ég segi það ekki nógu oft, en ég elska ykkur!
Curriculum Vitae

Soley Thordardottir was born in Neskaupstadur, Iceland on September 23rd, 1984. She attended a natural science-oriented track at the high-school in Neskaupstadur (Verkmenntaskóli Austurlands), where she graduated in 2003. In 2004, she moved to Reykjavik and started her education in biomedical sciences. During her studies she developed a strong interest in immunology, and performed her final internship studying the activation of the complement system following total hip replacement surgery at the Department of Immunology at the Landspítali – University hospital. She obtained her B.Sc. degree (summa cum laude) in biomedical science from Reykjavik University in 2008, whereafter she worked for one year as a laboratory technician. In 2009 she moved to Stockholm, Sweden, where she attended the biomedicine master program at Karolinska Institutet. She performed her major internship in the group of prof. dr. R. Holmdahl at the Medical Inflammation Research unit at the Department of Biochemistry and Biophysics at Karolinska Institutet. There she studied the involvement of major histocompatibility complex class II molecules and their presented peptides in rheumatoid arthritis animal models. After obtaining her M.Sc. degree in May 2011, she continued a stipend-supported internship in the same group for an additional four months. Thereafter, Soley pursued her scientific interest in immunology and translational medicine and started her PhD project in December 2011 at the Department of Laboratory Medicine, Laboratory of Hematology, at Radboud university medical center. During her PhD studies she was supervised by dr. H. Dolstra, dr. W. Hobo, prof. dr. T. Radstake and prof. dr. J. Jansen. She worked on the development and characterization of cancer immunotherapy strategies, with focus on development of protocols for generation of different dendritic cell subsets from hematopoietic stem and progenitor cells. These results are presented in this thesis. In April 2016, she continued her scientific career in the same group on a collaborative project with Merus N.V. on the pre-clinical evaluation of MCLA-117, a CLEC12AxCD3 bispecific antibody for therapy of patients with acute myeloid leukemia.
List of publications

Ex vivo generation of interstitial and Langerhans cell-like dendritic cell subset-based vaccines for hematological malignancies.

The aryl hydrocarbon receptor antagonist StemRegenin 1 promotes human plasmacytoid and myeloid dendritic cell development from CD34+ hematopoietic progenitor cells.

Hematopoietic stem cell-derived myeloid and plasmacytoid DC-based vaccines are highly potent inducers of tumor-reactive T cell and NK cell responses ex vivo.

CLEC12A-mediated antigen uptake and cross-presentation by human dendritic cell subsets efficiently boost tumor-reactive T cell responses.

The aryl hydrocarbon receptor antagonist StemRegenin 1 improves in vitro generation of highly functional natural killer cells from CD34+ hematopoietic stem and progenitor cells.

Association of MicroRNA-618 expression with altered frequency and activation of plasmacytoid dendritic cells in patients with systemic sclerosis.

Activation of complement following total hip replacement.
Positional identification of RT1-B (HLA-DQ) as susceptibility locus for autoimmune arthritis.

Natural polymorphisms in Tap2 influence negative selection and CD4ratioCD8 lineage commitment in the rat.
**PhD portfolio**

**Name PhD candidate:** Soley Thordardottir  
**Department:** Department of Laboratory Medicine, Laboratory of Hematology  
**Graduate School:** Radboud Institute for Molecular Life Sciences

**PhD period:** 01-12-2011 – 31-12-2015  
**Promotors:** Prof. Dr. J.H. Jansen and Prof. Dr. T.R.D.J. Radstake  
**Co-promotors:** Dr. H. Dolstra and Dr. W.A. Hobo

### TRAINING ACTIVITIES

**a) Courses & workshops**

- RIMLS introductory course  
  - 2012  
  - 2012-2014  
- Technical fora  
  - 2012  
- Laboratory Animal Science (part on Dutch legislation only)  
  - 2013  
- European network of immunology institutes (ENii) summer school*  
  - 2014  
- Scientific integrity  
  - 2014  
- Academic writing  
  - 2014  
- Career guidance for international PhDs  
  - 2014  
- Achieving your goals  
  - 2014

**b) Seminars & lectures**

- MACHT meetings*  
  - 2011-2014  
- RIMLS lecture series + seminars  
  - 2011-2015  
- Hematology theme meetings*  
  - 2011-2015  
- Laboratory of Hematology research retreat  
  - 2013-2015  
- Radboud Research Rounds  
  - 2014-2015  
- RIHS lecture series  
  - 2015

**c) Symposia & congresses**

  - 2012-2015  
- Radboud New Frontiers Symposium (2013*)  
  - 2012-2015  
- NVVI annual (December) Congress (2013*)  
  - 2011, 2013, 2014  
- Dutch Tumor Immunology Meeting (2013*, 2015*)  
  - 2012-2015  
- Radboud Science day (2013*)  
  - 2012-2014  
- Dutch Hematology Congress (2014*, 2015*)  
  - 2014-2015  
- NVVI voorjaar symposium Lunteren  
  - 2012  
- N4i Summer Frontiers Innate Immunity Symposium  
  - 2012  
- European Congress of Immunology, Glasgow  
  - 2012  
- Cell Therapy Symposium  
  - 2013  
- Tumor Immunology Symposium (RIMLS)  
  - 2013  
- International Dendritic Cell Symposium, Tours*  
  - 2014  
- Keystone Tumor Immunology Meeting, Banff, Canada*  
  - 2015

**d) Other**

- Journal clubs*  
  - 2011-2015

### TEACHING ACTIVITIES

**e) Lecturing**

- Lecture for honorary bachelor students  
  - 2015  
  - 0.2

**f) Supervision of internships / other**

- Supervision of master student literature thesis  
  - 2013-2014  
  - 0.25  
- Supervision of bachelor student (3 months)  
  - 2013-2014  
  - 1  
- Supervision of master student (6 months)  
  - 2014  
  - 2  
- Supervision of master student (8 months)  
  - 2014-2015  
  - 2.5

**TOTAL**  
39

^Oral or poster presentations are indicated * and #, respectively
**List of abbreviations**

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## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ITIM</td>
<td>Immunoreceptor tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>K</td>
<td>Killer immunoglobulin-like receptor</td>
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<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
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<td>LAA</td>
<td>Leukemia-associated antigen</td>
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<td>LAG-3</td>
<td>Lymphocyte-activating gene 3</td>
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<td>LAM-1</td>
<td>Lysosomal-associated membrane protein 1</td>
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<td>LC</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LUMC</td>
<td>Leiden University Medical Center</td>
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<td>M</td>
<td>Myeloid dendritic cell</td>
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<tr>
<td>MDP</td>
<td>Monocyte-dendritic cell progenitor</td>
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<tr>
<td>MDS</td>
<td>Myelodysplastic syndrome</td>
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<tr>
<td>MEP</td>
<td>Megakaryocyte-erythrocyte progenitor</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>MICL</td>
<td>Myeloid inhibitory C-type lectin-like receptor</td>
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<td>mkG</td>
<td>Mouse IgG</td>
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<td>MiHA</td>
<td>Minor histocompatibility antigen</td>
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<td>MLR</td>
<td>Mixed leukocyte/lymphocyte reaction</td>
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<td>MM</td>
<td>Multiple myeloma</td>
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<td>MoDC</td>
<td>Monocyte-derived dendritic cell</td>
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<td>MPA</td>
<td>Mycophenolic acid</td>
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<td>MPP</td>
<td>Multi-potent progenitor</td>
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<td>Natural cytotoxicity receptor</td>
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<td>Natural killer cell</td>
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<td>PBGD</td>
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<td>Poly I:C</td>
<td>Polynosinic:polycytidylic acid</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>Penicillin and streptomycin</td>
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<td>Q</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
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<td>RM</td>
<td>Repeated measures</td>
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<td>R848 + Poly I:C</td>
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<td>sc</td>
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<td>SCF</td>
<td>Stem cell factor</td>
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<td>Stem cell transplantation</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>siRNA</td>
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<td>StemRegenin 1</td>
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<td>ssRNA</td>
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<td>TAA</td>
<td>Tumor-associated antigen</td>
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<td>Transforming growth factor β</td>
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<td>TRAIL</td>
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<td>Tscm</td>
<td>Stem cell memory T cell</td>
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<tr>
<td>TCB</td>
<td>Umbilical cord blood</td>
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