Expression of CD45RB functionally distinguishes intestinal T lymphocytes in inflammatory bowel disease

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Abstract: The importance of CD45RB expression on T cells was already shown in mice where CD45RBhigh expression determines pathogenic potential. In this study, we analyzed the expression of CD45RA, CD45RB, and CD45RO on CD4+ T lymphocytes in the intestinal mucosa and in the circulation of patients with inflammatory bowel disease (IBD). In addition, we studied the cytokine profile of these cells. In the circulation, virtually all CD4+CD45RBhigh T cells expressed the naive marker CD45RA, and circulating CD4+CD45RBlow cells expressed the memory marker CD45RO in IBD patients and a control patient population. In contrast, the intestinal CD4+ CD45RBhigh T cells are in normal controls for 90% CD45RO+. However, in IBD, 27.7% [Crohn’s disease (CD)] and 49% [ulcerative colitis (UC)] of the intestinal CD4+ CD45RBhigh T cells are CD45RA+. This special CD4CD45RA+ T cell in IBD can be found in the lamina propria as well as in lymphoid follicles (confocal laser-scanning microscopy). The CD4+CD45RBhigh T lymphocytes produce significantly less interleukin (IL)-10 and IL-4 and produce more tumor necrosis factor α than CD45RBlow T lymphocytes in control patients. CD4+CD45RBlow T cells from IBD patients produced less IL-10 than CD4+CD45RBlow T lymphocytes of controls, and interferon-γ production by both T lymphocyte subsets was decreased in IBD. These data indicate that CD and UC are characterized by an influx of CD4+CD45RBhigh T lymphocytes. These CD4+CD45RBhigh T lymphocytes seem to be important in the pathogenesis of IBD, as they produce more proinflammatory cytokines and less anti-inflammatory cytokines compared with CD4+CD45RBlow T lymphocytes. J. Leukoc. Biol. 75: 1010–1015; 2004.

Key Words: cytokines · inflammation · human

INTRODUCTION

Crohn’s disease (CD) and ulcerative colitis (UC) are complex, multifactorial human inflammatory bowel diseases of which the etiology remains poorly understood. Recently, a genetic defect in the gene encoding a protein involved in the recognition of microbes, NOD2, was identified [1]. This links a dysregulated immune response to enteric bacteria to disease development in the immune susceptible host. Pathogenic CD4+ T lymphocytes are believed to play a major role in the pathogenesis of inflammatory bowel disease (IBD).

To study the mechanism of disease development, several mouse models of experimentally induced or spontaneously occurring colitis are available (for review, see ref. [2]). One of these models involves the adoptive transfer of a pathogenic CD4+CD45RBhigh T cell subpopulation into an immuno-deficient recipient. Transfer of CD4+CD45RBhigh T cells from donor mice into genetically immuno-deficient (recombination activating gene-2−/−) mice or severe combined immunodeficiency mice results in a spontaneous chronic and severe inflammation of the large intestines [3]. In contrast, cotransfer of the CD4+CD45RBlow T cell subset protects against disease [4] or results in a later onset of disease [5].

The level of expression of CD45RB on mouse CD4+ T cells is believed to distinguish naïve (CD45RBhigh) from activated/memory (CD45RBlow) cells [6].

In humans, two other isoforms of CD45, CD45RA and CD45RO, are used to distinguish naïve and primed/memory T cells, respectively [7, 8]. However, there is also a reverse CD45RB expression on CD45RA and CD45RO cells in human peripheral blood [9]. In the peripheral blood, CD45RA-positive T cells express high levels of CD45RB, whereas CD45RO cells express little CD45RB. Hitherto, the role of CD45RB expression in human intestinal inflammation has not been evaluated in detail.

The expression of CD45 is essential for the activation of T lymphocytes via the T cell receptor. The intracellular domain of CD45 contains a protein tyrosine phosphatase that regulates the intracellular proteins p56lck and p59fyn on T cell activation [10]. T lymphocytes express different isoforms of the extracellular domain of CD45 [7]. The expression of the isoforms varies between subpopulations of T lymphocytes and changes upon stimulation and differentiation. The CD45 isoforms display different abilities to support T cell activation [11].

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Conflicting reports exist concerning the distribution of memory and naïve cells in the peripheral blood cells of IBD patients. The number of memory cells was reported to be elevated in the peripheral blood of CD patients [12], but these observations were not confirmed in another report [13]. In the mucosa of the gut, almost all CD4-positive cells express the CD45RO marker and are therefore considered memory cells.

In view of the functional differences of T lymphocytes that express the various CD45 isoforms, we investigated the expression of CD45RA, CD45RO, and CD45RB by peripheral- and gut-derived T lymphocytes from IBD patients and controls. To further study the functional difference between CD45RBhigh and CD45RBlow cells, peripheral- and gut-derived T lymphocytes were sorted and stimulated with CD3/CD28. The pro- and anti-inflammatory cytokine profile of these subsets was determined.

MATERIALS AND METHODS

Patients

Mucosal lymphocytes were isolated from surgical resection specimens from patients with active CD or UC who underwent partial resection of the intestines. Eight CD patients (two male and six female) with a mean age of 34.8 years (range, 21–71 years) and median disease duration of 6.25 years (range, 1–12 years) were included. Four patients had ileal disease, and four had colonic disease. All patients were on corticosteroids. The mean age of the eight UC patients (four males and four females) was 34.8 years (range, 22–48 years), and mean disease duration was 4 years (range, 1–9 years). Six of these patients were on corticosteroids. Control mucosal lymphocytes were obtained from six patients undergoing a resection for cancer (three males and three females). Mean age of this group was 61.8 years (range, 40–78 years). The disease was located in the rectum (two) and in the colon (four), and noninvolved bowel segments were used for this study.

Cell preparation of mucosal specimen

Freshly obtained mucosal specimens (3 cm×0.5 cm) were homogenized using an automated, mechanical tissue desecration device (Medimachine system, Dako, Denmark). Cell suspensions were centrifuged with Ficoll (Pharmacia, Uppsala, Sweden), and the mononuclear cells were subsequently passed through a 40-μm filter cell strainer (Becton Dickinson Transduction Laboratories, Franklin Lakes, NJ). Subsequently, cells were washed with fluorescence-activated cell sorter buffer (phosphate-buffered saline, containing 0.5% bovine serum albumin, 0.3 mmol/L EDTA, and 0.01% sodium azide) and were kept on ice for the rest of the procedure.

Flow cytometry

Four color FACSCalibur analysis was used to determine the expression of CD45RA and CD45RO within the CD45RBhigh and CD45RBlow subgroups. One million cells per well (96-well microplate, Greiner B.V. Labor Techniek, Alphen aan de Rijn, The Netherlands) were incubated with anti-CD45RB (Southern Biotechnology Associates, Birmingham, AL) and anti-immunoglobulin G–peridinin chlorophyll protein (Per CP) as secondary antibody. Subsequently, cells were incubated with anti-CD45RO–allophycocyanin (APC)-, anti-CD45RO–phycoerythrin (PE)-, and anti-CD45RA–fluorescein isothiocyanate (FITC)-labeled antibody (Immunotech, Marseille, France). Isotype controls were used to determine the negative gates.

Analysis was done on a FACSCalibur in conjunction with the CellQuest Pro (BD Biosciences, Pharmingen, San Diego, CA) software, and 5000 cells were counted. CD4+CD45RBhigh and CD4+CD45RBlow subsets were defined as 40% of the highest and 40% of the lowest CD4+CD45RB-expressing cells.

Sorting of peripheral blood mononuclear cells (PBMC) and gut-derived T cells

Blood was drawn into a tube containing heparin (final concentration, 10 U/ml). PBMC were obtained using Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient centrifugation (400 g, 20 min). Subsequently, the cells residing at the interface were washed three times and resuspended in Iscove’s medium supplemented with 10% pooled human serum (BioWhittaker, Belgium) and ciproxin (10 μg/ml; Gibco-BRL, Life Technologies, Rockville, MD). Gut-derived T cells were isolated as described above under sterile conditions and resuspended in Iscove’s medium supplemented with 10% pooled human serum and ciproxin. For staining, per 1×106 cells, 20 μl unlabeled mouse anti-human-CD45RB antibody was added, and cells were incubated for 30 min on ice. After washing with sterile medium (Iscove’s medium supplemented with 10% pooled human serum (BioWhittaker)) and ciproxin (10 μg/ml), a secondary goat anti-mouse–FITC-labeled antibody (Immunotech) was added and incubated for 20 min on ice in the dark. Subsequently, cells were washed, and a 10-min blocking step with normal mouse serum (CLB, Amsterdam, The Netherlands) without preservative was performed before staining with 10 μl mouse anti-human–CD4–PE–Cy5-labeled antibody (Immunotech) per 1×107 cells for 20 min on ice in the dark. Cells were sorted by use of the FACS-Vantage (BD Biosciences, Pharmingen) under sterile conditions. CD4+CD45RBhigh and CD4+CD45RBlow subsets were sorted and defined as 40% of the highest and 40% of the lowest CD4+CD45RB-expressing cells. After sorting, the subpopulations were reanalyzed on a FACScan or FACS-Calibur (BD Biosciences, Pharmingen), and only the populations more than 95% pure were used. The sorted cells were washed and resuspended in RPMI 1640 (BioWhittaker), supplemented with 5% pooled human serum (BioWhittaker) and antibiotics (Gibco-BRL, Life Technologies). Sorted cells were stimulated (concentration, 1×105/well) with anti-human anti-CD3 and anti-CD28 antibodies (both 1:1000, CLB) for 72 h at 37°C.

Cytokines

 Supernatant of the stimulated peripheral blood cells was aspirated, and the concentration of interleukin (IL)-4, IL-10, interferon-γ (IFN-γ), and tumor necrosis factor α (TNF-α) was analyzed using enzyme-linked immunosorbent assay (CLB), according to the instruction of the manufacturer. For cytokine determination in the supernatants of the stimulated gut-derived T cells, the Cytometric Bead Array kit (BD Biosciences, Pharmingen) was used, according to the manufacturer’s instructions.

Immunofluorescence staining

Double-staining was performed as described previously with some modifications [14]. Brieﬂy, cryostat fragments of colon tissue were cut into 4–6 μm sections, air-dried overnight, and ﬁxed in acetone for 10 min at room temperature. The slides were ﬁrst incubated with 5% (vol/vol) normal goat serum (CLB), then with optimal dilutions of CD45RA–FITC (BD Biosciences, Pharmingen), CD4–PE (BD Biosciences, Pharmingen), rabbit anti-PE (Biosensis, UK), and Cy3-conjugated goat anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, PA). For each ﬂuorochrome label, negative-control antibodies were included. Fluorescence was analyzed using a Leica TCS SP (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser combination.

Statistics

Data are provided as mean and SEM. The Mann-Whitney U-test and the Wilcoxon test were used where appropriate (SPSS for Windows, version 7.5). A double-sided P < 0.05 was considered to represent a signiﬁcant difference.

RESULTS

Differential expression of CD45RA and CD45RO within the mucosal CD4+CD45RBhigh T cell subpopulation

To examine the distribution of naïve (CD45RA) and memory cells (CD45RO) within the CD45RBhigh and the CD45RBlow subgroups, four-color flowcytometric analysis was performed on peripheral blood and mucosal lymphocytes of controls, CD, and UC patients (Fig. 1A). In peripheral blood of controls,
99.6 ± 0.4% of the CD45RBhigh cells were CD45RA+, whereas the CD45RBlow subgroup almost exclusively contained CD45RO cells (93.4±1.2%). No differences were observed among UC, CD, and controls (Table 1).

Within the gut mucosa, the number of CD4+ CD45RBhigh+ T cells in IBD is significantly increased compared with controls (Fig. 1B). In controls, 2.5 ± 1.2% of the CD4+ T cells are CD45RBhigh compared with 18 ± 5% in CD and 41 ± 9.3% in UC. Also, the distribution of CD45RO and CD45RA within the CD45RBhigh and CD45RBlow CD4+ populations differed from peripheral blood (Fig. 1C). The CD4+ CD45RBlow cells from the gut, in contrast, were almost exclusively CD45RO-positive, and no differences were seen among CD, UC, or normal, control, gut-derived T cells (Table 1). However, within the CD4+ CD45RBhigh+ population of gut-derived CD4+ T cells, CD45RA-positive and CD45RO-positive cells were found (Table 1). In CD patients, the CD45RA/RO ratio was 27.7/59.7 and in UC, 49.0/34.3, compared with 4.5/90.0 in controls. This indicates that in IBD, the majority of the gut-derived CD4+ T cells is in fact CD45RBhigh CD45RA+.

To determine the localization of these CD4+/CD45RA+ cells in the intestinal mucosa, cryostat sections of IBD patients were stained for CD45RA and prepared for confocal laser-scanning microscopy (Fig. 2). In IBD patients, CD45RA-positive cells were found in the lamina propria and in follicular structures.

Cytokine profile of CD45RB cells in peripheral blood cells

To investigate functional differences between the CD4+ CD45RBhigh and CD4+ CD45RBlow T cell subsets, PBMC were sorted for the expression of this marker. After sorting, cells were stimulated for 72 h by anti-CD3/anti-CD28 double-stimulation, and the production of IFN-γ, IL-10, TNF-α, and IL-4 was determined (Fig. 3). CD4+ CD45RBlow T cells of normal controls produced fivefold more IL-10 than CD4+ CD45RBhigh T cells (92.6 pg/ml vs. 187.8 pg/ml in CD4+ CD45RBhigh T cells). In contrast, CD4+ CD45RBhigh T cells produced more TNF-α (1122.3 pg/ml vs. 2617.9 pg/ml) and IL-4 was determined (Fig. 3). CD4+ CD45RBhigh T cells produced more IL-10 than cells isolated from normal controls (200.6 pg/ml vs. 147.9 pg/ml in CD4+ CD45RBhigh T cells), and IL-4 production of peripheral blood CD4+ CD45RBhigh and CD4+ CD45RBlow T cells was significantly lower

**Table 1. CD45RA, CD45RB, and CD45RO Expression on Gut- and Peripheral Blood Derived CD4+ T Lymphocytes in UC or CD and Control Patients**

<table>
<thead>
<tr>
<th>CD4+ lymphocytes</th>
<th>CD45RBlow CD45RO</th>
<th>CD45RBhigh CD45RA</th>
<th>CD45RBhigh CD45RO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CD45RBlow CD45RO</td>
<td>CD45RBhigh CD45RA</td>
<td>CD45RBhigh CD45RO</td>
</tr>
<tr>
<td>N = 6</td>
<td>Gut-derived</td>
<td>98.1 ± 0.7%</td>
<td>4.5 ± 1.0%</td>
</tr>
<tr>
<td>UC</td>
<td>Peripheral blood</td>
<td>93.4 ± 1.2%</td>
<td>96.5 ± 0.4%</td>
</tr>
<tr>
<td>N = 8</td>
<td>Gut-derived</td>
<td>94.8 ± 5.6%</td>
<td>49.0 ± 9.2%*</td>
</tr>
<tr>
<td>CD</td>
<td>Peripheral blood</td>
<td>90.1 ± 1.1%</td>
<td>27.7 ± 6.6%*</td>
</tr>
<tr>
<td>N = 8</td>
<td>Gut-derived</td>
<td>97.9 ± 1.3%</td>
<td>69.1 ± 1.5%</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>CD45RBlow CD45RO</td>
<td>94.4 ± 1.2%</td>
<td>86.4 ± 4.2%</td>
</tr>
</tbody>
</table>

From patients with UC or CD and control patients, the gut-derived and peripheral blood lymphocytes were gated. From this population, the 40% CD4+ CD45RBlow and CD4+ CD45RBhigh T cells were gated as described in Materials and Methods. In both subgroups, the percentages of CD45RA and CD45RO expression were determined. Data are presented as mean and se; * significant as compared with controls (P < 0.05).
cantly reduced in CD patients compared with normal controls. In addition, the IFN-γ production of CD4⁺CD45RB<sup>high</sup> cells of UC patients was significantly decreased. There was a trend toward higher production of IL-4 in CD patients (P=0.083), whereas in UC patients, the IL-4 production was significantly decreased (398.1 pg/ml vs 116.2 in CD and 162.1 pg/ml in UC). No differences in TNF-α production were found among the three patient groups. The lymphocytes isolated from the gut of UC and CD patients demonstrated an enhanced CD69, CD134, CD44, and human leukocyte antigen-DR expression compared with control lymphocytes (results not shown), indicating an activated phenotype. This did not result in an enhanced, spontaneous cytokine secretion, as in the absence of CD3 and CD28, these cells did not secrete detectable levels of cytokines.

**Cytokine profile of CD45RB gut-derived T cells**

Cells were isolated from surgical resection specimen and sorted for the expression of the CD4⁺CD45RB<sup>high</sup> and CD4⁺CD45RB<sup>low</sup> T cells. After sorting, the cells were stimulated for 72 h by anti-CD3/anti-CD28 double-stimulation, and the production of IFN-γ, IL-10, TNF-α, and IL-4 was determined (Fig. 4). The results were similar to those found in peripheral blood. CD4⁺CD45RB<sup>low</sup> T cells produced more IL-10 and less TNF-α as compared with the CD4⁺CD45RB<sup>high</sup> subgroup. CD4⁺CD45RB<sup>high</sup> T cells isolated from a normal, control patient produced very little. TNF-α (CD4⁺CD45RB<sup>low</sup> 48.4 pg/ml and CD4⁺CD45RB<sup>high</sup> 320.4 pg/ml in controls). CD4⁺CD45RB<sup>low</sup> T cells from CD patients produced 504.0 ± 226.1 pg/ml and CD4⁺CD45RB<sup>high</sup> 1933 ± 1128 pg/ml. In contrast to the low TNF-α production, control-derived cells produced high amounts of IL-10 compared with cells derived from CD patients. Control CD4⁺CD45RB<sup>low</sup> T cells produced 691.3 pg/ml, as compared with 219.0 ± 73.8 pg/ml in CD patients. The CD4⁺CD45RB<sup>high</sup> and CD4⁺CD45RB<sup>low</sup> subgroups produced similar amounts of IFN-γ (mean production in CD: 5051 ± 2002 pg/ml in CD45RB<sup>high</sup> and 4444 ± 1419 pg/ml in CD45RB<sup>low</sup>).

**DISCUSSION**

Transfer of CD4⁺CD45RB<sup>high</sup> expressing T lymphocytes into an immunodeficient mouse induces severe intestinal inflammation [2, 3], and cotransfer with lymphocytes expressing CD4⁺CD45RB<sup>low</sup> T cells prevents disease development [4–6]. Because of this functional importance of CD45RB expression in mouse intestinal inflammation, we studied the role of intestinal CD4⁺CD45RB<sup>T</sup> cell expression in patients with IBD. In normal human mucosa, all CD4⁺ T cells have the memory
controls T lymphocyte activation and survival probably differs. In CD, but not in UC, lamina propria T lymphocytes are less sensitive to apoptosis as a result of a change in the Bax/Bcl-2 ratio [20, 21]. Our data suggest that this resistance to apoptosis in CD is not caused by a defective switch from CD45RBhigh to CD45RBlow and probably is a result of events further downstream.

Sorting experiments further emphasized the functional significance of the CD45RBhigh and CD45RBlow expression in human intestinal inflammation. Peripheral blood and intestinal T cells were sorted for CD45RB expression, and subsequently, the cytokine pattern was analyzed. In peripheral blood and intestinal cells, different cytokine profiles were observed between CD4+CD45RBlow and CD4+CD45RBhigh. CD4+CD45RBlow cells produce high amounts of IL-4 and IL-10, whereas the CD4+CD45RBhigh T cells produce high amounts of TNF-α. The markedly different cytokine profile of the CD45 subsets is reminiscent of analog mouse T lymphocyte subsets. In the mouse transfer model of colitis, the CD45RBlow subset protects against development of colitis, which is mediated by high production of IL-10 [22]. We now demonstrate that human CD4+CD45RBlow T cells produce significantly more IL-10 than CD4+CD45RBhigh T cells. It is interesting that in our study, the CD4+CD45RBlow subset from IBD patients produced significantly less IL-10, which has an immune-regulatory role in IBD. The pivotal importance of IL-10 as a regulatory cytokine within the mucosal environment was shown in IL-10−/− mice, which develop severe colitis [23]. IL-10 was identified as a differentiation factor for a subset of T lymphocytes, regulatory T lymphocytes (Tr1 cells). These Tr1 cells have immune-suppressive properties and were found to suppress experimental colitis induced by transfer of pathogenic CD4+/CD45RBhigh T cells [24, 25]. The source of IL-10 important for the development of regulatory T cells is not defined yet, but special regulatory dendritic cell populations secreting high levels of IL-10 could play a major role [26–28]. Future studies will elucidate their role in IBD.

It is unexpected that CD4+CD45RBlow T cells isolated from UC patients produced low amounts of IL-4 compared with CD patients and controls. However, it was previously reported that IL-5 and not IL-4 plays an important role in UC [29]. Although it was reported that CD45RBhigh cells produce more IFN-γ [15], we did not observe differences in IFN-γ production between CD45RB subsets obtained from controls. However, the IFN-γ production by the CD4+CD45RBhigh subset of IBD patients was decreased. Furthermore, the role of IFN-γ in intestinal inflammation is controversial, as IFN-γ-deficient and IFN-γ receptor-deficient mice are susceptible to experimental 2,4,6-trinitrobenzene sulphonic acid-induced colitis [30, 31].

In summary, we here report marked differences in the expression of CD45RBhigh by T lymphocytes present in the inflamed mucosa of patients with CD and UC. The CD45RBhigh and CD45RBlow differentiation could be of clinical importance, as we also observed an important, functional difference between both subgroups, not depending on CD45RA or CD45RO expression. These results indicate that CD45RB could play an important role in the maintenance of the intestinal inflammation by T cell activation.

Fig. 4. Cytokine production of gut-derived T cells sorted for CD4+CD45RBhigh (H) and CD4+CD45RBlow (L) expression and subsequently stimulated with CD3/CD28 for 72 h. (A) IL-10 production. (B) IL-4 production. (C) TNF-α production. (D) IFN-γ production. Solid bars represent a normal control; hatched bars represent CD patients.
REFERENCES


