IFN-γ-Stimulated Neutrophils Suppress Lymphocyte Proliferation through Expression of PD-L1

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Abstract

During systemic inflammation different neutrophil subsets are mobilized to the peripheral blood. These neutrophil subsets can be distinguished from normal circulating neutrophils (CD16bright/CD62Lbright), based on either an immature CD16dim/CD62Lbright or a CD16bright/CD62Ldim phenotype. Interestingly, the latter neutrophil subset is known to suppress lymphocyte proliferation ex vivo, but how neutrophils become suppressive is unknown. We performed transcriptome analysis on the different neutrophil subsets to identify changes in mRNA expression that are relevant for their functions. Neutrophil subsets were isolated by fluorescence-activated cell sorting from blood of healthy volunteers that were administered a single dose of lipopolysaccharide (2 ng/kg i.v.) and the transcriptome was determined by microarray analysis. Interestingly, the CD16bright/CD62Ldim suppressive neutrophils showed an interferon-induced transcriptome profile. More importantly, IFN-γ, but not IFN-α or IFN-β stimulated neutrophils, acquired the capacity to suppress lymphocyte proliferation through the expression of programmed death ligand 1 (PD-L1). These data demonstrate that IFN-γ-induced expression of PD-L1 on neutrophils enables suppression of lymphocyte proliferation. Specific stimulation of neutrophils present at the inflammatory sites might therefore have a pivotal role in regulating lymphocyte-mediated inflammation and autoimmune disease.

Introduction

Neutrophils represent the highest proportion of circulating leukocytes in the peripheral blood. Following invasion of microorganisms, these cells are recruited to the site of infection where they use their antimicrobial capacity to clear invading pathogens [1]. In the last decade, this “conventional” view of neutrophils in the immune response has changed substantially. Besides their capacity to kill invading pathogens, neutrophils have been shown to modulate the immune system on various levels [2]. The first evidence that neutrophils can modulate the response of other immune cells was found in their interaction with dendritic cells (DCs). Neutrophils were shown to induce maturation of monocyte-derived DCs and boost DC cytokine production, resulting in T-cell proliferation and polarization towards a Th1 phenotype [3,4].

Recent studies have shown that during experimental human endotoxemia i.e. systemic inflammation elicited by LPS administration in healthy volunteers or severe trauma changes the heterogeneity of the circulating neutrophils pool dramatically [5,6]. Three different neutrophil subsets can be distinguished based on their expression of CD16 and CD62L. CD16dim/CD62Lbright neutrophils appear to be released from the bone marrow and are characterized by a banded nuclear morphology and immature antimicrobial capacity. CD16bright/CD62Ldim neutrophils display a hypersegmented nucleus, increased functional antimicrobial capacity and, strikingly, exhibit the capacity to suppress lymphocyte proliferation. This novel immune regulatory mechanism for neutrophils was shown to be dependent on hydrogen peroxide release and expression of integrin MAC-1 (αMβ2) [6]. To date, it is unclear how this CD16bright/CD62Ldim subset of neutrophils acquires the ability to suppress lymphocyte proliferation. Knowledge on the regulation of this process could have important implications in the modulation of lymphocyte-mediated disease pathology.

Previously, we have shown that the total pool of circulating neutrophils during experimental human endotoxemia has a specific transcriptome profile that was reminiscent to a profile induced by a combination of direct activation by inflammatory cytokines and the influx of young neutrophils from the bone marrow [7]. In the current study, we further investigated the transcriptome of the different neutrophil subsets that emerge in the circulation during experimental human endotoxemia, based on the expression of CD16 and CD62L in order to identify factors involved in generation of suppressive neutrophils. Additionally, we
explored the mechanisms behind IFN-γ-induced neutrophil-mediated lymphocyte suppression.

Materials and Methods

Subjects and experimental human endotoxemia model

The neutrophil subset transcriptome was studied in 4 healthy male volunteers who participated in a human endotoxemia trial (Clinical Trial Register number NCT01374711, placebo group). The study protocol was approved by the Ethics Committee of the Radboud University Medical Centre and complies with the Declaration of Helsinki including current revisions and the Good Clinical Practice guidelines. Written informed consent was obtained from all study participants.

The experiments were performed according to a strict clinical protocol as described previously [8]. Subjects were screened before the start of the experiment and had a normal physical examination, electrocardiography, and routine laboratory values (including serology on HIV and hepatitis B). Subjects with febrile illness during the two weeks before the experiment were excluded. Subjects were not allowed to take any prescription drugs and asked to refrain from caffeine and alcohol intake 24 hours before the start of the experiment. Furthermore, subjects refrained from food 12 hours before the start of each endotoxemia experiment. After admission to the research intensive care unit of the Radboud University Nijmegen Medical Centre, purified LPS (US Standard Reference Endotoxin Escherichia Coli O113) obtained from the Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD) was administered at a dose of 2 ng/kg body weight. In all subjects, heart rate (5-lead electrocardiogram) and blood pressure (20-gauge radial artery catheter) were monitored starting 2 hours before administration of LPS until discharge 8 hours after LPS administration. A cannula was placed in an antecubital vein to permit infusion of prehydration fluid (150 mL/hour during 8 hours after LPS administration) to ensure optimal hydration status. Body temperature was measured using an infrared tympanic thermometer (FirstTemp Genius, Sherwood) once and stained for CD274 and CD279 were analyzed using a flow cytometer (FACSCalibur or FACS LSR II, BD Biosciences). The course of endotoxin-induced inflammation was determined with an automated system (ELEKTA, FBF-110, Sweden). Erythrocytes were lysed in isotonic ice-cold NH4Cl solution (8.3 g/L NH4Cl, 1 g/L KHCO3 and 37 mg/L EDTA) followed by centrifugation at 4°C as described previously [10]. After isolation, granulocytes (>95% pure with eosinophils as major contaminant) were washed in PBS and resuspended in RPMI buffered RPMI 1640 supplemented with 10% FCS. After Ficoll-Paque centrifugation, PBMCs fraction was washed twice with PBS and resuspended in RPMI supplemented with 2 mM L-Glutamine and 10% heat-inactivated human serum.

CD274, CD273 and CD279 expression experiments

Neutrophils were suspended in HEPES-buffered RPMI supplemented with 10% FCS to a concentration of 5.10^6/mL and incubated with 100 ng/mL TNF-α (BD Biosciences), 50 ng/mL G-CSF (R&D systems), 50 ng/mL GM-CSF (Sanquin), 200 ng/mL IFN-α-2a (Roche), 100 U/mL IFN-β-1A (Intrivogen), 1 to 1000 ng/mL IFN-γ (Sigma), 50 ng/mL LPS (Invitrogen) for 18–20 hours at 37°C+5% CO2. For short IFN-γ exposure, neutrophils were stimulated 15 minutes or 2 hours, where after IFN-γ was washed away and neutrophils were further incubated in HEPES-buffered RPMI supplemented with 10% FCS till 18–20 hours at 37°C+5% CO2. For kinetic experiments, neutrophils were stimulated 0, 2, 4, 6, 8 or 20 hours. Neutrophils were washed once and stained for αCD274, αCD273 or αCD279 (BD Biosciences) for 30 minutes at 4°C. Expression of CD274, CD273 and CD279 was analyzed using a flow cytometer (FACSCalibur or FACS LSR II, BD Biosciences).

For CD274 mRNA expression analysis, neutrophils were stimulated 0, 2, 4, 6 and 24 hours with IFN-γ, washed once with PBS and dissolved in RLT lysis buffer containing 1% β-mercaptoethanol and immediately frozen at –80°C.

Neutrophil survival

Neutrophils were suspended in HEPES-buffered RPMI supplemented with 10% FCS to a concentration of 5.10^6/mL and incubated with 100 ng/mL TNF-α (BD Biosciences), 50 ng/mL G-CSF (R&D systems), 50 ng/mL GM-CSF (Sanquin), 200 ng/mL IFN-α-2a (Roche), 100 U/mL IFN-β-1A (Intrivogen), 100 ng/mL IFN-γ (Sigma), 50 ng/mL LPS (Invitrogen) for 18–20 hours at 37°C+5% CO2. Apoptosis was determined by annexin-V binding (BD Biosciences). After staining the cells with annexin-V for...
15 min in the dark at room temperature in annexin-binding buffer, 10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂, 7-AAD were added. Cells were analyzed using a flow cytometer (FACSCalibur or FACS LSR II, BD Biosciences).

**Lymphocyte proliferation assay**

Neutrophils were suspended in HEPES-buffered RPMI supplemented with 10% FCS to a concentration of 5.10⁶/mL and stimulated with either recombinant 50 ng/mL GM-CSF, 200 ng/mL IFN-α (Roche), 100 U/mL IFN-β (In Vitrogen), 100 ng/mL IFN-γ (Sigma) or left untreated for 18–20 hours at 37°C with 5% CO₂. PBMCs from same donor were loaded with 5 μM CFSE (Sigma) and incubated 18–20 hours in HEPES-buffered RPMI supplemented with 10% pooled human AB-serum (Sigma) at 37°C with 5% CO₂. Neutrophils were washed twice with PBS, resuspended in HEPES-buffered RPMI supplemented with 10% pooled human AB-serum (Sigma) and added in various ratios to the CFSE-loaded PBMCs. Proliferation was stimulated with 5 μg/mL PHA (Sigma), CD3 (0.15 μg/mL)/CD28 (1 μg/mL) (Sanquin) or heat-killed *Candida albicans* (1.10⁶ CFU/mL) and measured by flow cytometry after 3 days (PHA and CD3/CD28) or 7 days (C. albicans) with the gating strategy as described in supplemental figure S3. Blocking studies were performed with 10 μg/mL 2CD274 (clone MH11, eBiosciences), 10 μg/mL 2CD11b (clone 44a, gift Prof. Leo Koenderman) or 10 μg/mL 2PAF-1 (clone 11A4, Cayman Chemicals) as isotype control that were present through the 3 days incubation.

**Neutrophil-PBMC interaction**

Neutrophils were suspended in HEPES-buffered RPMI supplemented with 10% FCS to a concentration of 5.10⁶/mL and stimulated with IFN-γ (Sigma) or left untreated for 18–20 hours at 37°C with 5% CO₂. Washed neutrophils were added to a concentration of 2.10⁶/mL in RPMI supplemented with 2 mM L-Glutamine and 10% heat-inactivated human serum. PBMCs from the same donor were incubated 18–20 hours at 37°C with 5% CO₂. Washed neutrophils were added to a concentration of 2.10⁶/mL in RPMI supplemented with 2 mM L-Glutamine and 10% heat-inactivated human serum. PBMCs from the same donor were incubated 18–20 hours at 37°C with 5% CO₂. Washed neutrophils were added to a concentration of 2.10⁶/mL in RPMI supplemented with 2 mM L-Glutamine and 10% heat-inactivated human serum. Neutrophils and PBMCs were mixed 2:1, stimulated with 5 μg/mL PHA (Sigma) in the presence or absence of 10 μg/mL 2CD11b (clone 44a) blocking antibody and Calcein-Blue and Calcein-AM double positive events were measured by flow cytometry (FACSCalibur or FACS LSR II, BD Biosciences).

**Statistics**

Statistical analysis was performed by using Graphpad prism 5. Reported values are shown as mean with standard error of the mean (SEM). We used a t-test, one-way ANOVA with Tukey post hoc test or two-way ANOVA with Bonferroni multiple comparisons. P values of <0.05 were considered statistically significant.

**Results**

Neutrophil subsets mobilized during experimental human endotoxemia display distinctive transcriptome profiles

Experimental human endotoxemia was used to induce systemic inflammation for mobilization of different neutrophil subsets into the circulation. Granulocytes were gated ( gating strategy described in supplemental figure S1) based on FSC/SSC, negative for CD14, and showed a large population of CD16bright/CD62Ldim neutrophils, a small population of CD16bright/CD62Lbright neutrophils and CD16neg/CD62Lhigh eosinophils (Figure 1A, upper panel). Four hours after a single intravenous dose of LPS (2 μg/kg body weight), 3 neutrophil subsets could be easily identified based on their expression of CD16 and CD62L (Figure 1A, lower panel). Hereafter, the different neutrophil phenotypes were isolated by FACS [5]. These subsets represented on average a CD16bright/CD62Lbright subset (62%), a CD16dim/CD62Lhigh (19%) and a CD16bright/CD62Ldim (19%) phenotype (Figure 1B). Microarray analyses of these neutrophil subsets revealed a clear response to LPS administration with 819 (CD16bright/CD62Lhigh), 998 (CD16bright/CD62Ldim) and 1108 (CD16dim/CD62Ldim) genes differentially expressed at least 2-fold relative to neutrophils isolated prior to LPS administration (Table S1). A total number of 690 genes were persistently higher expressed throughout the neutrophil subsets with lowest expression in CD16dim/CD62Lhigh, intermediate expression in CD16bright/CD62Ldim and highest expression in CD16bright/CD62Ldim neutrophils. Gene ontology (GO)-term enrichment analysis of this set of genes showed overrepresentation of genes involved in regulation of immune responses and apoptosis, but also the regulation of leukocyte proliferation (Figure 1C). The top 50 genes increased in CD16bright/CD62Ldim neutrophils compared to normal CD16bright/CD62Ldim neutrophils were enriched for interferon signaling, which included increased expression of *CXCL10*, *IDO1*, *IL1A*, *CCR2* and *CD274* (Figure 1D). Interestingly, from this list, expression of CD274 (the gene encoding for Programmed Death-Ligand 1, PD-L1), was highly increased in the suppressive CD16bright/CD62Ldim neutrophils compared to the CD16bright/CD62Ldim and CD16dim/CD62Ldim neutrophils. PD-L1 is a surface expressed ligand known to interact with its receptor PD-1 on various cell types to suppress cellular responses and proliferation [11]. We measured PD-L1 surface expression on the different neutrophil subsets 4 and 6 hours post-endotoxin administration. The surface protein expression of PD-L1 was significantly higher higher on CD16bright/CD62Ldim compared to normal CD16bright/CD62Ldim neutrophils and, intermediate on CD16dim/CD62Ldim neutrophils (Figure 1E).

**Ex vivo stimulation of neutrophils with IFN-γ induces expression of PD-L1**

With regard to the pronounced IFN-induced profile in CD16bright/CD62Ldim neutrophils, we further investigated the role of IFN signaling in the generation of suppressive neutrophils by stimulating freshly isolated neutrophils with various cytokines or LPS. Interestingly, especially IFN-γ, and to a lesser extend IFN-α or IFN-β, but not G-CSF, GM-CSF, TNF-α, LPS, increased PD-L1 expression as measured by flow cytometry (Figure 2A, and representative histograms in supplemental figure S2). Expression of PD-L1 could not be directly related to increased survival of the neutrophils by IFN-γ, since GM-CSF and G-CSF did not increase expression of PD-L1, but did increase survival (Figure 2B).

Subsequently, we assessed the dynamics of PD-L1 expression on IFN-γ-stimulated neutrophils. Expression of PD-L1 was especially apparent on annexin-V negative neutrophils (Figure 2C). Treatment with 1 ng/mL IFN-γ was sufficient to induce PD-L1 expression, reaching a plateau at 10–100 ng/mL (Figure 2D). Stimulation of neutrophils with IFN-γ for only 15 minutes was sufficient to induce PD-L1 expression after 18–20 hours (Figure 2E). Stimulation of neutrophils with IFN-γ increased CD274 mRNA expression starting at 2 hours, and reached
maximum after 6 hours (Figure 2F). Subsequently, we determined expression of PD-L1 on neutrophils in time. Stimulation of neutrophils with IFN-γ-induced PD-L1 surface expression slightly after 6 hours, which increased after 8 and 20 hours (Figure 2G), whereas this was not detected for CD273 (PD-L2) (Figure 2H) or CD279 (PD-1) (Figure 2I).

IFN-γ-stimulated neutrophils suppress lymphocyte proliferation

We determined the capacity of neutrophils that were stimulated with different types of interferons or GM-CSF to suppress lymphocyte proliferation (Gating strategy described in supplemental figure S3). Untreated, GM-CSF-, IFN-α- and IFN-β-stimulated neutrophils showed modest suppression of phytohemagglutinin (PHA)-induced lymphocyte proliferation, whereas IFN-γ-stimulated neutrophils showed a robust, up to 70%, inhibition of proliferation (Figure 3A–B and supplemental figure S3). Suppression of proliferation was also observed when lymphocytes were activated by CD3/CD28 (Figure 3C) or Candida albicans (Figure 3D), which points toward a general mechanism of suppression. To exclude a role for other leukocytes such as monocytes or eosinophils in the suppression of lymphocyte proliferation, neutrophils (CD16positive, CD14negative, CD3negative) and lymphocytes (CD16negative, CD14negative, CD3positive) were sorted by FACS. IFN-γ-stimulated purified neutrophils showed increased capacity to suppress PHA-induced purified lymphocyte proliferation compared to untreated neutrophils (Figure 3E). Although the induction of PHA-induced proliferation was decreased (data not shown), the level of suppression was comparable with total cell populations, indicating that this process is not dependent on the presence of other cell types such as monocytes.
Subsequently, lymphocyte phenotype after co-culture with neutrophils was analyzed. There was no significant difference in the distribution of CD4 and CD8 lymphocytes after co-stimulation with either IFN-γ-stimulated or control neutrophils (Figure 3F).

IFN-γ-induced PBMC suppression is dependent on cell-cell contact and PD-L1

Next, we investigated whether neutrophil-mediated T-cell suppression was dependent on cell-cell contact between lympho-
cytes and IFN-γ-stimulated neutrophils using a transwell system separating both cell suspensions. In this system, neutrophils lost their suppressive capacity indicating that cellular proximity is needed between neutrophils and lymphocytes (Figure 4A).

Previously it was postulated that CD16bright/CD62Ldim neutrophils form a synapse with lymphocytes wherein integrin MAC-1 (CD11b) plays a pivotal role [6]. We hypothesized that, next to the formation of a synapse by integrin MAC-1 on neutrophils, expression of PD-L1 would contribute to the suppressive function on lymphocyte proliferation because expression of this molecule on other cell types enables suppression of lymphocyte activation and proliferation [11]. Blocking MAC-1 using monoclonal antibody 44a in our co-cultures showed a modest decrease in suppressive capacity (Figure 4B). As hypothesized, PD-L1 showed to be absolutely essential for the IFN-γ-induced suppressive effect, because blocking PD-L1 attenuated suppression to the level of unstimulated neutrophils, whereas the isotype control antibody showed no effect (Figure 4B). In order to evaluate the role of MAC-1 and IFN-γ on the interaction between neutrophils and lymphocytes, we loaded neutrophils with Calcein-Blue and PBMCs with Calcein-AM. Interactions between these cell types were visualized as double positive events by flow cytometry. The percentage double positive events increased to more than 20% in 120 minutes of co-culture, which decreased to 15% in the presence of IFN-γ (Figure 4C). However, no differences were found between IFN-γ-, GM-CSF-stimulated or unstimulated neutrophils. Therefore, we conclude that interactions between neutrophils and lymphocytes occur independently of stimulation, but only in the presence of PD-L1 expression neutrophils inhibit lymphocyte proliferation.

**Discussion**

In the present study, we show that the neutrophil subsets that appear in the circulation during systemic inflammation elicited by experimental human endotoxemia, have distinct gene expression profiles. Our gene expression data indicate that for a significant amount of genes, the expression increases on a gradual scale with lowest expression in CD16dim/CD62Lbright neutrophils, intermediate expression in CD16bright/CD62Ldim neutrophils and the
highest expression in CD16bright/CD62Ldim neutrophils. With regard to their pronounced inflammatory gene expression pattern and hypersegmented nucleus, it can be suggested that CD16bright/CD62Ldim neutrophils are representative of a later phase in the lifespan of neutrophils. The origin of these CD16bright/CD62Ldim neutrophils is currently unknown. Since CD62L is shed from activated neutrophils, this marker has limitations in defining a homogenous subset of neutrophils [12]. The CD16bright/CD62Ldim neutrophil subset, obtained during experimental human endotoxemia, is clearly able to suppress lymphocyte proliferation in contrast to the CD16dim/CD62Ldim neutrophils [6].

We sought to investigate the factors involved in the generation of CD16bright/CD62Ldim suppressive neutrophil subset during systemic inflammation in vivo based on their transcriptome. Our gene expression data showed upregulation of various IFN-induced genes during endotoxemia, which was most pronounced in the CD16bright/CD62Ldim neutrophil subset. Previously, it has been shown that stimulation of whole blood with IFN-γ + GM-CSF induces expression of IFN-γ-regulated genes CXCL10, IDO1, IL1α, CCRL2 and CD274 [13], a profile that resembles the transcriptome of CD16bright/CD62Ldim neutrophils.

The moment of increased surface expression of PD-L1 on neutrophils during experimental human endotoxemia (6 hours post LPS), compared to our ex vivo experiments (6–8 hours post IFN-γ), which suggests that neutrophils are exposed to IFN-γ shortly after LPS infusion. The main producers of IFN-γ are CD4+ Th1 lymphocytes, CD8+ cytotoxic lymphocytes and natural killer (NK) cells [14]. These cell types typically do not respond directly to LPS, therefore, an indirect effect of LPS on the release of IFN-γ appears more likely. For instance, it was recently shown that flagellin-induced rapid IL-1β release from dendritic cells, which induced IFN-γ release from memory CD8+ T cells within 2 hours [15]. However, whether a similar indirect mechanism is responsible for IFN-γ release after LPS administration remains to be determined. Currently, we have no evidence that IFN-γ induces PD-L1 expression on neutrophils in vivo, or whether PD-L1 on CD16bright/CD62Ldim neutrophils is essential for the suppressive capacity observed by Pillay and co-workers [6]. The data we present here do support an important role for PD-L1 on neutrophils in lymphocyte proliferation in vitro.

The induction of PD-L1 on IFN-γ-stimulated neutrophils is likely de novo synthesis since a recent study did not detect this protein in the granules [16]. Expression of PD-L1 on circulating neutrophils has been shown in patients with active tuberculosis [17]. This is especially interesting because neutrophils from patients with active tuberculosis also exhibit an IFN-γ-induced transcriptome profile, including increased expression of CXCL10 and CD274 [18]. To date, no studies have investigated PD-L1 expression on neutrophils during systemic inflammatory diseases. Interestingly, during sepsis, increased expression of PD-L1 on monocytes has been suggested to play an important role in sepsis-induced immunosuppression [19,20].

To our knowledge, we are the first to identify an immune-suppressive effect of IFN-γ through expression of PD-L1 on neutrophils. Although originally defined as an agent with direct antiviral activity, the properties of IFN-γ also include regulation of several neutrophil functions such as stimulation of the respiratory burst [21], increased ex vivo survival [22] and antigen presentation [23]. We demonstrate that the IFN-γ-induced suppression of lymphocyte proliferation is dependent on increased expression of PD-L1. Under steady state conditions, expression of PD-L1 on neutrophils is very low [23] and these neutrophils show only a minor suppressive phenotype ex vivo. The suppressive phenotype on lymphocyte proliferation was independent of the stimulation method, as similar findings were observed for PHA, CD3/CD28 and Candida albicans stimulation. The suppressive capacity of the IFN-γ-stimulated neutrophils, as shown by neutrophil lymphocyte co-culture in transwell experiments, occurred in a cell-cell contact dependent manner. IFN-γ has been shown to increase expression of PD-L1 on various cell types [24] resulting in suppressive activity through ligation with PD-1 on target cells [25]. By blocking PD-L1 on neutrophils we verified that suppression of lymphocyte proliferation was dependent on PD-L1–PD1 signaling.

This study shows for the first time that suppressive neutrophils can be generated using IFN-γ, which could be used as a novel approach to modulate inflammation. For instance, during influenza infections, the tissue damage that is associated with...
Whole blood was shocked and labeled with antibodies. Subsets. (PDF) CD62L expression (lower panels). Then neutrophil subsets were selected based on CD16 and (upper panels). Then CD14- granulocytes were selected (mid panels). First, granulocytes were gated based on forward/sideward scatter (upper panels). Then CD14- granulocytes were selected based on CD16 and CD662L expression (lower panels). (PDF)

Figure S2 Neutrophil CD274 expression gating strategy. (A) Neutrophils were selected on the basis of their FSC/SSC. (B) MFI of the whole granulocyte population was determined. (C) Overlay of PD-L1 expression of unstimulated, IFNα, IFNβ and IFN-γ-stimulated neutrophils. (PDF)

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Author Contributions

Conceived and designed the experiments: JDL SdK. Performed the experiments: JDL JL SdK. Analyzed the data: JDL SdK. Contributed reagents/materials/analysis tools: JL MK MN PP LK. Wrote the paper: SdK JDL GF PP PH.

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