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Cytokine induced expression of programmed death ligands in human neutrophils

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1. Summary

Recent evidence indicates that human neutrophils can serve as non-professional antigen presenting cells (APC). Although expression of MHC class II and co-stimulatory molecules on human neutrophils is limited, these molecules can be significantly induced following *in vitro* exposure to the cytokines IFN γ and GM-CSF. Since professional APCs such as dendritic cells express both co-stimulatory and co-inhibitory molecules for activation and regulation of adaptive immunity, we determined whether cytokines induce increased expression of specific co-signaling molecules on human neutrophils. We report here that circulating human neutrophils express co-inhibitory molecules such as immunoglobulin-like transcript (ILT) 4 and 5, and also comparatively low and highly variable levels of ILT2 and 3, but the expression of these ILTs was not significantly changed by cytokine treatment. In contrast, we demonstrate for the first time that human peripheral blood neutrophils, although do not express the co-inhibitory molecule, programmed death ligand (PD-L) 1 on their surface, can express this molecule at moderate levels following cytokine exposure. Although moderate PD-L1 levels on healthy volunteers' neutrophils were not inhibitory to T cells, our findings do not exclude a possible robust increase in neutrophil PD-L1 expression in pathological conditions with immunosuppressive functions. These results suggest a possible immunoregulatory role for human neutrophils in adaptive immunity.

Keywords

Human neutrophil; PD-L; ILT; Cytokine

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2. Introduction

Although the role of polymorphonuclear neutrophils (PMN) in innate immunity is well established, their role in stimulating or regulating T cell functions is not well characterized [1,2]. In order to maintain homeostatic balance in the adaptive immune response, professional antigen presenting cells (APC) such as monocytes (MO) and dendritic cells (DC) express MHC class II and co-stimulatory molecules for signaling T cell activation and co-inhibitory molecules for inhibiting T cell activation [3,4]. In contrast to the well accepted role of MO and DC in the initiation and maintenance of adaptive immunity, the participation of human neutrophils, the most abundant circulating leukocyte population, in adaptive immune responses as accessory cells remains controversial. Recent studies have established the interaction of neutrophils with DCs during DC maturation resulting in enhanced antigen specific T cell responses [5,6]. Another recent report provides evidence for an *in vivo* role of neutrophils in antigen cross-presentation and naïve T cell cross-priming [7]. MHC class II (e.g., HLA-DR) and co-stimulatory molecules (e.g., CD86), important components of MO and DC's antigen presenting cell activity, have been extensively studied and found to be only minimally expressed on circulating PMN. However, these molecules can be significantly induced on human neutrophils in a variety of diseases and by *in vitro* activation with cytokines, such as IFN γ , and GM-CSF [8–10]. In contrast, neutrophil expression of co-inhibitory molecules such as the immunoglobulin-like transcript (ILT) and programmed death – ligand (PD-L) in disease or in response to cytokine activation has not been well characterized.

Immunoglobulin-like transcripts (ILTs, also known as leukocyte immunoglobulin-like receptors, LIR, CD85 family) represent a novel immunoglobulin super-family of inhibitory and stimulatory receptors expressed on myeloid cells such as monocytes and DCs [11,12] and non-professional APCs such as endothelial cells [13], involved in immune surveillance. Variable constitutive expression of several co-inhibitory ILTs [ILT2 (CD85j), ILT4 (CD85d), and ILT5 (CD85a)] has been reported in circulating human neutrophils, while the expression of ILT3 (CD85k) is absent [14–16]. Since the balance between co-stimulatory versus co-inhibitory molecule expression determines the strength of APC/accessory cell signal, characterization of concurrent expression of co-stimulatory and co-inhibitory molecules on cytokine treated human neutrophils and circulating neutrophils from patients with diseases will be critical to understand the role of neutrophils as accessory cells for T cell activation.

In contrast to the immunoglobulin-like transcripts, there has been no report of human neutrophil or other granulocyte expression of programmed death ligand 1 (PD-L1, B7-H1, CD274), an inhibitory molecule widely expressed on MO, DC [17], B cells, activated T cells [18] and even non-leukocytes such as epithelial cells [19]. The co-inhibitory role of PD-L1 is well documented while PD-L2 (CD273), the other PD-1 ligand, has been assigned both co-stimulatory and co-inhibitory functions [20,21]. Induction of PD-L1 expression on DCs has been reported to alter their co-stimulatory function providing more regulatory interactions with T cells, thus demonstrating the critical role of balanced expression of co-stimulatory versus co-inhibitory molecules on APCs [22]. Expression of PD-L1 is reported as increased on airway epithelial cells and mast cells after activation with IFN γ +GM-CSF (I+G) [23]. Since I+G delays neutrophil apoptosis and increases expression of several co-signaling molecules, we tested the effect of these cytokines on human neutrophil expression of co-inhibitory ILT and PD-L1 molecules. We also assessed the ability of these cytokine activated neutrophils to act as accessory cells for T cells and tested the role of specific co-signaling molecules in neutrophil modulation of T cell activation.

Here, we report that neutrophil expression of co-inhibitory ILT2, 3, 4 and 5 is not significantly altered after activation with I+G, although levels of ILT3 and 5 are slightly reduced. We demonstrate for the first time that PD-L1, though not expressed on untreated human

neutrophils, is induced on their surface concomitant to induction of HLA-DR and CD86 expression. Thus, pathological conditions or even therapeutic intervention that induces increased *in vivo* cytokine production may result in increased expression of PD-L1 by neutrophils.

3. Materials and methods

3.1. Reagents

Recombinant human IFN γ and GM-CSF were purchased from Peprotech Inc (Rocky Hill, NJ). Staphylococcal Enterotoxin A (SEA), rabbit SEA polyclonal antibody (PAb) and PE labeled goat anti-rabbit antibody were obtained from Sigma - Aldrich (St. Louis, MO). Monoclonal antibodies (MAb) against human CD1a (clone HI 149), CD14 (clone M Φ P9), CD9 (clone M-LI3, 8–10), HLA-DR [clone L243 (G46-6)], CD86 [clone 2331(FUN-1)] and their isotype controls were purchased from BD Biosciences (San Jose, CA). Antibodies against CD11b (clone BEAR) and CD66b (clone 80H3) were from Beckman Coulter (Fullerton, CA). MAbs against human PD-L1 (clone MIH1) and PD-L2 (clone MIH18) (biotinylated formulations for flow cytometry and functional formats for blocking experiments) and also CD16 (clone CB16) were purchased from eBioscience (San Diego, CA). Abs against ILT2 (clone 292305), 3, 4 (biotinylated goat anti-human antibody) & 5 (clone 222821) were purchased from R&D Systems (Minneapolis, MN). Biotinylated Annexin V was purchased from BD Biosciences. Neutralizing (functional) antibodies against HLA-DR [clone L243 (G46-6)] & CD86 (clone IT 2.2) antibodies and isotype controls were purchased from BD Biosciences.

3.2. Isolation of human neutrophils, T cells and monocytes and cell culture

Blood (60–90mls) was drawn into heparinized tubes from healthy volunteers for isolation of granulocytes, monocytes and T cells. Informed consent was obtained from every volunteer prior to blood collection, and the study was approved by the Institutional Review Board at the University of Rochester Medical Center. Granulocytes and T cells were isolated by Ficoll density centrifugation followed by dextran sedimentation (for granulocytes) and resetting with sheep red blood cells (for T cells) as described [24,25]. Monocytes (MOs) were separated from granulocyte and T cell depleted population by use of Miltenyi column and their negative isolation kits according to the manufacturer's instruction. The granulocyte preparation contained ~99% CD66b⁺ cells with < 0.3% monocyte contamination. Neutrophil (CD66b⁺ CD16^{high} CD9^{low}) purity in the granulocyte preparation ranged from 92–99% with variable ranges of (1–8%) eosinophil (CD66b⁺ CD16^{low} CD9^{high}) contamination. For functional assays, neutrophil populations were further depleted of contaminating eosinophils by using goat anti-mouse IgG bound magnetic beads and mouse anti-human CD9 MAb as described [26]. Isolated MO populations were ~90% CD14⁺ and T cells were ~95% CD3⁺.

Cells were suspended in RPMI 1640 medium containing 10% FBS, 50 units/ml of penicillin, 50 μ g/ml of streptomycin and gentamycin, 2-mM L-glutamine, 5-mM HEPES and 5×10^{-5} M 2-mercaptoethanol (complete RPMI) (Gibco BRL). Cell viability was confirmed by trypan blue exclusion. Cells were cultured at 37° C with humidified 5% CO₂ atmosphere. Freshly isolated granulocytes (described as neutrophils being the predominant population) (Day 0) were cultured in the presence of cytokines [IFN γ (100 U/ml) and GM-CSF (20 ng/ml) (I+G)] for 18–20 hrs (Day 1) and also for 40–44 hrs (Day 2), and were subsequently harvested for analysis of surface marker and functional study.

For differentiation of MOs to immature dendritic cells (iDC), MOs were cultured in presence of IL-4 (50 ng/ml) + GM-CSF (50 ng/ml) for 5 days and then cultured for additional 2 days in presence of TNF α (20 ng/ml) + IL-1 β (10 ng/ml) for generation of mature DC (mDC) [27]. Spent media and IL-4 + GM-CSF were replaced every 2–3 days. iDC and mDC were used as

positive controls for PD-L1 expression. To test the neutralizing effects of PD-L antibodies, iDCs were treated with IL-10 (50 ng/ml) for 2 days to obtain tolerogenic DC. Tolerogenic DCs were then incubated for 1hr with neutralizing antibodies against PD-L1, PD-L2 or isotype control (each 10 μ g/ml) and the allogeneic T cells were added to DC at 20:1 ratios and the MLR activities of DCs were assessed by [³H]-thymidine ([1 μ Ci/well] incorporation during the last 18 hrs of 6 day co-culture [27].

3.3. Assay for neutrophil accessory function

Neutrophils (1 $\times$ 10⁵/100 μ l) were pulsed with 20 ng of SEA for 3 hrs and unbound SEA was removed by two washes using PBS and 100 μ l of complete medium was added to each well. Autologous or allogeneic T cells (1 $\times$ 10⁵/100 μ l) were added to control and SEA pulsed neutrophils and the co-cultures were maintained for 3 days, then pulsed with [³H]-thymidine ([1 μ Ci/well]). The cells were harvested after 18–20 hrs for assessment of proliferation. In some experiments, after the neutrophils were pulsed with SEA and washed, cells were first incubated for 1hr with different neutralizing antibodies or isotype controls before addition of autologous T cells.

3.4. SEA binding Assay

SEA binding assay was performed as described [28]. In brief, 2 $\times$ 10⁵ neutrophils were cultured alone or treated with 100 ng of SEA for 40 minutes at 37° C. Cells were washed twice with ice-cold PBS containing 0.02% sodium azide and 2% FBS (FACS buffer). Cells were then blocked with human IgG for 15 minutes at 4° C; divided into 2 groups (1 $\times$ 10⁵ cells/group) and incubated for 30 minutes at 4° C with rabbit anti-SEA antibody or in FACS buffer alone (used for background binding subtraction). Cells were washed and incubated for 30 minutes at 4° C with PE labeled anti-rabbit antibody. Cells were then washed, fixed with Cytotfix buffer (BD Bioscience) and analyzed by FACS Calibur flow cytometer (BD Bioscience).

3.5. Neutrophil Phenotypic analysis by Flow cytometry

Neutrophils (1 $\times$ 10⁵ cells) were stained and analyzed by flow cytometry as described [27, 29]. For staining with biotinylated antibodies such as ILT3, ILT4, PD-L1 and PD-L2, cells were first stained with these antibodies and further labeled with Streptavidin-PerCP before fixation of the cells. Cells were also stained with 7AAD for dead cell discrimination and gating guidance for viable cells. Apoptosis was measured by incubating the cells for 30 min at 4° C with biotinylated Annexin-V in Annexin-V binding buffer (BD Bioscience) followed by staining with Streptavidin – PerCP and analyzed by flow cytometry. Data are represented as % positive cells and net mean fluorescence intensity (MFI) [as calculated by subtraction of the mean fluorescence intensity of the isotype control from that of the specific antibody].

3.6. Statistical analysis

Data are presented as mean $\pm$ SEM. A one-way ANOVA with Bonferroni correction was used to test whether each of the measured parameters varied over time. When this measurement was significant, the difference between the time points was assessed using Student's t test for paired samples. Two-tail hypothesis was considered, and the difference between two time points was considered statistically significant if p<0.05. Nonparametric statistics (Wilcoxon) were also performed where the data were either not normally distributed or had unequal variance.

4. Results

4.1. Differential expression of ILT2, 3, 4 and 5 on resting and cytokine activated human neutrophils

Although human circulating neutrophils (CD66b⁺ CD16^{high}CD9^{low}) express very low levels of MHC Class II (e.g., HLA-DR) and co-stimulatory molecules (e.g., CD86) as compared to professional APCs such as monocytes and DCs, they can express significantly increased levels of these molecules when their spontaneous apoptosis is delayed by exposure to IFN γ and GM-CSF (I+G) (Figure 1A–D and published reports) [8–10]. In addition to the expression of MHC class II and co-stimulatory molecules, professional APCs also express significant levels of ILT family co-inhibitory molecules ILT 2, 3, 4 and 5 [11, 12]. Expression of individual co-inhibitory ILTs on circulating neutrophils is relatively less characterized and the effects of cytokines on neutrophil ILT expression is not described [9, 14–16]. Fig. 2A and B demonstrates highly variable expression of ILT2 and 3 on unstimulated neutrophils among healthy controls (0.2 – 53% ILT2-expressing PMN with mean 21.6% and median 18.3%; ILT2 MFI: 0 – 7.19 with mean 3.09 and median 2.12; and 0 – 45% ILT3-expressing PMN with mean 15.4% and median 12.75%, ILT3 MFI: 0 – 6.1 with mean 3.09 and median 3.22). The levels (MFI) of expression of ILT2 and ILT3 were not significantly changed and still remained low even after I+G treatment. In contrast to high variations in expression of ILT 2 and 3, expression of ILT4 (Fig. 2C and D) and ILT5 (Fig. 2E and F) was uniformly high on almost all circulating neutrophils at baseline with a slight but not significant reduction only in ILT5 after exposure to I+G.

4.2. Cytokine - activated human neutrophils express PD-L1

In spite of a number of previous studies on ILT expression on human neutrophils, there still exists no report of human neutrophil expression of PD-L1, a functional co-inhibitory molecule expressed on professional APCs. We tested PD-L1 expression on human neutrophils isolated from 16 healthy donors. We could not detect surface expression of PD-L1 on neutrophils (CD66b⁺ CD16^{high} CD9^{low}) isolated from any of the donors (Fig. 3A, C, F, and H). When human monocytes were differentiated *in vitro* to DCs with high levels of functional APC activity, expression of PD-L1 was also significantly increased (data not shown). Therefore, we tested whether cytokine-activated human neutrophils expressed PD-L1 concomitant to elevated induction of HLA-DR and CD86. As depicted in Figure 3F and H, neutrophils cultured in the presence of I+G expressed significantly increased levels of surface PD-L1 than untreated neutrophils. Since there is no report of PD-L1 expression on other granulocytes and the neutrophil population had 1–8% eosinophil contamination, we also determined surface PD-L1 expression on those contaminating eosinophils. Similar to untreated neutrophils, PD-L1 expression was extremely low on untreated contaminating eosinophils (CD66b⁺ CD16^{low}CD9^{high}) (Fig. 3A, B, and D). However, in contrast to significant PD-L1 induction on cytokine activated neutrophils, eosinophil expression of PD-L1 was unaltered and still remained very low even after exposure to I+G. Thus, we demonstrate here that cytokine activation not only induces expression of MHC Class II and co-stimulatory CD 86, but also induces expression of the co-inhibitory molecule, PD-L1, on human neutrophils. We also tested the neutrophil expression of PD-L2 that is reported both as co-stimulatory as well as co-inhibitory molecule [20, 21]. As shown in Fig. 3G and H, PD-L2 expression was not detected on untreated neutrophils but cytokine activation induced significant increase in expression of this molecule. However, as compared to cytokine-induced PD-L1 levels, PD-L2 induction was very minimal (Fig. 3F, G, and H). Similar to PD-L1 levels, the untreated eosinophil population did not express any PD-L2 and cytokine treatment could not induce PD-L2 on those cells (Fig. 3D and E).

4.3. Cytokine activated neutrophils increase SEA binding and T cell accessory functions

Simultaneous induction of MHC class II (HLA-DR) and co-stimulatory (CD86) with co-inhibitory molecule (PD-L1) in cytokine activated neutrophils suggests a possible functional role for neutrophils as accessory cells for T cell activation or inhibition. In order to use pure neutrophils for functional assays, granulocyte populations were depleted of contaminating eosinophils (as detailed in method section). Purified neutrophils, untreated as well as cytokine-activated, were first tested for antigen capture by determining binding of SEA. As shown in Fig. 4A, SEA binding was highly increased in I+G treated neutrophils.

We then determined the ability of SEA pulsed (captured) neutrophils to activate autologous and allogeneic T cells. As depicted in Fig. 4B, the ability of cytokine activated (both Day 1 and 2) APC-like neutrophils to present SEA by inducing proliferation of autologous as well as allogeneic T cells was significantly increased as compared to untreated neutrophils. As compared to control Day 0 neutrophils, control Day 1 or 2 neutrophils (without SEA pulsing) induced an increase in proliferation of autologous or allogeneic T cells but levels of proliferation were very low (CPM <2000). Since cytokine treatment induced a simultaneous increase in the expression of HLA-DR and CD86 (that may enhance APC functions) and PD-L1 and PD-L2 (co-inhibitory molecules that may inhibit APC activity), we tested the critical functional roles of these molecules during T cell proliferation assay induced by SEA presented by neutrophils. Cytokine treatment did not have any significant effect on ILT expressions and therefore not tested for their critical regulatory roles in this study. Since the difference in T cell proliferation induced by day 1 and day 2 neutrophils was very minimal, we performed neutralization experiments only with unstimulated (day 0) and day 1 neutrophils. To test the critical functional role of HLA-DR and CD86, we first incubated SEA pulsed neutrophil population with neutralizing antibodies against HLA-DR and CD86 or their combination (isotype control combination kept as experimental control) for 1 hour before addition of autologous T cells (Fig. 4C). Similar experiments were performed with anti-PD-L1 or anti-PD-L2 neutralizing antibodies to assess their critical regulatory role (Fig. 4C). As expected, antibodies against HLA-DR or CD86 (most significant when used in combination) reduced T cell proliferation but neutralizing antibodies against PD-L1 and PD-L2 or their combination failed to induce any increase in T cell proliferation. The neutralizing activity of the PD-L1 antibody used in our experiments, is repeatedly reported. Moreover, in one experiment, we induced tolerogenic DC by IL-10 as described in methods. IL-10 treatment highly increased the DC expression of PD-L1 and PD-L2 (Control DC: PD-L1 MFI → 210.42 and PD-L2 MFI → 52.10; IL-10 treated tolerogenic DC: PD-L1 MFI → 334.44 and PD-L2 MFI → 69.43) concomitant to reduction in expression of CD86 (Control DC: CD86 MFI → 38.34; IL-10 treated tolerogenic DC: CD86 MFI → 8.13). As can be seen in Fig. 4D, T cell proliferation induction capacity of tolerogenic DC was highly reduced as compared to control DC (Mean CPM: Control DC → 53,654 versus tolerogenic DC → 20,652). Addition of PD-L1 neutralizing antibody to tolerogenic DC, greatly restored T cell proliferation (Mean CPM: Tolerogenic DC + anti-PD-L1 antibody → 41,998) while PD-L2 neutralizing antibody only slightly restored MLR activity (Mean CPM: Tolerogenic DC + anti-PD-L2 antibody → 33,615; Tolerogenic DC + isotype control for PD-L1/2 → 29,996). These data indicate that PD-L1 neutralizing antibody used in our experiments is functional. Minimal neutralizing effects of PD-L2 indicate limited inhibitory activity of PD-L2. In fact, PD-L2 is no longer considered as an inhibitory molecule for APCs. Although aberrantly high expression of PD-L1 (similar to IL-10 treated tolerogenic DC) can be highly inhibitory, our data with healthy volunteers' neutrophils indicate that moderate levels of PD-L1 expressed on cytokine induced human neutrophils do not have any inhibitory effects on T cell functions.

5. Discussion

Professional APCs express both co-stimulatory and co-inhibitory molecules for activation and regulation of immune responses. We demonstrate that the ILT family co-inhibitory molecules ILT2, 3, 4 and 5 have variable expression on unstimulated human neutrophils and their expression levels are not significantly unaltered by cytokines that delay apoptosis in neutrophils with an increase in expression of HLA-DR and CD86. We report here for the first time that human neutrophils can express surface PD-L1 and 2 following exposure to IFN γ +GM-CSF (I+G).

There have been a number of reports examining expression of different co-inhibitory ILT family molecules on untreated granulocyte populations but the individual ILT expression patterns reported are highly variable in published literature [14–16]. For example, Colonna et al. showed high ILT5 expression on granulocytes while ILT2, 3 and 4 were absent [16]. In contrast, Tedla et al. reported low levels of ILT2 with high levels of ILT4 and ILT5 on human neutrophils, while ILT3 was not detected at all [15]. Because of the wide variations in the ILT expression data in the literature, we first assessed the expression of these molecules on untreated neutrophils from healthy human subjects. Our data demonstrate consistent high level expression of ILT4 and ILT5 on neutrophils isolated from all of the healthy subjects. In contrast, we detected a much lower and more highly variable level of expression of ILT2 among the eleven donors tested. Variation in detection of ILT2 expression could reflect a marked heterogeneity in the ability of individual donor subjects to express the protein as reported for MHC class II [9]. Additionally, low expression levels of ILT2 may also reflect detection sensitivity of the antibody utilized. We also report detectable but highly variable levels of expression of ILT3 in human donor neutrophils using the biotin conjugated antibody and Streptavidin-PerCP amplified detection procedure. These results suggest that ILT2 and ILT3 may be excluded from the repertoire of human neutrophils for accessory cell functions or that it is induced in response to signals generated during different pathological conditions. Addition of I+G to neutrophils had no significant effect on neutrophil expression of ILT2, 3, 4, or 5 despite a marked induction of MHC class II and CD86.

Although human neutrophil expression of ILT family co-inhibitory molecules has previously been reported, we report here for the first time the expression of PD-L1 (a highly characterized co-inhibitory molecule) on neutrophils stimulated *in vitro* with I+G. The response of cultured neutrophils to I+G was more robust and specific to PD-L1 as compared to PD-L2. Increased expression of PD-L1 has been described in airway epithelial cells after activation with GM-CSF or IFN γ [23]. PD-L1 expression on untreated HL-60 cells was reported while studying leukemic cells isolated from patients and leukemia cell lines [30]. Interestingly, Tamura et al. could not detect PD-L1 expression on unstimulated HL-60 cells, but could induce low levels of PD-L1 in those cells after stimulation with IFN γ [31]. Since HL-60 cells are a promyelocytic cell line, they express many proteins that are also expressed in monocytes. Monocytes express PD-L1 [17,18]. HL-60 cells differentiate into neutrophil – like cells when they are cultured in presence of stimulants such as dimethyl sulfoxide [32]. Therefore, low levels of PD-L1 detected in undifferentiated HL-60 cells by Tamura et al. may not reflect the expression of PD-L1 on differentiated neutrophils. In fact, even after using biotinylated monoclonal antibody followed by Streptavidin – PE, we failed to detect any surface PD-L1 expression on freshly isolated human neutrophils, thus indicating the absence of surface PD-L1 on these untreated cells.

Our data demonstrate that cytokine treatment not only induces HLA-DR and CD86 expression, but also moderate levels of PD-L1 and PD-L2. T cell activating capacity of neutrophils was highly reduced while neutralizing antibodies against HLA-DR and CD86 (particularly their combination) were used. However, these antibodies (particularly anti-CD86) could not completely block SEA induced (presented by neutrophils) T cell proliferation since many other

well characterized APC function related molecules such as HLA-DP, HLA-DQ, CD80, CD40 etc. could also be involved in neutrophil APC activity. Interestingly, although cytokine activated neutrophils demonstrated a significant increase in accessory cell function as compared to untreated cells, expression of potent co-inhibitory molecule PD-L1 was actually increased. We hypothesized that the observed T cell activating ability of cytokine activated neutrophils may be partially inhibited by PD-L1 and/or PD-L2. However, use of neutralizing antibodies against PD-L1 or PD-L2 alone or their combination could not significantly alter neutrophil directed T cell proliferation. The inability of the neutralizing antibody to augment T cell activation may be due to the fact that PD-L1 expression in the cytokine activated neutrophils has not reached the threshold to significantly impact co-stimulatory effects or disrupt the balance between the co-stimulatory and co-inhibitory activity. In fact, DCs express PD-L1 at a much higher level than what we observed in cytokine treated neutrophils experiments but they are still the best known APCs with very high T cell stimulatory property [3,4]. However, tolerizing stimuli such as interleukin-10 can make DC as inhibitory cells due to induction of extremely high levels of PD-L1 [33]. Therefore, moderate levels of PD-L1 observed on healthy control neutrophils in our study may not be actually high enough to induce T cell inhibition.

Induction of HLA-DR and PD-L1 on cytokine - activated neutrophils demonstrated in this study may have a significant role in human diseases. High PD-L1 expressing DCs have been reported in inflammation [34] and cancer [35]. Leukemic and even solid tumor cells of epithelial origin express high levels of PD-L1 and cause immune suppression [31]. High HLA-DR expressing neutrophils are reported in many human diseases such as Wegener's granulomatosis [36], rheumatoid arthritis [37], abdominal sepsis [38] and periodontal disease [39]. Our data would suggest the possibility of PD-L1 expression on neutrophils in those pathological conditions where neutrophils express HLA-DR. Moreover, our novel findings certainly suggest a new area for further investigation to characterize and define the role of possible high expression of co-inhibitory molecules such as PD-L1 on human neutrophils, and their functional role in immune suppression observed in different human diseases such as cancer, rheumatoid arthritis, sepsis and trauma.

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References

1. Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev* 2007;219:88–102. [PubMed: 17850484]
2. Herrmann JM, Bernardo J, Long HJ, Seetoo K, McMenamin ME, Batista EL Jr, et al. Sequential chemotactic and phagocytic activation of human polymorphonuclear neutrophils. *Infect Immun* 2007;75:3989–98. [PubMed: 17526745]
3. Steinman RM. Dendritic cells: understanding immunogenicity. *Eur J Immunol* 2007;37 (Suppl 1):S53–60. [PubMed: 17972346]
4. Adler HS, Steinbrink K. Tolerogenic dendritic cells in health and disease: friend and foe! *Eur J Dermatol* 2007;17:476–91. [PubMed: 17951127]
5. van Gisbergen KP, Sanchez-Hernandez M, Geijtenbeek TB, van Kooyk Y. Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN. *J Exp Med* 2005;201:1281–92. [PubMed: 15837813]

6. Megiovanni AM, Sanchez F, Robledo-Sarmiento M, Morel C, Gluckman JC, Boudaly S. Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. *J Leukoc Biol* 2006;79:977–88. [PubMed: 16501052]
7. Beauvillain C, Delneste Y, Scotet M, Peres A, Gascan H, Guernonprez P, et al. Neutrophils efficiently cross-prime naive T cells in vivo. *Blood* 2007;110:2965–73. [PubMed: 17562875]
8. Gosselin EJ, Wardwell K, Rigby WF, Guyre PM. Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN-gamma, and IL-3. *J Immunol* 1993;151:1482–90. [PubMed: 8335942]
9. Reinisch W, Lichtenberger C, Steger G, Tillinger W, Scheiner O, Gangl A, et al. Donor dependent, interferon-gamma induced HLA-DR expression on human neutrophils in vivo. *Clin Exp Immunol* 2003;133:476–84. [PubMed: 12930377]
10. Radsak M, Iking-Konert C, Stegmaier S, Andrassy K, Hansch GM. Polymorphonuclear neutrophils as accessory cells for T-cell activation: major histocompatibility complex class II restricted antigen-dependent induction of T-cell proliferation. *Immunol* 2000;101:521–30.
11. Cella M, Dohring C, Samaridis J, Dessing M, Brockhaus M, Lanzavecchia A, et al. A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing. *J Exp Med* 1997;185:1743–51. [PubMed: 9151699]
12. Ju XS, Hacker C, Scherer B, Redecke V, Berger T, Schuler G, et al. Immunoglobulin-like transcripts ILT2, ILT3 and ILT7 are expressed by human dendritic cells and down-regulated following activation. *Gene* 2004;331:159–64. [PubMed: 15094202]
13. Kim-Schulze S, Seki T, Vlad G, Scotto L, Fan J, Colombo P, et al. Regulation of ILT3 gene expression by processing of precursor transcripts in human endothelial cells. *Am J Transplant* 2006;6:76–82. [PubMed: 16433759]
14. Munitz A, Bachelet I, Eliashar R, Moretta A, Moretta L, Levi-Schaffer F. The inhibitory receptor IRp60 (CD300a) suppresses the effects of IL-5, GM-CSF, and eotaxin on human peripheral blood eosinophils. *Blood* 2006;107:1996–2003. [PubMed: 16254138]
15. Tedla N, Bandeira-Melo C, Tassinari P, Sloane D, Samplaski M, Cosman D, et al. Activation of human eosinophils through leukocyte immunoglobulin-like receptor 7. *Proc Natl Acad Sci U S A* 2003;100:1174–9. [PubMed: 12529506]
16. Colonna M, Nakajima H, Navarro F, Lopez-Botet M. A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. *J Leukoc Biol* 1999;66:375–81. [PubMed: 10496306]
17. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192:1027–34. [PubMed: 11015443]
18. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008;26:677–704. [PubMed: 18173375]
19. Waeckerle-Men Y, Starke A, Wuthrich RP. PD-L1 partially protects renal tubular epithelial cells from the attack of CD8+ cytotoxic T cells. *Nephrol Dial Transplant* 2007;22:1527–36. [PubMed: 17339272]
20. Liu X, Gao JX, Wen J, Yin L, Li O, Zuo T, et al. B7DC/PDL2 promotes tumor immunity by a PD-1-independent mechanism. *J Exp Med* 2003;197:1721–30. [PubMed: 12810690]
21. Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2:261–8. [PubMed: 11224527]
22. Lehner T. Special regulatory T cell review: The resurgence of the concept of contrasuppression in immunoregulation. *Immunol* 2008;123:40–4.
23. Oei E, Kalb T, Beuria P, Allez M, Nakazawa A, Azuma M, et al. Accessory cell function of airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L318–31. [PubMed: 15246982]
24. Sheth K, Friel J, Nolan B, Bankey P. Inhibition of p38 mitogen activated protein kinase increases lipopolysaccharide induced inhibition of apoptosis in neutrophils by activating extracellular signal-regulated kinase. *Surgery* 2001;130:242–8. [PubMed: 11490356]
25. Bandyopadhyay G, De A, Laudanski K, Li F, Lentz C, Bankey P, et al. Negative signaling contributes to T-cell anergy in trauma patients. *Crit Care Med* 2007;35:794–801. [PubMed: 17255857]

26. Martin U, Bock D, Arseniev L, Tornetta M, Ames R, Bautsch W, et al. The human C3a receptor is expressed on neutrophils and monocytes, but not on B or T lymphocytes. *J Exp Med* 1997;186:199–207. [PubMed: 9221749]
27. Laudanski K, De A, Miller-Graziano C. Exogenous heat shock protein 27 uniquely blocks differentiation of monocytes to dendritic cells. *Eur J Immunol* 2007;37:2812–24. [PubMed: 17823891]
28. Fanger NA, Liu C, Guyre PM, Wardwell K, O'Neil J, Guo T, et al. Activation of human T cells by major histocompatibility complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid. *Blood* 1997;89:4128–35. [PubMed: 9166855]
29. De AK, Miller-Graziano CL, Calvano SE, Laudanski K, Lowry S, Moldawer L, et al. Selective activation of peripheral blood T cell subsets by endotoxin infusion in healthy human subjects corresponds to differential chemokine activation. *J Immunol* 2005;175:6155–62. [PubMed: 16237112]
30. Salih HR, Wintterle S, Krusch M, Kroner A, Huang YH, Chen L, et al. The role of leukemia-derived B7-H1 (PD-L1) in tumor-T-cell interactions in humans. *Exp Hematol* 2006;34:888–94. [PubMed: 16797416]
31. Tamura H, Dan K, Tamada K, Nakamura K, Shioi Y, Hyodo H, et al. Expression of functional B7-H2 and B7.2 costimulatory molecules and their prognostic implications in de novo acute myeloid leukemia. *Clin Cancer Res* 2005;11:5708–17. [PubMed: 16115907]
32. Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. *Proc Natl Acad Sci U S A* 1978;75:2458–62. [PubMed: 276884]
33. Steinbrink K, Wolf M, Jonuleit H, Knop J, Enk AH. Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 1997;159:4772–80. [PubMed: 9366401]
34. Kanai T, Totsuka T, Uraushihara K, Makita S, Nakamura T, Koganei K, et al. Blockade of B7-H1 suppresses the development of chronic intestinal inflammation. *J Immunol* 2003;171:4156–63. [PubMed: 14530338]
35. Curiel TJ, Wei S, Dong H, Alvarez X, Cheng P, Mottram P, et al. Blockade of B7-H1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 2003;9:562–7. [PubMed: 12704383]
36. Iking-Konert C, Vogt S, Radsak M, Wagner C, Hansch GM, Andrassy K. Polymorphonuclear neutrophils in Wegener's granulomatosis acquire characteristics of antigen presenting cells. *Kidney Int* 2001;60:2247–62. [PubMed: 11737598]
37. Iking-Konert C, Ostendorf B, Sander O, Jost M, Wagner C, Joosten L, et al. Transdifferentiation of polymorphonuclear neutrophils to dendritic-like cells at the site of inflammation in rheumatoid arthritis: evidence for activation by T cells. *Ann Rheum Dis* 2005;64:1436–42. [PubMed: 15778239]
38. van der Meer W, Stephen Scott C, Verlaet C, Gunnewiek JK, Warris A. Measurement of neutrophil membrane CD64 and HLA-Dr in a patient with abdominal sepsis. *J Infect* 2006;53:e43–6. [PubMed: 16253335]
39. Bisson-Boutelliez C, Miller N, Demarch D, Bene MC. CD9 and HLA-DR expression by crevicular epithelial cells and polymorphonuclear neutrophils in periodontal disease. *J Clin Periodontol* 2001;28:650–6. [PubMed: 11422586]

Fig. 1.

Cytokine induced expression of HLA-DR and CD86 in human neutrophils with delayed apoptosis. A) Neutrophil purity in the isolated granulocyte population was assessed by flow cytometry using CD66b-FITC, CD16-PE, CD9-APC antibody. B) Neutrophils cultured for 18–20hrs in medium alone (unstimulated) and in presence of IFN γ (100U/ml) + GM-CSF (20 ng/ml) (I+G) were stained for biotinylated Annexin V in binding buffer followed by labeling with streptavidin – PerCP and assessed for Annexin V binding (indicator of apoptosis) by flow cytometry. Representative of 4 experiments. C & D) Untreated neutrophils isolated from peripheral blood (Day 0), treated with I+G for 18–20 hrs (Day 1) and for 40–44 hrs (Day 2), were assessed for CD86 and HLA-DR respectively by flow cytometry. Data are expressed as % positive and MFI. N=13; *p<0.05, **p<0.01, ***p<0.001 as compared to Day 0 data and #p<0.05, ##p<0.01, ###p<0.001 as compared to Day 1 data.

Fig. 2.

Human neutrophils express co-inhibitory molecules ILT2, 3, 4 and 5, and their expressions are not significantly altered by I+G exposure. Untreated neutrophils isolated from peripheral blood (Day 0), treated with I+G for 18–20 hrs (Day 1) and for 40–44 hrs (Day 2) were assessed by flow cytometry for expression of ILT2 (A; n=11), ILT3 (B; n=19), ILT4 (C – representative data, D – all donors' data; n=20) and ILT5 (C – representative data, D – all donors' data; n=11). Data are expressed as MFI and/or % positive cells. Gates were set up according to isotype control data. No statistical significance was observed after cytokine exposure.

Fig. 3. Cytokine induces PD-L1 expression in neutrophils but not in contaminating eosinophils. A) Forward/side scatter of neutrophil and contaminating eosinophil population. Day 1 data is used as a representative of the time points (Day 0, 1 & 2). B&C) Cell types in the indicated gated population were characterized by flow cytometry. R1 gate consists of neutrophils (CD66b⁺ CD16^{high} CD9^{low}) whereas R2 gate represents eosinophils (CD66b⁺ CD16^{low} CD9^{high}). Day 0, 1 & 2 cell populations were assessed for expression of PD-L1 [(D&F - representative data and H - data of all donors (n=16)] and PD-L2 [(E&G - representative data and H - data of all donors (n=11)] by flow cytometry. Gates were set up according to isotype control data. Grey area - isotype control and open area - specific antibody. *p<0.05, ***p<0.0001 as compared to Day 0 data and #p<0.05, ###p<0.001 and ####p<0.0001 as compared to Day 1 data.

Fig. 4.

Cytokine exposure to neutrophils increases binding of SEA and its presentation to autologous and allogeneic T cells. A) Untreated neutrophils isolated from peripheral blood (Day 0), treated with I+G for 18–20hrs (Day 1) and for 40–44hrs (Day 2) were assessed for SEA binding by incubation of the cells in absence or presence of 100ng of SEA for 40 minutes at 37°C. Cells were washed, blocked and stained with rabbit anti-SEA antibody or in FACS buffer alone (to be used for background binding subtraction). Cells were washed, stained with PE labeled anti-rabbit antibody, fixed and analyzed by flow cytometry. SEA binding is expressed as MFI. SEA binding data of SEA treated Day 0, 1 and 2 neutrophils from the same donor (as indicated by arrows) are overlaid. Representative of 3 experiments. B) Day 0, 1 and 2 neutrophils

(1×10^5) were kept as controls or pulsed with 20 ng of SEA for 3hrs followed by co-culture with autologous (n=4) and allogeneic T cells (1×10^5) (n=6) for 3 days. Cells were then pulsed with [3 H]-thymidine for 18–20 hrs and harvested. T cell proliferation is expressed as counts per minute [CPM] of triplicate cultures. * $p < 0.05$, ** $p < 0.01$ as compared to Day 0 data. C) Day 0 and 1 neutrophils (1×10^5) were first pulsed with 20 ng of SEA for 3hrs followed by incubation for 1hr with different neutralizing antibodies or isotype controls [anti-HLA-DR or anti-CD86 (each 5 μ g/ml) or their combination; anti-PD-L1 or anti-PD-L2 (each 10 μ g/ml) or their combination]. Neutrophils were then co-cultured with autologous T cells (1×10^5) (n=3) for 3 days. T cell proliferation was measured as above. * $p < 0.05$ as compared to APC activity of SEA – pulsed neutrophil alone. D) MOs were cultured in presence of IL-4 (50 ng/ml) + GM-CSF (50 ng/ml) for 5 days for their differentiation into immature DC and then cultured for 2 more days in presence [tolerogenic (Tol.) DC] or absence [control (Cont.) DC] of IL-10 (50 ng/ml). Tolerogenic DCs were then incubated for 1hr with neutralizing antibodies against PD-L1, PD-L2 or isotype control (each 10 μ g/ml) and the allogeneic T cells were added to DC at 20:1 ratios and the MLR activities of DCs were assessed by [3 H]-thymidine ([1 μ Ci/well] incorporation during the last 18 hrs of 6 day co-culture [27].