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Cytokine induced expression of programmed death ligands in human neutrophils

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ABSTRACT

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 IFNγ 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 ILT3, 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.

1. 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 IFNγ, 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, characteriza-

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tion 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 IFNγ + 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, ILT3, ILT4 and ILT5 is not significantly altered after activation with I + G, although levels of ILT3 and ILT5 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.

2. Materials and methods

2.1. Reagents

Recombinant human IFNγ 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 MΦP9), 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) and 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)] and CD86 (clone IT 2.2) antibodies and isotype controls were purchased from BD Biosciences.

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

Blood (60–90 ml) 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 rosetting 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% to 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 μg/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% CO_2 atmosphere. Freshly isolated granulocytes (described as neutrophils being the predominant population) (Day 0) were cultured in the presence of cytokines [IFNγ (100 U/ml) and GM-CSF (20 ng/ml) (I + G)] for 18–20 h (Day 1) and also for 40–44 h (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 TNFα (20 ng/ml) + IL-1β (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 1 h with neutralizing antibodies against PD-L1, PD-L2 or isotype control (each 10 μg/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 μCi/well] incorporation during the last 18 h of 6-day co-culture [27]).

2.3. Assay for neutrophil accessory function

Neutrophils (1×10^5/100 μl) were pulsed with 20 ng of SEA for 3 h and unbound SEA was removed by two washes using PBS and 100 μl of complete medium was added to each well. Autologous or allogeneic T cells (1×10^5/100 μl) were added to control and SEA pulsed neutrophils and the co-cultures were maintained for 3 days, then pulsed with [^{3}H]-thymidine (1 μCi/well). The cells were harvested after 18–20 h for assessment of proliferation. In some experiments, after the neutrophils were pulsed with SEA and washed, cells were first incubated for 1 h with different neutralizing antibodies or isotype controls before addition of autologous T cells.

2.4. SEA binding assay

SEA binding assay was performed as described [28]. In brief, 2×10^5 neutrophils were cultured alone or treated with 100 ng of SEA for 40 min 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 min at 4 °C; divided into two