On the potential involvement of CD11d in co-stimulating the production of interferon-γ by natural killer cells upon interaction with neutrophils via intercellular adhesion molecule-3

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ABSTRACT

Interaction between neutrophils and other leukocytes plays a variety of important roles in regulating innate and adaptive immune responses. Recently, we have shown that neutrophils amplify NK cell/6-sulfo LacNAc$^+$ dendritic cells (slanDC)-mediated cytokine production, by potentiating IL-12p70 release by slanDC via CD18/ICAM-1 and directly co-stimulating IFNγ production by NK cells *via* ICAM-3. Herein, we have identified additional molecules involved in the interactions among neutrophils, NK cells and slanDC. More specifically, we provide evidence that: i) the cross-talk between neutrophils and NK cells is mediated by ICAM-3 and CD11d/CD18, respectively; ii) slanDC potentiate the production of IFNγ by NK cells via CD11a/CD18. Altogether, our studies shed more light on the role that adhesion molecules play within the neutrophil/NK cell/slanDC network. Our data also have potential implications in the pathogenesis of diseases driven by hyperactivated leukocytes, such as Sweet's syndrome, in which a neutrophil/NK cell co-localization is frequently observed.

Key words: CD11d, neutrophil, NK cell, slanDC, IFNγ, Sweet's syndrome

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Introduction

Cells belonging to the innate immune system are programmed to provide rapid and optimal responses when the host is challenged by environmental insults. While performing their specific activities, innate immune cells continuously interact to modulate each others' activities and adapt their response to the specific characteristics of the insult. The role of neutrophils in shaping the developing immune response is increasingly being recognized, not only for their cross-talks with components of the innate immune system, such as monocytes and macrophages,[1] dendritic cells (DC)[2] and natural killer (NK) cells,[3] but also with unpolarized/polarized T lymphocytes.[4] Recently, we have discovered and have started characterizing a novel network within the innate immune response in which human neutrophils can directly cooperate with both NK cells and one of the major DC subsets present in the blood and tissues, known as 6-sulfo LacNAc$^+$ DC (slanDC), to ultimately amplify the production of IFNγ by NK cells upon activation with LPS plus the IL-15/IL-18 combination.[5] Accordingly, we showed that neutrophils promote the release of IL-12p70 by slanDC via a CD18/ICAM-1 interaction, and that such IL-12p70 stimulates NK cells to produce IFNγ: the latter, in turn, has been shown to further potentiate the interaction between neutrophils and slanDC and the release of slanDC-derived IL-12p70, thus creating a positive feedback loop. On the other hand, we also showed that neutrophils could directly co-stimulate the production of IFNγ by NK cells *via* ICAM-3,[5] but its counter-receptor has not been identified. Originally, ICAM-3 was functionally identified as the third CD11a/CD18 (LFA-1) ligand, based on the inability of ICAM-1 and ICAM-2 neutralizing antibodies (Abs) to completely block LFA-1-dependent adhesion, and thereafter cloned.[6,7] Additional receptors for ICAM-3 have been subsequently identified, specifically CD11d/CD18[8] and dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN).[9]

Herein, we report that the specific counter-receptor for ICAM-3 in our system is likely represented by CD11d/CD18,

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and not by CD11a/CD18 or DC-SIGN. We also show that neutrophils contribute to this receptor-ligand interaction with ICAM-3, whereas NK cells contribute with CD11d/CD18. Finally, we show that these interactions might occur in Sweet's syndrome disease, in which neutrophils and NK cells are frequently observed in close contact.

Design and Methods

Isolation and co-cultures of neutrophils, NK cells and DC

Highly purified neutrophils, NK cells and slanDC were isolated and cultured for 18 h as previously described,[5] with or without 100 ng/mL LPS plus the IL-15/IL-18 combination (both at 10 ng/mL), in the presence of 10 μg/mL αCD11a/CD18 efalizumab, a humanized IgG1 antibody derived from the murine MHM24 clone[10] which, in preliminary experiments, confirmed its ability to prevent the binding of CD11a to either ICAM-1[11] or ICAM-3[12] in a specific T-cell adhesion assay[13], 10 μg/mL αCD18 or αCD18 F(ab')$_2$ fragments [clone IB4 (mouse IgG2a)], 10 μg/mL αDC-SIGN [clone 120507 (mouse IgG2b)[14] or clone MR-1 (mouse IgG1)[15]], as well as corresponding isotype control antibodies. Monocyte-derived dendritic cells (moDC) were generated as previously described.[16] All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use. Patients provided their informed consent before samples were taken. The study was approved by the Local Ethics Committee.

Neutrophil, NK cell and slanDC characterization

Analysis of cytokine production, cytofluorimetric analysis, laser confocal microscopy, and co-stimulation assays were performed exactly as previously described.[5] The Abs specifically used in this study were: αDC-SIGN [clone 120507, mouse IgG2b from Abcam], αCD11a (clone HI111, mouse IgG1 from BD BioSciences), and αCD11d (rabbit IgG) Abs.

Immunohistochemistry

Formalin-fixed paraffin embedded skin sections were obtained from 4 cases of Sweet's syndrome and immunostained for CD15 (clone MMA, mouse IgM, 1:50 dilution, Thermo Scientific), neutrophil elastase (clone NP57, mouse IgG1, 1:150 dilution, Dako), CD56 (clone 123C3.D5, mouse IgG1, 1:30 dilution, Thermo Scientific), CD3 (rabbit monoclonal, 1:100 dilution; Thermo Scientific) and IFNγ (clone 25718, mouse IgG2a, 1:250 dilution, R&D Systems). Upon appropriate antigen retrieval, reactivity was revealed using EnVision Mouse (Dako, Glostrup, Denmark), followed by diaminobenzidine. After completing the first immune reaction, the second was visualized using Mach 4-AP (Biocare Medical, Concord, CA, USA) and the reaction was developed with Ferangi Blue as chromogen (Biocare Medical). Digital images taken using the Olympus BX60 microscope were captured using a DP-70 Olympus digital camera and processed using Analysis Image Processing software (Olympus).

Statistical analysis

Bar graphs represent the means ±SEM of the number of experiments indicated in the figure legends. Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

Results and Discussion

CD11a/CD18, but not DC-SIGN, mediates the release of IL-12p70 and IFNγ by NK cell/slanDC co-cultures

To identify the specific counter-receptors of ICAM-3 that mediate the production of IFNγ by activated neutrophil/NK cell/slanDC co-cultures[5] we initially focused on CD11a that binds both ICAM-1 and ICAM-3,[17] since it was found to be constitutively expressed at high levels in neutrophils, NK cells and slanDC (Figure 1A). αCD11a mAbs did not affect the release of IL-12p70 by NK cell/slanDC co-cultures, but significantly inhibited the production of IFNγ (Figure 1B). In contrast, a moderate but not statistically significant inhibition of the neutrophil-dependent potentiation of IL-12p70 (by about 30%) as well as IFNγ (by about 15%) release by NK cell/slanDC co-cultures was observed using αCD11a mAbs (Figure 1B). Corresponding isotype-matched control antibodies were

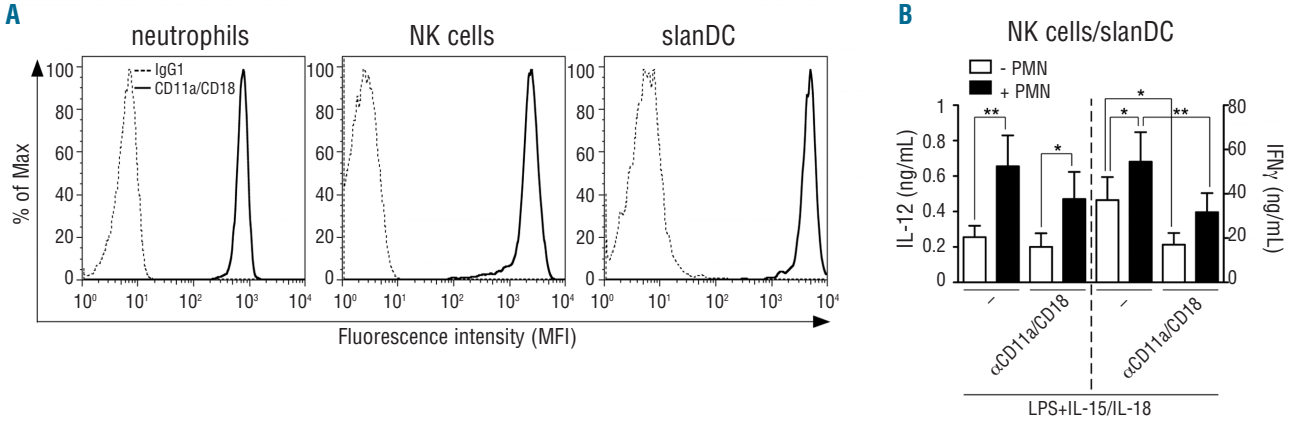

Figure 1. CD11a/CD18 is expressed by neutrophils, NK cells and slanDC and is involved in the release of IFNγ by NK cell/slanDC co-cultures. (A) Analysis for CD11a/CD18 expression by flow cytometry (as described in *Design and Methods*) in freshly isolated neutrophils, NK cells and slanDC. Cells were also stained with isotype control mAbs (gray histograms). One representative experiment out of 3 with similar results is shown. (B) NK cell/slanDC co-cultures were incubated with or without neutrophils and then treated with LPS plus IL-15/IL-18, in the absence or presence of αCD11a/CD18 mAbs (efalizumab). After 18 h, both extracellular IFNγ and IL-12p70 were measured by ELISA (n=3). $P<0.05$ (*) and $P<0.01$ (**) by one-way ANOVA of paired samples.

without effect under any experimental conditions (*data not shown*). The results shown in Figure 1B recall our previous findings, again confirmed in parallel experiments (*data not shown*), demonstrating that, while diminishing the production of IFNγ, but not IL-12p70, by NK cell/slanDC co-cultures,[5] αICAM-1 mAbs significantly inhibit the ability of neutrophils to potentiate the release of IL-12p70 and IFNγ by NK cell/slanDC co-cultures.[5]

Subsequently, we focused on DC-SIGN. However, in line with previous results showing that only a small subset of peripheral blood DC, characterized by the expression of CD14, CD16 and CD1c and corresponding to 0.02% of total PBMC, expresses DC-SIGN,[18] this could not be detected in freshly isolated slanDC (Figure 2A). By comparison, high levels of DC-SIGN were detected in moDC (Figure 2B), as expected.[9] In addition, DC-SIGN was also undetectable in slanDC cultured for 18 h with NK cells, with or without LPS plus IL-15/IL18-stimulation, either in the absence (F. Calzetti, C. Costantini and M.A. Cassatella, unpublished data, September 2010) or the presence of neutrophils (Figure 2C). Consistent with the absence of DC-SIGN, two different neutralizing mAbs (clones 120507 and MR-1), as well as the corresponding isotype control antibodies (*data not shown*), did not inhibit the neutrophil-dependent potentiation of IL-12p70 and IFNγ in the neutrophil/NK cell/slanDC co-cultures (Figure 2D and *data not shown*).

Taken together, our data suggest that CD11a/CD18, that binds ICAM-1 with high affinity,[17] likely mediates the effects of ICAM-1 within the neutrophil/NK cell/slanDC network, especially in the interaction between NK cells and slanDC. Finally, data suggest that a counter-receptor different from CD11a or DC-SIGN interacts with ICAM-3 in the neutrophil/NK cell/slanDC network.

ICAM-3 clusters and co-localizes with CD11d within the contact regions between neutrophils and NK cells

Since CD11a and DC-SIGN do not appear to interact with ICAM-3 (Figures 1 and 2), we subsequently evaluated whether CD11d, known to show a preferential binding to ICAM-3,[8] could instead function as ICAM-3 counter-receptor in our system. Because of the absence of commercially available Abs displaying CD11d blocking properties, we performed confocal microscopy studies and found a strong constitutive expression of CD11d by slanDC and NK cells, while neutrophils were nearly negative (Figure 3A). ICAM-3 was instead homogeneously expressed in the three cell types.[5]

We have previously shown that neutrophils, NK cells and slanDC are frequently found in direct contact upon incubation with LPS plus IL-15/IL-18,[5] and that ICAM-3 co-localizes with CD18 within the contact regions between neutrophils and NK cells.[5] By using a previously established fluorescence staining procedure to unequivocally identify polymorphonuclear neutrophils, NK cells and slanDC,[5] we found a co-localization between ICAM-3 and CD11d within the contact regions between neutrophils and NK cells (Figure 3B). Interestingly, an ICAM-3/CD11d co-localization was observed if neutrophils and NK cells were co-cultured in the absence of slanDC (*data not shown*), supporting the concept that neutrophils can directly function as accessory cells for NK cell activation. Since neutrophils appear to express very little CD11d (Figure 3A), it is likely that CD11d/CD18 on the NK cell side, and ICAM-3 on the neutrophil side, mediate the interaction between the two cell types. To unequivocally exclude the possibility that other receptors might be involved, we took advantage of an assay[19] that we had recently adapted,[5] showing that the release of IFNγ by NK cells is increased when either purified ICAM-3/Fc or

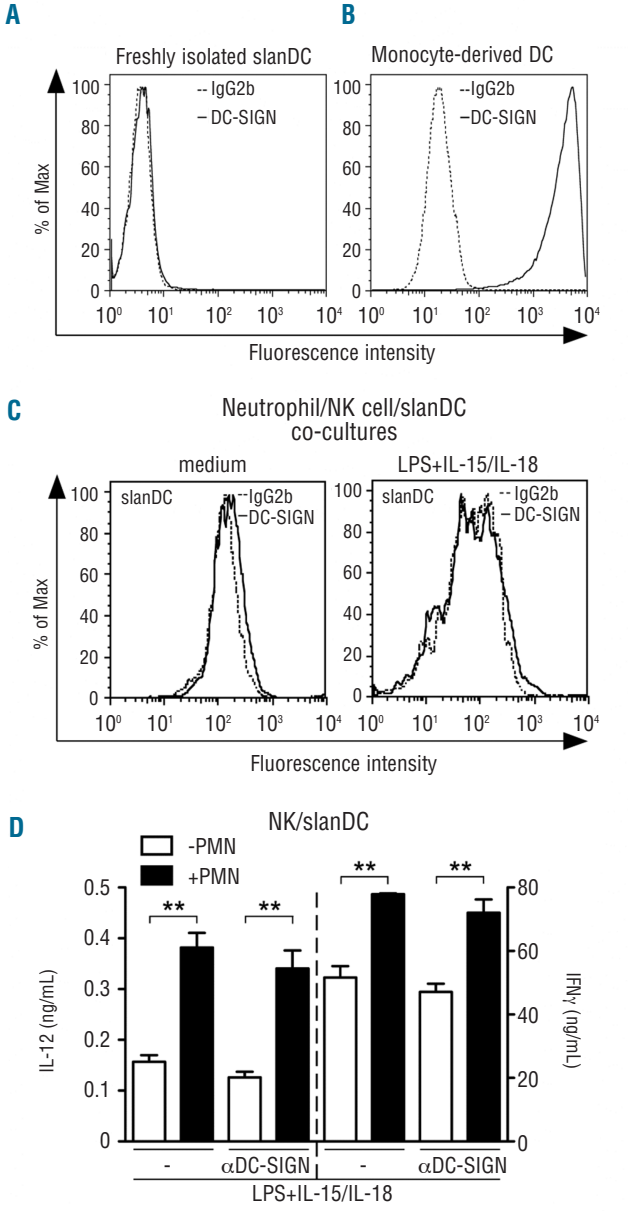

Figure 2. DC-SIGN does not mediate the production of IFNγ and IL-12 by NK cell/slanDC co-cultures in the presence or the absence of neutrophils. (A, C) Analysis for DC-SIGN expression by flow cytometry (as described in *Design and Methods*), in slanDC immediately after isolation (A) as well as after an 18 h co-culture with NK cells and neutrophils in the absence or the presence of LPS plus IL-15/IL-18. (C) One representative experiment out of 3 with similar results is shown. (B) DC-SIGN expression by moDC is shown as positive control. Cells were also stained with the corresponding isotype control mAbs (dashed lines). One representative experiment out of 3 with similar results is shown. (D) NK cell/slanDC co-cultures were incubated with or without neutrophils and then treated with LPS plus IL-15/IL-18, in the absence or presence of αDC-SIGN (clone MR-1) mAbs. After 18 h, both extracellular IFNγ and IL-12p70 were measured in culture supernatants (n=3). *P*<0.01 (**) by one-way ANOVA of paired samples.

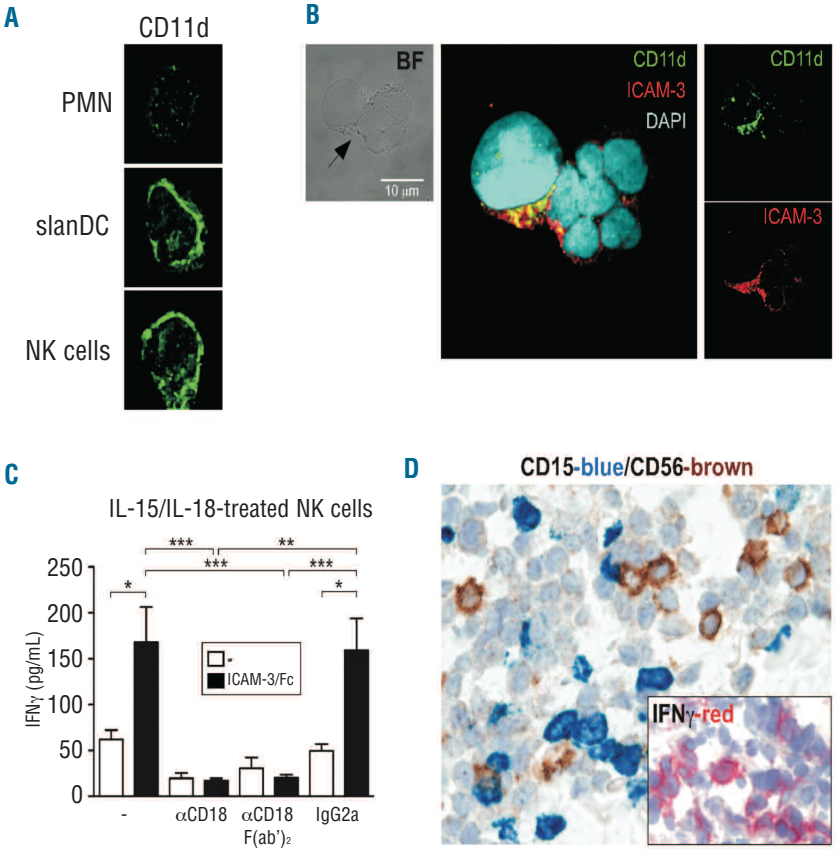

Figure 3. CD11d/CD18 co-localizes within the contact regions between neutrophils and NK cells. (A) Neutrophil/NK cell/slanDC co-cultures were incubated for 2 h, harvested, cytocentrifuged and finally co-stained with αCD11d/CD18 Abs, prior to confocal microscopy analysis (as described in *Design and Methods*). (B) Neutrophil/NK cell/slanDC co-cultures were incubated with LPS plus IL-15/IL-18 for 15 h prior to confocal microscopy analysis. The contact regions between neutrophils and NK cells are shown by a bright field image (BF) (left panels) and indicated by an arrow. Overlay of fluorescent images (middle panels and right panels) demonstrates the co-localization of CD11d (green) and ICAM-3 (red) within the contact regions between neutrophils and NK cells. (A-B) Representative images from 3 experiments with similar results are shown. (C) ICAM-3/Fc was immobilized on plates prior to the addition of NK cells and subsequent incubation in the presence of IL-15/IL-18 with or without αCD18 (clone IB4), its F(ab')$_2$ fragments or mouse IgG2a isotype-matched control. Extracellular IFNγ was measured in cell-free supernatants after 18 h of culture (n=4). Symbols stand for: $P<0.05$ (*), $P<0.01$ (**) or $P<0.001$ (***) by one-way ANOVA of paired samples. (D) Sections are from cases of SS (n=4), immunostained for CD15, CD56 (original magnification: 600X) and IFNγ (inset, original magnification: 400X).

αICAM-3 mAbs are immobilized on the culture plates prior to NK cell addition and their concurrent stimulation with IL-15 plus IL-18. By the same procedure, we found that the co-stimulatory effect of immobilized ICAM-3/Fc on the release of IFNγ by IL-15 plus IL-18-treated NK cells was completely prevented by αCD18, but not isotype-matched control, mAbs (Figure 3C). A similar degree of inhibition was obtained using αCD18 F(ab')$_2$ fragments (Figure 3C), thus definitively excluding any potential non-specific involvement of FcγR-mediated effects. Taken together, our results strongly suggest that NK cells express a functional CD11d/CD18 receptor and that neutrophils potentiate the production of IFNγ by NK cells via an ICAM-3/CD11d interaction.

Neutrophils co-localize with NK cells in Sweet's syndrome

Co-occurrence of NK cells and neutrophils was tested in Sweet's syndrome (SS), an acute febrile neutrophilic dermatosis which can follow viral infections, autoimmune diseases and hematologic malignancies.[20] SS is characterized by a dense dermal inflammatory infiltrate, mainly composed of mature neutrophils. By double staining tissue sections from our 4 cases of SS, we found that CD15$^+$Neutrophil Elastase$^+$ neutrophils were admixed with a variable number of CD56$^+$ (CD3$^-$) NK cells (Figure 3D and *data not shown*). Interestingly, we were able to detect numerous IFNγ-producing inflammatory dermal cells in the same areas (Figure 3D). By contrast, NK cells and neutrophils are regularly absent in normal skin (*data not shown*). These data suggest that a functional cross-talk between these two populations might occur in SS. Since neutrophil-survival cytokines, including IFNγ, have been associated with the etiology of the disease,[21] the presence of NK cells in Sweet's syndrome inflammatory infiltrate, likely producing IFNγ, provides new insights into the pathogenesis of this dermatosis and paves the way for novel therapeutic strategies.

Authorship and Disclosures

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