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TLR-Stimulated Neutrophils Instruct NK Cells To Trigger Dendritic Cell Maturation and Promote Adaptive T Cell Responses

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Polymorphonuclear neutrophils (PMNs) are innate effector cells with pivotal roles in pathogen recognition, phagocytosis, and eradication. However, their role in the development of subsequent immune responses is incompletely understood. This study aimed to identify mechanisms of relevance to the cross talk between human neutrophils and NK cells and its potential role in promoting adaptive immunity. TLR-stimulated PMNs were found to release soluble mediators to attract and activate NK cells in vitro. PMN-conditioned NK cells displayed enhanced cytotoxicity and cytokine production, and responded vigorously to ensuing stimulation with exogenous and endogenous IL-12. The neutrophil-induced activation of NK cells was prevented by caspase-1 inhibitors and by natural antagonists to IL-1 and IL-18, suggesting a role for the NOD-like receptor family pyrin domain containing-3 inflammasome. In addition, PMN-conditioned NK cells triggered the maturation of monocyte-derived dendritic cells, which promoted T cell proliferation and IFN-γ production. These data imply that neutrophils attract NK cells to sites of infection to convert these cells into an active state, which drives adaptive immune responses via maturation of dendritic cells. Our results add to a growing body of evidence that suggests a sophisticated role for neutrophils in orchestrating the immune response to pathogens. ***The Journal of Immunology*****, 2015, 195: 1121–1128.**

Neutrophils are the most abundant leukocytes in human blood and rapidly accumulate at sites of injury and infection (1). Traditionally, neutrophils are regarded as terminally differentiated, short-lived cells with the main purpose to ingest bacteria and kill them by the secretion of degrading enzymes and toxic reactive oxygen species (ROS) (2). However, albeit controversial, a recent report postulates that neutrophils may have a longer time span than previously appreciated (3). Furthermore, polymorphonuclear neutrophils (PMNs) are capable of de novo translation of chemokines and cytokines, and interact with and affect the function of, other immune cells (1, 4). Collectively, these studies suggest that the role for neutrophils in immunity is not limited to pathogen eradication.

With their wide range of pattern recognition receptors, including TLRs and others (5), neutrophils recognize structural patterns of several pathogens. The activated neutrophils release chemokines that may attract neutrophils and other immune cells, including NK cells, to sites of infection. NK cells were originally identified as large granular lymphocytes with capacity to kill malignant and virus-infected cells without prior sensitization (6), but in the last decade, several studies also point toward an immunoregulatory role of NK cells. The NK cell–mediated immunomodulation is, in part, conveyed by specifically directed cytotoxicity against immune cells, such as activated macrophages, immature dendritic cells (DCs), neutrophils, and T cells (7–10). By contrast, activated NK cells produce cytokines, which trigger the upregulation of HLA molecules, costimulatory molecules, and chemokine receptors on DCs (9, 11). By these actions, NK cells may promote the capacity of DCs to home to secondary lymphoid tissues to facilitate their function as efficient APCs, and thus induce adaptive T cell responses.

These previous findings suggest a role for innate immune cells in coordinating adaptive immune responses. Recent studies have addressed the relationship between PMNs and NK cells and potential bidirectional activation in inflamed tissue (12–14). Still, the importance of the cross talk between these cells is incompletely understood. In this study, we investigated whether initial innate interactions between NK cells and TLR-stimulated neutrophils may influence adaptive immune responses. We demonstrate that PMNs stimulated with TLR ligands attract and trigger NK cells to respond to endogenous and exogenous IL-12, and that this initial cross talk between NK cells and neutrophils drives DC maturation and promotes adaptive T cell responses. Our results demonstrate a role for PMN and NK cell cross talk for the development of subsequent adaptive immune responses.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; IL-18BP, IL-18 binding protein; IL-1RA, IL-1R antagonist; moDC, monocyte-derived DC; PMN, polymorphonuclear neutrophil; ROS, reactive oxygen species; SlanDC, 6-sulfo LacNAc$^+$ DC; YVAD-cmk, *N*-acetyl-Tyr-Val-Ala-Asp-chloromethylketone; z-VAD-fmk, benzyloxy-carbonyl-Val-Ala-Asp (OMe) fluoromethylketone.

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Materials and Methods

Culture medium and reagents

mAbs to CD4 FITC (clone SK3), HLA-DR PerCP (clone L243), CD56 PeCy7 (clone NCAM16.2), CD86 FITC (clone 2331), CD83 PE (clone HB15e), CD11b allophycocyanin (clone ICRF44), CD45RA allophycocyanin (clone HI100), and CD16 Pacific blue (clone 3G8) were obtained from BD Biosciences. Anti–IL-12 was purchased from R&D Systems (Oxon, U.K.) and anti–pro–IL-18 from MBL International Corporation (Nagoya, Japan). Anti-Slan (M-DC8) PE was purchased from Miltenyi Biotec (Bergisch GladBach, Germany), and allophycocyanin-conjugated goat anti-mouse IgG-γ3 was from Jackson Immunoresearch (West Grove, PA). Anti-CD69 (FST3) was produced in our laboratory.

Catalase (Worthington, Lakewood, NJ), GM-CSF (R&D Systems), elastase (Abcam, Cambridge, U.K.), IL-18 (MBL International Corporation), IL-4 and IL-12 (Peprotech, Rocky Hill, NJ), TNF-α and diphenyleneiodonium chloride (Sigma-Aldrich, St. Louis, MO), benzyloxy-carbonyl-Val-Ala-Asp (OMe) fluoromethylketone (z-VAD-fmk; Calbiochem, San Diego, CA), *N*-acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-cmk; Cayman Chemicals, Ann Arbor, MI), anti–ICAM-3 (ICAM-3, clone cal 3.10) and IL-1R antagonist (IL-1RA) (R&D Systems), anti-CD18 (clone 7E4; Beckman Coulter, Brea, CA), CellTrace Violet Cell Proliferation Kit (Molecular Probes, Eugene, OR), and IL-18 binding protein (IL-18BP; kind gift from Prof. Charles Dinarello) were all used in coculture experiments throughout the study.

The TLR ligands Clo97, Imiquimod, and R848 were obtained from Invivogen (San Diego, CA), and LPS serotype O111:B4 (LPS) was from Sigma-Aldrich.

In some experiments, the pan-caspase-inhibitor z-VAD-fmk (100 μM), caspase-1 inhibitor YVAD-cmk (100 μM), IL-1RA (1 μg/ml), IL-18BP (0.25 μg/ml), anti-CD18 (5 μg/ml), or anti–ICAM-3 (5 μg/ml) was used.

Isolation of human leukocytes

Buffy coats were obtained from healthy donors and mixed 1:1 with 2% dextran. After sedimentation of erythrocytes, granulocytes and PBMCs were separated by density gradient centrifugation. Remaining erythrocytes were lysed in distilled water as described elsewhere (8). The purity of granulocytes was >97%, with <1% contaminating monocytes. In control experiments, further purification of PMNs was performed using the EasySep Human Neutrophil Enrichment kit from Stemcell Technologies. These two neutrophil populations displayed equal capacity to activate NK cells, thus precluding a major role for contaminating monocytes (Supplemental Fig. 1A). NK cells, monocytes, 6-sulfo LacNAc$^+$ DCs (SlanDCs), and naive CD4 T cells were isolated from PBMCs by use of the corresponding MACS isolation kits (Miltenyi Biotec), according to the manufacturer's protocols.

Neutrophil and NK cell cocultures

NK cells (4×10^5/ml) and PMNs (2×10^5/ml) were cocultured in RPMI 1640, supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin and GM-CSF (100 U/ml) in the presence or absence of TLR ligands (Clo97: 1 μg/ml, R848: 1 μg/ml, Imiquimod: 1 μg/ml, LPS: 0.1 μg/ml, or IL-18: 8 ng/ml) for 24 h. Donors in whom NK cells were activated when treated with medium alone (7/47 donors) or responded to the TLR agonist (2/47 donors) were excluded from the analyses. An overview of the experimental setups is available in Supplemental Fig. 2. In indicated experiments, NK cells and PMNs were incubated in separate chambers of a transwell plate (0.4 μm; Corning Costar). After overnight incubation, the cultures were washed and stimulated with low-dose IL-12 (0.25 ng/ml), TNF-α (10 ng/ml), or incubated with autologous SlanDCs (NK:SlanDCs 8:1) for 24 h. The expression of surface markers was analyzed using flow cytometry.

Generation of DCs and coculture with PMN primed NK cells

To generate immature monocyte-derived DCs (moDCs), we cultured isolated monocytes in medium supplemented with IL-4 (500 U/ml) and GM-CSF (600 U/ml) as previously described (15). NK cell–PMN cocultures were washed extensively and incubated with moDCs for 24 h at a moDC/NK cell ratio of 5:1. moDCs stimulated with LPS (1 μg/ml) were used as positive control for DC maturation.

MLR and T cell proliferation and cytokine release

moDC-NK cell cocultures were washed extensively and resuspended in Iscove's medium supplemented with 10% human AB serum. MLR was conducted by culturing naive CD4$^+$ T cells, prestained with CellTrace Violet (Invitrogen), with NK cell–exposed moDCs at a moDC/T cell ratio of 1:10. After 4–5 d, the percentage of dividing T cells was determined and the supernatants were assayed for IFN-γ using ELISA. IFN-γ–secreting T cells were identified using an IFN-γ Secretion Assay (Miltenyi Biotec).

Cell death assays

NK cells and PMNs were incubated at a 2:1 ratio in the presence or absence of Clo97, GM-CSF, and endotoxin-purified catalase. After overnight culture, the fraction of dead NK cells was determined using the Far-Red Amine-Reactive Dead Cell Stain (FarVid) Kit (Invitrogen). For assessments of NK cell cytotoxicity, NK cells and PMNs were incubated in transwell chambers (0.4 μm) in the presence of Clo97, GM-CSF, and endotoxin-purified catalase. After 24 h, the upper well containing PMNs was removed. The NK cells in the lower chamber were washed and used in a killing assay against K562 cells (E:T ratio 1:1) for 4 h. Lysed leukemic cells were identified using FarVid (16).

Detection of cytokines/chemokines

Supernatants from cell cultures were collected and the amount of released IFN-γ (MBL International Corporation), IL-12, and TNF-α (R&D Systems) was determined using ELISAs according to the manufacturer's protocol. PMNs were cultured with or without Clo97 and GM-CSF. After incubation, intracellular staining for pro–IL-18 (MBL International Corporation) was determined using flow cytometry, and the levels of soluble IL-8, MIP-1α, MIP-1β, IL-18 (R&D Systems), and IL-1β (Biolegend, San Diego, CA) were measured in supernatants using ELISAs.

Chemotaxis assay

Conditioned medium from Clo97/GM-CSF–stimulated PMNs was diluted 1:20 and used as a chemoattractant in a migration assay (5 μm; Corning Costar). NK cells were added to the upper well and the number of migrated cells was determined after 4-h incubation using an Accuri C6 flow cytometer. The specific migration of NK cells was determined by normalizing against a positive (all cells in lower chamber) and negative control (migration towards medium).

Detection of active caspases

PMNs stimulated with Clo97 for 3 h were stained with the FAM-Fluorescent Labeled Inhibitor of Caspases (ImmunoChemistry Technologies, Bloomington, MN) according to manufacturer's protocol and analyzed using imaging cytometry.

Proteolytic processing of IL-18

rIL-18 (8 ng/ml) was treated with elastase in serum-free media at 37˚C. After 1 h, the media were analyzed for IL-18 using ELISA. In parallel, NK cells were incubated with processed IL-18 for 10 h before NK cell activation (CD69 staining) was assessed.

PMN-mediated ROS production

ROS production by TLR-stimulated neutrophils was determined using isoluminol-ECL (CL) as described elsewhere (17). The emitted light was recorded by a FLUOstar Omega plate reader (BMG LabTech).

Flow and imaging cytometry

Samples were collected using a two-laser BD Accuri C6 (488 and 640 nm), a three-laser BD FACSAria (405, 488, and 633 nm), or a four-laser BD LSRFortessa SORP (405, 488, 532, and 640 nm; BD Biosciences). For imaging cytometry, a two-laser Amnis Imagestream X was used. Data were analyzed with the FACSDiva Version 6-8 software (BD Biosciences) or Amnis Imagestream IDEAS software v. 6.0.

Statistics

For multiple comparisons within a data set, one-way ANOVAs with Bonferroni's post tests were performed. For single comparisons, Wilcoxon matched-paired tests or paired samples *t* tests were used. All reported *p* values are two-sided: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

TLR-activated PMNs release chemokines and ROS

In a first series of experiments, we stimulated neutrophils overnight with TLR agonists and determined the concentration of chemokines in collected supernatants. In accordance with previous reports (18), costimulation of neutrophils with GM-CSF was needed to generate high amounts of IL-8, MIP-1α, and MIP-1β in response to the TLR-7/8 agonist Clo97 (Fig. 1A–C). The collected supernatants

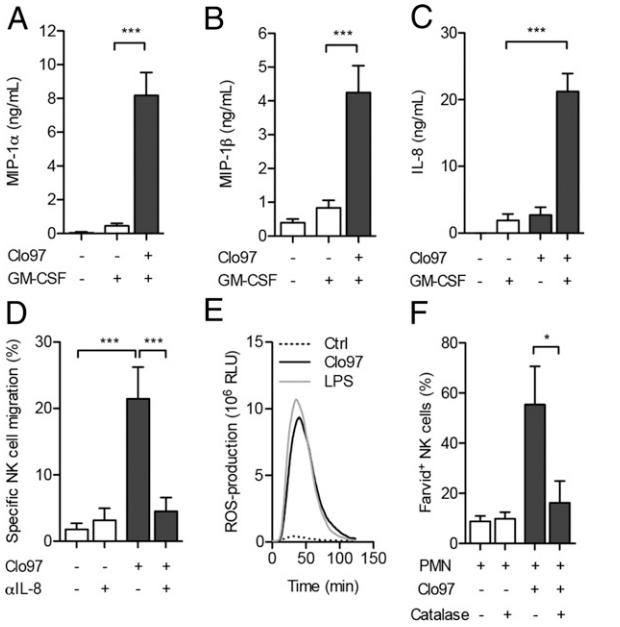

FIGURE 1. TLR stimulation of PMNs triggers chemokine and ROS release. (**A**–**C**) Levels of MIP-1α [(A), mean ± SEM, $n = 8$], MIP-1β [(B), mean ± SEM, $n = 8$], and IL-8 [(C), mean ± SEM, $n = 7$] in supernatants from neutrophils stimulated as indicated. (**D**) Neutrophil supernatants were used in NK cell chemotaxis experiments in the presence or absence of anti–IL-8 (mean ± SEM, $n = 6$). (**E**) The production of ROS by PMNs stimulated with Clo97 or LPS. (**F**) Percentage of dead NK cells after coculture with Clo97-activated PMNs (mean ± SEM, $n = 6$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

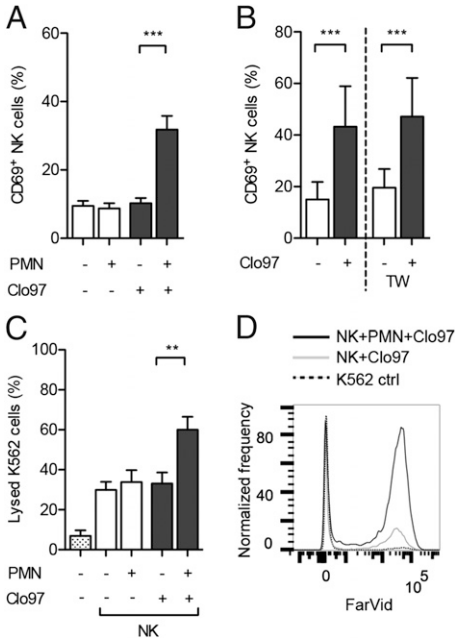

FIGURE 2. Clo97-stimulated PMNs induce NK cell activation. (**A**) NK cell expression of CD69 as determined by flow cytometry after indicated 24-h cocultures (mean ± SEM, $n = 40$). (**B**) Percentage of $CD69^+$ NK cells after 24-h cultures with PMNs unseparated or separated by a 0.4-μM transwell membrane (TW; mean ± SEM, $n = 7$). (**C**) NK cell cytotoxicity against K562 cells (E:T ratio 1:1) using NK cells recovered from indicated transwell cocultures (mean ± SEM, $n = 6$) and (**D**) a representative histogram of K562 Live/Dead staining (FarVid) after exposure to PMN-conditioned NK cells. $^{**}p < 0.01$, $^{***}p < 0.001$.

attracted NK cells by chemotaxis, which was completely inhibited by a blocking IL-8 Ab, thus suggesting a predominant role for IL-8 in attracting NK cells (Fig. 1D).

Neutrophil activation is commonly accompanied by the assembly of the NADPH oxidase and the subsequent release of ROS. We therefore determined whether TLR stimulation induced release of ROS from neutrophils. As shown in Fig. 1E, Clo97 and LPS triggered a robust, extended release of ROS. NK cells are highly sensitive to ROS (19), and accordingly Clo97-stimulated neutrophils triggered extensive NK cell death (Fig. 1F). The effect was mediated by ROS, because PMN-induced NK cell death was inhibited by addition of the hydrogen peroxide–degrading enzyme, catalase.

Stimulated neutrophils activate NK cells in an antioxidative environment

During acute inflammation, the release of immunosuppressive ROS is likely to be counteracted by increased levels of antioxidants (20). To enable controlled mechanistic studies of interactions between TLR-stimulated PMNs and NK cells, we performed the following experiments in the presence of catalase. Thus, NK cells were exposed to Clo97-stimulated PMNs overnight in the presence of catalase, and the percentage of NK cells expressing the early activation Ag CD69 was determined by flow cytometry. In these cocultures, the presence of Clo97-stimulated neutrophils significantly triggered NK cell activation (Fig. 2A). In agreement with results in studies of chemokine release, the PMN-induced NK cell activation was potentiated in the presence of GM-CSF (Supplemental Fig. 1B), and in all subsequent experiments, the culture media were supplemented with GM-CSF.

Previous studies on interactions between neutrophils and NK cells have implicated a role for the cell-surface receptors CD18 (14) and NKp46 (8), respectively. In the next series of experiments, we therefore determined whether PMN activation of NK cells was dependent on cell–cell contact. As shown in Fig. 2B, a transwell insert separating NK cells from PMN did not inhibit the activation of NK cells by Clo97-stimulated PMNs, indicating that soluble mediators released by stimulated neutrophils triggered NK cell activation.

Next, we sought to determine whether TLR-stimulated PMNs impacted on NK cell cytotoxicity. In these experiments, NK cells from PMN-NK transwell cultures were assayed for cytotoxicity against the NK cell–susceptible leukemic cell line K562. As shown in Fig. 2C and 2D, the capacity of NK cells to kill K562 cells was significantly increased after transwell culture with Clo97-stimulated PMNs.

TLR-stimulated PMNs promote NK cell responses to SlanDCs and exogenous IL-12

We next investigated whether TLR-stimulated neutrophils affected subsequent interactions between NK cells and other immune cells. In a recent study, Costantini et al. (14) demonstrated that a subset of myeloid cells, SlanDCs, potentiated NK cell activation in vitro by releasing IL-12. Thus, NK cells cocultured with PMNs were harvested and recultured with culture media, IL-12, or autologous SlanDCs. As shown in Fig. 3, the expression of CD69 on NK cells after SlanDC coculture was significantly higher when NK cells were preactivated by TLR-stimulated neutrophils. In line with their high expression of CD69, the PMN-conditioned NK cells also produced significant amounts of IFN-γ in response to SlanDCs (Fig. 3). Similar results were obtained in experiments where SlanDCs were replaced with low-dose exogenous IL-12. Thus, restimulation with low-dose IL-12 significantly increased NK cell expression of CD69 (Fig. 3C) and the production of IFN-γ (Fig. 3F) by NK cells preactivated by TLR-stimulated PMNs.

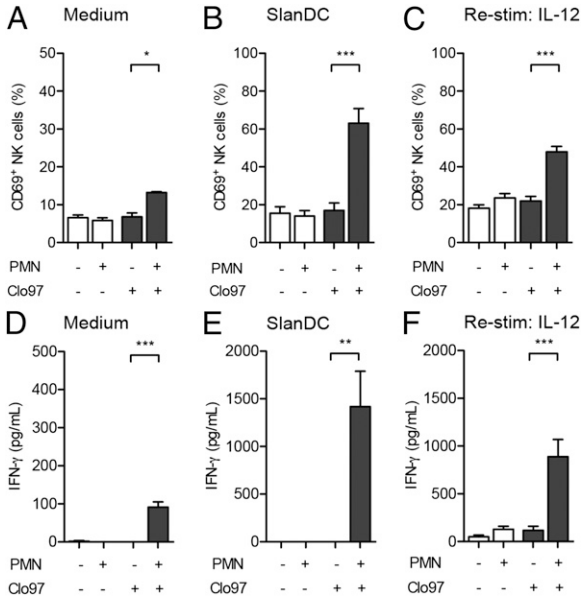

FIGURE 3. PMN-conditioned NK cells are primed for subsequent interaction with SlanDCs or stimulation with IL-12. In parallel experiments, NK cells pretreated as indicated were restimulated after 24 h with medium, autologous SlanDCs, or exogenous IL-12 (mean ± SEM, $n = 3$–6). NK cell upregulation of CD69 (**A**–**C**) and the levels of IFN-γ (**D**–**F**) are shown after 24 h in the restimulation cultures. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

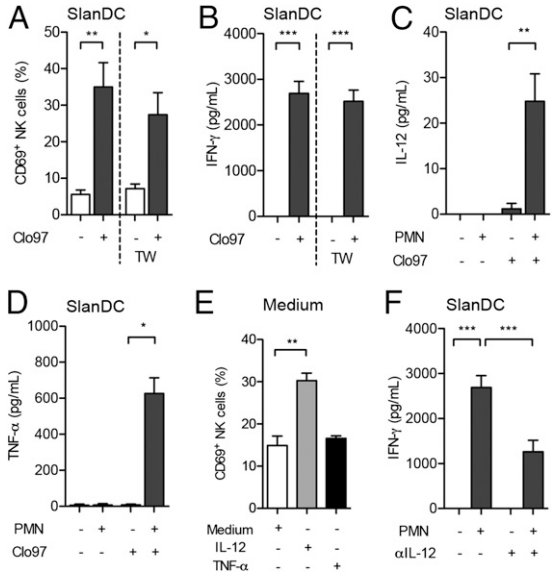

FIGURE 4. Restimulation of NK cells by SlanDCs is mediated by IL-12. (**A** and **B**) NK cell CD69 expression and IFN-γ production after cultures with TLR-stimulated PMNs in normal or transwell (TW) plates for 24 h and restimulation with SlanDCs (mean ± SEM, $n = 3$). (**C** and **D**) The secretion of IL-12 (mean ± SEM, $n = 9$) and TNF-α (mean ± SEM, $n = 3$) was analyzed in NK-PMN cell cultures after restimulation with SlanDCs. NK-PMN cocultures were restimulated with IL-12 or TNF-α [(**E**), mean ± SEM, $n = 3$] and (**F**) the levels of IFN-γ in PMN-pretreated NK-SlanDC cultures were measured in the presence or absence of a blocking IL-12 Ab (mean ± SEM, $n = 3$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Similar results were obtained in experiments where Clo97 was replaced with the TLR-4 agonist LPS (Supplemental Fig. 3A, 3B) or the TLR-7/8 agonist R848, but not by the TLR-7 agonist imiquimod, suggesting that Clo97 and R848 stimulate neutrophils via TLR-8 (Supplemental Fig. 1D). Previous studies have demonstrated roles for CD18 and ICAMs in the cross talk between NK cells and neutrophils (14). However, under these experimental conditions, neither the presence of blocking Abs to CD18 nor ICAM-3 negatively affected the PMN conditioning of NK cells (Supplemental Fig. 1C).

PMN-conditioned NK cells trigger cytokine release from SlanDCs

Next, we set out to clarify the mechanisms underlying the SlanDC-mediated restimulation of PMN-conditioned NK cells. In a first set of experiments, we found that also NK cells recovered from PMN-NK transwell experiments responded to SlanDC restimulation (Fig. 4A, 4B), suggesting that the restimulation by SlanDCs was independent of interactions between SlanDCs and neutrophils. Supernatants from the NK-SlanDC cultures, in which NK cells had been precultured with TLR-stimulated PMNs, contained elevated levels of IL-12 and TNF-α (Fig. 4C, 4D, respectively). These results incited us to compare the restimulation of PMN-conditioned NK cells with exogenous IL-12 and TNF-α. As shown in Fig. 4E, restimulation with IL-12, but not with TNF-α, induced enhanced CD69 expression in NK cells prestimulated with TLR-stimulated PMNs. Furthermore, in the presence of neutralizing Abs to IL-12, the levels of IFN-γ in NK-SlanDC cultures were significantly decreased (Fig. 4F). Collectively, these results strongly suggest that SlanDC-derived IL-12 was responsible for NK cell reactivation.

Neutrophil conditioning of NK cells is caspase dependent and involves IL-1β and IL-18

Stimulation of myeloid cells with TLR ligands commonly results in inflammasome formation (21). In an attempt to define the mechanisms underlying the ability of TLR-stimulated neutrophils to activate NK cells, we investigated the potential role of the NOD-like receptor family pyrin domain containing-3 inflammasome in TLR-8–stimulated neutrophils. Freshly isolated PMNs were stimulated with Clo97, and the upregulation of active caspases was visualized by imaging flow cytometry. As shown in Fig. 5A, the presence of Clo97 triggered PMNs to activate intracellular caspases as compared with unstimulated control PMNs. Furthermore, addition of the pan-caspase inhibitor z-VAD-fmk or the caspase-1 inhibitor YVAD-cmk to Clo97-stimulated NK-PMN cocultures inhibited the upregulation of CD69 expression in NK cells reactivated with SlanDCs (Fig. 5B) or IL-12 (Fig. 5D). In line with these findings, the amount of IFN-γ measured from the same coculture supernatants was significantly decreased if z-VAD-fmk or YVAD-cmk had been present in the initial NK-PMN cocultures (Fig. 5C, 5E). Collectively, these results suggest a role for caspase-processed cytokines of the IL-1 family in NK cell–PMN cross talk.

To clarify which caspase-dependent cytokines were involved in the PMN-induced activation of NK cells, we measured the levels of soluble IL-1β and IL-18 in supernatants from Clo97-stimulated PMNs. Overnight TLR stimulation resulted in significant production of IL-1β by PMNs (Fig. 6A), whereas no increased levels of IL-18 were observed (data not shown). In parallel, we investigated whether the presence of endogenous antagonists to IL-1 and IL-18, IL-1RA and IL-18BP, during the NK-PMN culture affected the subsequent NK cell activation in response to restimulation with SlanDCs or IL-12. Blockade of IL-1 or IL-18 significantly decreased neutrophil conditioning of NK cells, manifested by reduced IFN-γ release upon restimulation with SlanDCs or IL-12 (Fig. 6B, 6C). These contradictory results regarding IL-18 incited us to perform additional experiments. Short-time stimulation with Clo97 triggered IL-18 secretion by PMNs; but in agreement with previous studies (22), the concentration of IL-18 rapidly returned to baseline levels (Fig. 6D). However, TLR

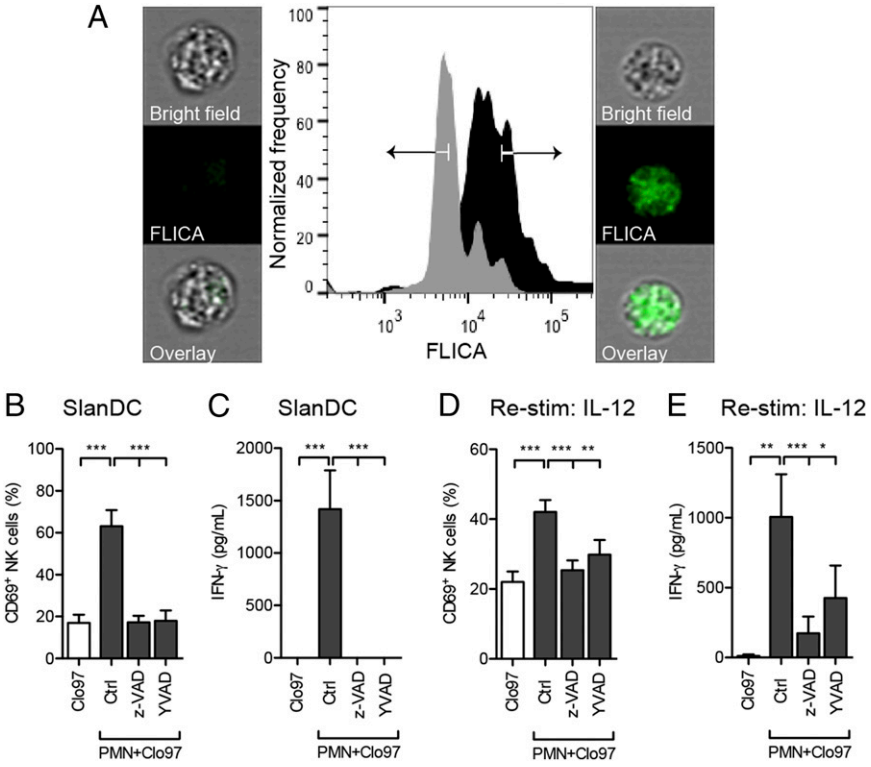

FIGURE 5. PMN conditioning of NK cells is caspase dependent. (**A**) The FAM-Fluorescent Labeled Inhibitor of Caspases (FLICA) intensity of unstimulated (gray) or Clo97-stimulated (black) populations of PMNs as determined by imaging flow cytometry. Two representative cells were selected by the respective median fluorescence intensities (marked by the two arrows). (**B**–**E**) NK cells pretreated as indicated for 24 h were restimulated with SlanDCs (B and C) or exogenous IL-12 (D and E). (B) NK cell expression of CD69 (mean ± SEM, $n = 6$) and (C) levels of IFN-γ (mean ± SEM, $n = 5$) after 24-h restimulation with SlanDCs. (D) NK cell expression of CD69 (mean ± SEM, $n = 9$–16) and (E) levels of IFN-γ (mean ± SEM, $n = 7$; z-VAD; $n = 5$; YVAD) after restimulation with IL-12. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

stimulation also resulted in enhanced intensity of intracellular pro–IL-18 in PMNs after 2 h (Fig. 6E). Furthermore, when we activated NK cells with supernatants collected from Clo97-stimulated PMNs, addition of IL-18BP completely inhibited NK cell IFN-γ production (Fig. 6F). These experiments suggest that overnight cultures of TLR-stimulated PMNs may contain species of IL-18 that are not detected by the ELISA, which is in line with previous reports demonstrating that neutrophil-derived proteases generate low m.w. species of IL-18 (23). To address this issue, we exposed rIL-18 to the neutrophil enzyme, elastase. As shown in Fig. 6G, increasing elastase concentrations processed IL-18 to become undetectable by ELISA. Importantly, the elastase-processed IL-18 was still capable of triggering NK cell activation (Fig. 6H).

PMN-conditioned NK cells can promote T cell proliferation by maturating DCs

Previous reports have demonstrated that NK cells, under certain conditions, induce DC maturation and promote adaptive T cell responses (9, 24–26). These findings led us to investigate whether PMN-activated NK cells affected moDCs. PMN-conditioned NK cells and moDCs were coincubated overnight, and the DC expression of the maturation markers CD83, CD86, and HLA-DR was determined using flow cytometry. As shown in Fig. 7, the presence of PMN-conditioned NK cells greatly enhanced the expression of all three DC markers compared with control NK cells (Fig. 7A–C and Supplemental Fig. 3C for Clo97 and LPS, respectively) and increased the release of IFN-γ, TNF-α, and IL-12 (Fig. 7D–F).

To exclude the possibility that DC maturation was induced by remaining neutrophils, we performed the same experiments using NK cells separated from neutrophils in a transwell system. As shown in Supplemental Fig. 4, similar results were obtained using these NK cells, demonstrating that the DC maturation is indeed induced by the conditioned NK cells. The capacity of PMN-conditioned NK cells to trigger moDC activation was cell–cell contact dependent, because separating the cells with a transwell membrane almost completely blocked the induced expression of moDC maturation markers (Supplemental Fig. 4E, 4F).

Cytokine-treated NK cells have been shown to exert helper functions and promote DC-induced $CD4^+$ T cell responses (25, 27). To assess whether PMN-conditioned NK cells impacted on the capacity of DCs to trigger adaptive immune responses, we assessed $CD4^+$ T cell proliferation in an allogeneic MLR. MoDCs were incubated overnight with PMN-conditioned NK cells or corresponding control NK cells. After washing, naive autologous $CD4^+$ T cells were added to the allogeneic DCs. After 4–5 d of culture, the T cell proliferation and IFN-γ production were significantly increased when the moDCs had been exposed to PMN-conditioned NK cells as compared with moDCs exposed to corresponding control NK cells (Fig. 8A–C and Supplemental Fig. 3D, 3E for Clo97 and LPS, respectively). The induction of T cell proliferation was not affected by contaminating PMNs, because moDCs exposed to NK cells from PMN-NK transwell experiments were equally efficient inducers of T cell proliferation (Supplemental Fig. 4B, 4D). Next, we used an IFN-γ secretion assay to detect the IFN-γ–producing cells. These experiments demonstrated that the percentage of IFN-γ–secreting T cells was significantly increased when cultured in the presence of moDCs being exposed to PMN-conditioned NK cells compared with unconditioned NK cells (Fig. 8D and Supplemental Fig. 3F). The NK cells also responded by increasing the fraction of IFN-γ–secreting cells from 1 to 5% (data not shown), but given the high T:NK cell ratio, the NK cell contribution was negligible in the culture (>150 IFN-γ^+ T cells per IFN-γ^+ NK cell).

Discussion

The interactions between NK cells and neutrophils are complex and multifaceted. ROS produced by the neutrophil NADPH oxidase, which are pivotal for elimination of phagocytosed microbes, are also potent suppressors of NK cells. Thus, both normal and leukemic neutrophils, obtained from patients with chronic myeloid

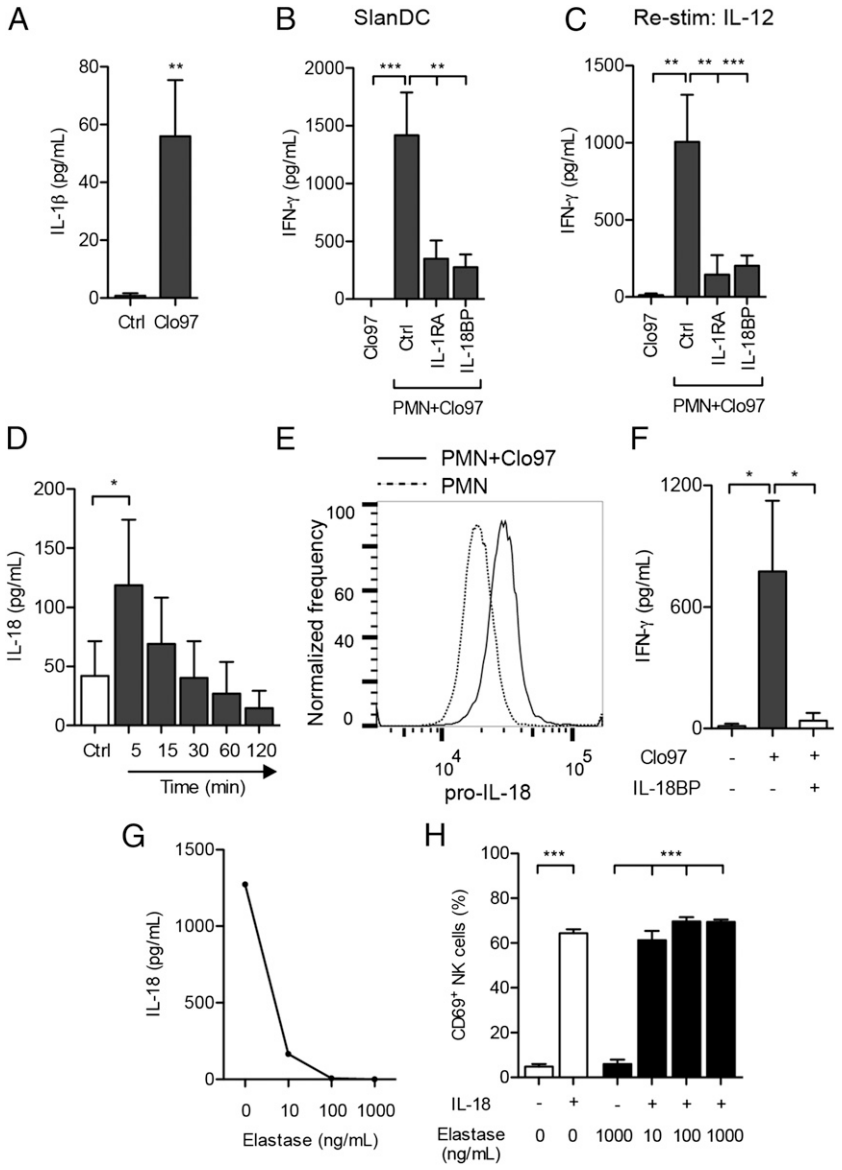

FIGURE 6. PMN conditioning of NK cells involves IL-1β and IL-18. (**A**) Levels of IL-1β in supernatants from Clo97-stimulated PMNs after 24 h (mean ± SEM, $n = 5$). (**B** and **C**) NK cells pretreated as indicated for 24 h were restimulated with SlanDCs (mean ± SEM, $n = 6$) or exogenous IL-12 (mean ± SEM, $n = 6$ for IL-18BP; $n = 5$ for IL-1RA), respectively. (**D**) IL-18 levels after different time points in response to Clo97 stimulation. (**E**) A representative experiment of intracellular staining of pro–IL-18 in Clo97-stimulated PMNs after 2 h. (**F**) NK cell IFN-γ release after restimulation with IL-12, when NK cells had been precultured for 24 h in PMN supernatants in the presence or absence of IL-18BP (mean ± SEM, $n = 4$). (**G**) Levels of IL-18 after pretreatment with elastase as determined by ELISA and (**H**) NK cell activation after 10 h in the presence of elastase-processed IL-18 (mean ± SEM, $n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

leukemia, can inactivate NK cells and trigger ROS-dependent parthanatic cell death in NK cells (28–30). By contrast, we have recently demonstrated that NK cells promote neutrophil apoptosis by Fas and NKp46-dependent mechanisms (8). Furthermore, recent interim reports from the European Stop Tyrosine Kinase Inhibitor Study and Stop Imatinib (STIM) trials, aiming at evaluating the possibility to stop tyrosine kinase inhibitor therapy in chronic myeloid leukemia, suggest that intact NK cell function is pivotal for a favorable outcome (31, 32). In addition, neutrophils were recently shown to be crucial for NK cell development in mice (33), and several studies highlight the relevance of cross talk between NK cells and neutrophils for ensuing immune response (12, 14, 34, 35).

In this study, we found that TLR-agonist stimulation of neutrophils triggered release of ROS and increased levels of parthanatos in coincubated NK cells. In subsequent experiments, we added catalase to the culture medium to enable mechanistic studies of interactions between TLR-stimulated PMNs and NK cells. This may reflect the situation in an inflammatory tissue where there is likely to be increased levels of antioxidants present, such as catalase from erythrocytes, histamine from mast cells, and scavenging intracellular structures from ruptured cells (20). We show that TLR-stimulated neutrophils activate NK cells through the following series of events. First, TLR-stimulated neutrophils released chemoattractants that triggered NK cell chemotaxis. Second, recruited NK cells were conditioned by the inflammatory neutrophils to become more efficient killers, to produce proinflammatory cytokines, and to respond more vigorously to IL-12 produced by other immune cells. Third, the neutrophil-conditioned NK cells acquired the capacity to induce maturation of DCs, manifested by upregulation of MHC class II and costimulatory molecules, which, in turn, made these DCs more efficient in generating an adaptive T cell response. Thus, our data suggest that signals initially derived from TLR-stimulated neutrophils may be important modulators of adaptive immune responses.

It was previously reported that cytokine-stimulated NK cells and neutrophils exchange contact-dependent activation signals, which involve CD18, ICAM-1, and ICAM-3 (14). In contrast, the activation of resting NK cells by TLR-stimulated neutrophils described in this study was predominantly mediated by soluble mediators. Thus, we did not observe a substantial impact of Abs to CD18 and ICAM-1 on neutrophil-induced NK cell activation. In our hands, NK cells were activated and retained the capacity to

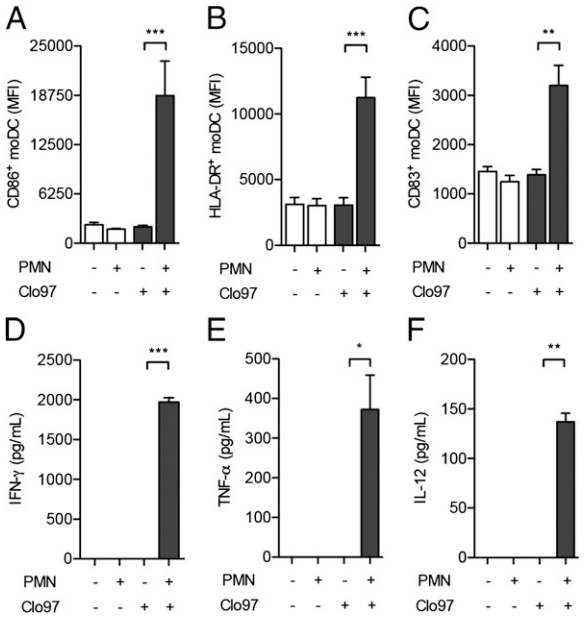

FIGURE 7. PMN-conditioned NK cells induce DC maturation. NK cells pretreated as indicated for 24 h were incubated with moDCs. (**A**–**C**) moDC expression of CD86, HLA-DR, and CD83 after 24-h culture (mean ± SEM, $n = 12$). (**D**–**F**) Levels of IFN-γ, TNF-α, and IL-12 were determined in the moDC-NK cocultures, respectively (mean ± SEM, $n = 3$). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

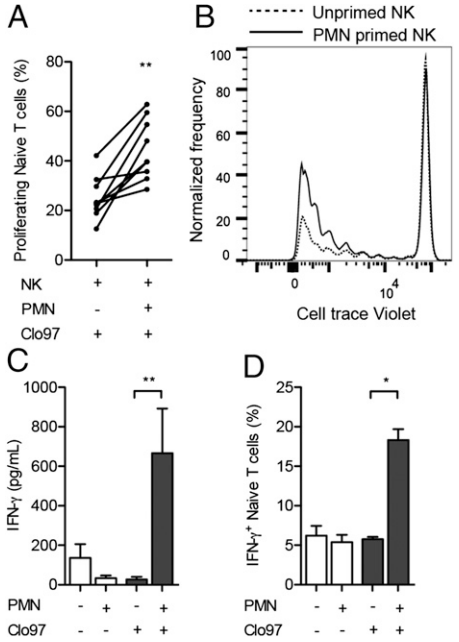

FIGURE 8. Allogeneic DCs matured by PMN-conditioned NK cells trigger $CD4^+$ T cell proliferation and IFN-γ release. (**A**) Percentage of dividing naive $CD4^+$ T cells after 4- to 5-d incubation with moDCs pre-incubated with PMN-conditioned NK cells (mean ± SEM, $n = 9$). (**B**) A representative proliferation experiment. Levels of IFN-γ in supernatants from NK-DC-$CD4^+$ T cell cultures are presented in (**C**) (mean ± SEM, $n = 9$). (**D**) Percentage of IFN-γ–secreting T cells was determined after 5 d of coculture (mean ± SEM, $n = 3$). $*p < 0.05$, $**p < 0.01$.

induce DC maturation also when separated from the TLR-stimulated neutrophils by a cell-impermeable membrane. Importantly, NK cells cultured with the TLR-7/8 agonist, Clo97, alone did not acquire this capacity, precluding that the obtained results are a consequence of direct TLR-mediated effects on NK cells or on moDCs.

The experiments aimed at defining the underlying mechanisms for NK cell activation by TLR-stimulated neutrophils suggested crucial roles for caspase-1 and members of the IL-1 family. Caspase-1 is a central component of the NOD-like receptor family pyrin domain containing-3 inflammasome, which converts the inactive proforms of IL-1 family cytokines into their corresponding active counterparts (36, 37). The ability of the inflammasome to form in response to TLR stimulation alone has been a source of debate. However, recent studies suggest that TLR stimulation is sufficient for caspase-1 activation to occur in primary myeloid cells (38). Accordingly, we found that Clo97-stimulated neutrophils released IL-1β and IL-18 in the presence of GM-CSF.

In accordance with previous studies, mature IL-18 was only transiently detectable in neutrophil supernatants (22), despite that TLR stimulation upregulated intracellular pro–IL-18 in neutrophils. This may be the result of neutrophil reuptake of released IL-18 or degradation by released proteases. In a study by Robertson et al. (23), release of proteases from activated neutrophils was shown to result in several small m.w. species of IL-18. In line with this finding, we did not detect substantial amounts of IL-18 in supernatants obtained from TLR-stimulated neutrophils after 24 h. However, these supernatants induced priming NK cells in an IL-18–dependent fashion. These findings suggest that neutrophils generate small variants of IL-18 with intact capacity to trigger responses in NK cells.

Both the natural antagonists to IL-18 and IL-1β, that is, IL-18BP or the IL-1RA, blocked downstream events mediated by PMN-conditioned NK cells. These results are in line with studies in mice suggesting a role for neutrophil-derived IL-18 in cross talk between NK cells and neutrophils (12). IL-18 is implicated in the induction of a specific NK cell phenotype characterized by expression of CCR7 and CD25 (24). These NK cells are reportedly only weakly cytotoxic, but support DC production of IL-12 and the induction of Th1 T cell responses. In this study, we did not observe a substantial induction of these markers in PMN-conditioned NK cells (data not shown), and these NK cells displayed enhanced cytotoxicity against K562 cells. This discrepancy may be related to relatively low levels of IL-18 being present in the neutrophil–NK cell cultures or to the impact of other soluble mediators, such as IL-1β, on NK cells.

In our study, we observed enhanced DC maturation and significantly increased T cell proliferation when DCs had been exposed to PMN-conditioned NK cells as compared with nonconditioned NK cells. Neutrophils and NK cells modulate DC function by different mechanisms. For example, activated neutrophils recruit DCs via the release and/or maturation of chemokines, by inducing DC maturation, and by providing DCs with ingested pathogen Ags (39, 40). Activated NK cells, in contrast, may promote DC maturation and ensuing adaptive immune responses by releasing stimulatory cytokines or by selectively killing immature DCs in a process known as DC editing (9, 25, 26, 41). In this study, the stimulatory effects on DCs were predominantly exerted by NK cells, because NK cells separated from neutrophils during the initial culture using transwell plates remained capable of inducing DC maturation. Thus, signals originating from TLR-stimulated PMNs shape NK cells so that they subsequently can promote adaptive immunity by triggering DC maturation.

In summary, studies conducted during the last decade point to a role of neutrophils in immunity that is far more sophisticated than previously appreciated. A growing pile of evidence thus points to an important role for neutrophils in initiating and regulating immune responses. The findings presented in this article extend the

role of neutrophils in immunity and suggest that TLR-driven cross talk between neutrophils and NK cells may play a key role in initiating the adaptive immune response. Future studies are warranted to identify therapeutic strategies to manipulate this cross talk, to stimulate adaptive immune responses to infectious agents and malignancies, but also to dampen autoreactivity in various autoimmune diseases.

Disclosures

A. Moretta is a founder and shareholder of Innate-Pharma (Marseille, France). The other authors have no financial conflicts of interest.

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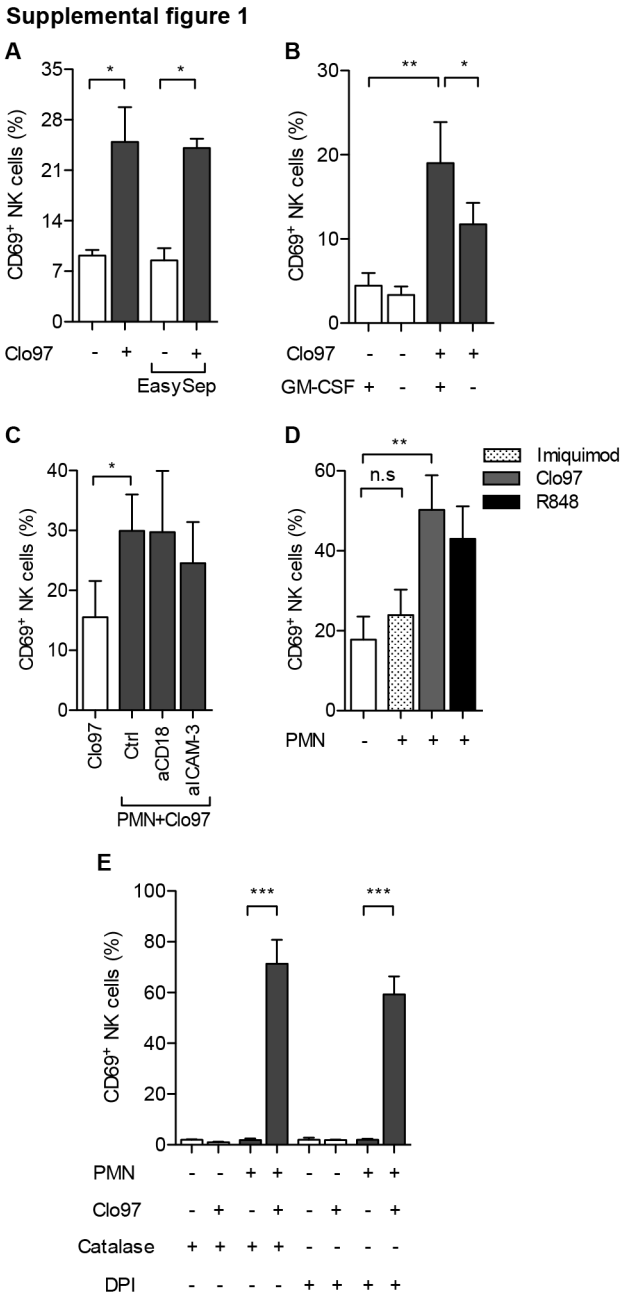
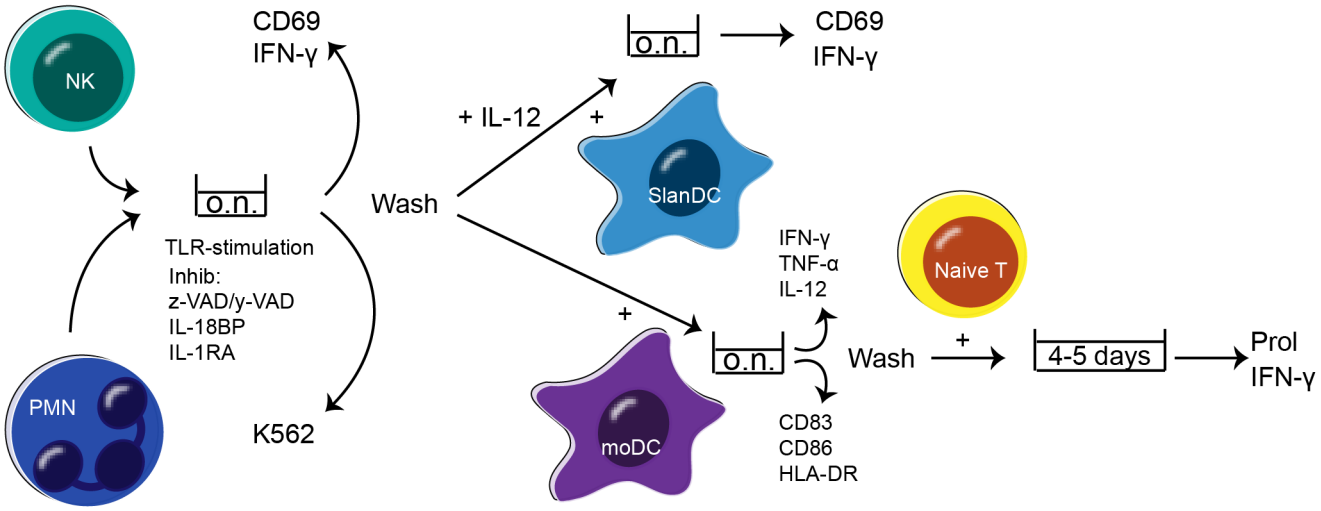


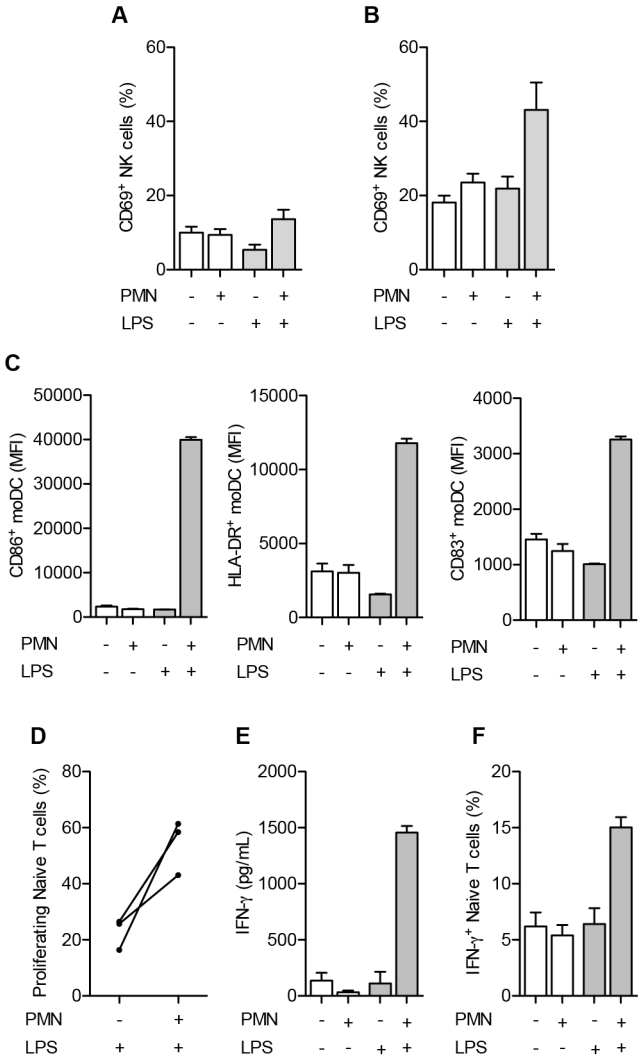
Supplemental figure 1. Panel A shows NK cell CD69 expression after 24h culture with either traditionally or highly purified (EasySep) PMNs in the presence of Clo97 and after reactivation with IL-12 (mean ± SEM, *n* = 3). In B, CD69-positive NK cells are presented after 24h incubation of TLR-stimulated PMN-NK cell cultures and re-stimulation with IL-12 in the presence or absence of GM-CSF (mean ± SEM, *n* = 8). In panel C, NK-PMN cultures were incubated in the presence of blocking antibodies against CD18 or ICAM-3, re-activated with IL-12 and analyzed for NK cell CD69 up-regulation (mean ± SEM, *n* = 7). Graph D represents corresponding TLR-stimulated PMN-NK cell co-culture experiments and the expression of NK cell CD69 after reactivation with IL-12 (mean ± SEM, *n* = 6). In panel E, TLR-stimulated PMN-NK cell cultures were incubated in the presence of either catalase or DPI and NK cell activation was analyzed after 24 hours (mean ± SEM, *n* = 3).

Supplemental figure 2



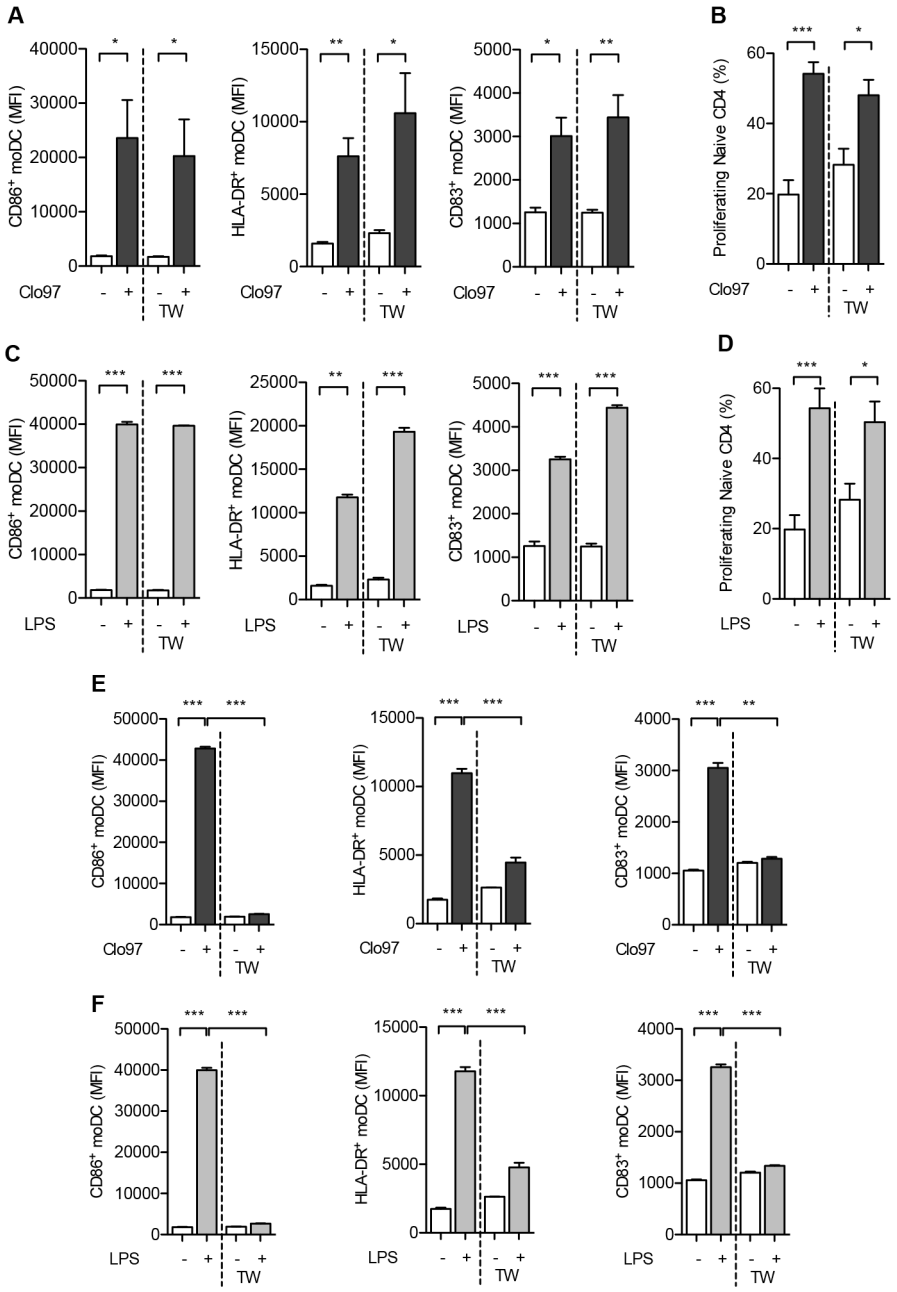
Supplemental figure 2. Schematic illustration of the experimental setups. O.n indicates over-night culture. Cells were washed extensively after each over-night culture before being used in subsequent co-cultures.

Supplemental figure 3



Supplemental figure 3. Graph A and B show NK cell CD69 expression after 24h incubation (A) and after subsequent restimulation with IL-12 (B) (mean ± SEM, *n* = 13). In C, the moDC expression of CD86, HLA-DR and CD83 are shown after incubation with stimulated NK-PMN co-cultures (mean ± SEM, *n* = 3). Panels D-F show proliferation (D), levels of IFN-γ (E) and IFN-γ secretion (F) by naïve T cells after MLRs with moDCs in the presence of PMN-conditioned NK cells (mean ± SEM, *n* = 3).

Supplemental figure 4



Supplemental figure 4. Panels A and C show moDC expression of CD86, HLA-DR and CD83 after 24h co-culture with NK cells pretreated with Clo97 (A) or LPS (C) activated PMNs in co-cultures or in transwell (TW) cultures, where NK cells and PMNs were kept separated by a membrane (mean ± SEM, $n = 4$). In B and D, the percentage of proliferating T cells is shown after co-culture with moDCs activated by NK cells, which were conditioned by PMNs in either contact or transwell systems (mean ± SEM, $n = 3$). Panels E and F present moDC maturations markers CD86, HLA-DR and CD83 expressed by moDCs cultured in transwell systems with NK cells pretreated with Clo97- or LPS-stimulated PMNs (E and F, respectively; mean ± SEM, $n = 3$).