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Reciprocal regulation of Natural Killer cells and macrophages associated with distinct immune synapses

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Abstract

NK cells directly lyse tumor or viral-infected cells but also, an important role for NK cell cytotoxicity in regulating the extent of immune responses is emerging. Here, we show that autologous human macrophages activated NK cell proliferation, cytokine secretion, increased expression of activating receptors and primed NK cell cytotoxicity against susceptible target cells. Ligation of NK cell 2B4, and not NKp30 known to be important for DC-mediated NK cell activation, is critical for this macrophage-mediated NK cell activation. Reciprocally however, NK cells regulated macrophage activity by directly killing macrophages stimulated by high doses of LPS. Cytolysis was triggered by NKG2D recognition of stress-inducible class I MHC-like ligands: High doses of LPS induced transcription and surface expression of ULBP1, 2 and 3, and surface expression of constitutively transcribed MICA. Thus, these data suggest a new function for NK cell cytotoxicity in eliminating over-stimulated macrophages. Additionally, these interactions define, for the first time, two distinct activating NK cell synapses; lytic and non-lytic. Triggering NK cell proliferation and cytokine secretion, but not cytolysis, specifically associated with synaptic accumulation of macrophage f-actin and NK cell 2B4, while macrophages were killed when NK cell f-actin and macrophage ICAM-1 accumulated around a central cluster of NKG2D/DAP10.
Introduction

Natural Killer (NK) cells are important effector cells of the innate immune response through their production of cytokines and lysis of transformed or infected cells without prior sensitization. NK cells exert killing by sensing “missing self” and/or by triggering of activating receptors upon interaction with specific ligands. One of the best characterized activating receptors is NKG2D, expressed on NK, NKT, and T cells, that recognises stress-inducible class I MHC-like proteins. Other activating receptors, Natural Cytotoxicity Receptors (NCR), are expressed almost exclusively on NK cells. Ligands for activating NK cell receptors are found on many cancer cell lines and cells infected with bacteria or viruses.

Immunoregulatory cross-talk between NK cells and dendritic cells (DC) has emerged as important in both innate and adaptive immune responses. However, the extent of cross-talk between human NK cells and macrophages has been less studied. Macrophages are also important effector cells of the innate immune response, exerting their function by using a range of receptors that recognize pathogen molecules such as bacterial lipopolysaccharide (LPS). LPS is a powerful endotoxin that activates macrophages, though at high doses macrophages become refractory to further stimulation, i.e. endotolerant. Here, we set out to examine the potential for immunoregulatory cross-talk between human NK cells and macrophages or macrophages activated with LPS and probe the molecular basis for this.

At the intercellular contact between immune cells, proteins are commonly seen to segregate into central and peripheral supramolecular activating clusters (c- and p-SMAC) at the immunological synapse (IS). Functions of the NK cell IS could be to
provide a framework for establishing checkpoints for cellular activation and/or directing secretion of lytic granules or cytokines in some circumstances\textsuperscript{13,14,15}. Here, we define for the first time, two distinct NK cell-activating synapses. The macrophage/NK cell IS associated with priming but not triggering of NK cytolysis and the IS between NK cells and macrophages treated with a high dose of LPS that triggers NK cytolysis.
Materials and Methods

Generation of macrophages and DC. PBMCs were isolated by density gradient centrifugation (Ficoll-Paque Plus, Amersham Pharmacia Biotech, Piscataway, NJ). Serum was collected, heat inactivated for 30 min at 56°C and filtered. PBMCs were incubated for 2 h in plastic plates previously coated overnight with 2% gelatin (Sigma-Aldrich, St. Louis, MO). After 2 h, the flask was washed intensively to remove the non-adherent cells. After 24 h of incubation in serum free media (X-vivo 10, Bio-Whittaker, Walkersville, MD) with 1% autologous serum, monocytes were washed with cold PBS and 98% of cells were positive for CD14, as assessed by flow cytometry (CELLQuest™, Becton Dickinson, San Jose, CA). CD14-expressing cells were then cultured in X-vivo media with 1% autologous serum for 10-12 days to generate macrophages. For activation when appropriate, macrophages were incubated with LPS from Salmonella Minnesota (Sigma-Aldrich, St. Louis, MO) for 48 h.

DC were generated by incubating 2 x 10^6 CD14-expressing cells/ml in RPMI containing 5% human serum (Type AB, Sigma-Aldrich, St. Louis, MO), 150 ng/ml rhIL-4 and 150 ng/ml rhGM-CSF (R&D systems Inc., Minneapolis, MN). The medium was replenished after 3, 5 and 7 days, and then after 10 days floating cells were harvested and confirmed to be DC. The phenotype of monocytes, macrophages and DC after preparation is shown in Figure S1.
**Isolation and culture of NK cells.** NK cells were isolated from PBMC by negative selection using magnetic activated cell sorting (Human NK cell Enrichment Cocktail, Stem Cell Technologies, Vancouver, Canada) and monitored to be CD3- and CD56+. NK cells were grown in DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% human serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 1 mM penicillin-streptomycin, 1 mM MEM nonessential amino acids and 20 µM β-mercaptoethanol. Freshly isolated NK cells were used unless stated that cultured NK cells were used, in which case 100U/ml human recombinant IL-2 (Roche, Basel, Switzerland) was added to the media and NK cells were used 8 days after restimulation with human recombinant IL-2 (i.e. when resting), unless stated otherwise.

**Cytotoxicity assays.** The cytolytic activity of NK cells was assessed in standard 5 h $^{35}$S release assays. For blocking experiments, NK cells were incubated with 20µg/ml anti-NKG2D mAb, or an isotype-matched control mAb, for 30 min on ice and washed prior to assaying their cytolytic activity. To assess NK cell cytotoxicity after co-incubation with APC, NK cells were first dissociated from APC by incubation in 5mM EDTA/0.2% BSA/PBS for 30 min at 4°C and free-floating NK cells were then harvested and washed with cold PBS before use. The purity of isolated NK cells was 98% as determined by being CD56+ and CD3- by flow cytometry, and viability was confirmed by cells being unstained by Trypan blue.

**Flow cytometric analysis.** For analysis of macrophages, after activation with LPS cells, cells were scraped gently in cold PBS. For analysis of NK cells after co-culture with APC, cells were first dissociated from APC as described for cytotoxicity assays.
Isolated NK cells or macrophages were then incubated with 10% human serum/1% BSA/0.01% azide/ PBS for 30 min on ice and washed 3 times. Then, cells were incubated with the appropriate mAb in 1% BSA/0.01% azide/ PBS for 45 min on ice, washed 3 times and incubated for 45 min on ice with FITC or PE -conjugated secondary antibody. Finally, cells were washed 3 times and analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

**Antibodies.** The following mAb were used: anti-CD14 (61D3, IgG1; eBioscience, San Diego, CA), anti-class I MHC (W6/32, IgG2a; American Type Collection Culture), anti-CD154 (TRAP-1, IgG1; Santa Cruz Biotechnology, Santa Cruz, CA), anti-2B4 (C1.7, IgG1; IMMUNOTECH S.A.S, Marseille, France). Anti-ULBP1 (170818), -ULBP2 (165903), -ULBP3 (166510) and anti-MICA/B (159207) (IgG2a, all for flow cytometry, R&D Systems Inc., Minneapolis, MN), A anti–ULBP1 (M295), -ULBP2 (M311), -ULBP3 (M551) -MICA (for western blot; M673) (all IgG1; Amgen, Thousand Oaks, CA). Anti-MICA (for flow cytometry; BAM195, IgG1; as described previously4), anti-NKG2D (for blocking and flow cytometry, 149810, IgG1; R&D Systems Inc., Minneapolis, MN; and for immunofluorescence staining, BAT221, IgG1; as described previously4), anti-NKp30 (A76, IgG1, for flow cytometry or F252, IgM, for blocking)16. Anti-NKp44 and anti-NKp46 were kind gifts from A. Moretta, University of Genova, Italy. Other antibodies used were anti-HLA-DR (L243, IgG2a), -CD54 (HA58, IgG1), -CD44 (515, IgG1), -CD3 (UCHT1, IgG1), -CD56 (B159, IgG1), -CD3ζ (8D3, IgG1), -CD11c (B-ly6, IgG1), -CD1a (HI149, IgG1), -CD48 (TÜ145, IgM), -actin (C4, IgG1), -perforin (δG9, IgG2b), -CCR7 (3D12, IgG2a), -CD86 (FUN1, IgG1), -CD69 (FN50, IgG1), -CD25 (M-A251, IgG1), IgG2a control (G155-178), IgG2b control (MPC-11), IgG1 control (MOPC-21), IgM control
(R6-60.2) (all from BD-Pharmingen, Franklin Lakes, NJ). FITC or PE – conjugated secondary antibodies were used (Jackson Immunoresearch, West Grove, PA). Finally, we generated a rat anti-DAP10 mAb (4E1, IgG2a) using a synthetic peptide corresponding to DAP10 intracytoplasmic domain. Cytokine secretion by macrophages was assayed by flow cytometry (Cytometric Bead Array - Human Inflammation kit, BD-Pharmingen, Franklin Lakes, NJ). For Western blotting, macrophages were incubated with different doses of LPS for 48 h, lysed and blotted with the indicated antibodies.

**Assays for NK cell responses after co-culture with macrophages, DC or monocytes.** Freshly isolated NK cells (10⁵ per well) were incubated with macrophages or DC, derived from 10-12 days culture, or monocytes, freshly isolated from the same donor, at the indicated ratio in media used to culture NK cells (without IL-2) in 96-well flat-bottom plates. To test for IFN-γ secretion after 48 h of co-culture, supernatants were assayed by ELISA using anti-IFNγ capture mAb (NIB42), biotinylated anti-IFNγ detection mAb (4S.B3) and streptavidin-HRP (all BD-Pharmingen). To test for proliferation after 24 h of co-culture, 1 µCi [³H] thymidine (ICN Biomedicals, Irvine, CA) was added to each well and NK cells were harvested the following day (Harvester Mach IIIM, Tomtec, Hamden, CT) and tested for incorporated [³H] thymidine (1540 MicroBeta TriLux, PerkinElmer, Boston, MA). For blocking experiments, APC were incubated with 10% human serum for 30 min washed and co-cultured for 48 h with NK cells previously incubated with 4µg/ml anti-2B4, anti-LFA1, anti-CD40L, anti-NKp30, isotype-matched control IgG1 or IgM mAbs for 30 min on ice. Alternatively, 4µg/ml anti-CD48 or an isotype-matched
control mAb were added to each APC for 30 min on ice and washed prior to co-culture with NK cells for 48 h.

**RT-PCR.** Macrophages were incubated with 50, 100 or 200ng/ml LPS for 24 or 48 h. Total RNA was extracted from macrophages (RNeasy Kit, Qiagen, UK) and samples were treated with DNase I (Ambion, UK) before 4µg RNA were used to synthesise first strand cDNA (SUPERSCRIPT™, Invitrogen, UK). 1 µl of the resulting cDNA was used in a 20 µl PCR reaction using AmpliTaq Gold (Applied Biosystems, UK). Primers and conditions used for PCR of each MIC/ULBP gene are given in Supplementary Table 1. PCR for GAPDH was used as a control for cDNA synthesis. PCR reactions were run in parallel with negative (water) and positive control reactions. HT1080 cell cDNA was used as a positive control for ULBP3, RAET1E, RAET1G, MICA and MICB expression and Jurkat cDNA was used as a positive control for containing ULBP1 and ULBP2 transcripts. For RAET1L, a pooled Universal cDNA library was used (Stratagene, UK). PCR products were cloned using a pGEM-T Easy kit (Promega, Wisconsin, USA) and sequenced to confirm their identity.

**Confocal microscopy.** For imaging, macrophages were derived from monocytes on coverslips, over ~10 days culture, and then incubated with autologous IL-2 cultured NK cells (resting NK cells) for 5, 10 and 20 min in a ratio of 1:5 (macrophage:NK cells). Cells were fixed (Cytofix/Cytoperm, BD-Pharmingen, Franklin Lakes, NJ) at 4°C, washed 3 times with 0.1% Tween-20/PBS and incubated with the indicated antibodies, as described previously17. For imaging f-actin, cells were stained with 5U/ml Fluor488-conjugated phalloidin (Molecular-Probes, Eugene, Oregon) for 1 h at
4°C and washed 3 times with 0.1% Tween-20/PBS. When appropriate, macrophages were first incubated with 200 ng/ml LPS for 48 h. For ATP depletion or cytoskeletal inhibition, cells were incubated respectively with 50 mM sodium azide (Sigma-Aldrich, St. Louis, MO) for 2 h, or 10 µM cytochalasin D (Sigma-Aldrich, St. Louis, MO) for 1 hour. Greater than 85% depletion of ATP was confirmed by a luciferase based assay (ATPLite™-M, Packard Bioscience, Netherlands). Cell conjugates were imaged under a 63x oil immersion objective using a laser scanning confocal microscope (TCS SP2; Leica). Conjugates were scanned in the xy-direction every 0.3 µm throughout the z-plane. The face of the IS was then reconstructed using a maximum intensity projection (Volocity, Improvision). Protein was considered clustered at the IS if the fluorescence intensity of labeled proteins at the intercellular contact was at least greater than twice that of the intensity at unconjugated regions of the same cell membrane.

**Statistical analysis.** Data were analyzed by ANOVA and the student t test for unpaired values unless stated otherwise. P values less than 0.05 were considered significant. The results are expressed as the mean ± SE.
Results

Macrophages can stimulate autologous NK cells to proliferate, secrete IFN-γ and augment NK cytotoxicity against susceptible target cells

Pure populations of macrophages and DC were prepared (Figure S1, available at the Blood website; see the Supplemental Figure link at the top of the online article) and blood from the same donor was then taken ~10 days later to prepare autologous NK cells and monocytes. In this manner, autologous NK cells could be co-incubated with macrophages or DC derived from monocytes over ~10 days of culture, or monocytes that were freshly isolated from the same donor. In addition, macrophages were stimulated for 48 h with 100 or 200 ng/ml LPS, hereafter denoted macrophages_{low LPS} or macrophages_{high LPS} respectively, during the co-incubation with NK cells.

After co-incubation for 48 h, the rate of NK cell proliferation was measured by thymidine incorporation (Figure 1A). Additionally, the total extent of NK cell proliferation was assessed by counting the number of live NK cells, unstained by trypan blue, present after 48 h of co-culture (Figure 1 B). Counting adherent APCs after co-incubation with NK cells confirmed that they did not proliferate (data not shown). By either assay, it was clear that NK cells had been activated to proliferate when co-incubated with either macrophages or DC, but not monocytes. Macrophages activated by LPS caused even greater NK cell proliferation. There was no increase in NK cell proliferation when NK cells were incubated with LPS in the absence of macrophages (Figure S2, available at the Blood website; see the Supplemental Figure link at the top of the online article). Additionally, IFN-γ was detected by ELISA in the supernatant of NK cells co-cultured with macrophages, LPS-activated macrophages or...
DC but not monocytes (Figure 1C). Thus, both macrophages and DC can induce NK cell proliferation and cytokine secretion.

We next set out to determine how NK cell cytotoxicity against susceptible target cells would be influenced by prior co-culture with APC. After co-culture, NK cells were dissociated from APC and harvested to 98% purity. NK cell cytotoxicity was tested against the MHC class I-negative EBV-transformed B cell line 721.221 (Figure 1D) or the erythroleukemic cell line K562 (Figure 1E). The extent of lysis of either target cell was dramatically increased by prior incubation of NK cells with macrophages. Unexpectedly however, NK cells co-incubated with macrophages_{low LPS} were subsequently able to kill target cells to a far greater extent than cells that were incubated with macrophages_{high LPS}.

**NK cell 2B4 recognition of macrophage CD48 is critical for triggering NK cell proliferation and IFN-γ secretion**

To probe the molecular basis of NK cell activation by macrophages, we tested if various mAb would block the NK cell response after co-culture with monocytes, DC, macrophages, or macrophages activated with LPS for 48 h, at a 10:1 ratio (NK:APC). We found that proliferation of NK cells (Figure 2A) and secretion of IFN-γ (Figure 2B) triggered by co-incubation with macrophages, or macrophages_{low LPS}, could be efficiently blocked with antibodies against NK cell 2B4 or its ligand CD48 on macrophages. However, while anti-2B4 or anti-CD48 mAb were efficient at blocking NK cell proliferation and IFN-γ secretion in response to macrophages or macrophages_{low LPS}, they had no effect on the response of NK cells co-cultured with
macrophages^{high LPS} (Figure 2). This further suggested that a distinct NK cell response occurs to macrophages stimulated with high doses of LPS.

We found that neither NK cell proliferation nor IFN-γ secretion were influenced by mAb against NKp30, whereas blocking this receptor did abrogate NK cell proliferation and cytokine secretion triggered by DC (Figure 2), consistent with previous studies\textsuperscript{18}. NK cell proliferation was also unaffected by mAb blocking of CD40L or LFA-1. Thus, the interaction of 2B4 and CD48 between NK cells and macrophages is critical in triggering NK cell proliferation and IFN-γ secretion, whereas NKp30 is critical in triggering NK cell activation by DC.

**Altered expression of NK cell surface proteins after incubation with macrophages**

We next examined whether co-culture with APC altered the expression of NK cell surface proteins. After co-culture with monocytes, DC, macrophages, or macrophages activated with LPS for 48 h, at a 10:1 ratio (NK:APC), NK cells were isolated and the cell surface expression of several proteins assessed by flow cytometry. NK cells in co-culture with DC, macrophages or LPS-activated macrophages expressed increased levels of the activating receptors NKp30, NKp44 and NKG2D. Thus to some extent, DC and macrophages show a broadly similar ability to activate NK cells. However, NK cells co-cultured with macrophages or LPS-activated macrophages, but not those cultured with DC, expressed higher levels of the activating receptor NKp46 (Figure 3). Particularly striking, was the increased level of 2B4 expression on NK cells co-incubated with macrophages or LPS-activated macrophages but not DC (Figure 3). Surprisingly, intracellular staining revealed dramatically less perforin in NK cells co-
incubated with macrophages$^{\text{high LPS}}$, consistent with the lower cytolytic activity of those NK cells against susceptible targets (Figure 1, D-E).

**Macrophages treated with high doses of LPS are directly lysed by NK cells**

We next set out to assay for NK cell cytolytic activity against macrophages or macrophages activated with different doses of LPS. We found that (i) freshly isolated NK cells, (ii) resting IL-2 cultured NK cells, last stimulated 8 d earlier with IL-2, or (iii) IL-2 activated NK cells stimulated 1 d earlier with IL-2, all could directly lyse autologous macrophages$^{\text{high LPS}}$ (Figure 4). Also, macrophages incubated with 200 ng/ml LPS for a shorter time, i.e. 12 or 24 h or macrophages incubated with a lower dose of LPS, i.e. 100 ng/ml (data not shown), were not lysed by autologous NK cells. Thus, NK cells specifically killed macrophages stimulated with a high dose of LPS. Exhaustion in NK cell cytotoxicity explains the loss of perforin (Figure 3) and the decreased lysis of 721.221 or K562 by NK cells previously co-incubated with macrophages$^{\text{high LPS}}$ (Figure 1).

**High doses of LPS trigger a distinct macrophage phenotype, including expression of stress-inducible ligands for NKG2D**

We next set out to compare the phenotypes of macrophage$^{\text{low LPS}}$ and macrophage$^{\text{high LPS}}$. ICAM-1 and CD44 expression (Figure 5A) and IL-10 secretion (Figure 5B) increased upon stimulation by LPS in a dose dependent manner. However by contrast, 200 ng/ml LPS triggered less secretion of TNF-α or IL-6 (Figure 5B). The high dose of LPS used did not trigger less cytokine secretion due to apoptosis of macrophages, since macrophages$^{\text{high LPS}}$ remained unstained with PI or Annexin V (data not shown). Thus, a distinct macrophage phenotype is induced by high doses of LPS.
Most importantly, transcription of NKG2D ligands ULBP1, 2 and 3, but not RAET1E, G and L, was triggered in macrophages only upon stimulation with 200 ng/ml LPS for 48 h and not with 100 ng/ml LPS (Figure 5C) or with any dose of LPS for 24 h (data not shown). Transcripts of NKG2D ligands MICA and MICB were detected constitutively in macrophages but expression of MICA protein was only found in macrophages incubated with 200 ng/ml LPS (Figure 5D). Importantly, ULBP1, 2, and 3, and MICA were detected at the surface of macrophages by flow cytometry (Figure 5E). Expression of each NKG2D ligand was confirmed with alternative mAb of different isotypes and macrophage expression of class I MHC protein or CD48 (Figure 5E) did not change after LPS activation. Thus, de novo transcription of ULBPs and cell surface expression of both ULBPs and MIC is specifically triggered in macrophages by a high dose of LPS.

NK cell-mediated lysis of macrophages could be abrogated by the addition of 20 µg/ml anti-NKG2D mAb, and not an isotype-matched control mAb (Figure 5F). The low NK cytotoxicity against macrophages was unaffected by anti-NKG2D mAb (data not shown). The addition of anti-NKG2D mAb only slightly reduced NK cell proliferation (Figure 5G) and IFN-γ secretion (Figure 5H) after co-incubation with macrophages. Thus, it is possible that other receptor/ligand interactions may also be important in macrophages triggering NK cell proliferation and IFN-γ secretion, such as the very recently reported interaction between NKp80 and AICL. Nevertheless, blocking of lysis by anti-NKG2D mAb was potent and thus, macrophages stimulated with high doses of LPS are lysed by NK cells via NKG2D recognition. NK cell cytotoxicity can therefore
serve to eliminate over-stimulated macrophages that would perhaps otherwise contribute to an immunopathology or endotoxic shock.

**Accumulation of f-actin at the macrophage/NK cell IS**

Interactions with macrophages or macrophages^{high LPS} define two distinct NK cell-activating synapses, one triggering only proliferation and cytokine secretion with the other triggering lysis, and thus we set out to compare their structures. Conjugates between NK cells and autologous macrophages or macrophages^{high LPS} were fixed and labeled with phalloidin, marking f-actin, and imaged by confocal microscopy. The fluorescence intensity around one cell in the conjugate showed, for example, a three-fold increase of f-actin at the synapse compared to elsewhere at the cell membrane (Figure 6A). Such accumulation of f-actin at the macrophage-NK cell synapse is not merely an increase in cell membrane at the synapse since there was no increase in the amount of MHC class II protein at the synapse (Figure 6A).

A greater than two-fold increase in phalloidin staining was seen at 45% of the intercellular contacts between NK cells and unstimulated macrophages, compared with unconjugated membrane. The arrangement of f-actin at the contact was ring-shaped in 45% of conjugates where f-actin did accumulate between NK cells and macrophages (Figure 6B). No differences in the frequency of f-actin accumulation or ring-shaped arrangements of f-actin were seen when cells were co-incubated together for 5, 10, 20 or 30 minutes before fixation (data not shown). Thus, assembly of a ring-shaped distribution of f-actin at the NK cell/macrophage IS is likely to be an early and/or long-lasting event. Strikingly however, f-actin was seen to accumulate at 85% of intercellular contacts between macrophages^{high LPS} and NK cells and in 65% of
these conjugates f-actin was in a ring-shaped arrangement. Thus, f-actin accumulates more frequently at the IS between macrophages^high LPS^ and NK cells.

Optical microscopy is unable to resolve directly from which cell type f-actin accumulated at the IS. To test this, NK cells and/or macrophages were treated before co-culture, with either cytochalasin D to inhibit actin polymerization or azide to inhibit ATP-dependent processes. For synapses involving either macrophages or macrophages^high LPS^, treatment of both NK cells and macrophages with either drug reduced the frequency of f-actin accumulation at the IS to ~10% (Figure 6 C-D). When only the macrophages were treated with either cytochalasin D or azide, the frequency of f-actin accumulating at the IS between NK cells and macrophages was also reduced to ~10% (Figure 6C). Treatment of only the NK cells had no effect on the frequency of f-actin at this non-cytolytic IS. In contrast, only when NK cells were treated with either cytochalasin D or azide, the accumulation of f-actin at the IS between NK cells and macrophages^high LPS^ was abrogated (Figure 6D). Thus, distinct synapses are assembled to facilitate disparate outcomes of NK cell interactions with macrophages or macrophages^high LPS^.

Trafficking of specific surface proteins and signaling adaptors to the NK cell/macrophage IS

Next, we compared the extent of recruitment and the specific supramolecular organization of proteins key to the outcome of NK cell/macrophage communication. After 20 min of co-incubation, DAP10 was recruited only to the NK cell IS with
macrophages$^{\text{high LPS}}$ (Figure 7A). Similarly, NKG2D accumulated at 76% of synapses with macrophages$^{\text{high LPS}}$ while very rarely accumulating at the IS with macrophages (Figure 7B). It is particularly striking that such a large fraction of NK cell NKG2D and DAP10, on average ~50% of the total amount, is accumulated at the IS. This results in an average ~2-fold increase in the amount of these proteins at the IS compared to the unconjugated membrane, implying that the contact between NK cells and macrophages is far larger than other NK synapses, e.g. with transformed B cells. Specific accumulation of NKG2D and DAP10 at the NK cell synapse with macrophages$^{\text{high LPS}}$ further demonstrates the key role of these proteins in triggering cytotoxicity.

After 20 min of co-incubation we found that another signaling adaptor protein, CD3$\zeta$ also accumulated at 55% of synapses with macrophages$^{\text{high LPS}}$, though rarely at contacts with macrophages (Figure 7C). Thus, activating NK cell receptors associated with CD3$\zeta$ could also play a role in the recognition of LPS-activated macrophages. Alternatively, CD3$\zeta$ may be brought to the cytolytic NK cell/macrophage IS if other activating NK receptors co-cluster with NKG2D.

In contrast to the specific accumulation of NKG2D, DAP10 and CD3$\zeta$ at synapses with macrophages$^{\text{high LPS}}$, 2B4 clustered at 55% or 56% of synapses with macrophages$^{\text{high LPS}}$ respectively, and at only 10% of synapses with macrophages$^{\text{low LPS}}$ (Figure 7D). Again, a strikingly large fraction, on average close to 50%, of NK cell 2B4 is recruited to the IS. Thus, complementing the effect of blocking mAb against 2B4 and CD48 (Figure 2), these imaging data provide a further line of
evidence for the importance of 2B4 in macrophage-mediated NK cell activation and surprisingly not in the lysis of macrophages \(^{\text{high LPS}}\).

**En face organization of the IS**

3D reconstructions of conjugates fixed after different times of co-incubation were used to reveal the spatial-temporal organization of proteins at the NK cell IS with macrophages \(^{\text{high LPS}}\). By 10 min of co-incubation, we could readily observe clustering of ICAM-1 at the IS, but only after 20 min of co-incubation could both ICAM-1 and DAP10 readily be seen to accumulate at the IS (Figure 7E). ICAM-1 was clearly seen in a ring-shaped p-SMAC around a central patch of DAP10 at the c-SMAC. Commonly, NKG2D was co-localized with DAP10 in the c-SMAC (Figure 7F). The observation that the activating receptors accumulate in the c-SMAC is reminiscent of the ‘prototypical’ T cell IS and could be important role in augmenting or balancing signaling\(^{13}\).
Discussion

There has been a great deal of interest in cross-talk between DC and NK cells\textsuperscript{9,10}, while less attention has been given to communication between NK cells and other cells of the innate immune system. Here, we found that macrophages prime NK cell cytotoxicity and trigger NK cell cytokine secretion and proliferation. In different situations, 2B4 can act as an activating or an inhibitory NK cell receptor, though in most situations it acts as a costimulatory receptor augmenting signals from other activating receptors\textsuperscript{20,21}. Here, we found that the interaction between NK cell 2B4 and macrophage CD48 is critical in triggering NK cell proliferation and IFN-\textgreek{g} secretion. Moreover, macrophages induced increased expression of 2B4 on NK cells, and 2B4 specifically clustered at the NK cell/macrophage IS associated with NK cell priming. It is surprising that, in interactions with macrophages, 2B4 triggers NK cell proliferation and not cytotoxicity since 2B4 triggers cytotoxicity in other interactions\textsuperscript{22,23}.

High doses of LPS for 48 h induced a distinct macrophage phenotype characterized by low levels of secretion of TNF-\textgreek{g} and IL-6, perhaps related to the phenotype of ‘tolerant’ macrophages\textsuperscript{24,25} and/or the macrophage response in septic patients\textsuperscript{26}. Most importantly, we also found that NK cells directly lysed macrophages\textsuperscript{high LPS} via NKG2D recognition. NKG2D ligands ULBP1, 2 and 3, but not RAET1E, G and L, were transcribed and expressed at the cell surface in macrophages treated with 200 ng/ml LPS, demonstrating that Toll-Like Receptor signaling can lead to NKG2D ligand expression. Transcription of MICA and MICB occurred constitutively in unactivated and LPS-activated macrophages alike but protein expression was only detected in macrophages\textsuperscript{high LPS}. Up-regulation of NKG2D ligands upon LPS-
activation has also been reported for mouse macrophages and conservation of this immune cell interaction is perhaps indicative of its importance.

The observation that NK cell f-actin clusters at the IS formed with LPS-activated macrophages, but not unactivated macrophages, is reminiscent of the specific accumulation of f-actin at the cytolytic synapse with EBV-transformed B cell lines lacking expression of MHC class I protein. The cytolytic synapse between NK cells and LPS-activated macrophages likely requires a ring-shaped accumulation of f-actin to facilitate directed granule secretion, as recently described for CTL-mediated lysis. NKG2D and the signaling adaptor protein associated with NKG2D, DAP-10, specifically accumulated at the IS involving LPS-activated macrophages consistent with the role for NKG2D in triggering NK cell lysis. The activating NK cell adaptor protein CD3ζ was also seen to accumulate in the cytolytic IS. CD3ζ is important in NK cell activation and is known to associate with CD16, NKp46 and NKp30. It is possible that an activating receptor that uses CD3ζ to signal may also be important in the cytolytic response of NK cells to macrophages. Alternatively, since it is established that there is cross-talk between activating receptors on NK cells, it is possible that they are constitutively clustered together such that CD3ζ is brought to the IS through cis interactions between activating receptors.

At the cytolytic synapse between NK cells and LPS-activated macrophages, ICAM-1 was readily seen in a ring-shaped structure at the IS after 10 min of co-incubation. Thus, the initial interaction between these cells resembles the antigen-independent cytotoxic T cell IS. Then, after 20 min of co-incubation of cells, DAP10 accumulates in the c-SMAC while ICAM-1 remains within the p-SMAC. The ‘bulls-
eye’ arrangement of activating receptors, signaling adaptors and integrins is reminiscent of the ‘prototypical’ activating T cell synapse \(^{33-37}\). Thus, the function of the specific arrangement of proteins at the NK/macrophage IS would be analogous to the possible functions of the T cell IS which include facilitating directed secretion, establishing checkpoints for activation, and/or balancing signaling \(^{13}\).

Together these data beg the question where would macrophage/NK cell interactions be important in vivo? Interaction between NK cells and macrophages could prime NK effector functions in organs such as the spleen or liver \(^{38}\) as well as cooperate together in inflammation sites in rheumatoid arthritis patients \(^{39}\) or in \textit{Plasmodium falciparum} infection \(^{40}\). It is also possible that macrophages could play a role in recruiting NK cells to lymph nodes, as previously determined for activated DC in mice \(^{41}\). In addition, both NK cells and macrophages are found in abundance in the human deciduas \(^{42-44}\), and could interact to produce appropriate cytokines important for pregnancy.

NK cell-mediated lysis of LPS-activated macrophages may be important in dysfunction of the immune system during septic shock following infection by gram-negative bacteria and/or the release of bacterial products. Our data suggest that the presence of high levels of LPS would render macrophages susceptible to NK cell-mediated lysis and indeed, there is considerable evidence that NK cells are important in the pathogenesis of septic shock. For example, liver NK cells were found to be the principal source of IFN-\(\gamma\) in a murine model of human septic peritonitis \(^{45}\). In addition, since our data suggest that NK cells participate in elimination of over-activated macrophages, in the absence of cytotoxicity, activated macrophages would not be
killed leading to hemaphagocytosis and other inflammatory syndromes. Thus, impaired NK cell-mediated immunoregulation of macrophages likely contributes to the pathology of several diseases associated with a loss of cytolytic activity, e.g. hemophagocytic lymphohistiocytosis (HLH)\textsuperscript{46,47}, macrophage activation syndrome\textsuperscript{48}, Hermansky-Pudlak syndrome type 2\textsuperscript{49} and Griscelli syndrome\textsuperscript{50}. 
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**Figure Legends**

**Figure 1.** Human macrophages trigger NK cell proliferation, IFN-γ secretion and prime NK cell cytotoxicity to susceptible target cells. Autologous NK cells were co-incubated for 48 h with monocytes, DC, macrophages, or macrophages in the presence of 100 ng/ml (macrophage<sub>low LPS</sub>) or 200 ng/ml (macrophage<sub>high LPS</sub>) LPS at the ratios shown. (A) 3H-TdR incorporation by NK cells. The first data point shows the extent of 3H-TdR incorporation in NK cells cultured in media alone. (B) Viable NK cells, unstained by Trypan blue, after 48 h of co-incubation with antigen presenting cells at the ratios indicated. Cells were counted using a haemocytometer. The first bar indicates the number of viable NK cells after culture in media alone. (C) IFN-γ secretion from NK cells co-cultured with the indicated APC, at the ratios shown, measured by ELISA. NK cells were isolated after co-incubation with indicated APC and tested for cytolytic activity against (D) the EBV-transformed B cell line 721.221 and (E) the myeloid leukemia cell line K562. *, $P < 0.05$; **, $P < 0.01$, by Student's $t$ test for unpaired values. The results are expressed as the mean ± SE of at least three independent experiments, using cells from different donors, are shown.

**Figure 2.** 2B4 and CD48 are critical for macrophage-mediated activation of NK cell proliferation and IFN-γ secretion. (A) Proliferation and (B) IFN-γ secretion of NK cells during 48 h of co-incubation alone or with monocytes, DC, macrophages, macrophages activated with 100ng/ml (macrophage<sub>low LPS</sub>) or 200ng/ml (macrophage<sub>high LPS</sub>) LPS at a 10:1 ratio. NK cells were pulsed with anti-2B4, anti-LFA1, anti-CD40L, anti-NKp30 (F252, IgM) or isotype-matched control mAbs prior
to co-culture with APC for 48 h. Alternatively, APCs were pulsed with anti-CD48 or isotype-matched control mAb (IgM; APC) prior to co-culture with NK cells for 48 h. *, P < 0.05; **, P < 0.01, by Student’s t test for unpaired values. The results are expressed as the mean ± SE of at least three independent experiments, using cells from different donors.

**Figure 3.** Phenotype of NK cells after co-culture with autologous APC. NK cells were stained for different surface markers after co-incubation for 48 h alone or with monocytes, DC, macrophages, macrophages activated with 100 ng/ml (macrophage\textsuperscript{low LPS}) or 200 ng/ml (macrophage\textsuperscript{high LPS}) LPS at 10:1 ratio and analyzed by flow cytometry. Flourescence intensities (FI) were calculated as the geometric mean fluorescence intensity of the sample - geometric mean fluorescence intensity after isotype-matched control mAb staining. The mean ± SD from three independent experiments, using cells derived from different donors, is shown.

**Figure 4.** Macrophages stimulated with a high dose of LPS were lysed by NK cells. IL-2 activated NK cells (i.e. NK cells 1 d after re-stimulation with human recombinant IL-2), resting NK cells (i.e. NK cells expanded with IL-2 but used 8 d after re-stimulation) or freshly isolated NK cells, were tested for their cytolytic activity against autologous unstimulated macrophages, or autologous macrophages incubated with 100 ng/ml (macrophage\textsuperscript{low LPS}) or 200 ng/ml (macrophage\textsuperscript{high LPS}) LPS for 48 h. NK cells and target cells were incubated at the ratios indicated and specific lysis was measured by a standard \(^{35}\)S release assay. In each experiment the spontaneous release of \(^{35}\)S was less than 20% of the total. The mean of at least three independent experiments, using cells from different donors, is shown.
Figure 5. High doses of LPS caused a distinct macrophage phenotype including protein expression of ULBPs and MIC that trigger NK cell-mediated lysis. (A) Macrophages, and macrophages incubated with 100 or 200 ng/ml LPS for 48 h (denoted 0, 100, 200 respectively) were stained with anti-CD54 mAb or anti-CD44 mAb and analyzed by flow cytometry. (B) Cytokines secreted into the supernatant by macrophages stimulated with different doses of LPS for 48 h. (C) Macrophages that were incubated with different amounts of LPS for 48 h, were probed for transcripts of different NKG2D-ligand genes by RT-PCR of cDNA. M, Marker. 0, 50, 100, 200; amount of LPS in ng/ml. -, water. +, positive control. (D) Macrophages incubated with different amounts of LPS for 48 h were tested for expression of ULBP3 and MICA by Western blotting. (E) Macrophages after incubation with 0 (green line), 100 (macrophage\textsuperscript{low LPS}; black), 200 (macrophage\textsuperscript{high LPS}; blue) ng/ml LPS were stained for NKG2D ligands, class I MHC protein or CD48 and analyzed by flow cytometry. Expression of each NKG2D ligand was confirmed by using alternative mAb as shown (clone names given in brackets). Isotype-matched control mAb were used for comparison (shown in each panel for macrophage\textsuperscript{high LPS}; grey filled histograms). (F) NK cells were pre-incubated with 20 µg/ml of anti-NKG2D mAb or an IgG1 isotype-matched control for 45 min before testing their cytotoxicity against macrophages incubated with 200 ng/ml LPS for 48 h. NK cells and target cells were incubated at the ratios indicated and specific lysis was measured by a standard \textsuperscript{35}S release assay. In each experiment the spontaneous release of \textsuperscript{35}S was less than 20% of the total. Proliferation (G) and IFN-γ secretion (H) of NK cells after 48 h incubated alone (No APC) or co-incubated with macrophages, or macrophages activated with 100ng/ml (macrophage\textsuperscript{low LPS}) or 200ng/ml (macrophage\textsuperscript{high LPS}) LPS at a 10:1 ratio. NK cells
were pulsed with anti-2B4 (4 µg/ml), anti-NKG2D (20 µg/ml) or isotype-matched control mAbs prior to co-culture with APC for 48 h. *, P < 0.05; **, P < 0.01, by Student's t test for unpaired values. The results are expressed as the mean ± SE of at least three independent experiments, using cells from different donors.

**Figure 6.** Accumulation of f-actin at the NK cell/macrophage IS. (A) NK cells and autologous macrophages were co-incubated, fixed and then stained with anti-MHC class-II mAb (red) and with Alexa Fluor 488-conjugated phalloidin (green) to mark the location of f-actin. Analysis of the fluorescence intensity around the cell membrane demonstrates a three-fold increase in the amount of f-actin, but not MHC class II protein, at the synapse. (B) The organization of f-actin at the IS was often ring-shaped as shown. Bar chart shows the frequency of a clearly ring shaped-distribution of f-actin at the IS between NK cells and unstimulated macrophages or macrophages high LPS. (C) The percent of NK cell/macrophage conjugates where f-actin clearly accumulated at the IS was scored and then compared when each cell type was pre-treated with cytochalasin D (Cyto.) or sodium azide (Azide). (D) The percent of NK cell/macrophage high LPS conjugates where f-actin clearly accumulated at the IS was scored and then compared when each cell type was pre-treated with cytochalasin D or sodium azide. The mean ± SD from three independent experiments are shown with the total number of cells indicated above each bar. Scale bars, 10µm.

**Figure 7.** Two distinct NK cell-activating immune synapses. (A-D) NK cells were co-incubated with macrophages or macrophages high LPS for 20 min and conjugates were fixed, stained and imaged. Figure shows (in left panels) the frequency of conjugates where the indicated protein accumulated at the IS, with the number of conjugates
imaged indicated above each bar; (in middle panels) the fold increase of the fluorescence intensity at the synapse relative to elsewhere at the cell membrane, determined from 3D reconstructions of conjugates; (in right panels) the percent of fluorescence at the IS as a fraction of the total cell fluorescence in the cell, calculated from 3D reconstructions of conjugates. Figures show data for (A) DAP10 and ICAM-1, (B) DAP 10 and NKG2D, (C) DAP 10 and CD3ζ and (D) DAP 10 and 2B4. In (A-C), measurements of fluorescence intensities (middle and right panels) were made on synapses with macrophages$^{\text{high LPS}}$, whereas in (D) calculations of 2B4 and DAP10 recruitment (middle and right panels) are made for synapses with (unstimulated) macrophages (Mac.) or macrophages$^{\text{low LPS}}$ (Mac. $^{\text{low LPS}}$). For 3D reconstructions, at least 27 synapses were analyzed in each case. (E) NK cells and macrophages$^{\text{high LPS}}$ co-incubated for different times were fixed and stained for ICAM-1 and DAP10. Reconstructions of the distribution of ICAM-1 (green) and DAP-10 (red) at the face of the IS are shown, with the frequency of such distributions marked. 30 synapses were analyzed for each time-point over three independent experiments. (F) NK cells were co-incubated for 20 min with macrophages$^{\text{high LPS}}$ and conjugates were fixed, stained and imaged. Top row shows the bright field image, and associated fluorescence within the boxed area marking NKG2D and DAP10. Reconstructions of the distribution at the face of the IS are shown (lower rows), with the frequency of such distribution marked. For the distribution at the face of the IS, ~30 synapses were analyzed over three independent experiments. Scale bars, 10µm.
References


dendritic cells activate resting natural killer (NK) cells and are recognized via
2006;7:1334-1342.
20. McNerney ME, Guzior D, Kumar V. 2B4 (CD244)-CD48 interactions provide
a novel MHC class I-independent system for NK-cell self-tolerance in mice.
Molecular basis for positive and negative signaling by the natural killer cell
interactions in human NK cell recognition: the role of 2B4-CD48. Eur J
characterization of NK cell activation-inducing ligand, a counterstructure for
24. Wysocka M, Robertson S, Riemann H, et al. IL-12 suppression during
experimental endotoxin tolerance: dendritic cell loss and macrophage
25. Frankenberger M, Pechumer H, Ziegler-Heitbrock HW. Interleukin-10 is


Figure 1
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Figure 7
Reciprocal regulation of natural killer cells and macrophages associated with distinct immune synapses

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