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## Cutting Edge: Toll-Like Receptor Signaling in Macrophages Induces Ligands for the NKG2D Receptor<sup>1</sup>

Jessica A. Hamerman, Kouetsu Ogasawara, and Lewis L. Lanier<sup>2</sup>

*Macrophages recognize the presence of infection by using the Toll-like receptor (TLR) family of proteins that detect ligands on bacterial, viral, and fungal pathogens. We show that murine macrophages stimulated with pathogen products known to signal through TLRs express ligands for the NKG2D receptor, found on NK cells, activated CD8<sup>+</sup> T cells and activated macrophages. TLR signaling, through the MyD88 adaptor, up-regulates transcription of the retinoic acid early inducible-1 (RAE-1) family of NKG2D ligands, but not H-60 or murine UL16-binding protein-like transcript-1. RAE-1 proteins are found on the surface of activated, but not resting, macrophages and can be detected by NKG2D on NK cells resulting in down-regulation of this receptor both in vitro and in vivo. RAE-1-NKG2D interactions provide a mechanism by which NK cells and infected macrophages communicate directly during an innate immune response to infection. The Journal of Immunology, 2004, 172: 2001–2005.*

Macrophages are distributed throughout the body where they are poised to alert the innate immune system to infection. They directly sense a wide variety of microbes through pattern recognition receptors, including the Toll-like receptors (TLRs),<sup>3</sup> which are involved in the recognition of conserved molecular patterns on bacterial, viral, and fungal pathogens (1). Rather than detecting the pathogen itself, NK cells use a variety of activating and inhibitory receptors to detect alterations in the infected cells (2, 3). Some NK cell activating receptors directly recognize pathogen-encoded proteins expressed on infected cells, as is the case with the NK receptor Ly49H binding to the murine CMV-encoded protein m157 (4, 5). Alternatively, NK cells may recognize host proteins only expressed after infection. This has also been shown in viral infection, where ligands for the NK receptor NKG2D are expressed on CMV-infected cells (6, 7).

The NKG2D receptor is constitutively expressed on all NK cells. NKG2D has no intrinsic signaling capacity and therefore complexes with transmembrane signaling adaptors, DAP10

and in mice DAP12 (8–10). Mouse NKG2D binds to the retinoic acid early inducible-1 (RAE-1) proteins (RAE-1  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$ ), to the minor histocompatibility Ag H60, and to the murine UL16-binding protein-like transcript-1 (MULT-1) glycoprotein (11–14). The RAE-1 proteins are expressed during embryogenesis, but have not been detected in adult tissue (15, 16), while the H60 peptide complexed with MHC class I has been detected on macrophages in BALB.b, but not C57BL/6 (B6) mice (17). MULT-1 mRNA has been found in many tissues, but whether protein expression correlates with this is unknown (13, 14).

### Materials and Methods

#### Mice

BALB/c and B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and Charles River Breeding Laboratories (Wilmington, MA), respectively. Macrophages from MyD88<sup>-/-</sup> mice (18) on a B6 background were generously provided by Dr. T. DeFranco (University of California, San Francisco, CA) and Dr. A. Aderem (Institute for Systems Biology, Seattle, WA).

#### Activation of macrophages in vitro and in vivo

Resident peritoneal macrophages were plate-adhered overnight, and nonadherent cells were removed by washing. Macrophages were cultured with pathogen products or whole bacteria for 24 h or with IFN- $\gamma$  (10 U/ml; Life Technologies, Rockville, MD) for 48 h for RNA isolation and flow cytometric analysis. Macrophages were exposed to 100 ng/ml LPS (*S. Minnesota* R595; List Biological Laboratories, Campbell, CA), 100 ng/ml Pam<sub>3</sub>CSK<sub>4</sub> (Roche, Basel, Switzerland), or 100  $\mu$ g/ml poly(I:C) (Amersham, Arlington Heights, IL). Heat-killed *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Mycobacterium bovis* BCG (provided by Dr. A. Aderem), and zymosan (Molecular Probes, Eugene, OR) were used at 10 particles per macrophage. All macrophage activators, except LPS and *E. coli*, were treated with 10  $\mu$ g/ml polymixin B (Sigma-Aldrich, St. Louis, MO) for 1 h before addition to macrophages. For in vivo macrophage activation, mice were injected i.p. with 10  $\mu$ g of LPS.

#### Flow cytometry

FcR were blocked with 2.4G2 mAb (BD PharMingen, San Diego, CA) and macrophages were then stained with a fusion protein comprised of the extracellular domain of mouse NKG2D with the Fc domain of human IgG1 (NKG2D-Ig) (11), followed by PE-labeled goat anti-human Fc Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). For detection of RAE-1, macrophages were stained with a mAb that recognizes all known RAE-1 proteins (6) (186107; developed in collaboration with Dr. J. P. Houchins, R&D Systems, Minneapolis, MN). In vivo activated macrophages were costained with FITC-conjugated F4/80 mAb (Caltag Laboratories, Burlingame, CA).

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<sup>3</sup> Abbreviations used in this paper: TLR, Toll-like receptor; RAE-1, retinoic acid early inducible-1; MULT-1, murine UL16-binding protein-like transcript-1.

### Quantitative real-time PCR

Real-time PCR was performed using an ABI 7700 with Sequence Detector software (Applied Biosystems, Foster City, CA) as described (6, 19). The MULT-1 set included: probe 5'FAM-CCGAAAGCCCCTCACTCTGCA-3'-TAMRA, sense primer TTCACATAGTGCAGGAGACTAACACA, and antisense primer ACTGGCCACACACCTCAGC.

### NKG2D modulation in vitro and in vivo

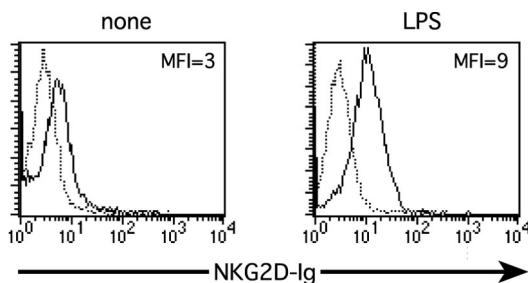
B6 peritoneal macrophages were activated, as above, with LPS or heat-killed *L. monocytogenes*. After 48 h, splenocytes depleted of T and B cells were added to the macrophages at a ratio of 2:1 in the presence of 200 U/ml human recombinant IL-2 (provided by the National Cancer Institute Biological Resources Branch Preclinical Repository). After 24 h of coculture with macrophages, NK cells were stained with mAb against NK1.1, CD3 and CD44 (BD Pharmingen) or NKG2D (CX5) (19) and analyzed by flow cytometry. For in vivo modulation of NKG2D, mice were injected with 10  $\mu$ g of LPS and at 1–7 days after injection, peritoneal cells were stained with mAb to DX5, CD3 and NK1.1 or NKG2D (CX5) and analyzed by flow cytometry.

## Results and Discussion

### NKG2D ligand expression on activated macrophages

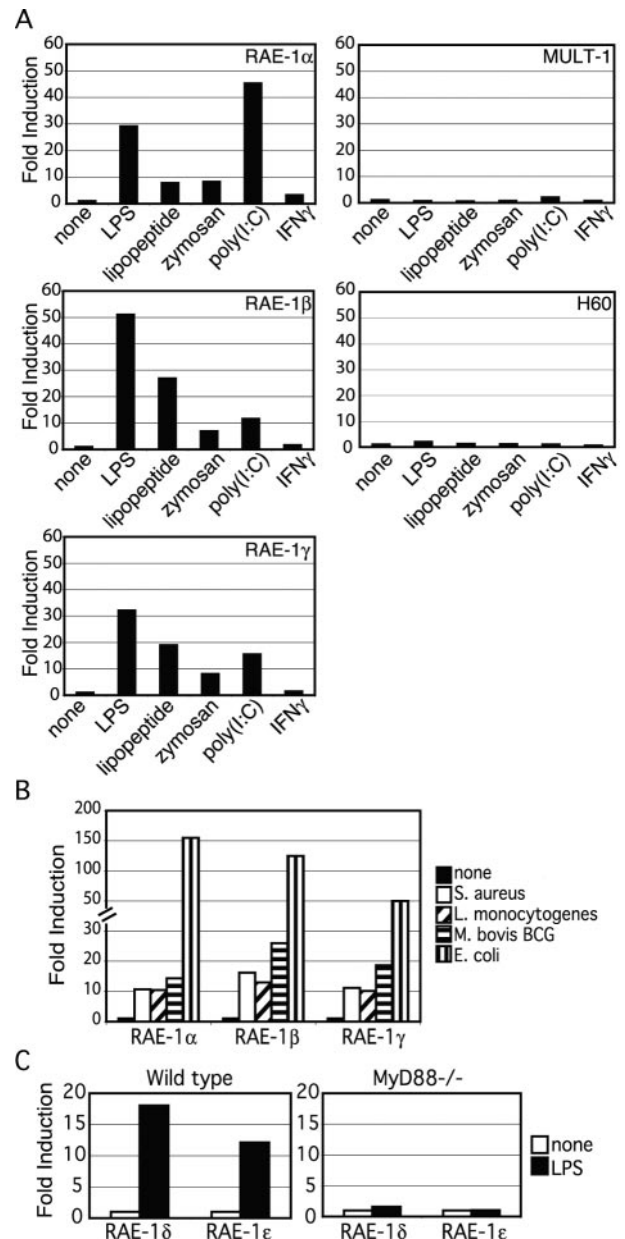
We investigated whether pathogenic stimulation of macrophages induces expression of NKG2D ligands. Peritoneal macrophages from BALB/c mice were activated in vitro with LPS, a potent macrophage activator from the surface of Gram-negative bacteria. After 24 h, the cells were stained with NKG2D-Ig that recognizes all ligands for this receptor (11). While very low levels of staining were seen on unstimulated macrophages, an increase in staining was seen on LPS-activated cells (Fig. 1).

*RAE-1 $\alpha$* , *RAE-1 $\beta$* , and *RAE-1 $\gamma$*  were initially described as three separate genes in 129 mice that were induced by retinoic acid treatment of the F9 teratocarcinoma (15, 16, 20). The proteins they encode were subsequently found to be ligands for mouse NKG2D, as were two additional members of this family, *RAE-1 $\delta$*  and *RAE-1 $\epsilon$*  (11, 12, 21). We used real-time PCR primer-probe sets that distinguish between the *RAE-1* cDNAs, along with sets designed to detect H60 and MULT-1, to amplify cDNA generated from mRNA from resting and activated macrophages to obtain quantitative information about the levels of transcripts. We used a panel of ligands that are derivatives of pathogens and known to signal through various TLRs to activate macrophages, as well as IFN- $\gamma$ , another potent macrophage activator that signals through a distinct pathway. These stimuli included LPS from Gram-negative bacteria (TLR4); the synthetic bacterial lipopeptide, Pam<sub>3</sub>CSK<sub>4</sub> (TLR2 + 1); zymosan, derived from yeast cell walls (TLR2 + 6); and poly(I:C), a synthetic double-stranded RNA (TLR3) (1).



**FIGURE 1.** NKG2D ligands are up-regulated on peritoneal macrophages in response to LPS. Resident peritoneal macrophages from BALB/c mice were cultured in medium alone or with LPS for 24 h. Macrophages were stained with NKG2D-Ig (solid line) or with control Ig (dotted line) and analyzed by flow cytometry. Results are representative of two independent experiments.

In BALB/c macrophages, *RAE-1* transcripts were induced by TLR ligands, but not by IFN- $\gamma$  (Fig. 2, *A* and *B*). In contrast, levels of mRNA for MULT-1 and H60 were unchanged in macrophages treated with microbial stimuli or IFN- $\gamma$ . We were interested in whether the TLR ligands had selective effects on the promoters of the different *RAE-1* genes. In BALB/c macrophages (Fig. 2*A*), LPS equivalently induced transcription of *RAE-1 $\alpha$* , *RAE-1 $\beta$* , and *RAE-1 $\gamma$* , while other stimuli had differential effects. Particularly striking was the strong induction of



**FIGURE 2.** *RAE-1* transcripts are induced in macrophages in response to TLR ligands. *A*, Resident peritoneal macrophages from BALB/c mice were cultured for 24 h in medium alone (none) or with TLR ligands or for 48 h with IFN- $\gamma$ . cDNA was subjected to quantitative real-time PCR. *RAE-1* transcription is expressed as the fold induction over untreated macrophages. SD for duplicate or triplicate samples was <1%. Data shown are representative of three to six experiments. *B*, Macrophages were cultured for 24 h alone or in the presence of LPS or heat-killed bacteria. Results are representative of two independent experiments. *C*, Resident peritoneal macrophages from MyD88<sup>-/-</sup> mice or from wild-type B6 controls were cultured alone or with LPS. Results are representative of three mice assayed in two experiments.



RAE-1 $\alpha$  mRNA by the TLR3 stimulus, poly(I:C), in comparison with the more modest effects of poly(I:C) on RAE-1 $\beta$  and  $\gamma$ . In fact, this was the only case in which a stimulus other than LPS gave the strongest induction of a *RAE-1* gene. We also examined the induction of RAE-1 mRNA by whole bacteria, each of which display a variety of TLR ligands. Bacteria, including the Gram-negative bacterium *E. coli*, the Gram-positive bacteria *S. aureus* and *L. monocytogenes*; and the mycobacterium *M. bovis* BCG, induced *RAE-1* transcription (Fig. 2*B*). These data suggest that the increase in NKG2D-Ig staining seen in LPS-activated macrophages (Fig. 1) was likely due to expression of the RAE-1 proteins and not MULT-1 or H60. Additionally, we have shown that infection of macrophages with murine CMV, a dsDNA virus, induces mRNA encoding all five known RAE-1 genes, though it is not clear whether this occurs through a TLR pathway or another mechanism (6). By contrast, IFN- $\gamma$  did not induce transcription of any NKG2D ligand genes.

Taken together, these results show that the different *RAE-1* genes, while all were induced by some microbial stimuli, are differentially regulated by particular agents. Differences in the promoters of the *RAE-1* genes resulting in differential regulation could be one explanation for why multiple genes which encode proteins with greater than 90% homology and similar affinities for NKG2D exist (22).

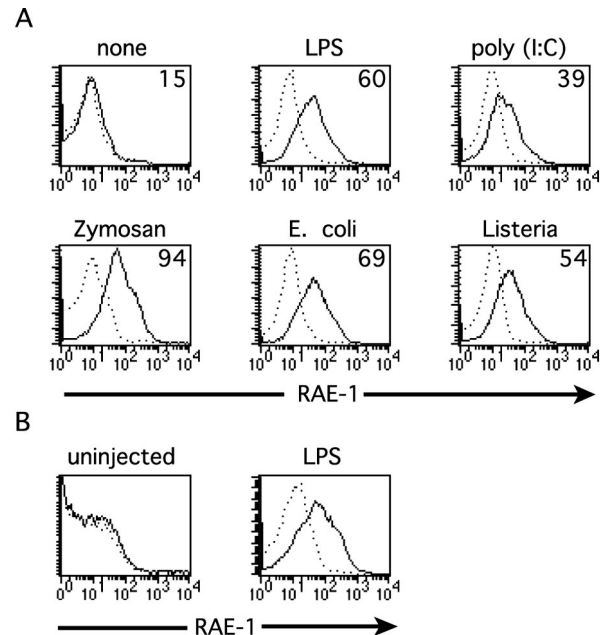
#### Induction of *RAE-1* mRNA is MyD88-dependent

While all *RAE-1* genes were induced transcriptionally in response to signaling through the TLRs analyzed (TLR1, TLR2, TLR3, TLR4, TLR6), there were differences in the magnitude of the response. This led us to analyze the mechanism by which TLR signaling resulted in RAE-1 expression. TLRs mediate signaling through recruitment of a set of TIR-domain-containing adaptor molecules. MyD88 is the best studied adaptor and is required for many TLR responses (1). MyD88 interacts with all TLRs through its TIR domain linking TLR ligation to downstream signaling pathways resulting in activation of NF- $\kappa$ B (1).

It was unclear whether induction of RAE-1 by LPS would require MyD88, as do the inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-6, or would be MyD88-independent as is costimulatory molecule induction in dendritic cells (1). To test whether induction of RAE-1 mRNA is dependent upon MyD88, we treated peritoneal macrophages from MyD88-deficient mice (18) on a B6 background with LPS. We found that the induction of RAE-1 $\delta$  and RAE-1 $\epsilon$  by LPS is completely dependent upon MyD88 signaling (Fig. 2*C*). Neither TNF- $\alpha$  nor IFN- $\alpha\beta$  are required for LPS-induced RAE-1 expression because gene-deficient mice for TNF- $\alpha$  or IFN- $\alpha\beta$  receptor demonstrated normal levels of RAE-1 induction in response to these stimuli (data not shown).

#### Induction RAE-1 surface expression by microbial stimuli

To examine whether the increase in mRNA for the RAE-1 proteins resulted in surface expression of these NKG2D ligands, we used a mAb that recognizes all known RAE-1 proteins (6). While untreated macrophages did not show RAE-1 surface expression, RAE-1 was detected on macrophages stimulated with LPS, poly(I:C), zymosan, *E. coli*, and *L. monocytogenes* (Fig. 3*A*). Because mAbs against H60 and MULT-1 are not available, we cannot exclude the possibility that the surface expression of H60 and MULT-1 are increased after TLR signaling, while the mRNA levels are constant (Fig. 2).

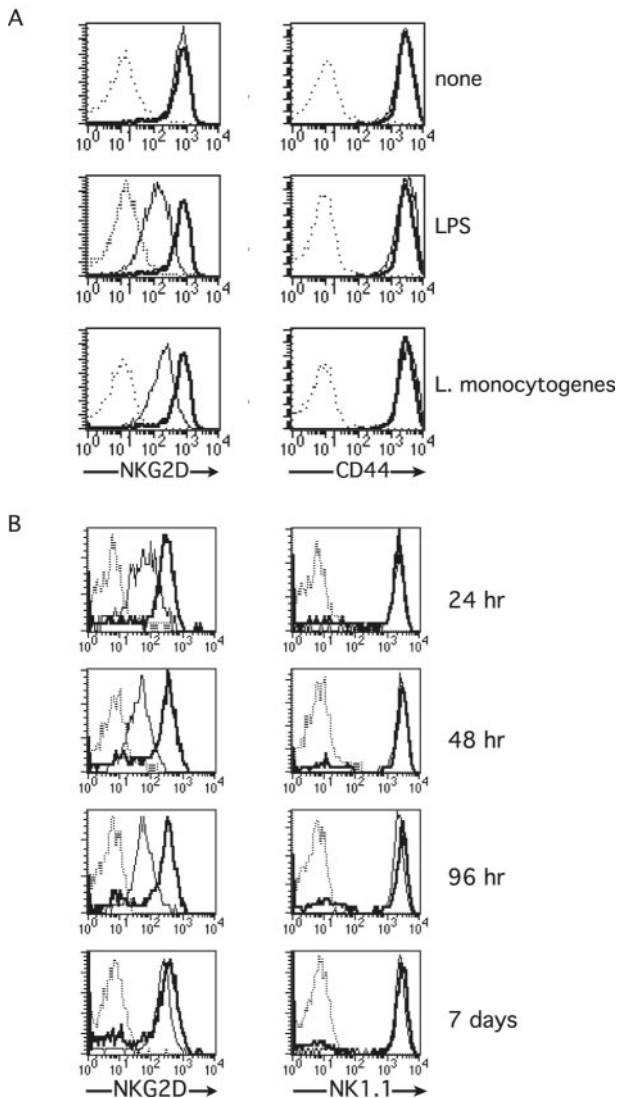


**FIGURE 3.** RAE-1 is found on the surface of macrophages treated with TLR ligands *in vitro* and *in vivo*. *A*, Resident peritoneal macrophages from B6 mice were cultured alone or treated with TLR ligands. After 48 h, the macrophages were stained with a pan-RAE-1 mAb and analyzed by flow cytometry. The number in the upper right corner is the mean fluorescence intensity of the cells stained with the anti-RAE-1 mAb. *B*, Peritoneal cells were harvested from uninjected mice or from mice injected i.p. 48 h previously with LPS. Staining of the pan-RAE-1 mAb is shown (gating on F4/80-positive macrophages). Data are representative of four independent experiments.

RAE-1 surface expression was also observed on macrophages activated *in vivo*. Freshly isolated resident peritoneal macrophages did not express RAE-1, while after i.p. injection of LPS, surface RAE-1 was detected on the macrophages in the peritoneal cavity (Fig. 3*B*). Thus, we have demonstrated that the RAE-1 family of ligands for mouse NKG2D are expressed on the surface of macrophages after signaling through TLRs, the initiating step in the immune response to many pathogens.

#### *RAE-1* on activated macrophages is recognized by NKG2D on NK cells *in vitro* and *in vivo*

We sought to determine whether the levels of RAE-1 protein found on the surface of activated macrophages were sufficient to be detected by NKG2D on NK cells. To do this, we took advantage of the fact that NKG2D is down-regulated on NK cells after interacting with its ligands (19). Unstimulated macrophages or macrophages activated for 24 h with LPS or *Listeria monocytogenes* were cultured overnight with NK cells and then NKG2D levels were assessed by flow cytometry. NKG2D levels were unchanged on NK cells cultured with unstimulated macrophages in comparison with NK cells cultured alone (Fig. 4*A*). In contrast, NKG2D levels were reduced on NK cells cultured with LPS or *Listeria monocytogenes*-treated macrophages. Surface levels of CD44 on the NK cells were unchanged these culture conditions (Fig. 4*A*). The modulation of NKG2D required cell-cell contact, as it did not occur when the NK cells and macrophages were separated during culture by a 0.4- $\mu$ M Transwell filter (data not shown), suggesting the numerous cytokines produced by TLR-activated macrophages were not responsible for the NKG2D down-regulation on the NK cells.



**FIGURE 4.** NKG2D on NK cells is down-regulated in response to RAE-1 on activated macrophages. *A*, Fresh NK cells were cocultured with macrophages from B6 mice that were untreated (none) or treated with LPS or heat-killed *L. monocytogenes* for 48 h before addition of NK cells. After 24 h, the NK cells were stained and analyzed by flow cytometry. The plots show the staining profiles of NK cells gated as NK1.1<sup>+</sup>CD3<sup>-</sup> for NKG2D (left panel) or CD44 (right panel). The thick line shows staining of NK cells cultured alone, the thin line of NK cells cultured with macrophages and the dotted line indicates staining with control mAb. *B*, B6 mice were injected i.p. with LPS. At indicated times after injection peritoneal cells were stained and analyzed by flow cytometry. The staining profiles for NKG2D (left panel) and NK1.1 (right panel) are shown for DX5<sup>+</sup>CD3<sup>-</sup> gated NK cells. The plots show the levels of NKG2D or NK1.1 on NK cells from an uninjected mouse (thick lines), an LPS-injected mouse (thin lines) or stained with control mAb (dotted lines). Three injected mice were assayed per experiment and the results shown are representative of three independent experiments.

Given that NK cells cultured with LPS-activated macrophages in vitro modulated NKG2D levels, we examined whether this happens in vivo. As early as 24 h after LPS injection, NKG2D was reduced on NK cells in the peritoneal cavity in comparison with the NKG2D staining seen on NK cells from uninjected mice (Fig. 4*B*). This modulation of NKG2D was not permanent, as NKG2D levels returned to normal within 7 days after injection. This may reflect the fact that RAE-1 expression on peritoneal macrophages after in vivo LPS

treatment peaked at 48 h and returned to background levels at 96 h after injection (data not shown). At all time points, the level of NK1.1, another NK cell activating receptor, had not changed. The efficient modulation of NKG2D on NK cells after LPS injection in vivo suggests that NK cells are responding to RAE-1-bearing cells, presumably including macrophages, in vivo. As NKG2D is also expressed on mouse CD8<sup>+</sup> T cells and activated macrophages (12, 23), it is possible that the RAE-1 on macrophages signals to these cell types as well. Ligand-induced modulation of NKG2D levels has been shown to depend upon signaling through the YXXM motif in DAP10, the NKG2D-associated signaling adaptor in the resting NK cells used in these experiments (19). Taken together, these data suggest that the levels of RAE-1 protein found on the surface of activated macrophages is sufficient to induce NKG2D signaling in NK cells, resulting in modulation of receptor levels.

In summary, RAE-1 expression on macrophages in response to pathogenic stimuli may contribute to both the innate and adaptive immune responses to infection. RAE-1-NKG2D interactions may delineate a mechanism by which NK cells and infected macrophages interact directly during infection, in addition to the well-defined indirect activity of macrophage-produced cytokines on NK cell IFN- $\gamma$  production. Indeed, we have seen that NK cells can kill LPS-activated macrophages in vitro (J. Hamerman, unpublished observations), and we are currently investigating the receptors involved in this function.

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