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The Rapid Induction of HLA-E Is Essential for the Survival of Antigen-Activated Naive CD4 T Cells from Attack by NK Cells

Sumiko Takao,* Takayuki Ishikawa,* Kouhei Yamashita,* and Takashi Uchiyama*†

Increasing evidence shows that NK cells regulate adaptive immunity, but the underlying mechanisms are not well understood. In this study, we show that activated human NK cells suppress autologous naive CD4 T cell proliferation in response to allogeneic dendritic cells (DCs) by selectively killing Ag-activated T cells. Naive CD4 T cells, which were initially resistant to NK cell-mediated cytotoxicity, became substantially susceptible to NK cells within a day after priming with DCs. Ag-activated T cells showed various degrees of susceptibility to NK cells. After 1 d of priming with LPS-matured DCs, T cells were less susceptible to NK cells than were T cells primed with TNF-α-matured DCs. Subsequently at day 3, Ag-activated T cells regained resistance to NK cells. The level of HLA-E expression on Ag-activated T cells was closely correlated with resistance to NK cells. HLA-E was highly expressed at day 1 by T cells primed with LPS-matured DCs but not by T cells primed with TNF-α-matured DCs. An Ab blockade revealed a critical role for the HLA-E–NKG2A interaction in the protection of Ag-activated T cells from NK cells. Collectively, this study demonstrates that NK cells impact adaptive immunity through the finely controlled kinetics of HLA-E expression on T cells. Thus, HLA-E may be a new target for immunoregulation.


NK cells are broadly distributed throughout lymphoid and nonlymphoid tissues and reciprocally interact with various types of cells, such as dendritic cells (DCs), macrophages, T cells, and endothelial cells (1, 13). In lymph nodes, NK cells reside in the parafollicular T cell areas (14), where additional NK cells are recruited in response to pathogen or tumor cell invasion (15, 16). In these areas, NK cells exist in close proximity to DCs (17, 18), facilitating NK cell modulation of T cell priming or activation of naive T cells by DCs.

Several mechanisms by which NK cells influence adaptive immune responses have been proposed. Activated NK cells have been found to induce the maturation of DCs through the secretion of cytokines, such as IFN-γ and TNF-α, or through direct cell–cell contact (19–21). Additionally, NK cells can kill immature DCs (22, 23) and can also directly promote a Th cell type 1 response through the secretion of IFN-γ (24). Finally, NK cells can inhibit T cell responses through the secretion of cytokines, such as TGF-β and IL-10 (25–27), p21-mediated cell cycle arrest (28), or cytotoxic activity against activated T cells (29–31).

It is well known that the nature of DC maturation signals determines the effector function of these cells, resulting in the regulation of the T cell activation status (32). As recently reported, signaling through pattern-recognition receptors such as TLRs can confer the capacity of DCs to differentiate functional effector T helper cells, whereas inflammatory mediators, such as TNF-α, cannot do so (33). However, whether these different types of DC maturation signals lead to distinct modes of NK cell-mediated regulation of DC-stimulated T cell responses remains unknown.

Thus, NK cells can enhance or restrict adaptive immune responses through a variety of mechanisms. The outcome of NK cell-mediated regulation is determined by a variety of factors, including the specific site of interaction, the nature of the triggering stimulus, the subtype of the NK cells, and the phase of the immune response (3). However, the precise mechanisms by which NK cells regulate adaptive immunity are not yet fully understood.

Our study examined the effects of NK cells on naive CD4 T cell responses in humans using an in vitro coculture system consisting
of naive CD4 T cells, allogeneic DCs, and NK cells. Our results indicate that naive CD4 T cells become susceptible to NK cell-mediated cytotoxicity following Ag stimulation and that Ag-activated T cells show various degrees of susceptibility to NK cells, depending on the type of activating DCs and the phase of activation. We also found that the Ag-activated T cell expression of HLA-E, which engages NKG2A, an inhibitory receptor on NK cells (34, 35), is a critical factor for determining T cell susceptibility to NK cells.

Materials and Methods

Blood and lymph node samples

In accordance with the Declaration of Helsinki, peripheral blood samples were obtained from healthy donors following written informed consent. Surgical samples of lymph nodes were obtained from healthy donors following written informed consent for their experimental use. This study was approved by the Institutional Review Board of Kyoto University.

Immunohistochemistry

Two lymph node specimens from patients with nonspecific lymphadenitis were studied. Tissue sections were prepared as described previously (36). For dual-color staining, anti-CD4 (clone IF6, 1/100 dilution; Novocastra Laboratories, Newcastle, U.K.) and anti-CD56 mAbs (clone B1, 1/100 dilution; Novocastra Laboratories) were used. Immunoreactive cells were visualized using combinations of peroxidase/diaminobenzidine and alkaline phosphatase/new fuchsin staining systems, respectively. Nuclei were counterstained with hematoxylin.

Reagents and Abs

Cell culture was performed in complete medium consisting of RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (ICN Biomedicals, Aurora, OH), penicillin G, and streptomycin (Invitrogen). The following mAbs and control Abs were obtained from eBioscience (San Diego, CA): PE-Cy7-conjugated anti-CD4 (L200), allophycocyanin-conjugated anti-CD25 (PC96), biotin-conjugated anti-CD25 (BC96), anti-HLA class I-related chain (MIC)A/MICB (6D4), and mouse IgG1, IgG2a, and IgG2b isotype controls. FITC-conjugated anti-CD25 (B1.409.9), allophycocyanin-conjugated F(ab′)2 goat anti-mouse IgG (GAM), anti-NKG2A (Z199), anti-CD158a (EB6.B), and anti-CD158b (GL183) were purchased from Beckman Coulter (Fullerton, CA). Anti-UL16-binding protein (ULBP)1 (AUMO12), ULBP2 (BUMO1), and ULBP3 (CUMO3) mAbs were obtained from BamOmaB (Munich, Germany). Anti-HLA-E mAbs included clones 4D12 (Medical & Biological Laboratories, Nagoya, Japan) and 3D12 (37) (provided by Dr. Daniel E. Geraghty, Fred Hutchinson Cancer Research Institute, Seattle, WA), and PE-conjugated anti-HLA-E mAb (clone 3D12) was purchased from eBioscience. An anti-HLA class I mAb (HP-1F7), which detects all classical and nonclassical HLA class I molecules (38), was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD3 mAbs (OKT3) were obtained from Janssen Pharmaceutical (Tokyo, Japan) and anti-CD28 mAbs (clone B7.20.1) were purchased from Beckman Coulter (Fullerton, CA). Anti-CD25 (B1.409.9), allophycocyanin-conjugated F(ab′)2 goat anti-mouse IgG (GAM), anti-NKG2A (Z199), anti-CD158a (EB6.B), and anti-CD158b (GL183) were purchased from Beckman Coulter (Fullerton, CA). Anti-UL16-binding protein (ULBP)1 (AUMO12), ULBP2 (BUMO1), and ULBP3 (CUMO3) mAbs were obtained from BamOmaB (Munich, Germany).

Preparation of naive CD4 T cells and NK cells

PBMCs were isolated by density gradient centrifugation using Lympholyte-H (Cedarslane Laboratories, Hornby, Ontario, Canada). Naive CD4 T cells were negatively selected from PBMCs using a naive CD4 T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with minor modifications. To remove CD25+ "regulatory" naive CD4 T cells, a biotinylated anti-CD25 mAb was added to the biotin-conjugated Ab mixture included with the kit. The purity of naive CD4 T cells was >90% and the frequency of contaminating CD25+CD4 T cells was <0.1%. Prior to culture, purified naive CD4 T cells were labeled with PKH26 dye (Sigma-Aldrich, St. Louis, MO), unless otherwise indicated. NK cells were isolated from PBMCs by negative selection using an NK cell isolation kit (Miltenyi Biotec). The NK cell purity was >90%. Purified NK cells were stimulated in complete medium containing 50 ng/ml GM-CSF (PeproTech) and 40 ng/ml IL-4 (R&D Systems, Minneapolis, MN). For the last 24 h of culture, the DCs were matured with 10 ng/ml TNF-α (TNF-DCs; PeproTech) or 100 ng/ml LPS (LPS-DCs; Sigma-Aldrich). Prior to their incubation with T cells, DCs were washed and the TNF-α or LPS was removed from the culture medium. In some experiments after 5 d of culture, a portion of the DCs were stimulated with TNF-α or LPS for a day and then used for the initial culture with T cells. The remaining DCs were exposed to maturation stimuli on the following day for use in cell restimulation.

Flow cytometric analysis

Flow cytometric analyses were performed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ) and flow cytometry data were analyzed with CellQuest software (BD Biosciences). To examine cell viability, cells were stained with FITC-conjugated annexin V (BD Biosciences). Naive CD4 T cells stimulated with allogeneic DCs were comprised of all allogene-specific activated and nonactivated T cells. For the detection of NKG2D ligands and HLA class I molecules on Ag-activated T cells, separately from those on nonactivated T cells, cell populations including PKH26-labeled CD4 T cells were first incubated with mAbs against each molecule and then stained with allophycocyanin-conjugated goat anti-mouse IgG. Cells were further treated with PBS (Invitrogen) supplemented with 5% mouse serum (Sigma-Aldrich), followed by staining with a PE-Cy7-conjugated anti-CD4 mAb and a FITC-conjugated anti-CD25 mAb. Ag-activated CD4 T cells were identified by gating on CD25+ CD4 T cells at day 1 and PKH26low CD4 T cells at day 3. Mean fluorescence intensity (MFI) value of the isotype control Ab was subtracted from the MFI value relative to each molecule (referred to as AMFI). In some experiments, naive CD4 T cells were treated with 100 ng/ml human IFN-α (PeproTech) or 20 ng/ml human IFN-β1a (ProSpec, Rehovot, Israel) in the coculture.

Proliferation assays

PKH26-labeled, naive CD4 T cells (1 × 10^6 cells/well) were cocultured with 2 × 10^6 allogeneic TNF-DCs (allo-TNF-DCs) or allo-LPS-DCs (referred to as allo-DCs) in 24-well plates (day 0) unless otherwise indicated. At day 1 or 3 after priming with DCs, 50 μl of IL-15/18-activated NK cells was added to the culture at the indicated NK/T ratio, which ranged from 0:10 to 4:10. At day 4, the cells were stained with an anti-CD4 mAb and the percentage of proliferating CD4 T cells, detected using the diluted PKH26 signals, was analyzed by flow cytometry (see Fig. 1A). To evaluate absolute cell numbers, an aliquot of the culture was added to a solution of fluorescent microspheres (Flow-Count Fluospheres; Beckman Coulter) at known concentrations, and viable cells and fluorescent microspheres were then counted based on their forward light scatter and side light scatter or forward light scatter and FL1 characteristics, respectively (see Fig. 1A). When NK cell-mediated T cell growth inhibition was compared, we evaluated the percentage inhibition of T cell proliferation defined as follows: 100 × (proliferation of control (stimulated in the absence of NK cells) – proliferation of target of background lysis). The percentage of background lysis was determined as follows: 100 × (proliferation in the presence of NK cells/percentage of background lysis). In some experiments, 10 μg/ml anti-NKG2A mAb, 10 ng/ml IFN-α (PeproTech), or 1, 10, or 100 ng/ml IL-2 (PeproTech) was added to the culture.

Cytotoxicity assays

PKH26-labeled, naive CD4 T cells were cocultured with allo-DCs as described for the proliferation assays, and then cells were harvested at day 1 or 3. To precisely analyze the lysis of Ag-activated T cells separately from that of nonactivated T cells, at day 1, CD25+, “Ag-activated” CD4 T cells and CD25-, “nonactivated” CD4 T cells were separated using differences in Ag-activated and nonactivated T cells were separated using differences in Ag-activation and nonactivation (Ag-activated T cells were <0.1%, prior to culture). The purity of naive CD4 T cells was >98%. Isolated Ag-activated and nonactivated T cells proliferating with IL-15/18–activated T cells were >98% and the percentage of background lysis was >98%. Isolated Ag-activated and nonactivated T cells were stimulated with TNF-α or LPS for a day and then used for the subsequent assay. To evaluate absolute cell numbers, an aliquot of the culture was added to a solution of fluorescent microspheres (Flow-Count Fluospheres; Beckman Coulter) at known concentrations, and viable cells and fluorescent microspheres were then counted based on their forward light scatter and side light scatter or forward light scatter and FL1 characteristics, respectively (see Fig. 1A). When NK cell-mediated T cell growth inhibition was compared, we evaluated the percentage of specific lysis of T cell lysis defined as follows: 100 × [(proliferation of control (stimulated in the absence of NK cells) – proliferation in the presence of NK cells – proliferation in the presence of background lysis)]/([proliferation of control (stimulated in the absence of NK cells) – proliferation in the presence of background lysis]). When comparing blocking experiments, NK cells and CD4 T cells were pretreated with 10 μg/ml mAb in each cytotoxicity assay. Briefly, NK cells were incubated with either an anti-

of T cells, defined as follows: 100 × (proliferation of control (stimulated in the absence of NK cells) – proliferation in the presence of NK cells – proliferation in the presence of background lysis). The percentage of background lysis was determined as follows: 100 × (proliferation in the presence of NK cells/percentage of background lysis). In some experiments, 10 μg/ml anti-NKG2A mAb, 10 ng/ml IFN-α (PeproTech), or 1, 10, or 100 ng/ml IL-2 (PeproTech) was added to the culture.
NK2G2A mAb, anti-NKG2D mAb, or a control Ab, and then T cells were treated with a combination of anti-CD158a and anti-CD158b mAbs (referred to as anti-CD158a/b mAbs), anti-HLA class I mAb, or control Ab for 25 min. When the effects of blocking mAbs on NK cell-mediated T cell lysis were compared, we evaluated changes in specific lysis, defined as the difference in the percentage specific lysis between the blocking mAb-treated T cells and control Ab-treated T cells.

ELISA

Monocyte-derived immature DCs were seeded at a concentration of $1 \times 10^6$ cells/ml and stimulated with 10 ng/ml TNF-α or 100 ng/ml LPS for 24 h. The supernatants were then collected and the concentration of IFN-α and IFN-β was measured using human IFN-α ELISA kits and IFN-β ELISA kits (PBL Interferon Source), in accordance with the manufacturer’s instructions.

Statistical analysis

Statistical significance was determined using a Wilcoxon signed-rank test. The p values <0.05 were considered to be statistically significant.

Results

The proliferation of naive CD4 T cells is markedly inhibited by activated NK cells

We first assessed whether CD4 T cells and NK cells colocalized within human inflamed lymph nodes using donated human patient samples. Immunohistochemical staining showed that CD56+ and CD4+ cells residue in close proximity in the parafollicular T cell areas of the lymph nodes (Supplemental Fig. 1), indicating that CD4 T cells and NK cells can interact within secondary lymphoid organs in vivo. We then examined the effects of NK cells on the alloantigen-induced proliferation of naive CD4 T cells in vitro. PKH26-labeled, naive CD4 T cells, depleted of CD25+ regulatory naive CD4 T cells, were cocultured with allo-TNF-DCs. The following day (day 1), autologous NK cells activated with IL-15 and IL-18 were added to the coculture at NK/T ratios of 0:10, 0.5:10, or 1:10. Four days after priming with DCs (day 4), CD4 T cell proliferation was analyzed by flow cytometry (Fig. 1A, 1B). The results indicate that the percentage and absolute number of proliferating CD4 T cells, as defined by the PKH26 intensities, were significantly decreased by the addition of activated NK cells in a cell dose-dependent manner (Fig. 1B–D). In contrast, the number of nonproliferating T cells that retained high PKH26 intensity was unaffected by the addition of activated NK cells (Fig. 1C). Interestingly, resting NK cells could not inhibit T cell proliferation (data not shown).

We next investigated the mechanisms underlying the inhibition of T cell proliferation by activated NK cells. First, we performed an experiment using a Transwell culture system. The results showed that the inhibition of T cell proliferation required direct cell contact between T and NK cells (data not shown), indicating that this suppression was unlikely to be mediated by soluble factors. Consistent with this, the inhibition of T cell proliferation by NK cells was not reversed by the addition of various blocking mAbs, such as anti–IFN-γ and anti–TNF-α (data not shown), nor by the addition of different concentrations of IL-2 (up to 100 ng/ml) (Supplemental Fig. 2A) or IL-15 (data not shown). Second, we examined whether the inhibition of T cell proliferation was mediated by the elimination of DCs. Consistent with the findings of previous studies (22, 23), mature TNF-DCs showed minimal cell death by flow cytometry following exposure to activated NK cells at an NK:DC ratio of 1:1 in a 4-h cytotoxicity assay (data not show). Additionally, even when increasing numbers of DCs were added to the coculture, where the number of DCs changed much more dynamically than did that of Ag-activated T cells, the number of proliferating T cells did not increase in correlation with the number of DCs (Supplemental Fig. 2B), indicating that the inhibition is unlikely to be mediated by direct cytotoxicity to DCs.

Third, even when naive CD4 T cells stimulated with TNF-DCs or LPS-DCs for 24 h were isolated by cell sorting and then incubated with activated NK cells, the proliferation of T cells was still inhibited (Supplemental Fig. 2C). This indicated that the interaction of NK cells with DCs is not involved in the inhibition of T cell proliferation. Collectively, our results suggest that growth inhibition of allo-DC–stimulated T cells is mediated by the direct interaction between T cells and NK cells and not through the secretion of regulatory cytokines, a deprivation of cytokines required for T cell growth, or the interaction between DCs and NK cells.

Alloantigen-activated CD4 T cells are killed by NK cells

To further investigate the mechanism underlying NK cell-mediated T cell growth inhibition, we first examined the T cell CD25 expression levels at day 1 and their correlation with cell proliferation. PKH26-labeled, naive CD4 T cells, depleted of CD25+ cells prior to culturing, were cocultured with allo-TNF-DCs. At day 1, a small fraction (~1–2%) of the T cells expressed CD25. By day 2, a variable but significant proportion of the CD25+ T cells showed reduced PKH26 intensities, indicating that they had begun...
to proliferate. At day 3, the CD25⁺ cell PKH26 intensity continued to decrease, whereas the PKH26 intensity of CD25⁻ T cells remained high (Supplemental Fig. 3). Moreover, we isolated both CD25⁺ as well as CD25⁻ T cells at day 1 and reincubated them with DCs from the original source for 3 further days. As shown in Fig. 2A, the vast majority of the CD25⁺ T cells vigorously proliferated, whereas the CD25⁻ T cells remained in a resting state. These observations demonstrate that in a coculture of naive CD4 T cells and allo-DCs, only a small fraction of T cells can recognize alloantigens on DCs and are specifically activated by allo-DCs (referred to as alloantigen- or Ag-activated T cells), and the vast majority of T cells cannot recognize alloantigens and are not specifically activated by allo-DCs (hence referred to as nonactivated T cells). Moreover, the expression of CD25 at day 1 and reduced PKH26 intensity at day 3 are reliable markers that can discriminate alloantigen-activated T cells from nonactivated T cells.

We next examined the effects of NK cells on alloantigen-activated, naive CD4 T cells using CD25 expression as a marker. We found that the percentage of CD25-expressing, alloantigen-activated T cells was significantly decreased at 1 d after the addition of activated NK cells (Fig. 2B, 2C). To determine whether the decrease of CD25⁺ alloantigen-activated naive CD4 T cells following NK cell incubation was mediated by NK cell-mediated cytotoxicity against T cells, PKH26-labeled, naive CD4 T cells were first cultured with allo-TNF-DCs for 24 h. Thereafter, CD25⁺ as well as CD25⁻ T cells were isolated and then incubated with activated NK cells at NK/T cell ratios of 1:1 and 10:1 for 4 h. Flow cytometric analysis using annexin V revealed that a substantial fraction of CD25⁺ CD4 T cells were killed by NK cells in an NK/T ratio-dependent manner (Fig. 2D, 2E). We additionally stained T cells with propidium iodide and for annexin V, and we confirmed that almost all propidium iodide-positive T cells were also annexin V-positive (data not shown). Annexin V staining was therefore used to detect dead cells thereafter. NK cells pretreated with concanamycin A inefficiently killed CD25⁺ T cells, indicating that the cell killing was mediated mainly by the release of lytic granules from NK cells (data not shown). In contrast, CD25⁻ nonactivated T cells were resistant to NK cell-mediated cytoxicity (Fig. 2D, 2E). Hence, NK cells exert their inhibitory effects on T cell proliferation by selectively killing alloantigen-activated T cells.

**Naive CD4 T cells stimulated with allo-LPS-DCs are relatively resistant to NK cells**

Different DC maturation signals induce distinct DC maturation states, resulting in distinct DC-mediated modes of T cell activation (32). When allo-LPS-DCs were used to stimulate naive CD4 T cells, the stimulated T cells were less susceptible to NK cell-mediated growth inhibition than were T cells stimulated with allo-TNF-DCs (Fig. 3A). In the absence of NK cells, allo-LPS-DC stimulation increased the fraction of alloantigen-activated T cells compared with allo-TNF-DCs (data not shown), resulting in increased T cell proliferation at day 4 (Fig. 3A). To compare the inhibitory effects of NK cells on T cell proliferation, we used the percentage inhibition of T cell proliferation as defined in Materials and Methods. As shown in Fig. 3B, the percentage inhibition of T cell proliferation was significantly lower in the allo-LPS-DC–stimulated T cells. Additionally, CD25⁺ T cells isolated from T cells stimulated with allo-LPS-DCs for 24 h were relatively resistant to NK cell-mediated cytoxicity compared with CD25⁺ T cells stimulated with allo-TNF-DCs (Fig. 3C, 3D). Thus, LPS-matured DCs not only activate a larger proportion of T cells, but they also confer increased resistance to NK cell-mediated cytoxicity on Ag-activated T cells.

**Alloantigen-activated CD4 T cells reestablish resistance to NK cells 3 d after stimulation**

As previously reported (41), we found that resting, naive CD4 T cells were resistant to NK cells (data not shown). Additionally, PHA-activated T cell blast cells have also been reported to be resistant to NK cells (41). Based on these findings, we hypothesized that T cell vulnerability to NK cells may be a transient phenomenon. To test this possibility, PKHlow alloantigen-activated and PKHhigh nonactivated T cells were isolated at day 3 and exposed to NK cells. Remarkably, PKHlow T cells exhibited a much...
lower level of cytolysis than did CD25⁺ alloantigen-activated T cells at day 1, showing only a marginal susceptibility to NK cell-mediated cytotoxicity at a high NK/T ratio. This resistance to NK cells developed by day 3 irrespective of whether T cells were stimulated with allo-TNF-DCs or allo-LPS-DCs. PKH high T cells showed complete resistance to NK cells (Fig. 4A). In accordance with the regained resistance to NK cells, the addition of activated NK cells to the coculture comprised of T cells and allo-DCs at day 3 only had a negligible effect on T cell growth (Fig. 4B).

The alloantigen-activated CD4 T cell HLA-E expression levels correlate with the resistance to NK cells

NK cell-mediated cytotoxicity is determined by a balance of signals from inhibitory and activating receptor ligands on target cells (42). HLA class I molecules, such as HLA-E, engage inhibitory receptors on NK cells and prevent NK cell-mediated cytotoxicity. Conversely, NKG2D ligands, such as MICA, MICB, ULBP1, ULBP2, and ULBP3, engage the activating receptor NKG2D and trigger NK cell-mediated cytotoxicity.

To further elucidate the mechanisms that regulate the susceptibility to NK cells, we analyzed by flow cytometry both the inhibitory and activating ligand expression levels on alloantigen-activated T cells during their coculture with allo-DCs. After PKH26-labeled, naive CD4 T cells were stimulated with either allo-TNF-DCs or allo-LPS-DCs for 1 d, CD25⁺ T cells were isolated and incubated with NK cells for 4 h at an NK/T ratio of 0:1 or 10:1. The percentage of annexin V-positive T cells was then compared between allo-TNF-DC–stimulated and allo-LPS-DC–stimulated sorted CD25⁺ T cells. A representative experiment is shown in C. The percentage of specific lysis at an NK/T ratio of 10:1 from 10 independent experiments is shown in D.

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activated CD4 T cells are defined as CD25+CD4 T cells at day 1 and alloantigen-activated CD4 T cells also from five independent experiments. The Δ MF of the indicated molecules on alloantigen-activated CD4 T cells on days 1 and 3 also on resting CD4 T cells on day 0 was evaluated. Each unique symbol represents an identical donor. A, Alloantigen-activated CD4 T cells are defined as CD25+CD4 T cells at day 1 and PKH26low CD4 T cells at day 3. The kinetics of MICA/B expression on alloantigen-activated T cells, the cells were stained with anti-CD4 mAb, anti-CD25 mAb, and Abs specific to those molecules in four-color flow cytometric analysis. The alloantigen-activated T cell subpopulation was identified by gating on CD25+ CD4 T cells on day 1 and PKH26low CD4 T cells on day 3. B, Representative data for HLA-E expression on alloantigen-activated CD4 T cells (filled histograms). The open histograms indicate background staining with the control IgG1 mAb. E, HLA-E expression kinetics on alloantigen-activated CD4 T cells also from five independent experiments.

The rapid induction of HLA-E in LPS-DC–stimulated, alloantigen-activated T cells plays a critical role in conferring a survival advantage to these cells in the presence of activated NK cells

To evaluate the functional role of HLA-E in T cell resistance to NK cell-mediated cytotoxicity, we performed cytotoxicity assays using ligand- or receptor-blocking mAbs as described previously (35, 43). We confirmed that control Abs do not affect NK cell-mediated cytotoxicity against T cells (data not shown). In the case of PKH26low alloantigen-activated T cells isolated from a coculture of T cells and allo-DCs at day 3, various blocking mAbs, including anti-CD158a, anti-CD158b, anti-NKG2A, and anti-HLA-class I mAbs, reduced T cell resistance to NK cells to different extents (Fig. 6A). Interestingly, the anti-NKG2A mAb rendered the PKHlow T cells more susceptible to NK cells as compared with the anti-CD158a/b mAbs, which block HLA-C–mediated inhibitory signals to NK cells (Fig. 6A). CD25+ alloantigen-activated T cells isolated at day 1 were next subjected to blocking experiments. As shown in Fig. 6B, the anti-NKG2A mAb enhanced specific lysis of the CD25+ T cells stimulated with allo-TNF-DCs or allo-LPS-DCs to almost the same extent as the anti–HLA class I Abs (Fig. 6A). CD25+ alloantigen-activated T cells isolated from a coculture of T cells and LPS-DCs with NK cells at day 1 almost completely rendered the PKHlow T cells more susceptible to NK cells as compared with the anti-CD158a/b mAbs, which block HLA-C–mediated inhibitory signals to NK cells (Fig. 6B). In contrast, the effects of anti-CD158a/b mAbs upon T cell susceptibility to NK cells were minimal (Fig. 6B). The importance of the NKG2A–HLA-E interaction was also confirmed in cell proliferation assays, in which the addition of an anti-NKG2A mAb to cocultures of T cells and LPS-DCs with NK cells at day 1 almost completely inhibited T cell proliferation by day 4 (Fig. 6D).

Rapid induction of HLA-E protects alloantigen-activated T cells from NK cell-mediated cytotoxicity

We tested the hypothesis that HLA-E expression is the major determinant of day 1 T cell susceptibility to NK cell-mediated cytotoxicity. We found that exogenous IFN-α enhances HLA-E expression in resting, naive CD4 T cells (data not shown) as well as in activated CD4 T cells from NK cell-mediated cytotoxicity.
as in CD25+ alloantigen-activated T cells stimulated with allo-TNF-DCs for 1 d (Fig. 7A). Consistent with these results, CD25+ T cells isolated at day 1 from naive CD4 T cells cocultured with allo-TNF-DCs in the presence of IFN-α showed a significantly increased resistance to NK cells (Fig. 7B). This resistance was largely abrogated by the addition of anti-NKG2A mAbs to the culture media (Fig. 7C). This finding was further supported by data showing that IFN-α partially prevents the inhibition of T cell proliferation by the addition of NK cells at day 1 to cocultures of naive CD4 T cells and allo-TNF-DCs (Fig. 7D, 7E).

**TCR signaling and type I IFNs are involved in the upregulation of HLA-E expression on Ag-activated T cells**

Finally, we examined the signals that regulate the kinetics of HLA-E expression in Ag-activated T cells. First, we assessed HLA-E expression in naive CD4 T cells stimulated with plate-
bound anti-CD3 plus soluble anti-CD28 mAbs. We found that the expression level of HLA-E in CD25+ activated T cells was slightly upregulated at day 1 and substantially elevated at day 3 (Supplemental Fig. 5A). This indicated that TCR signaling is mainly involved in the induction of HLA-E on Ag-activated T cells during the relatively late phase of activation. The addition of type I IFNs (IFN-β as well as IFN-α) induced higher levels of HLA-E expression on both CD25+ TNF-DC–activated T cells and CD25+ anti-CD3/28-activated T cells as early as day 1 (Fig. 7A and Supplemental Fig. 5B, 5C), indicating that type I IFNs contribute to the early-phase upregulation of HLA-E. We next compared type I IFN production in TNF-DCs and LPS-DCs by ELISA. Whereas IFN-α production was not detected in either TNF-DCs or LPS-DCs (data not shown), IFN-β production was observed in LPS-DCs but not in TNF-DCs (Supplemental Fig. 5D). Moreover, the level of HLA-E expression in CD25+ LPS-DC–activated T cells was partially reduced by blocking Abs against the IFN-α/βRs (Supplemental Fig. 5E).

**Discussion**

The activation and proliferation of Ag-specific, naive CD4 T cells is a crucial step in the induction of adaptive immune responses. The results of our study reveal that activated NK cells substantially inhibit Ag-specific, naive CD4 T cell growth by selectively killing Ag-activated T cells. Additionally, our data show that the kinetics of induced HLA-E expression on Ag-activated T cells determine whether T cells survive or are killed by exposure to activated NK cells.

The inhibitory effects of activated NK cells on T cell growth induced by allogeneic as well as Ag-bearing autologous DCs have been previously reported (25). Several mechanisms through which NK cells inhibit T cell proliferation have also been reported and include the elimination of DCs (22, 23), secretion of the regulatory cytokine IL-10 (25), and direct cytotoxicity against activated T cells (29–31). In our study, in which we used allogeneic DCs as stimulators, we found that proliferation of naive CD4 T cells is inhibited mainly through direct NK cell cytotoxicity. The killing of T cells by activated NK cells is specific to Ag-activated T cell subpopulations, as demonstrated by cytotoxicity assays where we isolated Ag-activated T cells and nonactivated T cells from cocultures of allo-DCs and T cells.

We used different NK/T ratios based on the results of titration experiments (Figs. 1D, 2E), which were 1:10 in proliferation assays and 10:1 in cytotoxicity assays. When comparing these experimental conditions for proliferation and cytotoxicity assays, one must note that the T cells were derived from different populations in each case. In the proliferation assays, we used the whole naive CD4 T cell population stimulated with allo-DCs, of which only a tiny fraction (~1–2%) was specifically activated by alloantigen at day 1. In contrast, in cytotoxic assays, we used T cells that had been sorted by their activation status, that is, CD25+ at day 1 and the PKH26 intensity at day 3. As shown in Fig. 2D and 2E, the cytotoxic activity of NK cells was considerably higher against CD25+ alloantigen-activated T cells compared with CD25− nonactivated T cells. This indicated that only a minor fraction of the whole T cell population at day 1 was a main target of the NK cells. In proliferation assays, considering that the number of added NK cells was calculated on the basis of that of the whole T cell population, the actual E/T ratio in the coculture at the NK/T ratio of 1:10 at day 1 was estimated to be ~5:1 to 10:1. Additionally, T cell lysis by NK cells was still observed at an NK/T ratio of 1:10 (data not shown). Taken together, and even though we could not perform cytotoxicity assays under identical conditions to the proliferation assays, we conclude from our data that the NK cell-mediated inhibition of T cell proliferation is primarily induced by the selective cytotoxicity of NK cells toward Ag-activated T cells.

We observed variations in the alloantigen-activated T cell susceptibility to NK cell-mediated cytotoxicity, which was dependent on the phase of activation and the type of DCs. NK cell-mediated cytotoxicity is regulated by a balance of activating and inhibitory signals from the target cells. NKG2D is one of activating receptors on NK cells and binds to a family of MHC class I-related molecules, including MICA, MICB, ULBP1, ULBP2, ULBP3, retinoic acid early transcript-1E, and retinoic acid early transcript-1G (44). In contrast, NK cells display several kinds of inhibitory receptors specific to HLA class I molecules, including killer cell Ig-like receptors (KIRs), NKG2A, and leukocyte Ig-like receptor B1 (45).

Recent studies have shown that while resting T cells only minimally express MICA, ULBP1, ULBP2, and ULBP3, activated T cells express these molecules at higher levels (30). Additionally, the induction of NKG2D ligand expression on activated T cells confers a susceptibility to NK cell-mediated cytosis (30, 31). In our study, we also confirmed that at day 1 after priming with allo-DCs, CD25+ alloantigen-activated CD4 T cells upregulate these NKG2D ligands and become susceptible to NK cell-mediated cytotoxicity. However, in contrast to the findings of a previous study (30), the addition of anti-NKG2D mAbs did not completely inhibit T cell cytosis, suggesting that other NK cell-activating pathways are involved. Furthermore, a recent study has reported that NKp46 ligands on Ag-expanded regulatory T cells partially contribute to the triggering of NK cell-mediated cytotoxicity (46). We also observed the partial involvement of NKp46 in triggering NK cell-mediated cytotoxicity in our system (data not shown). Thus, NKG2D ligands are important determinants of the triggering of NK cell-mediated cytotoxicity against Ag-activated T cells. However, signaling through other activating receptors also appears to be involved.

At day 1, alloantigen-activated T cells moderately augmented HLA class I molecule expression in tandem with NKG2D ligands, but became susceptible to NK cell-mediated cytotoxicity. Activating receptor signals seemed to have a more significant effect on NK cell activity against these cells. In contrast, nonactivated CD4 T cells show relatively low levels of HLA class I molecule expression at day 1, but are not killed by NK cells. The lack of activating receptor ligands likely underlies why nonactivated cells escape from NK cell-mediated cytotoxicity, regardless of their low level expression of HLA class I molecules.

At day 3, alloantigen-activated T cells show higher NKG2D ligand expression but are more resistant to NK cells compared with day 1. The addition of an anti-HLA class I mAb greatly enhanced NK cell-mediated cytotoxicity against alloantigen-activated T cells, indicating that these cells protect themselves from NK cells by expressing high levels of HLA class I molecules, among which HLA-E seems to be of some importance. At day 1, alloantigen-activated T cells stimulated with either TNF-DCs or LPS-DCs showed a differential susceptibility to NK cells, even though these cells expressed equivalent levels of NKG2D ligands. This suggests that the NKG2D ligand expression levels do not alone determine this susceptibility. In contrast, we found that HLA class I molecules play a significant role in determining the susceptibility of these T cells to NK cells. More specifically, we found that the kinetics of HLA-E expression, which is the sole ligand of NKG2A (34, 35), are particularly important and correlate well with the resistance to NK cell-mediated cytotoxicity. This finding is based on the following observations. First, the addition of an anti-NKG2A mAb increased the cytolysis of Ag-activated T cells at
day 1 to almost the same extent as anti-HLA class I mAb. Second, the rapid upregulation of HLA-E expression induced by LPS-DCs was found to be associated with decreased T cell susceptibility. Third, the increase in T cell cytolyis induced by an anti-NKG2A mAb was higher in LPS-DC-stimulated T cells than in TNF-DC-stimulated T cells. Finally, the IFN-α–mediated upregulation of HLA-E expression protects T cells from NK cell-mediated cytotoxicity. Given that the susceptibility of all-anti-activated T cells to NK cell-mediated cytotoxicity at day 1 led to the extent of proliferation inhibition by NK cells, we conclude that the rapid induction of HLA-E in LPS-DC-stimulated, all-anti-activated T cells plays a critical role in conferring a survival advantage to these cells in the presence of activated NK cells.

The significance of Qa-1, the murine homolog of HLA-E, in the regulation of CD4 T cell responses has been reported earlier (29). Specifically, Lu et al. (29) showed in their study that Qa-1–deficient CD4 T cells are short-lived and fail to undergo Ag-induced or homeostatic expansion in vivo due to their increased susceptibility to NK cells. Although the significance of a rapid induction of HLA-E expression on naïve CD4 T cells was not mentioned, this report supports the in vivo relevance of our study.

Based on the results presented in this study, we propose a mechanism for NK cell-mediated regulation of the T cell response. Our findings that LPS-DCs prime naïve CD4 T cells to rapidly upregulate HLA-E expression, resulting in an increased resistance to NK cells compared with TNF-DCs, implies that in the presence of activated NK cells, DCs that have directly contacted pathogens and can present pathogen-derived Ags can differentiate a broader repertoire of naïve CD4 T cells into effector T cells. In contrast, bystander DCs that have been activated only by inflammatory cytokines and that do not possess pathogen-derived Ags can differentiate only a limited repertoire of T cells. This discriminatory mechanism may be instrumental in preventing the promiscuous activation of naïve CD4 T cells and possible autoimmune responses. Moreover, as previously reported (47), we confirmed from our experiments that IFN-β is produced only by LPS-DCs and not by TNF-DCs. We additionally show that IFN-β secreted by LPS-DCs is an important factor responsible for high-level HLA-E expression during the early phase of activation and the increased resistance to NK cells in LPS-DC–stimulated, all-anti-activated T cells.

NK cells display a variegated expression pattern of inhibitory receptors. In terms of the NK cell–mediated killing of immature DCs, it has been reported that NKG2A*KIR* NK cells are the main effectors (48). Considering the high expression of classical HLA class I molecules on T cells and on DCs (data not shown), KIR-expressing NK cells seem to be unable to kill activated T cells. In the experimental system used in this study, NKG2A* KIR* NK cells were also found to be the likely main effectors in the killing of activated T cells. In lymph nodes, most NK cells are CD56brightCD16+, display an NKG2A*KIR* phenotype, and are thus poorly cytotoxic prior to stimulation (49, 50). These resident lymph node NK cells may be activated by cytokines derived from developing adaptive immune responses, and together with activated NK cells that are newly recruited from peripheral blood to lymph nodes, they may serve as effector cells in the regulatory mechanism presented in our study. This mechanism may contribute to the dampening and limiting of T cell responses to develop responses of optimal strength and duration. Second to allo-SCT, it is well known that peripheral blood NK cells are constituted quickly and have a unique phenotype, with NKG2A*, KIR+, and CD56bright cells predominating (51–53). It is therefore possible that post-allo-SCT NK cells suppress T cell responses and regulate graft-versus-host disease.

Recent reports have shown that the homozygous state for the HLA-E*0103 allele in allo-SCT recipients, which results in a higher cell surface expression of HLA-E, is associated with a lower incidence of transplant-related mortality (54) and an improved overall survival (55). These findings suggest a protective role for HLA-E against endothelial damage by NK cells. These reports also suggest that the regulation of NK cell activity by HLA-E occurs in vivo in humans, raising the possibility that the manipulation of HLA-E expression on T cells will lead to a regulation of the T cell responses.

In conclusion, the results of our study demonstrate that intricate and coordinated interactions among T cells, NK cells, and DCs regulate the adaptive immune responses through the finely controlled kinetics of HLA-E expression on Ag-activated T cells. HLA-E may therefore be a new immunoregulatory target in the contexts of transplantation, autoimmune disease, and antitumor immunity.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure Legends

Supplemental Figure 1. NK cells reside in close proximity to CD4+ T cells in the parafollicular T cell areas of swollen lymph nodes.

(A) A hematoxylin-eosin-stained biopsy of a swollen lymph node (×10). (B, C) The same specimen was immunohistochemically stained using anti-CD4 (brown) and anti-CD56 (red) antibodies. Representative images of parafollicular T cells areas at low (×10) (B) and high (×40) (C) magnification are shown.

Supplemental Figure 2. T cell growth inhibition is not restored by the addition of excess DCs or exogenous IL-2, or by isolation from DCs.

(A) PKH26-labeled, naive CD4 T cells were co-cultured with allo-TNF-DCs or allo-LPS-DCs. Activated NK cells were added the following day at an NK:T ratio of 0:10 or 1:10, together with different concentrations of IL-2 (0, 1, 10, or 100 ng/ml). At day 4 of DC-stimulation, the proliferating T cell numbers were evaluated. The data shown are representative of two independent experiments. (B) PKH26-labeled, naive CD4 T cells were applied at 1 x 10^5 cells/well, which is similar to other proliferation assays, and co-cultured with allo-TNF-DCs at a DC:T ratio of 1:5, 2:5, or 5:5. The
following day, activated NK cells were added to the culture at an NK:DC:T ratio of 1:1:5, 1:2:5, or 1:5:5. At day 4 of DC-stimulation, the numbers of proliferating (open bars) and non-proliferating (filled bars) CD4 T cells were assessed. (C) PKH26-labeled, naive CD4 T cells were co-cultured with allo-TNF-DCs or allo-LPS-DCs for one day. The whole population of CD4 T cells was then isolated from DCs using a cell sorter, and incubated with activated NK cells at an NK:T ratio of 0:10 or 1:10 for three additional days. The numbers of proliferating (open bars) and non-proliferating (filled bars) CD4 T cells were then assessed. The data shown are representative of two independent experiments.

Supplemental Figure 3. Kinetics of CD25 expression and analysis of the dilution of PKH26 signals in allo-DC-stimulated CD4 T cells.

PKH26-labeled, naive CD4+ T cells were co-cultured with allo-TNF-DCs. On days 1, 2, and 3 of DC-stimulation, the cells were harvested and stained with anti-CD4 and anti-CD25 mAbs. The CD4 T cell population was identified by gating on CD4+ PKH26+ cells and the CD25 expression levels and dilution of PKH26 signals were sequentially measured by flow cytometry.
Supplemental Figure 4. Kinetics of MICA/B, HLA class I, and HLA-E expression on non-activated CD4 T cells.

PKH26-labeled, naive CD4 T cells were stimulated with either allo-TNF-DCs or allo-LPS-DCs for three days. To examine the expression kinetics of MICA/B, HLA-E, and HLA class I molecules on non-activated T cells, cells were stained with anti-CD4 mAb, anti-CD25 mAb and antibodies specific to the molecules for 4-color flow cytometric analysis. The non-activated T cell subpopulation was identified by gating on CD25<sup>−</sup> CD4 T cells at day 1 or on PKH<sub>high</sub> CD4 T cells at day 3. The ∆MFI of the indicated molecules on non-activated CD4 T cells at days 1 and 3 as well as on resting CD4 T cells on day 0 was then evaluated. Each unique symbol represents an identical donor. (A) The kinetics of MICA/B expression on non-activated T cells in five independent experiments. (B) The kinetics of HLA class I molecules expression on non-activated T cells in three independent experiments. (C) The kinetics of HLA-E expression on non-activated T cells in five independent experiments.

Supplemental Figure 5. TCR signaling and IFN-β are involved in the upregulation of HLA-E on antigen-activated T cells.

(A) Unlabeled, naive CD4 T cells were stimulated with 100 μg/ml of plate-bound
anti-CD3 mAb plus 5 μg/ml of soluble anti-CD28 mAb (referred to as stimulation with anti-CD3/28 mAbs) for three days. To assess the kinetics of HLA-E expression on activated T cells, the cells were stained with PE-conjugated anti-HLA-E mAb together with PC7-conjugated anti-CD4 mAb and APC-conjugated anti-CD25 mAb in flow cytometric analysis, and the activated T cell population was defined by gating on CD25+ CD4 T cells. The ΔMFI of HLA-E on CD25+, activated CD4 T cells at day 1 and day 3 and on resting naive CD4 T cells on day 0 was evaluated in three independent experiments. (B) Unlabeled, naive CD4 T cells were stimulated with anti-CD3/28 mAbs in the presence or absence of 20ng/ml of IFN-β. HLA-E expression on CD25+, activated CD4 T cells was evaluated at day 1 by flow cytometry using PE-conjugated anti-HLA-E mAbs, as described for Supplemental Figure 5A, in three independent experiments. (C) Unlabeled, naive CD4 T cells were co-cultured with allo-TNF-DCs or allo-LPS-DCs in the presence or absence of 20ng/ml of IFN-β. At day 1, the cells were stained with unconjugated anti-HLA-E mAbs followed by staining with APC-conjugated GAM, PC7-conjugated anti-CD4 mAb and FITC-conjugated anti-CD25 mAb. HLA-E expression on alloantigen-activated CD4 T cells (defined as CD25+ CD4 T cells) was evaluated by flow cytometry in four experiments, two each for TNF-DC-activated T cells and LPS-DC-activated T cells. (D) Monocyte-derived immature DCs were seeded
at a concentration of $1 \times 10^6$ cells/ml and stimulated with 10 ng/ml of TNF-$\alpha$ or 100 ng/ml of LPS for 24 hours. The supernatants were then collected and the concentration of IFN-$\beta$ was measured by ELISA in three independent experiments. Each unique symbol represents an identical donor. (E) Unlabeled, naive CD4 T cells were co-cultured with allo-LPS-DCs in the presence or absence of 20 $\mu$g/ml of anti-IFN-$\alpha$/$\beta$ receptor blocking mAbs. HLA-E expression on CD25$^+$, alloantigen-activated CD4 T cells was evaluated at day 1 by flow cytometry using PE-conjugated anti-HLA-E mAbs, as described in Supplemental Figure 5A, in two independent experiments.
Supplemental Figure 1

A

B

C
Supplemental Figure 2

A

TNF-DC-Stim T cells

LPS-DC-Stim T cells

B

Non-proliferating T cells

Proliferating T cells

C

Non-proliferating T cells

Proliferating T cells

DCT: NK-1:0 1:1 2:1 5:1

DC:NK 1:0 1:1 2:1 5:1

NK-T: 0:10 1:10 6:10 1:10

TNF-DC-Stim

LPS-DC-Stim

Isolated T cells

Isolated T cells
Supplemental Figure 3

Day 1  Day 2  Day 3

PKH26  2.2%  2.4%  28.0%
Supplemental Figure 4

A

CD25+PKH26high T cells

MICA/B (ΔMFI)

Day 0 | TNF LPS | Day 1 | TNF LPS | Day 3

B

CD25+PKH26high T cells

anti-HLA class I (ΔMFI)

Day 0 | TNF LPS | Day 1 | TNF LPS | Day 3

C

CD25+PKH26high T cells

HLA-E (ΔMFI)

Day 0 | TNF LPS | Day 1 | TNF LPS | Day 3
Supplemental Figure 5

A. CD25+ CD3/28-act T cells

B. CD25+ CD3/28-act T cells

C. CD25+ TNF-DC-act T

D. CD25+ LPS-DC-act T cells

E. CD25+ LPS-DC-act T cells