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Potential Role of NKG2D/MHC Class I-Related Chain A Interaction in Intrathymic Maturation of Single-Positive CD8 T Cells

Sophie Hüe,* Renato C. Monteiro,† Sonia Berrih-Aknin,‡ and Sophie Caillat-Zucman2*

The nonclassical MHC class I molecule MHC class I-related chain A (MICA) interacts with the NKG2D receptor expressed at the surface of most peripheral CD8 T cells, γδ T cells, and NK cells. We investigated the role of MICA-NKG2D interactions in the selection or maturation of the T cell repertoire within the thymus using MICA tetramers and anti-MICA mAbs. MICA tetramers identified a small population of late stage CD8 single-positive, CD45RA+/CD62L+CCR7+CD69− thymocytes, a phenotype compatible with that of fully mature CD8+ cells ready to emigrate to the periphery as naive cells. MICA molecules were expressed in the outer layer of Hassal’s corpuscles within the medulla of normal thymus. In thymomas, an overexpression of MICA in cortical and medullar epithelial cells was observed. This was associated with a decreased percentage of NKG2D-positive thymocytes, which expressed a less mature phenotype than in normal thymus. These results indicate that CD8+ thymocytes up-regulate NKG2D as they complete their developmental program before leaving the thymic medulla to seed the periphery, and identify NKG2D as a potential regulator of the developmental processes in T cells that are essential for immune homeostasis. The Journal of Immunology, 2003, 171: 1909–1917.

Lymphocyte development in the thymus is a complex process essential for the formation of the immune system. Different developmental stages of T cells within the thymus can be defined by expression of the co-receptor molecules CD4 and CD8. Immature T cell precursors are found within the CD4−CD8− double-negative compartment. Cells that successfully rearrange their TCR β locus and express a functional pre-TCR proliferate rapidly and differentiate into CD4+CD8− double-positive cells. These cells undergo TCR α-chain gene rearrangements, and upon expression of a functional αβ TCR complex are subjected to the processes of positive and negative selection that generate CD4+ or CD8+ single-positive (SP) thymocytes (1, 2). In addition to these changes in expression of surface markers, thymocytes traverse through the thymus as they develop. Immature thymocytes reside in the cortex, and as they begin the selection process they traffic from the cortex to the medulla.

A key role of thymic epithelial cells in positive selection is to provide HLA/peptide ligands for the αβ TCR. In addition, the thymic medulla is likely to be involved in regulating postselection differentiation events at the level of SP thymocytes (3). Newly generated SP thymocytes reside in the medullar areas for up to 2 wk, during which they undergo changes in expression of a variety of cell surface molecules such as CD24, CD62L, and CD69, before they are exported to the periphery (4, 5). At the same time, positively selected thymocytes undergo an expansion phase that may act to increase numbers of T cells to aid the establishment of the peripheral T cell pool (6). However, the nature of signals required for emigration of newly matured T cells out of the thymus is still elusive.

The NKG2D-activating receptor is expressed at the surface of most circulating αβ CD8 T cells, γδ T cells, and NK cells in humans. NKG2D forms homodimers that pair with the transmembrane adaptor protein DAP10. Like the CD28 molecule, DAP10 contains an SH2-binding YxxM motif in the cytoplasmic domain that, upon tyrosine phosphorylation, recruits the p85 catalytic subunit of the phosphatidylinositol 3 kinase (7). Various families of NKG2D ligands have been identified, including the human MHC class I-related chains (MIC) A and B proteins (8, 9). These are nonclassical MHC class I molecules that have no role in Ag presentation, but function as signals of cellular distress, and trigger a range of effector mechanisms, including cellular cytotoxicity, cytokine secretion, and cellular proliferation (for review, see Refs. 10 and 11). In NK cells, MICA can transduce through NKG2D, an activating signal that can override a negative signal generated by engagement of inhibitory receptors for MHC class I (12–14). In CD8 αβ T cells, NKG2D/MIC engagement delivers a costimulatory signal that complements TCR-mediated Ag recognition on target cells (15). Recent data indicate that two splice variants of NKG2D may associate differently with the adaptor molecules DAP10 and DAP12 (16, 17). NK express both DAP10 and DAP12, and can therefore transmit both direct stimulatory and costimulatory signals. In CD8 T cells, due to the absence of DAP12, NKG2D interacts with DAP10 and thus delivers only a costimulatory signal to T cells. In addition, it has been shown that MIC is recognized by intraepithelial or tumor-infiltrating γδ T cells (18, 19) and is able to deliver both a TCR-dependent signal 1 and a NKG2D-dependent costimulatory signal 2 for a subset of Vδ1 γδ T cells (20).
At variance with the wide constitutive expression of conventional MHC class I molecules, expression of MICA molecules is low in adult tissues, is mostly restricted to the surface of intestinal epithelium (9), but is stress inducible in various epithelial cells. It is also considerably up-regulated in different types of tumors (19, 21) and in the context of intracellular viral (15) or bacterial infection (22, 23). In addition to gut epithelium, MICA expression has been suggested in a population of stellate cells located in the subcapsular thymic cortex (9). This remarkably restricted expression of MICA in two known sites of T lymphopoiesis suggests a possible role of MICA/NKG2D interaction in the selection or maturation of the T cell repertoire.

In this study, we show that MICA expression is observed in epithelial cells and Hassal’s corpuscles of the thymic medulla. Furthermore, NKG2D is expressed on a subpopulation of CD4+CD8+ medullary thymocytes that express a phenotype of CD45RA+CD69+,CD62L+,CCR7+ naive mature T cells. These data suggest that NKG2D may be a signal allowing newly matured CD8 T cells to emigrate to the periphery. Such hypothesis is sustained by observation that in thymoma, an overexpression of MICA molecules on tumor epithelial cells is accompanied with an abnormal maturation phenotype of NKG2D-positive thymocytes.

Materials and Methods

Cells

HT-29 (ATCC HTB-38, colon adenocarcinoma), HeLa (ATCC CCL-2, carcinoma of cervix), CaCo2 (ATCC HTB-37, colorectal adenocarcinoma), and Raji (ATCC CCL-86, lymphoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). M6 (melanoma cell line) was kindly provided by P. Paul (Marseille-Luminy, France). Cell lines were grown in RPMI 1640 supplemented with 10% FCS, glutamine, and antibiotics. Fresh PBMC from healthy adult volunteers were purified from whole blood by Ficoll density-gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ). Human umbilical cord blood cells were isolated, as described above, for whole blood specimens.

Human postnatal thymus specimens were obtained from 13 children during corrective cardiac surgery. The children were 2 days to 4 years old. All the procedures followed in the study were performed in accordance with the Declaration of Helsinki. Thymic samples were stored in saline and processed within 1 h of excision. Mononuclear cells were obtained by gentle teasing of thymus over wire mesh. Thymic cells were washed twice in RPMI before immunofluorescence staining. Recovered viable thymocytes (hereafter referred to as total thymocytes) were depleted in CD4-positive cells by CD4 magnetic bead depletion using the MACS system, according to the manufacturer’s protocol (Miltenyi Biotec, Auburn, CA). The CD4+CD8+ cells (up to 10% of total thymocytes) were >98% pure.

Construction, expression, and purification of soluble MICA and MICB molecules

Recombinant MICA and MICB were produced as secreted proteins in Sf9 insect cells using the pBAC4 Baculovirus expression system (Novagen, Madison, WI). The MICA and MICB expression vectors were constructed by PCR using the cDNA coding for the human MICA*001 and MICB*002 alleles (kindly provided by S. Bahram, Strasbourg, France). The coding region for the signal peptide and extracellular domains of the proteins (residues 1–276) fused to a DNA tag encoding a glycerine-serine linker and the recognition sequence for enzymatic biotinylation were amplified using the following primers: MICA forward, 5’-TCT GGA TCC ACC ATG GGG CTG GGC GCG GTG TCT GGC-3’; MICA reverse, 5’-CAT GAC TGC TCT ACA GAT TCC TCC AAC TCC ACC GGC GCT TTT CCC AGA GGG CAC AGG GTG AGT GAG GAG AG-3’. BambH and XhoI restriction enzyme sites used for cloning are in italic. The antisense primer annealed to codons corresponding to the first 2 aa of the transmembrane domain (bold), a glycine-serine linker, and the biotin protein ligase BirA recognition sequence 45 of Serratia (24) (underlined). The resulting fragments were cut with BamH and XhoI and cloned into pBACIX-11 upstream to a six-His coding sequence and a stop codon. The recombinant transfer vector was cotransfected with linearized mutant AcNPV genomes (Baculogold; BD PharMingen, San Diego, CA) into Sf9 cells using Insect-Plus liposomes (Invitrogen, San Diego, CA), according to the manufacturers’ instructions. Viruses in supernatants from transfected cells were harvested after 4 days and plaque purified using standard techniques. Plaque virus clones were tested for protein expression by protein immunoblotting of supernatants from cultures of infected cells using mouse anti-His(4) mAb (Qiagen, Valencia, CA). Appropriate viruses were expanded in complete TN-MFH medium (BioWhittaker, Walkersville, MD).

Large-scale preparations were carried in serum-free SF 900-II medium (Invitrogen). Supernatants were harvested by centrifugation at day 3 after infection. rMICA was concentrated from 80 to 10 ml in Amicon concentrator stir cell on BioMax membranes (Millipore, Bedford, MA). MICA and MICB molecules were affinity purified by Ni-NTA agarose (Qiagen) or His-Select HC Nickel affinity gel (Sigma-Aldrich, St. Louis, MO) chromatography, respectively, and concentrated to 2 mg/ml using Centricron ultrafilters (Amicon, Beverly, MA). MICB protein was further purified by gel filtration chromatography on a Superdex 200 10/30 column (Amersham Pharmacia Biotech) in 50 mM Tris, 150 mM NaCl, pH 8.0. MICA and MIB proteins were 98% pure, as determined by SDS-PAGE and Coomassie staining.

Generation of fluorescent MICA and MICB tetramers

Biotinylation of soluble recombinant MICA or MICB proteins was done for 16–24 h at room temperature using BirA-Ligase (Avidyn, Denver, CO), according to the manufacturer’s instructions, and was validated by immunoprecipitation with streptavidin-agarose beads (Pierce, Rockford, IL). Efficiency was always >90%. Protein concentration was measured by micro-bicinchoninic acid (Pierce). Tetramers were generated by mixing MICA or MICB monomers with fluorochrome-labeled streptavidin in a 4:1 ratio (streptavidin PE or streptavidin FITC; BD PharMingen) without further separation by gel filtration. For staining, tetramers were used at a concentration of 10 µg/ml of monomers.

Production of anti-MICA mAbs

Mice (BALB/c, female, 7 wk old) were primed by injecting 50 µg of purified rMICA emulsified in CFA (Sigma–Luzinb) in the footpad. Mice were then boosted five times at 3-day interval by footpad injection with 50 µg of rMICA emulsified in IFA or PBS. Eighteen hours after the sixth injection, lymphocytes were isolated from inguinal lymph nodes, and were fused with Ag8.653 myeloma cells (25) using 50% polyethylene glycol (Sigma–Aldrich). Hybridomas were grown in RPMI-10% FCS presence of peritoneal macrophages. Supernatants were screened for specific reactivity with rMICA by ELISA and Western blot, and by flow cytometry on HT-29 cells. A total of 38 of 180 hybridomas was reactive with MICA Ags by ELISA. Three hybridomas (SR99, SR104, SR116) were further selected on the basis of both reactivity against rMICA by ELISA and strong staining of HT-29 cells by flow cytometry. After subcloning, ascite fluid was harvested from mice that were pristane primed and inoculated i.p. with mAb-producing hybridoma clones. The isolated SR99 and SR104 Abs were of the IgG1 subclass, and SR116 mAb was of IgG2a subclass.

Immunocytometry analysis

Freshly isolated cells (1–2 × 107) were stained with combinations of PE-labeled MICA or MICB tetramers and directly conjugated mAbs. Cells were first incubated at 22°C for 90 min in the dark with MICA or MICB tetramers at concentration equivalent to 10 µg/ml of MICA protein. The following Abs were added directly for 20 min at 4°C: CD4 FITC, CD16 FITC, CD8 FITC, CD3 FITC, CD69 APC, CD45RA Tricolor, CD45RO FITC, CD19 Tricolor, and CD62L FITC were from Caltag (Burlingame, CA). Cells were fixed in PBS with 1% paraformaldehyde, and immediately analyzed on a FACSArray flow cytometer.

For cell surface staining of MICA molecules, cells were incubated first with 1 µg/ml of aggregated IgG (10 mg/ml) for 15 min at 4°C in the dark. Then incubated with 1 µg/ml of mAb against CD3, CD4, CD8, FcyR, and then with 10 µg/ml SR104 mAb, or with Ig isotype-matched control Ab, at 4°C for 30 min, washed, and stained with FITC–labeled goat anti-mouse IgG. Cells were then analyzed on a BD Biosciences FACS-Calibur flow cytometer (Mountain View, CA), and data were obtained using CellQuest software. The fine specificity of the mAbs was determined by blocking the staining of Hela cells by preincubation with a 10-fold excess of recombinant soluble MICA or MICB protein for 1 h at 4°C.
Thymocyte cultures

CD8⁺-purified thymocytes were cultured for 1–5 days in 24-well plates (Falcon; BD Biosciences) at 1 × 10⁶ cells/well in 1 ml RPMI 1640 supplemented with 10% human pooled AB serum and the following cytokines, either alone or in combination: IFN-γ (100 U), IL-2 (10 U/ml), IL-7 (10 ng/ml), IL-15 (50 ng/ml) (Sigma-Aldrich). Thymocytes were then harvested and assayed for cell surface markers by flow cytometry, as described above.

Quantitative PCR of TCRα rearrangement excision circles (TREC)

CD8-purified thymocytes were stained with PE-labeled MICA tetramers, and positively selected by anti-PE microbeads (Miltenyi Biotec). The number of TRECs was determined by quantitative real-time PCR using the 5'-nuclease (TaqMan) assay in an ABI7700 system (Applied Biosystems, Foster City, CA), as previously described (26). In brief, the sorted NKGD2-positive and NKGD2-negative CD8 thymocytes and total PBLs were lysed in 100 μg/ml proteinase K for 3 h at 56°C and then 20 min at 95°C. Each 25-μl reaction contained 5 μl cell lysate, and the final concentration of each component was as follows: 1× reaction buffer, 20 U/ml platinum Taq polymerase (Life Technologies), 3.5 mM MgCl₂, 0.2 mM dNTPs, 500 nM each primer, and 150 nM probe. PCR conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. A standard curve was plotted, and TREC values for samples were calculated using ABI7700 software. Samples were analyzed in duplicate.

Immunohistochemical analysis

Normal human thymuses were obtained as discard tissues from healthy children during corrective cardiac surgery (see above). In addition, thymus specimens were obtained from patients with thymoma who underwent curative thymectomy. Thymus samples were immersed in OCT compound (TissueTek; Bayer, Pittsburgh, PA) and snap frozen in liquid nitrogen. Cryosections were cut 5 μm thin onto slides. Sections were air dried for 1 h, fixed in ice-cold methanol for 1 h, and stored at −20°C. Double immunostaining was then performed using FITC-labeled MICA tetramers (100 μl at 50 μg/ml overnight at 4°C), followed by anti-CD8 mAb, 30 min at 22°C (DAKO, Carpentry, CA), plus rhodamine-labeled rabbit anti-rat IgG. In parallel, staining was performed with the anti-MICA SR99 mAb plus FITC-labeled goat anti-mouse IgG, and rhodamine-labeled human anti-pan-cytokeratin Ab. Isotype-matched Ig was used as control.

Alternatively, normal or pathologic thymic tissues were fixed in 10% Formalin for 4 h, washed twice with PBS, and immersed in 70% ethanol. Serial sections of the Formalin-fixed paraffin-embedded thymic tissue blocks were prepared. After deparaffinization and rehydration, sections were treated in citrate buffer for 5 min in a microwave, then treated with normal human serum for 20 min at room temperature, and incubated with a titrated solution of anti-MICA mAb for 1 h, or isotype-matched control Ig. Ab binding was visualized by using biotinylated goat anti-mouse Ig (DAKO). The peroxidase EnVision System (DAKO) was used to complete staining, followed by counterstain with hematoxylin.

Detection of soluble MICA by ELISA

To detect soluble MICA in the serum, two different anti-MIC mAbs were used in a sandwich ELISA. High-binding polystyrene plates (Greiner; Sigma-Aldrich) were coated with the capture SR99 Ab (5 μg/ml in PBS, 100 μl per well) for 12 h at 4°C, washed five times with PBS plus 0.05% Tween 20, blocked by addition of 100 μl of 5% BSA for 1 h at 22°C, and washed in PBS-0.05% Tween 20. The standard (serial dilutions of soluble MICA from 100 μg/ml to 0.1 ng/ml in PBS-0.05% Tween 20) and the serum samples (100 μl per well) were then added for 2 h at room temperature. After five washes, the biotinylated SR104 detection Ab (150 ng/ml in PBS, 100 μl per well) was added for 1 h at room temperature. Plates were washed and incubated for 20 min at room temperature with streptavidin-conjugated HRP (1/30,000; Amersham Pharmacia Biotech), washed, and reacted with tetramethylbenzidine substrate (Sigma-Aldrich) for 15 min at 37°C. Reaction was stopped with 0.5 M H₂SO₄ (50 μl per well). The absorbance was measured at 450 nm. Sera from 20 healthy individuals and 48 patients with thymoma were analyzed.

Results

Binding characteristics of MICA and MICB tetramers on PBLs

To study the interaction of MICA and MICB molecules with their receptor NKGD2, we generated fluorescently labeled tetrameric complexes of the extracellular domain of MICA and MICB. Soluble forms of the MICA and MICB protein were produced in insect cells and purified. MIC proteins migrated on SDS-PAGE in position consistent with the expected molecular size for the glycosylated form of the soluble protein (43 kDa).

To verify the binding specificity of MICA tetramers onto NKGD2-expressing cells, we used four-color flow cytometric analysis on human peripheral blood cells from healthy individuals. MICA tetramers stained most, if not all, freshly isolated αβ CD8⁺ T cells and γδ T cells (both Vγ2Vδ2 and Vγ1; data not shown), and the vast majority of CD16 NK cells (Fig. 1a). Staining of CD4 T lymphocytes and CD19 B lymphocytes was consistently negative. Therefore, the binding patterns of MICA tetramers fit the known expression of NKGD2 using specific mAbs (13, 14), and indicate that MICA tetramers can be used to identify NKGD2-positive cells. MICA tetramer binding was optimal following 90 min incubation of cells at room temperature. No staining was observed with biotinylated MICA monomers, even when prolonged incubation was performed. Therefore, although NKGD2/MICA interaction has been reported to be strong (dissociation constant $K_D$ of 0.3 μM; i.e., two orders of magnitude stronger than TCR/MHC class I interaction (27)), multimerization of the ligand appears to be required in vitro to stabilize interaction with its receptor.

![FIGURE 1. MICA tetramer binding to PBLs using four-color flow cytometric analysis. a. Freshly isolated PBLs were stained with streptavidin PE-complexed MICA tetramers and with Abs to CD4, CD8, CD16, γδ TCR, and CD19. Numbers in upper right quadrants of each dot plot indicate percentages of gated cells. Results are representative of five different experiments. b. Blocking of MICA (left panel) or MICB (right panel) tetramer staining with (open) or without (shaded) a 10-fold excess of unlabeled soluble MICA protein. c. Coincubation with PE-labeled MICA tetramers and APC-labeled MICB tetramers showed an equilibrated binding to CD8 cells.](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
Similar results were observed when staining peripheral blood cells with MICB instead of MICA tetramers. MICA or MICB tetramer binding was completely inhibited by previous incubation of cells with a 10-fold excess of soluble MICA protein (Fig. 1b). In addition, when cells were coincubated with PE-labeled MICA tetramers and APC-labeled MICB tetramers, equilibrated binding was observed, suggesting that MICA and MICB bind to the same (or overlapping) epitopes on their NKG2D receptor (Fig. 1c). Altogether, these results demonstrate that soluble MICA and MICB bind to NKG2D in a specific and apparently high-affinity interaction, and this binding of MIC molecules to NKG2D does not require additional components for stability. This is in agreement with crystallographic data showing that the MICA groove is not occupied by anything other than water (27).

Murine and human NKG2D ectodomains are 69% identical. Therefore, we tested whether MICA or MICB tetramers were able to bind NKG2D on murine splenocytes from 6- to 8-wk-old BALB/c mice. MIC tetramers did not stain CD8 T cells, while a significant staining was observed following 3-day incubation of splenocytes with 100 U/ml rIL-2 (data not shown). These results confirm that NKG2D is not expressed on resting, but only activated T lymphocytes in mice (28), and also indicate that MIC molecules are able to bind to the murine NKG2D receptor, suggesting that NKG2D may use different epitopes to recognize its various human or mouse ligands.

Expression of NKG2D, the receptor of MICA and MICB, on human thymocytes

NKG2D is expressed at the surface of all circulating CD8 T cells in humans. To determine whether and when developing thymocytes acquire NKG2D, we used flow cytometric analysis on thymocytes purified from 13 normal thymuses from 2-day- to 4-year-old children. Altogether, the mean percentage of total thymocytes that were specifically stained with the MICA tetramers was 1.44%.
ranging from 0.85 to 2.65%. Phenotypical analysis of NKG2D expression was conducted on different thymocyte stages according to expression of the CD4 and CD8 markers. Representative results are depicted in Fig. 2a. Following the intrathymic lineage relationships, we did not detect NKG2D+ thymocytes in CD4−CD8− double-negative cells, or CD4+CD8+ double-positive thymocyte subpopulation. Interestingly, MICA tetramer staining was clearly restricted to a small subset of thymocytes that were almost entirely CD3 bright, CD4+, and CD8+. When quantified within this subset of SP CD8 thymocytes, MICA tetramer-positive cells represented a mean of 11% of these cells. This percentage slightly varied according to the thymus analyzed (range 4.2–14.8%, median 11.9%), but did not appear to be developmentally regulated, because no correlation was observed with the age thymus was removed (between day 2 to year 4 after birth). Results were strictly similar when using MICB instead of MICA tetramers. Among cells committed to the CD8+ lineage, NKG2D expression was not detected in CD4−CD8− cells or in CD4lowCD8+ cells, but was only observed in CD4+CD8+ thymocytes. Thus, it appears that NKG2D expression is not stochastic, but is acquired as a postpositive selection event.

CD4−CD8+ thymocytes comprise developmentally heterogeneous subsets of cells. Some of them have not yet reached the stage at which functional competence is acquired and need to undergo postpositive selection differentiation before their migration to the periphery (5). We investigated whether the NKG2D-expressing CD8+ thymocytes were cells ready to be released from the thymus after finishing their maturation. CD4-depleted thymocytes were analyzed for NKG2D expression together with expression of different thymocyte maturation markers. The results (Fig. 2b) showed that NKG2D+ cells were all CD8+ (data not shown), CD45RA−, CD45RO−, and CCR7−, and predominantly CD62Llow, CD69low, a phenotype compatible with that of fully mature CD8+ cells ready to leave the thymus as naive cells. Altogether, 70% of NKG2D-positive CD8 thymocytes were CD62L+ and CD69+. The same results were obtained when similar staining assays were done with total thymocytes. To confirm that this subset of NKG2D-positive CD8 SP thymocytes represents a late stage of thymopoiesis and not a subset of peripheral memory T cells that recirculate to thymic medulla, we quantified TREC in NKG2D-negative and NKG2D-positive thymocytes compared with PBLs. TREC represent episomal DNA by-products of the TCR rearrangement process that occurs in ~70% of thymocytes destined to become mature αβ TCR T cells. TREC are stable, do not replicate during mitosis, and are therefore diluted out during cell divisions. High levels of TREC were detected in the same proportion in NKG2D-positive and NKG2D-negative CD8 thymocytes (~5,000/10,000 cells, i.e., the same order of magnitude as previously reported in CD8 SP thymocytes (29)). This TREC content was 10 times higher than that of peripheral blood T cells (~500/10,000 cells), confirming that the CD8−NKG2D+ subset of thymocytes does represent a late stage of thymopoiesis and not a subset of mature peripheral T cells that recirculate to the thymic medulla.

Because human cord blood T cells are composed almost exclusively of naïve T cells and are representative of recent thymic emigrants (30), we also analyzed NKG2D expression on cord blood cells. Flow cytometric analysis confirmed that >95% of CD8+ T cells in unstimulated cord blood carried the naïve CD45RA+CD62L+ phenotype, and showed that 90% of CD8 T cells (as well as 90% of NK and 67% of γδ TCR cells) were stained with MICA tetramer (Fig. 2c).

Altogether, these results indicate that acquisition of NKG2D characterizes a percentage of mature CD8 SP thymocytes ready to emigrate to the periphery.

Expression of MICA and MICB in the human thymus

To establish whether the acquisition of the NKG2D molecules on late stage thymocytes is related to the presence of their ligand in the thymus, we analyzed intrathymic MICA expression in the thymus specimens. To this aim, anti-MICA mAbs were produced by immunizing mice with soluble rMICA. Three mAbs (SR99, SR104, and SR116) were selected for their ability to react with MICA molecules on the surface of HT-29, HeLa, and CaCo2 epithelial cell lines by flow cytometry and with denatured rMICA molecules in ELISA. SR104 recognized MICA molecules only, while SR99 and SR116 mAbs recognized both MICA and MICB (data not shown). MIC molecules are highly polymorphic, with >60 alleles described to date (31–33). Therefore, there is a possibility that mAbs could be raised against allele-specific determinants, because the MICA*001 variant was used for immunizing mice. However, because of their reactivity with several lines expressing different alleles, such as HT-29 (MICA*004/016), HeLa (MICA*008), and CaCo2 (MICA*033) (34), our mAbs were likely to recognize a nonpolymorphic determinant on the MICA protein. In peripheral tissues, MICA molecules have been observed at the surface of gut epithelium (9). Indeed, as shown in Fig. 4a, the SR99 mAb significantly stained epithelial cells in normal gut sections.

To decipher the potential role of MICA in the acquisition of NKG2D maturation on SP CD8+ thymocytes, we examined the localization of MICA molecules on paraffin-embedded or frozen thymus sections (Fig. 4, b and c). MICA proteins were exclusively detected in the thymic medulla, and were located in cells forming the outer walls of Hassal’s corpuscles and in cells morphologically resembling mesenchymal cells (35). A very faint staining was observed in epithelial cells of the medulla, but not in cortical epithelial cells, endothelial cells, dendritic cells, or thymocytes. The epithelial nature of MICA-expressing cells in the thymus was confirmed by double immunostaining for MICA and cytokeratin (data not shown). These results indicate that MICA molecules in the thymic medulla are located within close proximity to mature CD8+ SP thymocytes.

Acquisition of NKG2D on human thymocytes

We wanted to determine whether cytokines affect the acquisition of NKG2D on late stage CD8 thymocytes. Total or purified SP CD8 thymocytes were cultured for 1–5 days in presence of IFN-γ, IL-2, IL-15, or IL-7 either alone or in conjunction, and were analyzed for expression of NKG2D by flow cytometry. Surprisingly, in absence of any cytokine, NKG2D-positive cells progressively disappeared within 5 days (17% of CD8 SP thymocytes at day 0,
3.1% at day 4, 1.3% at day 5). By contrast, in presence of IL-2 or IL-15, the percentage of NKG2D-positive cells was maintained or even increased throughout the culture (27 and 31%, respectively, at day 4; Fig. 5). Furthermore, the level of NKG2D on positive cells was 2-fold increased, as determined by mean fluorescence intensity derived from histograms. IL-7 alone was not able to maintain expression of NKG2D at normal levels (9.8% of CD8 SP cells at day 5), and its presence partially antagonized the effect of IL-2 or IL-15. None of the tested cytokines was able to induce NKG2D expression in culture among NKG2D-negative CD8 thymocytes.

Abnormal MICA expression in thymic epithelial tumors

Because MICA molecules are overexpressed in epithelial tumors of various origins (19, 21), it was of interest to determine whether MICA expression was altered in thymoma, a thymic epithelial tumor. Immunohistochemical analysis using anti-MICA mAb staining on cryostat sections from different thymoma specimens revealed a strong overexpression of MICA proteins in epithelial cells (Fig. 6a). At variance with what was observed in normal thymus, MICA was observed not only in epithelial cells from the medulla, but also in those from the cortex. In addition, we found that MICA tetramers stained a much smaller number of thymocytes within the tumor than in the adjacent normal thymus.

To determine whether overexpression of MICA was accompanied by modifications of NKG2D expression, thymocytes were purified from a freshly removed thymoma specimen. Flow cytometry analysis on thymocytes derived from the tumor part of the thymus and from the healthy tissue adjacent to the tumor showed striking differences in both percentage and phenotype of NKG2D+ CD8 SP thymocytes. Indeed, the proportion of intratumoral CD8 SP thymocytes stained by MICA tetramers was one-half the proportion observed among thymocytes isolated from the normal tissue outside the thymoma (18.4% vs 31%), confirming our observation in immunohistochemical analysis. More importantly, the phenotype of these intratumoral NKG2D-positive CD8 thymocytes was mostly CD45RO positive (43.7% compared with 22.5% in their normal counterpart), CD45RA negative (30.3% compared with 52%), and CD69 positive (69.1% compared with 49.7%) (Fig. 6b). This profile of a less mature phenotype may suggest that the maturation of CD8 SP thymocytes in presence of an excess of MICA protein is partially blocked in the tumor.

Classical and nonclassical HLA class I molecules exist in soluble forms in serum and other body fluids, and have been shown to bind to the same physiologic ligands as the membrane-anchored ones (36–38), thus functioning as modulators of the immune response. We therefore tested the presence of soluble MICA in the serum of patients with thymoma using a highly sensitive sandwich

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** In situ detection of NKG2D on cryostat sections of human normal thymus. a, Double staining with FITC-labeled MICA tetramers and rhodamine-labeled anti-keratin Ab localizes NKG2D-positive cells to the medullar areas. b, NKG2D-positive cells overlap with a small proportion of CD8-positive cells. Serial sections were incubated overnight at 4°C with FITC-labeled MICA tetramers, and 30 min at 22°C with anti-CD8 revealed with rhodamine-labeled rabbit anti-rat IgG. Original magnification, ×200.

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Immunohistochemical detection of MICA by staining with SR99 anti-MIC Ab in normal intestine (a) and normal thymus (b, c). Sections from a and b were from paraffin-embedded specimens; sections from c were from frozen thymus. Original magnification, ×160.
ELISA. Soluble rMICA was used as a standard, and was consistently detected at concentration of 0.1 ng/ml. Sera from 20 healthy individuals were all negative (below the positive detection threshold of 0.1 ng/ml). Among sera from 48 patients with thymoma, 4 sera only were slightly positive, with levels of soluble MICA ranging between 0.15 and 1 ng/ml.

**Discussion**

Thymic emigration of newly generated T cells is an essential process for seeding circulating T cells. CD8\(^{+}\) SP thymocytes comprise developmentally heterogeneous subsets of cells, some of which have not yet reached the stage at which functional competence is acquired. These cells reside in thymic medullar areas, where they must undergo postpositive selection differentiation before their migration to the periphery (4, 5). During this maturation stage, they show a down-regulation of CD69 and an up-regulation of CD62L on their way to becoming recent thymic emigrants. Emigration is controlled by mechanisms within the thymic medulla. However, identification of the accessory molecules that are expressed by thymic epithelial cells and are involved in this late phase of positive selection remains a key area of thymus biology. Recent experiments have outlined the role of a CCL-19- and CCR7-dependent pathway in emigration of T cells from the thymus in newborn mice (39).

We have found that acquisition of the C-lectin-like receptor NKG2D in thymus occurs after the Ag-dependent positive selection in CD4\(^{+}\)CD8\(^{+}\) cells, and characterizes a percentage of mature CD8\(^{+}\) CD69\(^{\text{low}}\) CD62L\(^{\text{high}}\) thymocytes ready to emigrate to the periphery, where NKG2D is expressed on the totality of CD8 T cells. The NKG2D-positive CD8\(^{+}\) SP thymocytes therefore resemble recent thymic emigrants, as confirmed by quantification of TREC levels.

Another important finding emerging from our study is the identification of cells that, because of their ability to express MICA and MICB, the ligands of NKG2D, might represent a potential signaling source for NKG2D-positive thymocytes. Interestingly, MIC molecules are exclusively detected in Hassal’s corpuscles and in a few epithelial cells from the thymic medulla. Hassal’s corpuscles are considered to be a part of the reticuloendothelial network constructing the thymic microenvironment that provides developing thymocytes with paracrine and juxtacrine signals to ensure their proper functional maturation during intrathymic lymphopoiesis. Reticuloendothelial cells usually undergo hypertrophy before their inclusion in the outer walls of the corpuscles. During development, Hassal’s corpuscles become infiltrated by granulocytes, thymocytes, and macrophages. The use of scanning electron microscopy has evidenced long cytoplasmic processes originating from reticuloendothelial cells and directly contacting thymic T lymphocytes (40). Thus, it appears that the Hassal’s corpuscles participate in the physiological activities of thymus and in thymic T cell development. The presence of MIC in the outer walls of Hassal’s corpuscles may suggest that a contact between these molecules and their
receptor NK2G2D is of functional importance for the final maturation and export of SP CD8 thymocytes. In addition, it has been postulated that Hassal’s corpuscles play an important role in removal and disintegration of dying cells within the thymus. In this context, MIC-expressing Hassal’s corpuscles might also reflect the high turnover of medullar thymic epithelial cells because of signaling provided by the interaction with NK2G2D-positive thymocytes. Other thymus-expressed receptor/ligand pairs are implicated in the control of thymocyte migration. The CCR9/CCL25 interaction is involved in the intrathymic trafficking of both αβ TCR⁺ and γδ TCR⁺ T cells (41), while CCR7-CCL19 interaction plays a role in the emigration of newly matured T lymphocytes (39).

In addition to MIC proteins, several other NK2G2D ligands have been identified. These include in the human the UL16-binding proteins (ULBP) (12, 42), and in the mouse the retinoic acid-inducible early genes protein family and the H-60 minor histocompatibility Ag (28, 43). Rodents lack any recognizable homologues of the MIC proteins, but murine NK2G2D ligands RAEB and H60, like ULBP’s, are homologous to the platform domains of the MIC class I proteins, and are anchored in the membrane by GPI linkages. Although it is unclear which NK2G2D ligands are more physiologically important, it is likely that different members of the ULBP and MIC families have distinct biological roles (44). ULBP mRNAs have been detected in thymus (12), but protein expression has not been analyzed. Crystallographic analyses of NK2G2D have revealed a surprising degree of ligand plasticity, as it can accommodate the platform domains of the distantly related MIC and ULBP class Ib molecules. In addition, ULBP1 and 2 are capable of binding to mouse NK cells (42), but conversely, human NK2G2D does not bind to retinoic acid-inducible early genes (45). It has been predicted that murine NK2G2D will bind MICB (46), and indeed we could observe a binding of MIC tetracmers to murine IL-2-activated splenocytes.

How CD8 SP thymocytes acquire NK2G2D remains unclear. CD8⁺ TCR αβ⁺ jejunal intraepithelial lymphocytes (IEL) express NK2G2D, but at levels consistently lower than those found on peripheral blood T cells (47). Because MICA is mostly expressed in gut epithelium, many IELs are continuously exposed to MICA and thus, conceptually, are in state of alert. Therefore, it is not surprising that IELs express low levels of NK2G2D, which is probably down-modulated to prevent chronic T cell stimulation and to limit autoreactive bystander T cell activation (48). Expression of NK2G2D on IELs is, however, restored to levels similar to those in PBMCs by exposure to IL-15, a cytokine secreted by intestinal epithelial cells and induced upon inflammation and viral infections. IL-15 exerts stimulatory effects similar to those of IL-2 and shares the same receptor. In NK cells, stimulation by IL-15 has also been shown to up-regulate NK2G2D expression (42, 44). Indeed, we observed that both IL-15 and IL-2 not only allowed NK2G2D-positive thymocytes to survive in vitro, but also increased the levels of NK2G2D molecules on CD8 SP thymocytes.

Human thymoma is a thymic epithelial cell tumor that often contains a large number of lymphocytes. Although thymomas have the capacity to generate mature T cells, intratumoral T cell maturation is abnormal in that the mature CD45RA⁺ T cell subset is decreased compared with normal thymus (49). Furthermore, mature intratumoral T cells often have an increased autoantigen-specific potential toward fragments of the α subunit of acetylcholine receptor, compared with T cells from normal thymus (50). This explains why thymoma is occasionally associated with autoimmune myasthenia gravis. It has been proposed that thymomas may generate autoantigen-specific T cells by a process of abnormal positive or negative T cell selection. Such T cells would leave the thymoma and contribute to the peripheral autoimmune cascade that leads to myasthenic symptoms of muscle weakness, following interaction between thymoma-derived autoantigen-specific T cells and autoantibody-producing B cells that reside entirely outside the tumor (51, 52). Because various epithelial tumors are known to considerably up-regulate MICA, it was particularly informative to have access to thymomas to analyze MICA expression and the consequences on NK2G2D acquisition. We observed a striking overexpression of MICA in thymic epithelial cells in thymoma. Surprisingly, this overexpression was accompanied by a decreased proportion of NK2G2D-positive cells among SP CD8 thymocytes compared with those in the healthy thymus. This observation mirrors that in normal gut, where strong expression of MICA on gut epithelium is associated with down-modulation of NK2G2D on IELs, and may suggest a defect in the maturation events associated with the tumoral process. Indeed, intratumoral NK2G2D-positive CD8⁺ thymocytes did not express a fully mature phenotype, as shown by the persistence of CD69 and the presence of CD45RO. An increased expression of MICA on epithelial cells might be accompanied by the release of soluble MICA shed from tumor cell death. At the time the present work was completed, Groh et al. (53) showed that soluble MIC is released by shedding from MIC-positive tumors, and causes down-regulation of NK2G2D at the surface of tumor-specific effector T cells. Sahil et al. (54) simultaneously reported the presence of soluble MIC in the serum of patients with gastrointestinal tumors. Although we observed the presence of very low levels of soluble MIC in only a minority of our patients, it is conceivable that soluble MIC is locally released from thymoma in the vicinity of thymocytes, leading to a down-regulation of NK2G2D and a blockade of CD8 thymocytes in an immature phenotype. Such blockade might prevent T cell export of mature CD8 SP thymocytes. Together with events triggering deregulation of cytokine networks, MICA might therefore represent a pathogenicity factor, which could exacerbate autoimmune conditions. Our results are in the same line of thinking as recent data that suggest that the maturation of the CD8⁺ lineage might be affected in a subset of thymomas, leading to some thymopoietic incompetence (55). Alternatively, we cannot exclude the possibility that part of these NK2G2D-positive cells represent effector memory T cells infiltrating the tumoral tissue.

In summary, we have identified the NK2G2D⁺ CD8 SP thymocyte population as a thymus-dependent late stage in CD8 T cell development. Mature CD8 SP T cells are able to seed the periphery once they have acquired the NK2G2D⁺ phenotype. Inability to up-regulate NK2G2D expression on thymocytes might cause them to remain in the thymus, as suggested by results in thymoma. Our data bring new lights in the nature of the thymic cross talk between the thymic medulla and SP thymocytes.

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References