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Intracellular Expression of MICA in Activated CD4 T Lymphocytes and Protection from NK Cell-Mediated MICA-Dependent Cytotoxicity

Luciana Lorena Molinero[1], Carolina Inés Domaica, Mercedes Beatriz Fuertes, María Victoria Girart, Lucas Ezequiel Rossi, and Norberto Walter Zwirner

ABSTRACT: MICA is a stress-regulated molecule recognized by the NK cell-activating receptor NKG2D. Previously, we demonstrated that MICA is induced on activated T cells but regulation by mitogenic cytokines and its biological consequences remain unexplored. Here, we show that IL-2, IL-4, and IL-15 but not TNF-α or IFN-α induced MICA expression in T lymphocytes present in peripheral blood mononuclear cells (PBMCs), as assessed by Western blot. IL-2 effect involved Jak3/STAT5, p38 MAPK, $p70^{56}$ kinase, Lck/fyn kinases, and NF-κB. MICA expression was also observed in Th1 and Th2 cells. However, surface expression was not detected. T lymphocytes present in PBMCs and isolated $CD4^+$ T lymphocytes stimulated with phorbol-12-myristate-13-acetate and ionomycin also induced MICA expression as assessed by Western blot, but only low levels were expressed at the cell surface. Activated but not resting $CD4^+$ T lymphocytes were lysed by IL-15- or IL-2-stimulated NK cells, and susceptibility was increased when HLA class I molecules were blocked. Also, cytokine-stimulated NK cells produced more IFN-γ after culture with activated $CD4^+$ T lymphocytes. However, the participation of MICA in these responses, if any, was marginal. Confocal microscopy revealed that MICA is retained mostly inside activated $CD4^+$ T cells. Our results suggest that low surface expression of MICA on activated $CD4^+$ T lymphocytes might be a safeguard mechanism to protect them from NK cells in an inflammatory, virus-infected, or tumor microenvironment, where NK and activated $CD4^+$ T cells are recruited. *Human Immunology 67, 170–182 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: MICA; MHC; T lymphocytes; NK cells

ABBREVIATIONS

MICA	MHC class I chain–related gene A
Sz	sulfasalazine
PBMCs	peripheral blood mononuclear cells
TCR	T-cell receptor
HLA	human leukocyte antigen
PE	phycoerithrin
FCS	fetal calf serum
PBS	phosphate-buffered saline
BSA	bovine serum albumin

INTRODUCTION

MICA is a -65-kDa polymorphic non-antigen-presenting molecule that is not associated with β_2-microglobulin and is encoded by a gene that maps to the HLA region [1–3]. Expression of MICA has been shown to be codominant [4] and stress regulated [2, 5–7]. MICA is expressed at the cell surface in different tumors [2, 3, 5, 8–12], in gastrointestinal epithelium [2], in endothelial cells, and in fibroblasts [3, 13]. It has been demonstrated that surface expression of MICA signals for recognition by the lectin-like NKG2D molecule that is expressed by

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γδ T lymphocytes, peripheral blood $CD8^+$ αβ T lymphocytes, and NK cells [14, 15]. This receptor recognizes not only MICA but also other specific ligands (NKG2DLs), some of which are surface molecules anchored to the cell membrane by glycophosphatidyl–inositol residues [16–19]. Upon engagement of NKG2D, these cells trigger a cytotoxic response to MICA-expressing cells and IFN-γ secretion, which has been suggested to contribute to the elimination of stressed/neotransformed cells [5–12].

We have demonstrated that MICA expression is induced on $CD4^+$ and $CD8^+$ T cells activated by allogeneic peripheral blood mononuclear cells (PBMCs) [20] in a process that involves the activation of multiple and simultaneously operating intracellular pathways triggered by microclustering of the TCR/CD3 complex and costimulation through CD28. These intracellular routes that connect T-cell activation with MICA gene expression include activation of Lck and Fyn kinases and signaling through MEK1/ERK, p38 MAPK, calcineurin, Jak/STATs, and $p70^{S6}$ kinase [21]. Full T-cell activation requires the activation of NF-AT, AP-1, and NF-κB among other transcription factors [22–24]. We recently demonstrated that MICA expression in activated T lymphocytes is regulated by NF-κB through binding of p65(RelA)/p50 heterodimers and p50/p50 homodimers to a putative κB binding site located in intron 1 of the MICA gene [25]. However, regulation of MICA expression in T lymphocytes by mitogenic cytokines and the biological consequences of the upregulated expression of MICA on activated T cells still remain unexplored.

Evidence that NK cells can interact with activated $CD4^+$ T cells has recently been provided [26], but the consequences of this interaction on NK cell immunobiology are still unknown. Our previous findings [20, 21, 25] suggested that the MICA–NKG2D system might be involved in this crosstalk. In addition, expression of MICA on the cell surface of activated $CD8^+$ T lymphocytes has been observed when these cells were cultured with anti-CD3 or anti-CD3 plus anti-NKG2D mAbs and IL-2 or IL-7 plus IL-15 for 7 days [27]. However, the functional consequences of this expression are not known.

Considering that we demonstrated that MICA is expressed by activated T cells and characterized some intracellular routes involved in this process, the aims of this work were to further investigate regulation of MICA expression by T-cell mitogenic cytokines, to address the functional consequences of MICA expression, and to further explore the role of this HLA-related antigen in the immunobiology of the T lymphocytes.

MATERIALS AND METHODS

Reagents and Antibodies

SB202190 and rapamycin were purchased from Calbiochem (La Jolla, CA, USA), AG490 (an inhibitor of Jak3) and PP1 (an *Src* kinase family inhibitor) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Sulfasalazine (Sz) was purchased from Sigma (St. Louis, MO, USA). These drugs were dissolved in dimethyl sulfoxide, Cyclosporin A (CsA) was kindly provided by Novartis Argentina (Buenos Aires, Argentina). Phytohemagglutinin (PHA-L), phorbol-12-myristate-13-acetate (PMA), and ionomycin were from Sigma. Interleukin (IL)-2 was from R&D Systems (Minneapolis, MN, USA), IL-4 and IL-12 were from BD (San Jose, CA, USA), and IL-15 was from PeproTech (Rocky Hill, NJ, USA). Anti-STAT5 (anti-STAT5) mAb, antiphosphorylated STAT5 polyclonal (anti-pSTAT5) mAb, anti-GATA3 polyclonal (anti-GATA3) Ab, and anti-T-bet (anti-T-bet) mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-IL-4 and anti-IL-12 mAbs were from BD. Anti-NKG2D mAb (clone 1D11) was from Santa Cruz Biotechnology. Anti-ULBPs1-3 mAbs were from R&D Systems. Polyclonal rabbit antibodies against MICA were obtained as described previously [3] and their reactivity was validated elsewhere [13, 20, 21, 28]. Anti-MICA/B mAb D7 was produced as previously described [25]. An isotype-matched mAb [29] was used as negative control (IC). Fluorochrome (FITC, PE, or SPRD)-labeled anti-CD56, anti-CD16, anti-CD4, or anti-CD3 mAbs were from Southern Biotech (Birmingham, AL, USA). FITC-labeled anti-CD25 mAb was from BD, and PE-labeled anti-IFN-γ mAb was from IQ Products (Gröningen, Netherlands). The pan-reactive anti-HLA class I mAb W6/32 was purified in our laboratory from hybridoma culture supernatants.

Peripheral Blood Mononuclear and $CD4^+$ T Cell Isolation and Stimulation

Peripheral blood mononuclear cells were isolated from blood donors of healthy human volunteers by Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ, USA), gradient centrifugation, washed with RPMI 1640 (Sigma), and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Nato-Cor, Córdoba, Argentina), sodium pyruvate, glutamine, and penicillin–streptomycin (RPMI/FCS). $CD4^+$ T lymphocytes were isolated by positive selection with magnetic beads (Dynabeads; Dynal, Oslo, Norway) or by negative selection with the RosetteSep isolation kit (Stem Cell, Vancouver, BC, Canada). Cells were stimulated for 3 days in 96-well, U-bottomed plates or in 24-well, flat-bottomed plates (Becton–Dickinson Labware) either with a stimulating anti-CD3 mAb (clone

SK-7; BD) as described [20, 21] or with 10 ng/ml of PMA and 0.5 μg/ml of ionomycin. In other experiments, PBMCs were stimulated for 18 hours with PHA (1 μg/ml), washed, and cultured in the presence of different doses of IL-2 (0.8 or 8 ng/ml), IL-4 (5 ng/ml), or IL-15 (10 ng/ml). In some experiments, PBMCs were preincubated for 30 minutes at 37°C with pharmacologic inhibitors of intracellular signaling molecules. Control experiments were performed, stimulating PBMCs with an isotype-matched, negative-control mAb or in RPMI 1640 medium alone. Cultured cells were then used for proliferation assays, Western blot analysis, or flow cytometry. For polarization experiments, PBMCs were cultured for 6 days with 0.5 μg/ml of PHA, 2 ng/ml of IL-12, and 100 ng/ml of anti-IL-4 mAb for Th1 polarization or with 1 μg/ml of PHA, 5 ng/ml of IL-4, and 2 μg/ml of anti-IL-12 mAb for Th2 polarization. Polarized cells were used for assessment of MICA expression by Western blot. Expression of GATA-3 and T-bet in polarized PBMCs was assessed after 3 days of culture.

Proliferation Assay

Cells were pulsed with 1 μCi/well methyl-^3H-thymidine (^{3}H-Thy; New England Nuclear Life Science, Boston, MA, USA) during the last 18 hours of cell culture and were harvested on glass-fiber filters using a Packard Filtermate cell harvester (Packard Instruments, La Grange, IL, USA). Incorporated radioactivity was measured in a liquid scintillation β-counter (Packard Instruments). Results are expressed as mean counts per minute (cpm) of triplicate wells ± SD.

SDS–PAGE and Western Blot

Cell lysates, SDS–PAGE, and Western blot were performed as previously described [20, 21]. Twenty micrograms of proteins from each sample were loaded onto the gels. Proteins were transferred to nitrocellulose membranes (Amersham) and equal loading was confirmed by Ponceau S staining or by probing for β-actin expression using a specific mAb (DAKO, Dakocytomation, Glostrup, Denmark). Blocked membranes were incubated with different polyclonal or monoclonal Abs. Bound Abs were detected with peroxidase-labeled antirabbit or antimouse IgG (Bio-Rad, Hercules, CA, USA), and chemiluminescent detection using the ECL detection reagent (Amersham Biosciences) and Kodak BioMax films. No bands were observed in Western blots incubated with normal rabbit sera or normal mouse IgG.

Flow Cytometry

Cell viability after culture with different drugs and inhibitors was checked by propidium iodide (PI) exclusion. Purity of isolated cell populations (CD4$^+$ T lymphocytes, NK cells) was analyzed using specific fluorochrome-labeled mAbs in a FACScalibur flow cytometer (BD). Surface MICA and ULBPs expression was analyzed by flow cytometry with the corresponding mAbs and antimouse IgG-RPE (DAKO). An isotype-matched mAb was used as negative control.

NK Cells and Stimulation with IL-15

Human peripheral blood-derived NK cells were isolated from healthy human volunteers using the RosetteSep NK cell enrichment reagent (Vancouver, BC, Canada) and Ficoll-Paque Plus gradient centrifugation. NK cells were washed with RPMI 1640, resuspended in RPMI/FCS, and used as effector cells for cytotoxicity assays or IFN-γ production. In some experiments, NK cells were stimulated with 10 ng/ml of IL-15 for 72 hours prior to use. Purity of NK cells was always above 94%, as assessed by flow cytometry with PE-labeled anti-CD56 and FITC-labeled anti-CD16 mAbs.

Cytotoxicity Assays

Five-hour standard ^{51}Cr release assays were performed using 5000 target cells labeled with ^{51}Cr/well (CD4$^+$ T cells stimulated for 72 hours with PMA and ionomycin) and NK cells (resting or stimulated with IL-15 for 72 hours) as effector cells, at E:T ratios of 10:1 or 5:1. For blocking experiments, targets and effector were cultured in the presence of 10 μg/ml of the anti-HLA class I pan-reactive mAb W6/32, the anti-NKG2D mAb 1D11, or an isotype-matched negative control mAb. The percentage of cytotoxicity (^{51}Cr release) was calculated as follows: 100 × [(experimental release − spontaneous release)/(maximum release − spontaneous release)]. Maximum release was obtained from target cells lysed with 2% Triton X-100. Spontaneous release was always below 15% of maximum release. Statistical analysis of data was performed using ANOVA with Dunnett's comparison test.

Intracellular IFN-γ Detection

Resting or IL-15-stimulated NK cells were cultured with syngeneic CD4$^+$ T lymphocytes stimulated with PMA and ionomycin, at an E:T ratio of 1:1 for 24 or 48 hours. Brefeldin A (10 μg/ml; Sigma) was added during the last 5 hours of culture, and then cells where used for triple color flow cytometry staining with anti-CD3-SPRD, anti-IFN-γ-RPE, and anti-CD56-FITC mAbs to analyze the intracellular expression of IFN-γ. Samples were acquired in a FACScalibur flow cytometer and results were analyzed with the WinMDI 2.8 software.

Indirect Immunofluorescence and Confocal Microscopy

Positively or negatively isolated CD4$^+$ T cells were stimulated with PMA and ionomycin for 72 hours. Stimulated cells were stained for surface MHC cells I molecules using the W6/32 mAb and Cy3-labeled donkey

antimouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA). Thereafter, cells were washed with PBS supplemented with 0.2% of bovine serum albumin (PBS/BSA), seeded onto polylysine-coated glass coverslips, and fixed with 2% of *p*-formaldehyde (pfa) in PBS for 5 min. After washing, cells were permeabilized with 0.05% saponin (Sigma) in PBS/BSA (PBS/BSA/saponin) for 10 min, and nonspecific binding sites were blocked by incubation with normal goat serum diluted 1/20 in PBS/BSA/saponin for 30 min. Thereafter, cells were incubated with 200 μg/ml of biotinylated anti-MICA mAb D7 or IC mAb for 30 min. Bound Abs were detected with FITC-labeled streptavidin (Pierce, Rockford, IL, USA) for 30 min. After washing, coverslips were mounted with DABCO on glass slides and observed in a digital Eclipse E800 Nikon C1 confocal microscope system with Nikon Plan Apo 60X/1.40 Oil and Nikon Plan Apo 40X/0.95 objectives. All procedures were performed at room temperature.

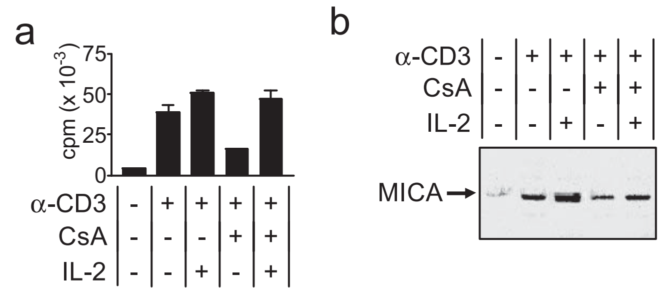


FIGURE 1 IL-2 regulates MICA expression in anti-CD3-activated T lymphocytes present in PBMCs. The effects of IL-2 on proliferation and MICA expression were investigated in PBMCs stimulated with anti-CD3 mAb for 72 hours in the absence or in the presence of 8 ng/ml IL-2, as indicated. Proliferation was assessed by [^{3}H]thymidine uptake (a) and MICA expression was evaluated by Western blot (b). Also, the effects of IL-2 on proliferation and MICA expression in PBMCs stimulated with anti-CD3 mAb and treated with 1 μM cyclosporine A (CsA) were evaluated. As control, PBMCs stimulated with the isotype control mAb were analyzed. Results are representative of three independent experiments performed with three different blood donors.

RESULTS

Mitogenic Cytokines Induce Upregulated Expression of MICA in T Lymphocytes

MICA is not expressed by resting T or B lymphocytes [3] but it is induced in activated $CD4^+$ and $CD8^+$ T cells [3, 20]. Since MICA expression appears to be partially dependent on a functional IL-2-dependent intracellular signaling route [21], we explored whether this cytokine induces MICA expression in T cells simultaneously stimulated with an activating anti-CD3 mAb and whether IL-2 can directly induce MICA expression in T cells present in PBMCs (Figure 1). We observed that IL-2 strongly enhanced the proliferation of PBMCs stimulated with an anti-CD3 mAb (Figure 1a) and induced higher levels of MICA expression, assessed by Western blot, than each stimulus alone (Figure 1b). CsA, which inhibits calcineurin, blocked T-cell proliferation and MICA expression as previously described [21], and this effect was reverted by exogenous IL-2. Thus, we conclude that IL-2 cooperates with CD3 engagement for the induction of MICA expression. To investigate whether IL-2 independently triggers MICA expression, resting PBMCs were stimulated with this cytokine for 3 or 5 days. It was observed that high doses (8 ng/ml) of this cytokine induced a proliferative response (Figure 2a) and MICA expression (Figure 2b). Since resting T cells do not express CD25 (the α chain of the high-affinity IL-2 receptor), PBMCs were preactivated with PHA for 18 hours and stimulated with IL-2 (Figures 2c–2e). As expected, preactivated T lymphocytes expressed CD25, which reached a plateau after 12 hours of stimulation and remained high for at least 48 hours (Figure 2c). Also, these preactivated cells became more responsive to exogenous IL-2 with regard to proliferation (Figure 2d) and MICA expression (Figure 2e). However, when surface MICA expression was investigated by flow cytometry, no consistent surface MICA expression could be detected (*not shown*).

Intracellular signaling routes involved in IL-2-driven MICA expression were investigated using pharmacologic inhibitors (Figure 3). The results of these experiments demonstrated that the Jak3 inhibitor AG490, the p38 MAPK inhibitor SB202190, the $p70^{S6}$ kinase inhibitor rapamycin, the *Src* kinase inhibitor PP1, and the NF-κB inhibitor sulfasalazine all inhibited proliferation in different degrees (Figure 3a) and strongly inhibited MICA expression (Figure 3b) in preactivated T cells present in PBMCs stimulated with IL-2. As a control of the experiment, we observed that AG490 and PP1 inhibited the phosphorylation of STAT5, as expected (Figure 3c). Also, Sz inhibited the formation of the NF-κB complexes in electrophoretic mobility shift assays performed with a consensus κB nucleotide (*not shown*).

To address whether other mitogenic cytokines that share the γ chain of their receptor with the IL-2R, such as IL-4 and IL-15, can induce MICA expression in T lymphocytes present in PBMCs, these cytokines were used to stimulate resting or preactivated PBMCs (Figure 4). In contrast to IL-2, the culture of quiescent PBMCs with IL-4 did not upregulate MICA at any doses (up to 25 ng/ml) and times (up to 10 days) examined (*data not shown*). However, preactivated cells stimulated with IL-4 (Figure 4a) or IL-15 (Figure 4b) showed a strong proliferation and marked MICA expression. Like IL-2, IL-4 collaborated with an activating anti-CD3 mAb for MICA

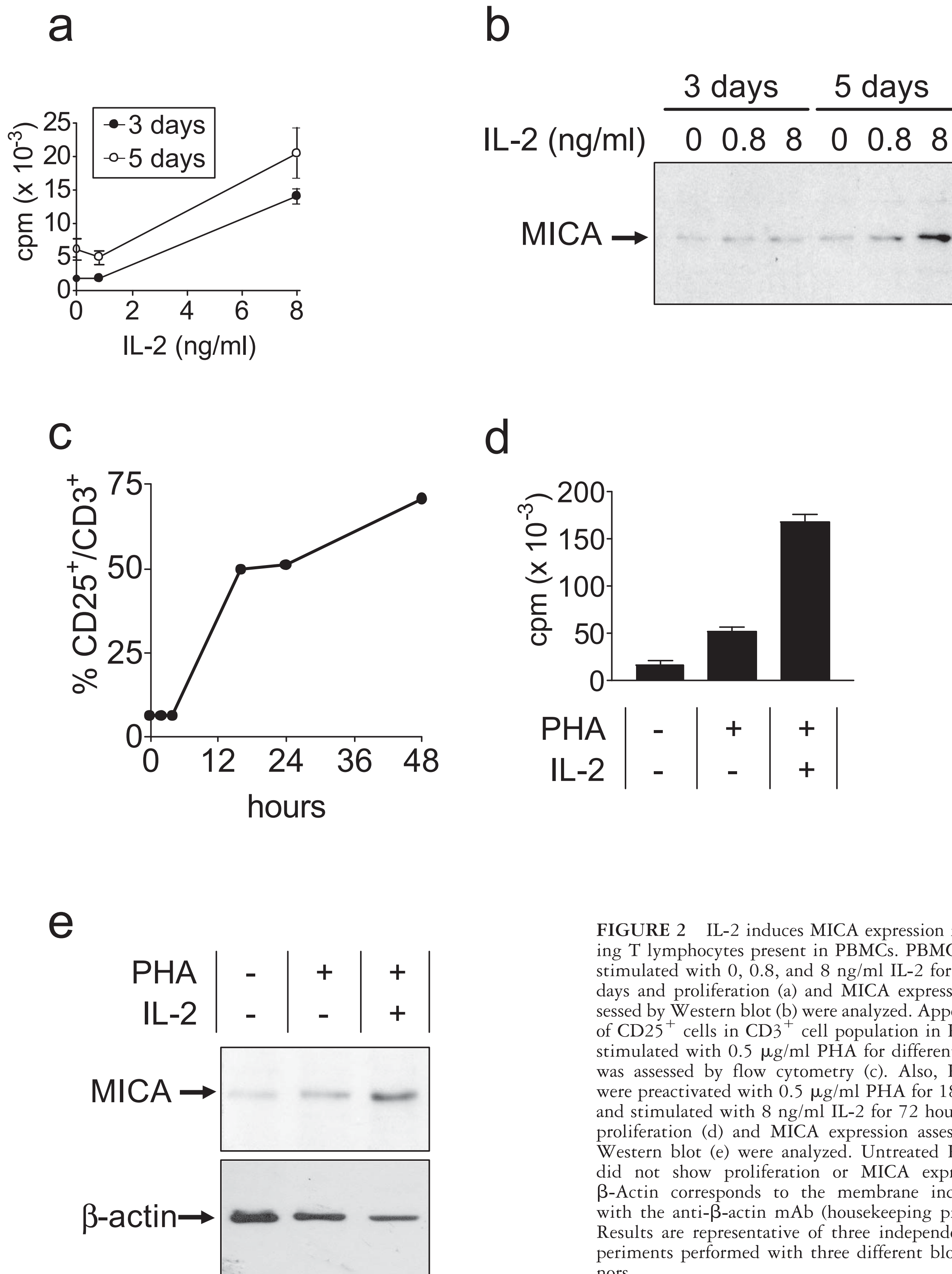
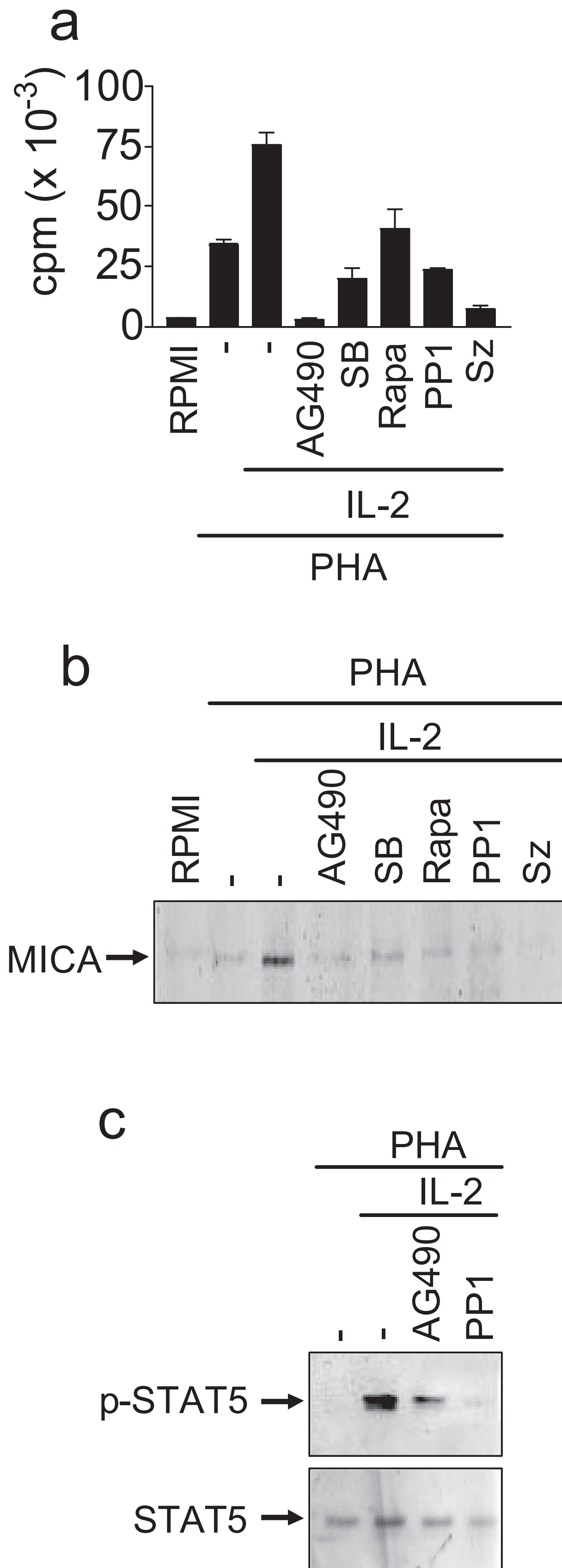


FIGURE 2 IL-2 induces MICA expression in resting T lymphocytes present in PBMCs. PBMCs were stimulated with 0, 0.8, and 8 ng/ml IL-2 for 3 or 5 days and proliferation (a) and MICA expression assessed by Western blot (b) were analyzed. Appearance of $CD25^+$ cells in $CD3^+$ cell population in PBMCs stimulated with 0.5 μg/ml PHA for different times was assessed by flow cytometry (c). Also, PBMCs were preactivated with 0.5 μg/ml PHA for 18 hours and stimulated with 8 ng/ml IL-2 for 72 hours, and proliferation (d) and MICA expression assessed by Western blot (e) were analyzed. Untreated PBMCs did not show proliferation or MICA expression. β-Actin corresponds to the membrane incubated with the anti-β-actin mAb (housekeeping protein). Results are representative of three independent experiments performed with three different blood donors.



expression (Figure 4c). Regarding IL-2, when surface MICA expression was investigated by flow cytometry, no surface MICA expression was detected (*not shown*). Conversely, nonmitogenic cytokines such as IFN-α and TNF-α did not induce MICA expression in T lymphocytes at different doses and times of stimulation tested, as assessed by Western blot (*not shown*).

From these experiments, we conclude that different mitogenic cytokines such as IL-2, IL-4, and IL-15 induce MICA in preactivated T cells. The effects observed with IL-2 require functional signaling routes that involve the Jak/STAT pathway, the p38 MAPK, the $p70^{S6}$ kinase, the *Src* kinases Lck and Fyn, and NF-κB.

Expression of MICA in Th1 and Th2 Cells

Since IL-2 and IL-4 are involved in the proliferation of Th1 and Th2 cells, we addressed whether they express MICA. Th1 and Th2 cells were obtained after *in vitro* polarization of PBMCs as described under Materials and Methods. Thereafter, proliferation was evaluated by thymidine uptake (Figure 5a) and MICA expression was assessed by Western blot (Figure 5b). As shown, MICA expression was induced on both cell populations and no major differences could be observed between Th1 and Th2 cells. An increase in T-bet expression confirmed polarization to a Th1 phenotype and an increase in GATA3 expression confirmed polarization to a Th2 phenotype (Figure 5b). However, we were unable to detect surface expression of MICA by flow cytometry on Th1 or Th2 cells (*not shown*).

Low Levels of Surface Expression of MICA on T Lymphocytes Stimulated with PMA and Ionomycin

Different modes of stimulation of T lymphocytes that induced engagement of surface receptors led to low or absent surface MICA expression [3, 20, and results here presented]. To become independent of these surface receptors, we explored the effects of PMA and ionomycin, which act together as a potent mitogen for human T lymphocytes (Figure 6). MICA expression was investi-

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FIGURE 3 Inhibition of Jak3, Lck, p38 MAPK, $p70^{S6}$ kinase, and NF-κB prevent expression of MICA in T lymphocytes present in PBMCs activated by IL-2. Proliferation (a) and MICA expression assessed by Western blot (b) were analyzed in PBMCs preactivated with 0.5 μg/ml PHA for 18 hours and cultured with 8 ng/ml IL-2 for 72 hours in the absence or in the presence of 50 μM AG490 (AG490), 10 μM SB202190 (SB), 1 μM rapamycin (Rapa), 10 μM PP1, or 0.5 mM sulfasalazine (Sz). As control, inhibition of STAT5 phosphorylation by 50 μM AG490 and 10 μM PP1 was analyzed by Western blot (c). Results are representative of three independent experiments performed with three different blood donors.

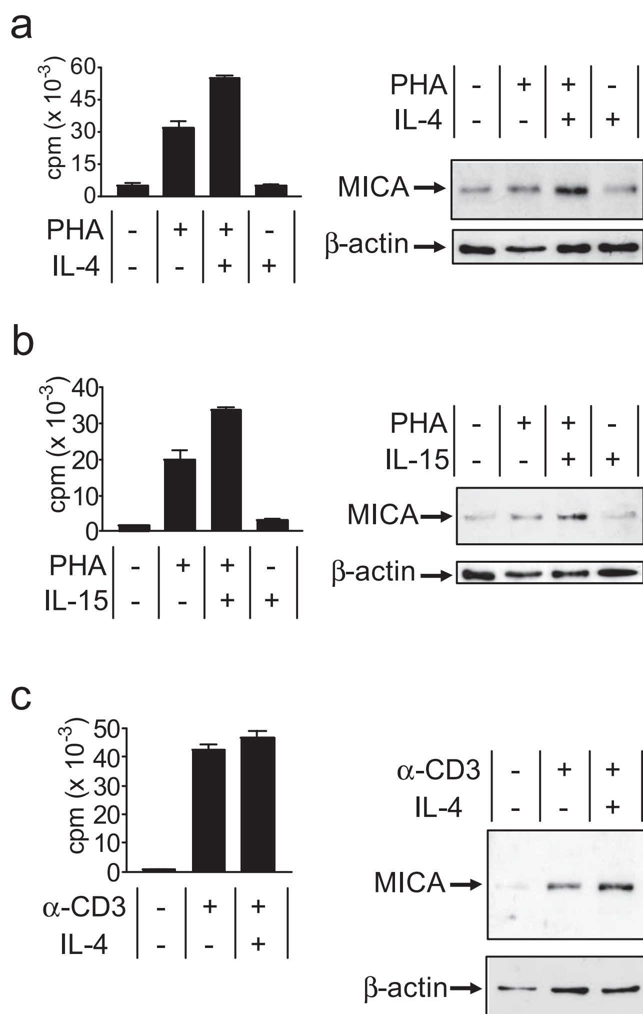

FIGURE 4 IL-4 and IL-15 induce the expression of MICA in T lymphocytes present in PBMCs. Proliferation (*left*) and MICA expression (*right*) analyses of PBMCs preactivated with PHA for 18 hours and cultured without or with 5 ng/ml IL-4 (a) or 10 ng/ml IL-15 (b) for 72 hours. Also, the effects of IL-4 on proliferation (*left*) and MICA expression (*right*) were investigated in PBMCs stimulated with anti-CD3 mAb for 72 hours in the absence or in the presence of 5 ng/ml IL-4 (c). As control, PBMCs stimulated with the isotype control mAb were analyzed. β-Actin corresponds to the membrane incubated with the anti-β-actin mAb (housekeeping protein). Results are representative of three independent experiments performed with three different blood donors.

gated by Western blot (Figure 6a) and flow cytometry (Figure 6b). We observed that MICA was consistently detected in lysates of these cells, but only low levels were expressed at the cell surface. Although we performed the flow cytometry studies with the mAb D7, which also detects MICB, this particular NKG2DL was not expressed by activated T lymphocytes when we used a MICB-specific mAb (*not shown*). Preliminary evidence obtained in our lab suggests that IL-10 or TGF-β secreted by activated T lymphocytes or other effects mediated by $CD4^{+}CD25^{+}$ regulatory T cells are not responsible for this low surface expression because neutralization of these cytokines with specific mAbs or depletion of $CD4^{+}CD25^{+}$ regulatory T cells with magnetic beads did not increase the levels of MICA detected by flow cytometry on $CD4^{+}$ T lymphocytes stimulated with PMA and ionomycin (C.I. Domaica, *personal communication*). These results indicate that activated T lymphocytes, in particular $CD4^{+}$ cells, upregulate MICA expression but that the levels reached at the cell surface remain quite low. Since other NKG2DLs have been described, we addressed their expression on resting and activated CD4 T cells. In these experiments, we were unable to detect expression of ULBP-1 and -3 on resting (*not shown*) and on $CD4^{+}$ T lymphocytes stimulated with PMA and ionomycin (Figure 6c), and we observed a very slight upregulation of ULBP-2 on activated T lymphocytes in only two of six donors (the other four donors did not upregulate ULBP-2).

Functional Consequences of MICA Expression in T Lymphocytes

Since stimulation with PMA and ionomycin induced low levels of surface MICA on stimulated $CD4^{+}$ T lymphocytes, we address its functional consequences. First, we investigated whether these cells become more susceptible to syngeneic NK cells. $CD4^{+}$ T cells stimulated for 72 hours with PMA and ionomycin were cultured with syngeneic NK cells or with syngeneic NK cells stimu-

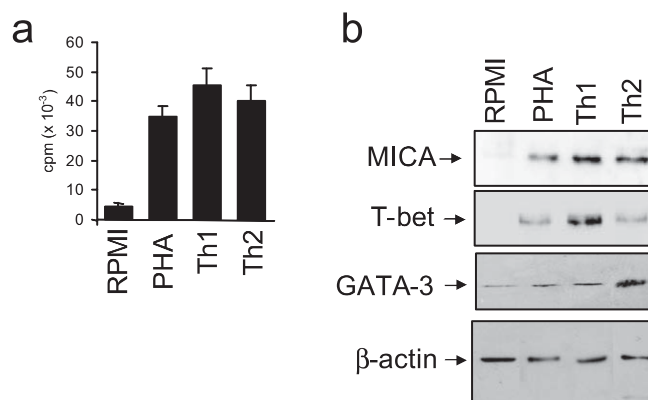

FIGURE 5 Th1 and Th2 cells express MICA. Proliferation (a) and MICA expression analysis (b) in T cells present in PBMCs preactivated with PHA for 18 hours and cultured with 0.5 μg/ml PHA, 2 ng/ml IL-12, and 100 ng/ml anti-IL4 mAb (Th1-polarizing conditions) or with 0.5 μg/ml PHA, 5 ng/ml IL-4, and 2 μg/ml anti-IL12 mAb (Th2-polarizing conditions). As control, expression of T-bet and GATA-3 transcription factors were analyzed. β-Actin corresponds to the membrane incubated with the anti-β-actin mAb (housekeeping protein). Results are representative of three independent experiments performed with three different blood donors.

lated with IL-15 for 72 hours. Thereafter, standard chromium release assays were performed (Figures 7a and b). We observed that resting NK cells were completely unable to lyse resting (*not shown*) or activated $CD4^+$ T lymphocytes but IL-15-activated NK cells were cytotoxic against activated $CD4^+$ T lymphocytes (Figures 7a and b). However, susceptibility of activated $CD4^+$ T lymphocytes to NK cell-mediated lysis was acquired when the interaction of KIRs with the HLA class I molecules was blocked with the W6/32 mAb (Figure 7b). Blockade with an anti-NKG2D mAb did not restore resistance to NK cell-mediated cytotoxicity, suggesting that MICA is not involved in the lysis of activated $CD4^+$ T lymphocytes by resting NK cells when engagement of NK cell-inhibitory receptors is prevented (*not shown*). Also, blockade of NK cell-inhibitory receptors with the W6/32 mAb strongly increased susceptibility of activated $CD4^+$ T lymphocytes to IL-15-stimulated NK cells (Figure 7b) but this susceptibility was not prevented by the anti-NKG2D mAb (*not shown*). These results suggest that activation renders $CD4^+$ T lymphocytes sensitive to destruction by activated NK cells but not by resting NK cells and that the MICA–NKG2D system does not participate in this biological response. Similar results were obtained when the experiments were performed with NK cells stimulated with IL-2 (*not shown*).

Engagement of NKG2D has been shown to trigger IFN-γ secretion by NK cells [10–12, 14]. Thus, we investigated whether NK cells or NK cells stimulated with IL-15 or IL-2 for 72 hours secrete IFN-γ upon interaction with resting or $CD4^+$ T lymphocytes stimulated for 72 hours with PMA and ionomycin. We observed the appearance of a slight percentage of IFN-γ-producing NK cells (defined as $CD3^-CD56^+IFN\text{-}\gamma^+$ cells) upon coculture with activated $CD4^+$ T lymphocytes, but this phenomenon was insensitive to NKG2D blockade using an anti-NKG2D mAb (Figure 7c, *top dot plots*). The percentage of IFN-γ-producing NK cells was strongly increased when NK cells were previously stimulated with IL-15 for 72 hours (Figure 7c, *bottom left dot plot*). The production of IFN-γ was slightly reduced by the anti-NKG2D mAb (Figure 7c, *bottom left dot plot*), but the amount of IFN-γ detected in the cells remained unchanged in the presence of this mAb (Figure 7d). These results suggest that the involvement of the MICA–NKG2D system in the production of IFN-γ by activated NK cells, if any, is marginal. Neither resting nor cytokine-stimulated NK cells spontaneously produced IFN-γ during these cultures (*not shown*). Similar results were obtained with NK cells stimulated with IL-2.

Confocal Microscopy

As mentioned above, MICA was consistently detected in cell lysates of PMA/ionomycin-activated $CD4^+$ T lymphocytes, but only low levels were expressed at the cell surface. To investigate whether MICA is retained inside the cell, confocal microscopy was performed (Figure 8). Here, we observed that MICA expression was confined to an intracellular compartment since the green fluorescence corresponding to MICA was detected inside the cells, mostly localized in the cytoplasmic region and clearly not located in the nucleus, which remained unstained. This cellular localization was clearly distinguishable from a cell surface localization like the red fluorescence obtained for the MHC class I molecules. Therefore, despite expressing some MICA on the cell surface, activated $CD4^+$ T lymphocytes retain the majority of the MICA pool of molecules inside the cell. From a functional point of view, this provides these activated T cells with a mechanism to prevent NKG2D-dependent cytotoxicity.

DISCUSSION

Previous evidence obtained by our lab indicated that MICA expression can be induced on activated T lymphocytes in a process that partially depends on IL-2 [21]. In the present work, we demonstrated that this cytokine cooperates with CD3 engagement for MICA expression (Figure 1) and that IL-2 can directly induce MICA expression in T cells present in PBMCs (Figure 2). This ability was accompanied by the strong mitogenic effect of IL-2 on T cells [22–24]. The effect of IL-2 was more pronounced when cells were preactivated for 18 hours with PHA due to the upregulation of CD25 and assembly of the high-affinity IL-2R [24]. However, no surface expression of MICA was observed. MICA induction by IL-2 was dependent on functional signaling routes that involve Jak3/STAT5, p38 MAPK, $p70^{S6}$ kinase, Lck/fyn kinases, and the transcription factor NF-κB (Figure 3), which is in line with the dependence on similar intracellular mediators previously demonstrated by us to be involved in activation-induced MICA expression in T lymphocytes stimulated with anti-CD3 mAb or anti-CD28 mAb plus PMA [21, 25].

Because we demonstrated that different mitogenic stimuli [20] induce MICA expression on T lymphocytes, we investigated the effect of other T-cell mitogenic cytokines that signal through receptors that share their γ chain with the IL-2R, such as IL-4 and IL-15 [30], and found that both cytokines also induced MICA expression (Figure 4). In contrast, IFN-α and TNF-α, like IFN-γ [3], did not induce MICA expression in T lymphocytes. Thus, cytokine-regulated expression of MICA in T lymphocytes appears to be restricted to mitogenic cytokines.

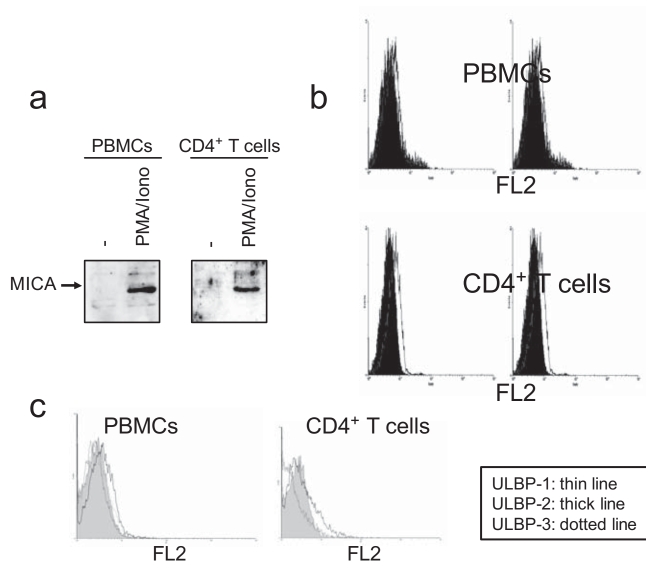

FIGURE 6 Expression of MICA in T lymphocytes present in PBMCs and in isolated CD4$^+$ T lymphocytes activated with PMA and ionomycin. MICA expression on T lymphocytes present in PBMCs and in isolated CD4$^+$ T lymphocytes not stimulated (−) or stimulated with PMA and ionomycin for 72 hours (PMA/Iono) was investigated by Western blot (a) and flow cytometry with the anti-MICA mAb D7 (b, open histograms). Also, expression of ULBPs-1, -2, and -3 was investigated by flow cytometry with specific mAbs in lymphocytes present in PBMCs and in isolated CD4$^+$ T lymphocytes stimulated with PMA/Iono for 72 hours (c). Gray histograms correspond to the IC mAb. Results are representative of five independent experiments performed with three different blood donors.

Since IL-2 and IL-4 are involved in the activation and proliferation of Th1 and Th2 cells, we investigated whether they express MICA. We found that indeed, MICA was induced on both cell types (Figure 5) but were unable to detect the molecule expressed on the cell surface.

MICA is recognized by the C-type lectin receptor NKG2D and this recognition triggers cytotoxicity against different MICA-expressing tumors or infected cells and IFN-γ secretion by NK cells [6, 7, 10–12, 14]. Also, mitogen-activated T lymphocytes are targets of IL-2-stimulated NK cells in a NKG2D-dependent process [12, 31] and IFN-γ secretion by CD4$^+$ T cells is induced upon coculture with IL-2-stimulated NK cells [26]. However, IFN-γ production by NK cells upon coculture with CD4 T cells was not investigated in this work. Previous work performed in our laboratory led to the observation that although MICA was induced on activated T lymphocytes by different mitogens or physiological stimuli, surface expression of this NKG2DL was not systematically detected or was even absent [3, 20, and results here presented]. To address the functional consequences of MICA expression in activated T cells and to become independent of the engagement of surface receptors, we stimulated PBMCs and isolated CD4$^+$ T lymphocytes with PMA and ionomycin. Here, we observed that MICA was consistently detected in lysates of these cells, but only low levels were expressed at the cell surface (Figure 6) compared to the levels expressed by different tumor cells [5, 8, 9, 11, 12]. IL-10 or TGF-β secreted by activated T lymphocytes or the presence of CD4$^+$CD25$^+$ regulatory T cells were not responsible for this low or absent surface expression (C.I. Domaica, *personal communication*). Currently, we are investigating the factors that may regulate surface expression of MICA on activated T lymphocytes. In addition, expression of other NKG2DLs such as MICB and the ULBPs-1 [16–19], -2, and -3 was not detected on resting or on CD4 T lymphocytes stimulated with PMA and ionomycin (only in T cells from two of six donors were a very slight basal expression and minor upregulation of ULBP-2 observed). Unfortunately, we could not assess surface expression of ULBP-4 and RAET1G because specific Abs are not available. Therefore, we considered that the low surface expression of MICA detected on the cell surface of activated CD4$^+$ T lymphocytes may not be enough to elicit cytotoxicity by NKG2D$^+$ cells.

To investigate the functional consequences of MICA expression on activated T lymphocytes, we analyzed their susceptibility to NK cell-mediated cytotoxicity and IFN-γ production by the NK cells. Although we detected cytotoxicity of IL-15- and IL-2-stimulated NK cells toward mitogen-stimulated CD4$^+$ T cells (Figure 7), the involvement of MICA in this process could not be demonstrated using a blocking anti-NKG2D mAb (since we did not observe expression of the ULBPs1-3 or MICB on activated T cells, any blockade of the anti-NKG2D mAb was likely due to interference with the interaction between this receptor and MICA). Likewise, cytokine-activated NK cells produced more IFN-γ than resting NK cells upon coculture with activated but not with resting CD4$^+$ T lymphocytes, but MICA does not appear to be involved in this process.

Our results indicate that the low surface expression of MICA on activated CD4$^+$ T lymphocytes may be a safeguard mechanism to protect them from resting NK cells, especially during a T cell-dependent immune response. These levels may not be enough to overcome the protective effects of the high levels of expression of HLA class 1 molecules that act as ligands of NK cell-inhibitory receptors such as KIR2DLs, KIR3DLs, and members of the ILT/LIR family [32]. Accordingly, susceptibility of activated CD4$^+$ T lymphocytes to NK cells was observed when the engagement of these inhibitory receptors was avoided with the blocking anti-HLA class I pan-reactive mAb W6/32. Intracellular retention of

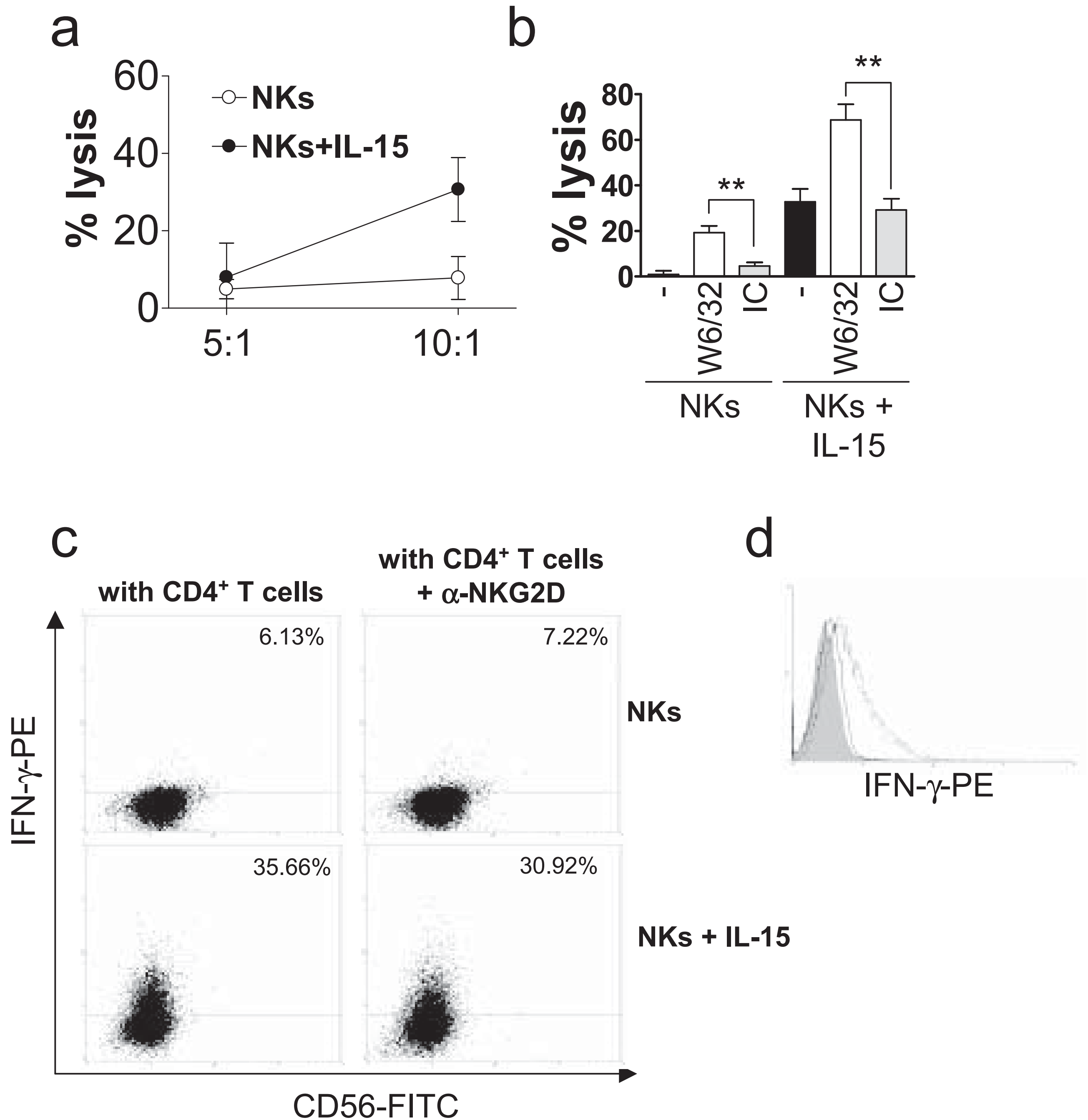

FIGURE 7 Cytotoxicity and IFN-γ secretion of resting and IL-15-stimulated NK cells in response to activated $CD4^+$ T lymphocytes. Standard ^{51}Cr release assays were performed using $CD4^+$ T lymphocytes stimulated for 72 hours with PMA and ionomycin as target cells and resting or IL-15-stimulated NK cells as effector cells at two E:T ratios (a). Also, blocking experiments with the anti-HLA class 1 mAb W6/32 or an isotype control (IC) mAb were performed using resting NK cells or NK cells stimulated for 72 hours with IL-15, at an E:T ratio of 10:1 (b). Standard error bars are shown in (a) and (b). $^{**}p < 0.01$. (c) Intracellular IFN-γ produced by resting (top) or IL-15-stimulated NK cells (bottom) after coculture for 24 hours with $CD4^+$ T lymphocytes stimulated for 72 hours with PMA and ionomycin, in the absence (*left*) or in the presence of the anti-NKG2D blocking mAb (*right*) was assessed by triple color flow cytometry. The numbers in the top right quadrant represent the percentage of IFN-γ-producing cells. Less than 1% of IFN-γ-producing $CD3^-CD56^+$ cells were detected in the absence of coculture with activated $CD4^+$ T cells. The relative amounts of IFN-γ detected in IL-15-stimulated NK cells after coculture with activated $CD4^+$ T lymphocytes, in the absence (*thin line*) or in the presence of the anti-NKG2D blocking mAb (*dotted line*) are also shown (d). No IFN-γ production was detected in IL-15-stimulated NK cells cultured in the absence of activated $CD4^+$ T cells (*thick line*). The gray histogram corresponds to the staining produced by the IC control mAb. Results are representative of three independent experiments performed with three different blood donors.

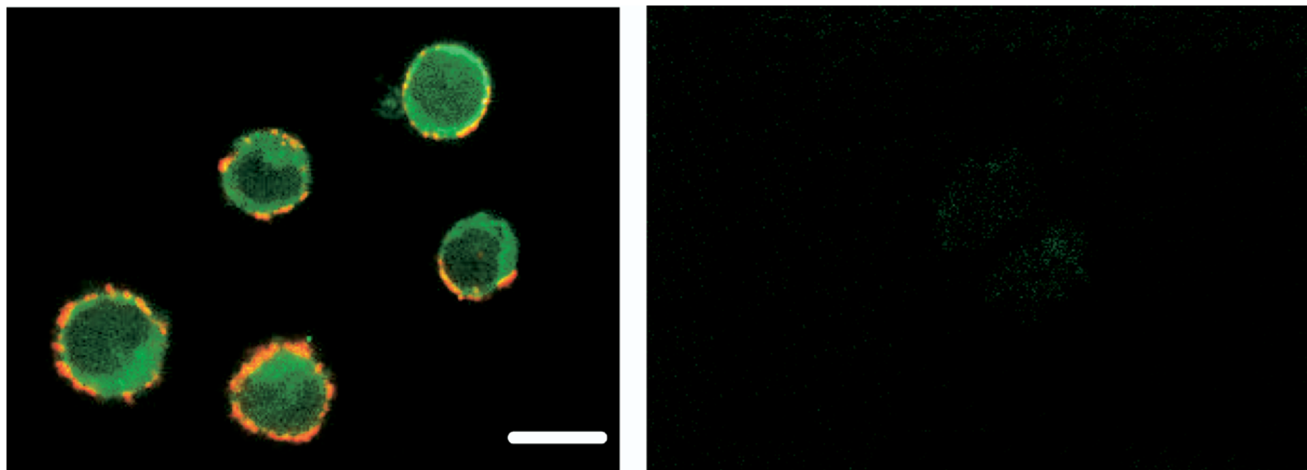

FIGURE 8 Confocal microscopy with activated $CD4^+$ T lymphocytes. Isolated $CD4^+$ T lymphocytes stimulated with PMA and ionomycin for 72 hours were stained for surface MHC class I molecules (using the W6/32mAb), fixed, permeabilized, and stained with biotinylated anti-MICA mAb D7 and streptavidin–FITC (*left*). As negative control, staining obtained with the corresponding IC mAbs is also presented (*right*). Cells were analyzed by confocal microscopy. Bar, 10 μm.

MICA in activated $CD4^+$ T lymphocytes was confirmed by confocal microscopy (Figure 8), which suggests that activated $CD4^+$ T cells have the potential to become targets of NKG2D-dependent cytotoxicity if surface expression of MICA surpasses the threshold imposed by the expression of the HLA class I molecules. We believe that this may provide the activated $CD4^+$ T lymphocytes with a mechanism to resist cytotoxicity of cytokine-activated NK cells in an inflammatory environment, in a virus-infected tissue, or in a tumor microenvironment, where NK and activated $CD4^+$ T cells are recruited and further stimulated with locally produced cytokines. The protection of activated $CD4^+$ T cells from NK cell-mediated cytotoxicity in such environments might be important to allow them to exert their effector functions (*i.e.,* cytokine secretion). Otherwise, lysis of recently arrived activated $CD4^+$ T cells by NK cells would lead to an inefficient antigen-elimination phase and early termination of the immune response.

Our findings raise the question of the biological significance of intracellular MICA in activated T lymphocytes. Two hypotheses can be proposed. On one hand, it is possible that intracellular MICA may have an additional, still unknown immunoregulatory function exerted through association with NKG2D. For example, MICA may regulate the surface levels of NKG2D by intracellular sequestration. On the other hand, it is also possible that, to become expressed on the cell surface on activated T cells, MICA needs an extra signal produced during the cross talk of activated T lymphocytes with other cell populations present in inflamed, virus-infected, or neotransformed tissues. A cross talk of activated CD4 T cells and NK cells has been demonstrated recently [26] but it is also possible that this putative extra signal may be provided by other cells present in such tissues. Regardless of the origin and nature of this hypothetical signal, it is possible that activated T lymphocytes rapidly express MICA at high levels at the cell surface by mobilization from intracellular deposits. Recently, it was observed that MICA can be expressed at the cell surface on $CD8^+$ T cells stimulated with anti-CD3 or anti-CD3 plus anti-NKG2D mAbs and cultured for 7 days in the presence of mitogenic cytokines such as IL-2 or IL-7 plus IL-15 [27], but so far we ignore the functional consequences of this surface expression. Although cytotoxicity of activated $CD4^+$ T lymphocytes by IL-15-activated NK cells was observed (Figure 7), we think that it is advantageous for an activated effector T lymphocyte to keep MICA inside the cell, especially in stressed tissues where high concentrations of IL-15 secreted by dendritic cells and macrophages induce NKG2D upregulation and cytotoxicity of NK cells against stressed target cells [30]. Once the termination phase of the immune response is reached due to antigen exhaustion, activated T lymphocytes need to be cleared from the body. In this case, surface expression of MICA may contribute to the elimination of these activated T lymphocytes.

In summary, we provide evidence that MICA is expressed by T lymphocytes stimulated with mitogenic cytokines such as IL-2, IL-4, or IL-15 and by Th1 and Th2 cells, but the molecule, being retained mostly inside the cell, is poorly transported to the cell surface. These cells resisted NK cell-mediated cytotoxicity, even though they expressed low levels of surface MICA. Although the timing of *in vivo* surface expression of MICA

on T lymphocytes in stressed tissues remains to be investigated, it is possible that under still unknown conditions, MICA may be rapidly expressed at high levels at the cell surface of activated T cells simply by mobilization from intracellular deposits. Accordingly, our results are of potential relevance in designing strategies to modulate NKG2D-mediated cytotoxicity by NK cells toward activated T lymphocytes in pathological situations, through the induction of surface expression of MICA.

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REFERENCES

1. Bahram S, Bresnahan M, Geraghty DE, Spies T: A second lineage of mammalian major histocompatibility complex class I genes. Proc Natl Acad Sci USA 91: 6259, 1994.
2. Groh V, Bahram S, Bauer S, Herman A, Beauchamp M, Spies T: Cell stress-regulated human major histocompatibility complex class I gene expressed in gastrointestinal epithelium. Proc Natl Acad Sci USA 93: 12445, 1996.
3. Zwirner NW, Fernández-Viña MA, Stastny P: MICA, a new polymorphic HLA-related antigen, is expressed mainly by keratinocytes, endothelial cells and monocytes. Immunogenetics 47: 139, 1998.
4. Molinero, LL, Marcos CY, Mirbaha F, Fainboim L, Stastny P, Zwirner NW: Codominant expression of the polymorphic MICA alloantigens encoded by genes in the HLA region. Eur J Immunogenet 29, 315–319, 2002.
5. Groh V, Steinle A, Bauer S, Spies T: Recognition of stress-induced MHC molecules by intestinal epithelial γδ T cells. Science 279, 1737–1740, 1998.
6. Groh V, Rhinehart R, Randolph-Habecker J, Topp MS, Riddell SR, Spies T: Co-stimulation of CD8αβ T cells by NKG2D via engagement by MIC induced on virus-infected cells. Nat Immunol 2, 255–260, 2001.
7. Das H, Groh V, Kuijl C, Sugita M, Morita C, Spies T, Bukowski JF: MICA engagement by human Vγ2Vδ2 T cells enhances their antigen-dependent effector function. Immunity 15, 83–93, 2001.
8. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T: Broad tumor-associated expression and recognition by tumor-derived γδ T cells of MICA and MICB. Proc Natl Acad Sci USA 96, 6879–6884, 1999.
9. Seliger B, Abken H, Ferrone S: HLA-G and MIC expression in tumors and their role in anti-tumor immunity. Trends Immunol 24, 82–87, 2003.
10. Pende D, Rivera P, Marcenaro S, Chang CC, Biassoni R, Conte R, Kubin M, Cosman D, Ferrone S, Moretta L, Moretta A: Major histocompatibility complex class I-related chain A and UL16-binding protein expression on tumor cell lines of different histotypes: analysis of tumor susceptibility to NKG2D-dependent natural killer cell cytotoxicity. Cancer Res 62, 6178–6186, 2002.
11. Menier C, Riteau B, Carosella ED, Rouas-Freiss N: MICA triggering signal for NK cell tumor lysis is counteracted by HLA-G1-mediated inhibitory signal. Int J Cancer 100, 63–70, 2002.
12. Pende D, Cantoni C, Rivera P, Vitale M, Castriconi R, Marcenaro S, Nanni M, Biassoni R, Bottino C, Moretta A, Moretta L: Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. Eur J Immunol 31, 1076–1086, 2001.
13. Zwirner NW, Dole K, Stastny P: Differential surface expression of MICA by endothelial cells, fibroblasts, keratinocytes, and monocytes. Hum Immunol 60, 323–330, 1999.
14. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier L, Spies T: Activation of NK and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285, 727–729, 1999.
15. Wu J, Song Y, Bakker ABH, Bauer S, Spies T, Lanier L, Phillips JH: An activating immunoreceptor complex formed by NKG2D and DAP10. Science 285, 730–732, 1999.
16. Bahram S, Inoko H, Shiina T, Radosavljevic M: MIC and other NKG2D ligands: from none to too many. Curr Opin Immunol 17, 505–509, 2005.
17. Bacon L, Eagle RA, Meyer M, Easom N, Young NT, Trowsdale J: Two human ULBP/RAET1 molecules with transmembrane regions are ligands for NKG2D. J Immunol 173, 1078–1084, 2004.
18. Vivier E, Tomasello E, Paul P: Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition? Curr Opin Immunol 14, 306–311, 2002.
19. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ: ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 14, 123–133, 2001.
20. Molinero LL, Fuertes MB, Rabinovich GA, Fainboim L, Zwirner NW: Activation-induced expression of MICA on T lymphocytes involves engagement of CD3 and CD28. J Leukoc Biol 71, 791–797, 2002.
21. Molinero LL, Fuertes MB, Fainboim L, Rabinovich GA, Zwirner NW: Up-regulated expression of MICA on activated T lymphocytes involves Lck and Fyn kinases, and signaling through MEK1/ERK, p38 MAP kinase and calcineurin. J Leukoc Biol 73, 815–822, 2003.
22. Rothenberg EV, Ward SB: A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation. Proc Natl Acad Sci USA 93, 9358–9365, 1996.
23. Kuo CT, Leiden JM: Transcriptional regulation of T lym-

phocyte development and function. Annu Rev Immunol 17, 149–187, 1999.

24. Ballard DW: Molecular mechanisms in lymphocyte activation and growth. Immunol Res 23, 157–166, 2001.
25. Molinero LL, Fuertes MB, Girart MV, Fainboim L, Rabinovich GA, Costas MA, Zwirner NW: NF-κB-regulates expression of the MHC class I related chain A gene (MICA) in activated T lymphocytes. J Immunol 173, 5583–5590, 2004.
26. Zingoni A, Sornasse T, Cocks BG, Tanaka Y, Santoni A, Lanier LL: Cross-talk between activated human NK cells and $CD4^+$ T cells via OX40-OX40 ligand interactions. J Immunol 173, 3716–3724, 2004.
27. Maasho K, Opoku-Anane J, Marusina AI, Coligan JE, Borrego F: NKG2D is a costimulatory receptor for human naive $CD8^+$ T cells. J Immunol 174, 4480–4484, 2005.
28. Zwirner NW, Marcos CY, Mirbaha F, Zou Y, Stastny P: Identification of MICA as a new polymorphic alloantigen recognized by antibodies in sera of organ transplant recipients. Hum Immunol 61, 917–924, 2000.
29. Goldbaum FA, Leoni J, Wallace JC, Fossati CA: Characterization of an 18-kilodalton *Brucella* cytoplasmatic protein which appears to be a serological marker of active infection of both human and bovine brucellosis. J Clin Microbiol 31, 2141–2145, 1993.
30. Hue S, Mention JJ, Monteiro RC, Zhang S, Cellier C, Schmitz J, Verkarre V, Fodil N, Bahram S, Cerf-Bensussan N, Caillat-Zucman S: A direct role for NKG2D/MICA interaction in villous atrophy during celiac disease. Immunity 21, 367–377, 2004.
31. Rabinovich BA, Li J, Shannon J, Hurren R, Chalupny J, Cosman D, Miller RG: Activated, but not resting, T cells can be recognized and killed by syngeneic NK cells. J Immunol 170, 3572–3576, 2003.
32. Trowsdale J: Genetic and functional relationships between MHC and NK receptor genes. Immunity 15, 363–374, 2001.