Activation-induced expression of MICA on T lymphocytes involves engagement of CD3 and CD28

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Abstract: MICA is an HLA-related cell stress-regulated antigen recognized by cytotoxic cells expressing the NKG2D molecule. Although resting lymphocytes do not express MICA, it can be induced on PHA-activated T cells. Here, we demonstrate by Western blot that MICA is induced on allogeneic-activated CD4^{+} and CD8^{+} T lymphocytes. Blocking activation with anti-HLA class I, anti-HLA-DR, or anti-CD86 mAb affected the expression of MICA slightly. When T cells were stimulated with anti-CD3 or anti-CD28 mAb plus PMA, a sustained up-regulation of MICA was observed by Western blot, RT-PCR, and flow cytometry. The expression of MICA reached a plateau at day 4 after CD3 engagement and at day 3 after anti-CD28/PMA stimulation. Conversely, the proliferative response reached a peak at day 4. Hence, CD3 or CD28 engagement induces MICA expression on T lymphocytes. This activation-induced expression might participate in NKG2D-mediated cytotoxicity toward activated T cells to maintain homeostasis during an ongoing immune response. *J. Leukoc. Biol.* 71: 791–797; 2002.

Key Words: *MHC · activation · RT-PCR · HLA*

INTRODUCTION

MICA is a polymorphic gene that maps to the human leukocyte antigen (HLA) region, which encodes for a 383 amino acid polypeptide with three extracellular domains ($\alpha1$, $\alpha2$, and $\alpha3$), one transmembrane region, and a cytoplasmic tail [1]. MICA does not bind peptide [2] and does not associate with β_2-microglobulin [3]. Although it is expressed by many cell types and tumors [3–6], MICA was not detected in peripheral blood CD4^{+}, CD8^{+}, or CD19^{+} lymphocytes [3].

MICA has been suggested to function as cell stress sensor because this antigen is up-regulated by heat shock [5] or oxidative stress [7] in colon adenocarcinoma cell lines. Then, these cells can be recognized and destroyed by intestinal $\gamma\delta$ T lymphocytes [8]. Although MICA has no apparent function in antigen presentation, infection with intracellular pathogens like cytomegalovirus and *Mycobacterium tuberculosis* results in substantial increases in surface expression of MICA [9, 10]. This allows the recognition of infected cells by cells expressing the NKG2D molecule [9–12] and the delivery of a cytotoxic response toward the infected cells.

Although the function of MICA is becoming apparent during the course of an immune response against intracellular pathogens, little is known about its role on activated peripheral blood CD4^{+} and CD8^{+} T lymphocytes [3]. There is still no evidence regarding the mechanisms involved in this up-regulated expression and the physiological stimuli and membrane receptor engagements that may induce this effect.

Because we have demonstrated previously that MICA is expressed on phytohemagglutinin (PHA)-activated T cells, in the present study we investigated physiological activation stimuli that could modulate MICA expression, as well as some of the receptors implicated in this phenomenon. We observed that MICA is induced on allogeneic-activated CD4^{+} and CD8^{+} T lymphocytes. This activation-induced expression involves CD3 or CD28 engagement, and these mechanisms appear to operate simultaneously. These results constitute the first insight into the mechanisms responsible for the expression of the novel HLA-related MICA alloantigen on activated T lymphocytes.

MATERIALS AND METHODS

Reagents

Phorbol-12-myristate-13-acetate (PMA; Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide at 25 μg/ml. PHA (Sigma Chemical Co.) was dissolved in phosphate-buffered saline (PBS) at 1 mg/ml. Stimulating mouse anti-human CD3 monoclonal antibody (mAb; clone SK7) and stimulating mouse anti-human CD28 mAb (clone L293) were obtained from Becton Dickinson (San Jose, CA). The anti-HLA class I monomorphic mAb W6/32 and the anti-HLA-DR monomorphic mAb were used as locally produced ascitic fluids.

Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated from blood donors from healthy human volunteers by Ficoll-Paque™ Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, washed with RPMI 1640 (Sigma Chemical Co.), and resuspended in RPMI 1640 supplemented with 10% heat-inactivated, pooled normal human AB serum, sodium pyruvate, glutamine, and penicillin-streptomy-

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cin (RPMI/PHS). Alternatively, for anti-CD3- or anti-CD28/PMA-stimulated PBMCs, cells were resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Gaithersburg, MD), sodium pyruvate, glutamine, and penicillin-streptomycin (RPMI/FCS).

Allogeneic stimulation of T cells and isolation of $CD4^+$ and $CD8^+$ T lymphocytes

PBMCs resuspended in RPMI/PHS were stimulated with mitomycin-treated allogeneic cells from fully HLA-mismatched donors (previously typed by SSOP at the Tissue Typing Section of our laboratory) and were cultured for 3–9 days in 96-well, "U"-bottomed polystyrene plates (Becton Dickinson Labware, Franklin Lakes, NJ). Blocking experiments were performed by adding the anti-HLA class I monomorphic mAb W6/32, the anti-HLA-DR monomorphic mAb L243 or an isotype-matched negative control mAb [13] to the cultures, as ascitis diluted 1/1000. Blocking experiments were also performed with an anti-CD86 mAb (clone FUN-1, Pharmingen, San Diego, CA) by preincubating the stimulator cells with the mAb at 20 μg/ml for 30 min at room temperature, after which responder cells were added and cultured for 7 days. Cultured cells were then used for proliferation assays, flow cytometry, or Western blot analysis. Percentage of inhibition of proliferation was calculated as $100 - 100 \times (cpm_x - cpm_{autologous})/(cpm_{allogeneic} - cpm_{autologous})$, where x is the mAb used for blocking. Statistical analysis was performed by applying the parametric ordinary analysis of variance test with Bonferroni's correction.

$CD4^+$ and $CD8^+$ T cells were isolated from allogeneic-activated PBMCs after 7 days of stimulation with anti-CD4 mAb-coated and anti-CD8 mAb-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway), respectively. Positively selected cells were washed and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

Mitogen and mAb stimulation of PBMCs

PBMCs resuspended in RPMI/PHS were stimulated with PHA (1 ng/ml), PMA (1 ng/ml), anti-CD3 mAb (12.5 μg/ml), anti-CD28 mAb (0.5 μg/ml), or anti-CD28 mAb (0.5 μg/ml) plus PMA (1 ng/ml) and were cultured for 1–9 days in 96-well, "U"-bottomed plates (Becton Dickinson Labware). Cultured cells were then used for proliferation assays or Western blot analysis. In some experiments, PBMCs were resuspended in RPMI/FCS, stimulated as before, and used for proliferation assays, flow cytometry, or Western blot analysis.

Proliferation assay

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

Reverse transcriptase-polymerase chain reaction (RT-PCR) and hybridization

RNA was extracted from resting and anti-CD3-, anti-CD28-, PMA-, and anti-CD28/PMA-activated T cells using Trizol® (Life Technologies) reagent, following procedures recommended by the manufacturer. Retrotranscription into cDNA was performed with the Advantage™ RT-for-PCR kit (Clontech, Palo Alto, CA), using oligo $(dT)_{18}$ primer and Moloney murine leukemia virus retrotranscriptase. PCR reactions were normalized with β-actin using the 5′ primer TGACGGGGTCACCCACACTGTGCCCATCTA and the 3′ primer CTAGAAGCATTTGCGGTGGACGATGGAGGG. PCR reactions were run in a PTC-100 thermocycler (MJ Research, Watertown, MA) with 2 mM $MgCl_2$ and 0.5 U Taq polymerase (T-plus, Tandil, Argentina) per tube. PCR conditions were as follows: 94°C for 5 min, 66°C for 5 min, 62°C for 2.5 min, 30 cycles at 94°C for 1 min, 66°C for 1 min, and 72°C for 1 min and final extension at 72°C for 5 min. MICA exons 2–3 were amplified by PCR using the primers MA109C (GAGCCCCACAGTCTTCGTTAT) and MA173 (CCTGACGTTCATGGCCAA). PCR reactions were performed with 1.5 mM $MgCl_2$ and 0.5 U Taq polymerase per tube. PCR conditions were as follows: 94°C for 3 min, 55°C for 2 min, 72°C for 5 min, 36 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min and final extension at 72°C for 15 min. PCR products were separated by electrophoresis on 1.5% agarose gels and blotted onto N^+-Hybond membranes (Amersham Pharmacia Biotech). UV-cross-linked membranes were hybridized with ^{32}P-labeled, MICA-specific probe (ACAGGGAACGGAAAGGACC) or β-actin-specific probe (CGCAAAGACCTGTACGCCAA) at 42°C. After washing at 58°C (MICA probe) or 60°C (β-actin probe), membranes were exposed to Kodak X-OMAT XK1 films (Kodak, Sao Paulo, Brazil).

Anti-MICA rabbit sera

Polyclonal rabbit antibodies against MICA were obtained as described previously [3]. Briefly, serum #620 was raised against a peptide corresponding to amino acid residues 42–60 of the translated sequence of MICA, and sera #621 and #622 were raised against a peptide corresponding to amino acid residues 140–160 of the translated sequence of MICA [1]. These sera have been shown to react with recombinant, soluble MICA by enzyme-linked immunosorbent assay [14] and Western blot (unpublished results). Sera #620 and #621 react with different cell lysates by Western blot [3, 4], and serum #622 reacts with different cells by flow cytometry [3, 4]. Specificity for MICA was confirmed by peptide-neutralization assays [4] and by detection of this protein by Western blot and flow cytometry in MICA-transfected COS-7 cells (unpublished results).

SDS-PAGE and Western blot

Cells were washed three times with PBS and lysed with 1% 3-[(3-chloramidopropyl)dimetylammonio]-1-propanesulfonate (Sigma Chemical Co.) in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, in the presence of a mixture of protease inhibitors (Sigma Chemical Co.). Protein concentration of lysates was measured with the Micro BCA kit (Pierce, Rockford, IL).

Denaturing (0.1% SDS), discontinuous gel electrophoresis (SDS-PAGE) under reducing conditions and Western blots were performed as described previously [3]. The same amount of proteins from different samples was loaded onto the gels (10 or 20 μg, depending on the experiment). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech), and equal loading was confirmed by Ponceau S staining. Blocked PVDF membranes were incubated with a pool of anti-MICA sera #620 and #621, diluted 1/10000. Bound Ab were detected with peroxidase-labeled anti-rabbit immunoglobulin G (Bio Rad, Hercules, CA) and chemiluminescent detection with the enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech) and Kodak BioMax films. No bands were observed in Western blots incubated with normal rabbit sera.

Films were analyzed with the Scion Image Analysis software (Scion Corp., Frederick, MD). Background was substracted from the images, and after tresholding and binary conversion, the intensity of each band was recorded and expressed as arbitrary units (AU).

Flow cytometry

Cell populations in allogeneic-activated cultures were analyzed using fluorescein isothiocyanate- or phycoerythrin-labeled commercial mAb specific for CD3, CD4, CD8, and CD14 (Becton Dickinson) and were read in an Ortho Cytoron flow cytometer (Ortho, Raritan, NJ). MICA expression on activated T cells was analyzed by flow cytometry with serum #622. Normal rabbit serum (NRS) was used as negative staining control. Dot plots of forward scatter versus green fluorescence staining (FL1=MICA expression) were obtained from data representing resting lymphocytes and blasts, which were gated from dot plots of side scatter versus forward scatter of whole PBMCs.

RESULTS

Because the HLA-encoded MICA alloantigen can be induced on peripheral $CD4^+$ and $CD8^+$ T lymphocytes after stimulation with PHA [3], we investigated whether a physiologically relevant stimulus may induce the same effect, assessing the expression of MICA after allogeneic stimulation between PBMCs from fully HLA-mismatched donors. As shown in **Figure 1a**, MICA expression was induced, showing a peak of expression at day 7 after stimulation. Resting PBMCs did not show

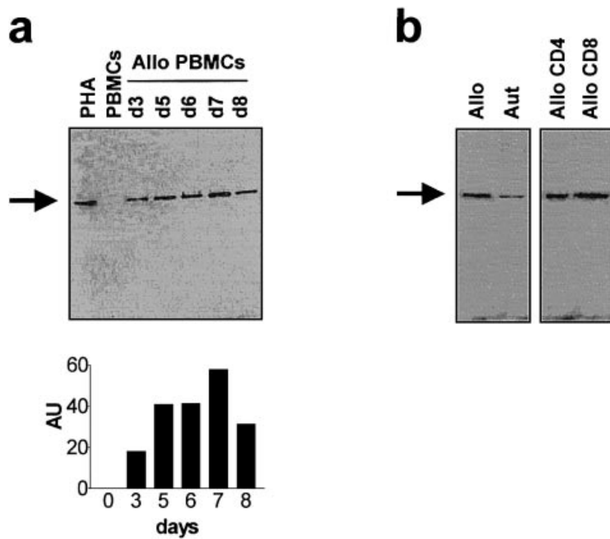


Fig. 1. Induction of MICA expression on allogeneic-activated CD4$^+$ and CD8$^+$ T cells. (a) Kinetic analysis of MICA expression in PBMCs stimulated with allogeneic PBMCs for 3–8 days (top panel). As controls, PHA-activated T cells (PHA) and unstimulated PBMCs (PBMCs) were also analyzed. The arrow indicates the ~65-kDa band corresponding to MICA. The intensity of each band was quantified by densitometric analysis, and results were expressed in AU (bottom panel). (b) Western blot of lysates of allogeneic-activated PBMCs (Allo), autologous-stimulated PBMCs (Aut), CD4$^+$ (Allo CD4), and CD8$^+$ (Allo CD8) T cells isolated with magnetic beads after 7 days of allogeneic stimulation. The arrow indicates the ~65-kDa band corresponding to MICA. The results shown are representative of three independent experiments performed with different blood donors and different allogeneic combinations.

MICA expression, and autologous cultures produced a very faint band (Fig. 1b), which we interpreted as a slight degree of stimulation by autologous cells. Proliferation measurements showed a SI of 31.1 (45,591±7612 cpm for the allogeneic culture and 1468±189 cpm for the autologous control) at day 6 of stimulation (not shown).

To investigate the cell subpopulations that contribute to MICA expression, we isolated CD4$^+$ and CD8$^+$ T cells using mAb-coated magnetic beads. The results show that CD4$^+$ and CD8$^+$ T-lymphocyte subpopulations are induced to express MICA after activation with allogeneic cells (Fig. 1b). Flow cytometry analysis of the cell populations in the allogeneic cultures after 7 days of stimulation showed ~87% of CD3$^+$ cells. Sixty-seven percent of these T cells were CD4$^+$ T cells, and the other 33% were CD8$^+$ T cells. Monocytes, which have been shown to express MICA [3, 4], are very rare cells in the cell suspension (<0.04% of CD14$^+$ cells; not shown).

To investigate surface receptors involved in this up-regulated expression, we performed blocking experiments using an anti-HLA class I monomorphic mAb (W6/32) and an anti-HLA-DR monomorphic mAb (L243) to block activation through signal 1 or an anti-CD86 mAb to block costimulation (signal 2). As expected, a profound inhibition of ^{3}H-Thy uptake was induced by each mAb (**Fig. 2, a** and **c**). In contrast, inhibition of proliferation was accompanied by only a weak inhibition of MICA expression when the anti-HLA class I mAb, the anti-HLA-DR mAb (Fig. 2b), or the anti-CD86 mAb (Fig. 2d) was used. These results apparently indicate that induction of MICA by allogeneic stimulation of T cells would be independent of T-cell receptor (TCR)/CD3 engagement and also independent of the interaction between CD28 and CD86. Nevetheless, it is likely that these surface molecules might actually trigger MICA expression and that their blockade might be compensated by engagement of other cell-surface molecules by their putative ligands on the surface of the antigen-presenting cells (APC).

Next, we investigated whether CD3 and CD28 engagement participate in the inducible expression of MICA. We analyzed

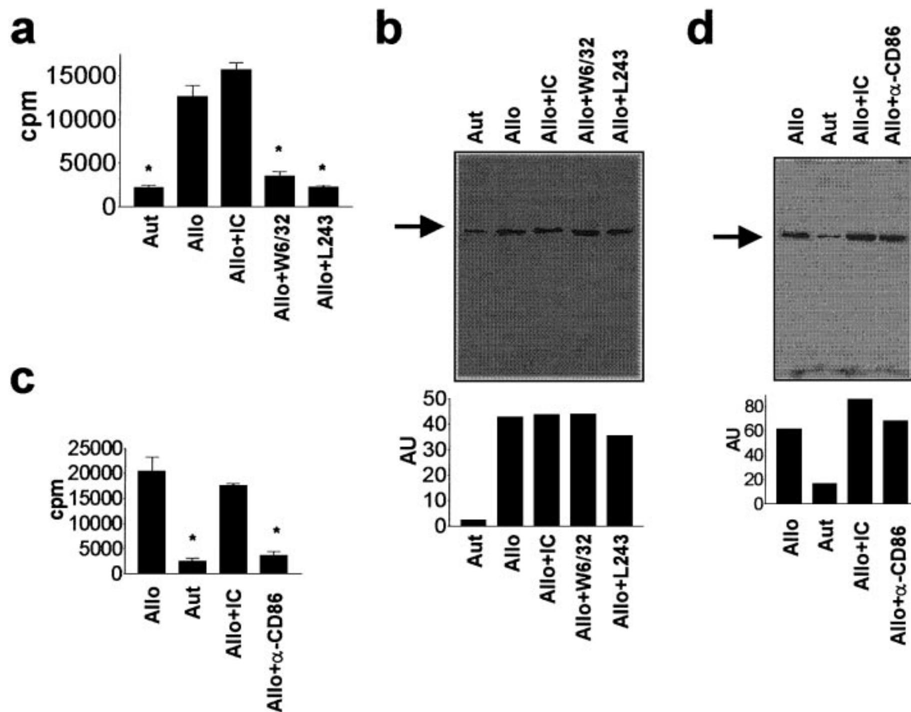
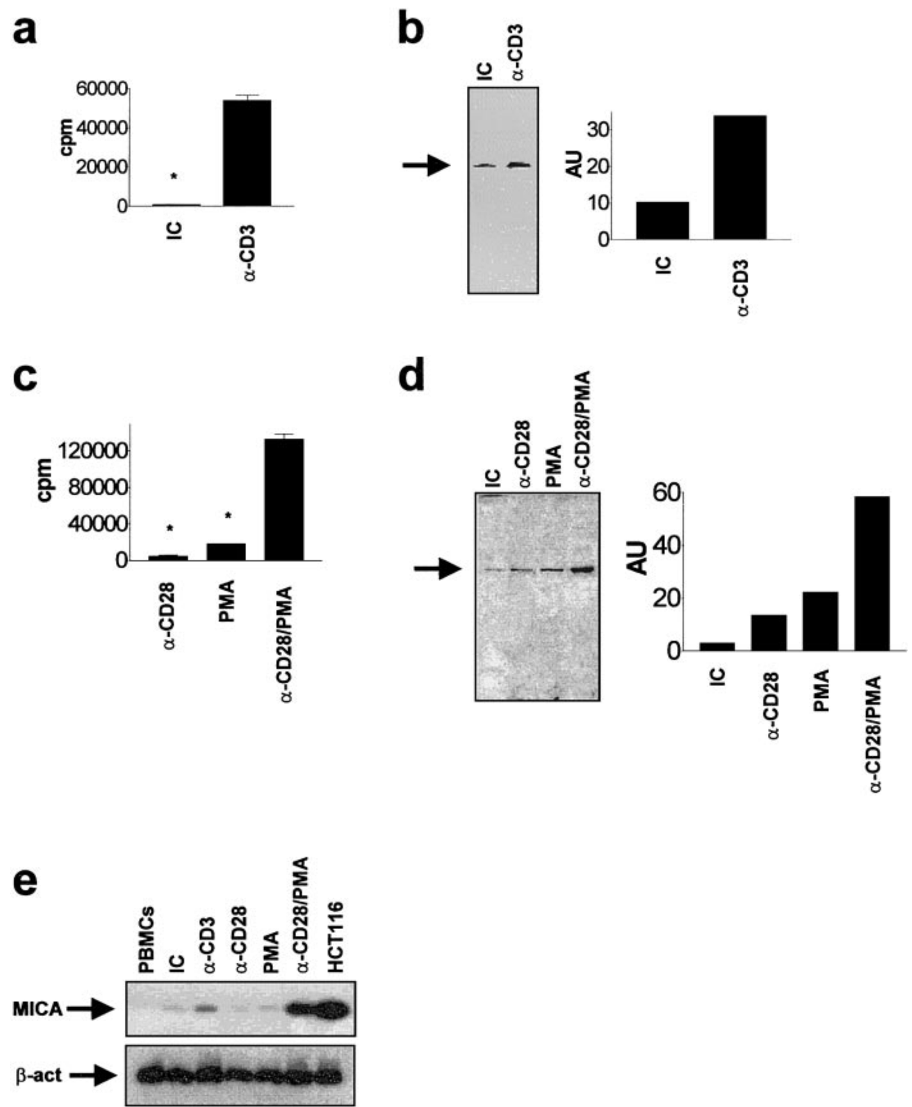


Fig. 2. Blocking of allogeneic activation affects MICA induction minimally. Effect of blocking signal 1 with an anti-HLA class I mAb (W6/32) or an anti-HLA-DR mAb (L243) on the proliferative response (a) or on MICA expression (b) on allogeneic-activated PBMCs (Allo). An isotype-matched mAb (IC) was used as negative blocking control, and autologous cultures (Aut) were used to assess background of ^{3}H-Thy uptake. Effect of blocking signal 2 (costimulation) with an anti-CD86 mAb (α-CD86) on the proliferative response (c) or on MICA expression (d) of allogeneic-activated PBMCs. The arrow indicates the ~65-kDa band corresponding to MICA. Western blots were quantified by densitometric analysis, and results were expressed in AU (bottom panel of each Western blot). The results shown are representative of three independent experiments performed with different blood donors and different allogeneic-stimulation combinations.

Fig. 3. Induction of MICA expression on anti-CD3- and anti-CD28/PMA-stimulated PBMCs. Proliferative response (a) and Western blot for MICA (b) on PBMCs stimulated with anti-CD3 mAb (α-CD3) or with the isotype-matched, negative control mAb (IC). Proliferative response (c) and Western blot for MICA (d) on PBMCs stimulated with anti-CD28 mAb (α-CD28), PMA, anti-CD28 plus PMA (α-CD28/PMA), or the isotype-matched, negative control mAb (IC). The arrow indicates the ~65-kDa band corresponding to MICA. The intensity of each band was quantified by densitometric analysis, and the results were expressed in AU (right panel). (e) RT-PCR and hybridization with the ^{32}P-labeled, MICA-specific probe on RNA extracted from isotype control (IC)-, anti-CD3 mAb (α-CD3)-, anti-CD28 mAb (α-CD28)-, PMA-, or anti-CD28 plus PMA (α-CD28/PMA)-stimulated T cells (top panel). PCR reactions were normalized against β-actin (bottom panel). As positive control for the RT-PCR, RNA from the HCT116 colon adenocarcinoma cell line, shown previously to express MICA, was analyzed. The results shown are representative of three independent experiments performed with three different blood donors.



its expression on T cells stimulated with an anti-CD3 mAb or an anti-CD28 mAb plus suboptimal doses of PMA in the presence of autologous accessory cells (**Fig. 3**). The rationale for using stimulations of PBMCs with anti-CD28 + PMA instead of stimulations with anti-CD28 + anti-CD3 relies on our purpose to investigate the role of signaling through CD28 in the absence of engagement of the TCR/CD3 complex. The results obtained show that both signals induced a strong proliferative response, with SI of 61.9 and 122.8 for anti-CD3 and anti-CD28/PMA (Fig. 3, a and c, respectively). Western blot analysis showed that engagement of CD3 and CD28 induced up-regulated expression of MICA protein (Fig. 3, b and d, respectively), which represents a 3.3-fold and a 19.4-fold increase over control cells incubated with a negative control mAb, respectively). The transcriptional activation of the MICA gene on stimulated T cells was confirmed by RT-PCR and hybridization with a MICA-specific probe (Fig. 3e). A kinetic analysis of the induction of the expression of MICA was also performed on PBMCs activated with anti-CD3 or anti-CD28/PMA (**Fig. 4**). Whereas the peak of proliferation was reached at day 4 in both cases (Fig. 4a), both stimuli produced a sustained induction of the expression of MICA, which reached a plateau at day 4 after CD3-mediated stimulation and at day 3 after CD28/PMA-mediated stimulation (Fig. 4, b and c). Taken together, these results suggest that T cells activate MICA transcription and that they express sustained levels of MICA protein after CD3 engagement or CD28 costimulation in the presence of autologous accessory cells, even when they ceased to incorporate the ^{3}H-Thy.

Finally, we addressed whether MICA expression is also induced on the cell surface of activated T lymphocytes, performing flow cytometry on unstimulated T cells and anti-CD3- and anti-CD28/PMA-stimulated T lymphocytes (**Fig. 5**). The results demonstrate that >45% of anti-CD3-stimulated T lymphocytes up-regulated expression of MICA at day 3 of stimulation (Fig. 5, c and d). In addition, >52% of anti-CD28/PMA-stimulated T cells increased MICA expression at day 3 of stimulation (Fig. 5, e and f). The scatter properties of MICA-expressing cells demonstrate that some of the anti-CD3-stimulated cells have a size compatible with the size of resting T lymphocytes, and some are blasting. Conversely, almost all MICA-expressing, anti-CD28/PMA-stimulated cells are blasts. These results confirm that up-regulated expression of MICA observed on activated T cells

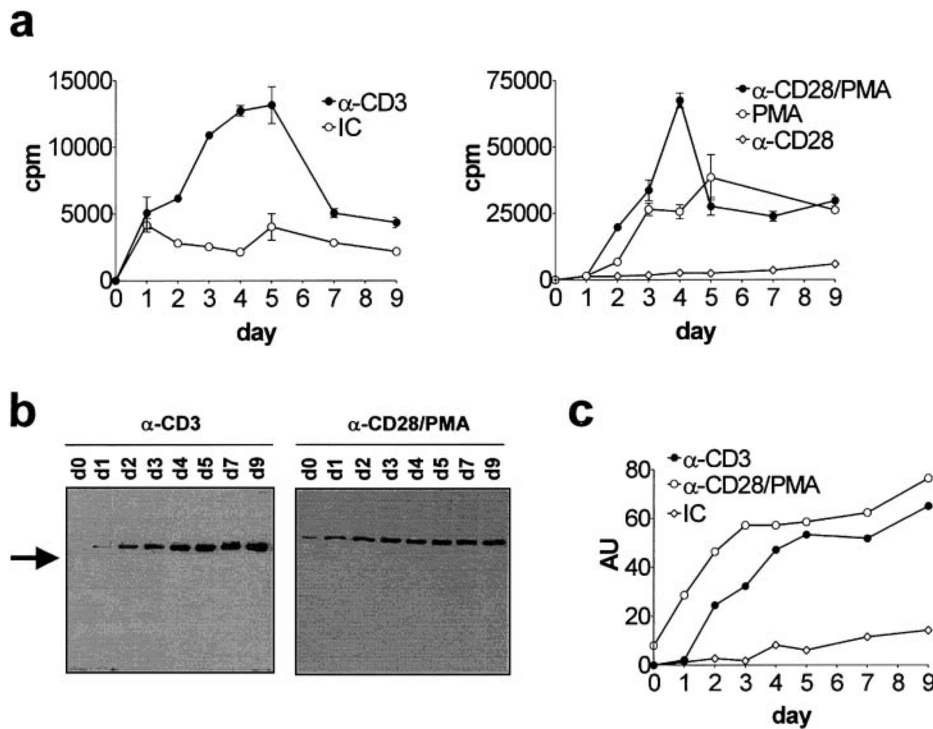


Fig. 4. Kinetic analysis of the expression of MICA in anti-CD3- and anti-CD28/PMA-stimulated PBMCs. (a) Proliferation of PBMCs stimulated with anti-CD3 mAb (α-CD3) or with the isotype-matched, negative control mAb (IC). (a, left panel) Proliferation of PBMCs stimulated with anti-CD28 mAb plus PMA (α-CD28/PMA), PMA, or anti-CD28 mAb (α-CD28) (right panel). (b) Western blot for the detection of MICA on PBMCs stimulated with anti-CD3 mAb (α-CD3, left panel) or with anti-CD28/PMA (α-CD28/PMA, right panel) for 1–9 days. Unstimulated cells (d0) were also analyzed. The arrow indicates the ~65-kDa band corresponding to MICA. (c) Densitometric analysis of the intensity of each band (expressed as AU) along the culture with anti-CD3 mAb (α-CD3), anti-CD28/PMA (α-CD28/PMA), or the isotype-matched, negative control mAb (IC). The results shown are representative of different experiments performed with three different blood donors.

by Western blots correlates with up-regulated expression of this molecule on the cell surface.

DISCUSSION

In the present study, we provide the first evidence showing that physiological stimuli such as TCR/CD3 engagement in the presence of autologous accessory cells and costimulation through CD28 up-regulate MICA expression on T cells. It has been demonstrated previously that the expression of MICA can be induced in PHA-activated $CD4^+$ and $CD8^+$ T lymphocytes [3]. However, it is well known that some biological responses elicited by mitogens are not equivalent to physiological stimuli for T cells [15]. By activating PBMCs using fully HLA class I and class II-mismatched allogeneic cells, we demonstrated that expression of MICA is strongly up-regulated on $CD4^+$ and $CD8^+$ T lymphocytes, with a peak of expression at day 7 after stimulation. This result is coincident with the peak of proliferation of T cells in allogeneic cultures [16]. The band corresponding to MICA was not detected in resting (unstimulated) PBMCs, although it has been detected previously in monocytes [3, 4]. This apparent discrepancy arises from the fact that monocytes constitute <10% of the cells in the PBMC preparation isolated by gradient centrifugation, and the amount of proteins derived form these monocytes loaded onto the gels used for the Western blots is below the detection limit of MICA (unpublished results). Furthermore, there are almost no monocytes after 7 days of culture (allogeneic stimulation), most likely because they remain attached to the plastic wells when cells were harvested for Western blot analysis. This was confirmed by flow cytometry, in which we detected <0.04% of $CD14^+$ cells in allogeneic-activated PBMCs harvested after 7 days of culture.

To investigate how receptor engagement modulates MICA expression, we performed blocking experiments using an anti-HLA class I mAb, an anti-HLA-DR mAb, and an anti-CD86 mAb. We did not perform blocking experiments with anti-CD80 mAb because CD80 is induced only after 3–4 days of stimulation, but CD86 is expressed constitutively on APC [17]. In addition, preliminary experiments confirmed that blocking CD80 reduced the proliferative response by only 20–30% (not shown), and blocking CD86 showed a pronounced effect (Fig. 2c). Hence, CD86 appears to be more important as a costimulatory molecule in our system, and the results presented support our hypothesis that multiple signaling pathways trigger MICA expression on T cells. In all cases, MICA expression remained unchanged, suggesting that MICA expression might be triggered by engagement of multiple receptors. Consequently, blockade of one of these receptors might be compensated by the engagement of other cell-surface molecules. In this sense, dissociation of the proliferative response from MICA expression may reflect the use of different but partially overlapped signaling routes responsible for cell-cycle progression and MICA expression. Direct stimulation through CD3 or CD28 in the presence of autologous APC induced transcriptional activation of the MICA gene and the expression of MICA protein on the cell surface of activated T cells, demonstrating clearly that signaling through these receptors actually participates in MICA expression. Nevertheless, it is likely that signaling through other costimulatory molecules such as ICOS [18], SLAM [19], CD154 [18], or other surface molecules may also contribute to MICA expression. The finding that cells expressed high levels of MICA even when they ceased to proliferate may reflect an accumulation of MICA, although it is likely that MICA expression may be a terminal event that "labels" the cell for destruction by cytotoxic NKG2D-expressing cells under still-unknown conditions.

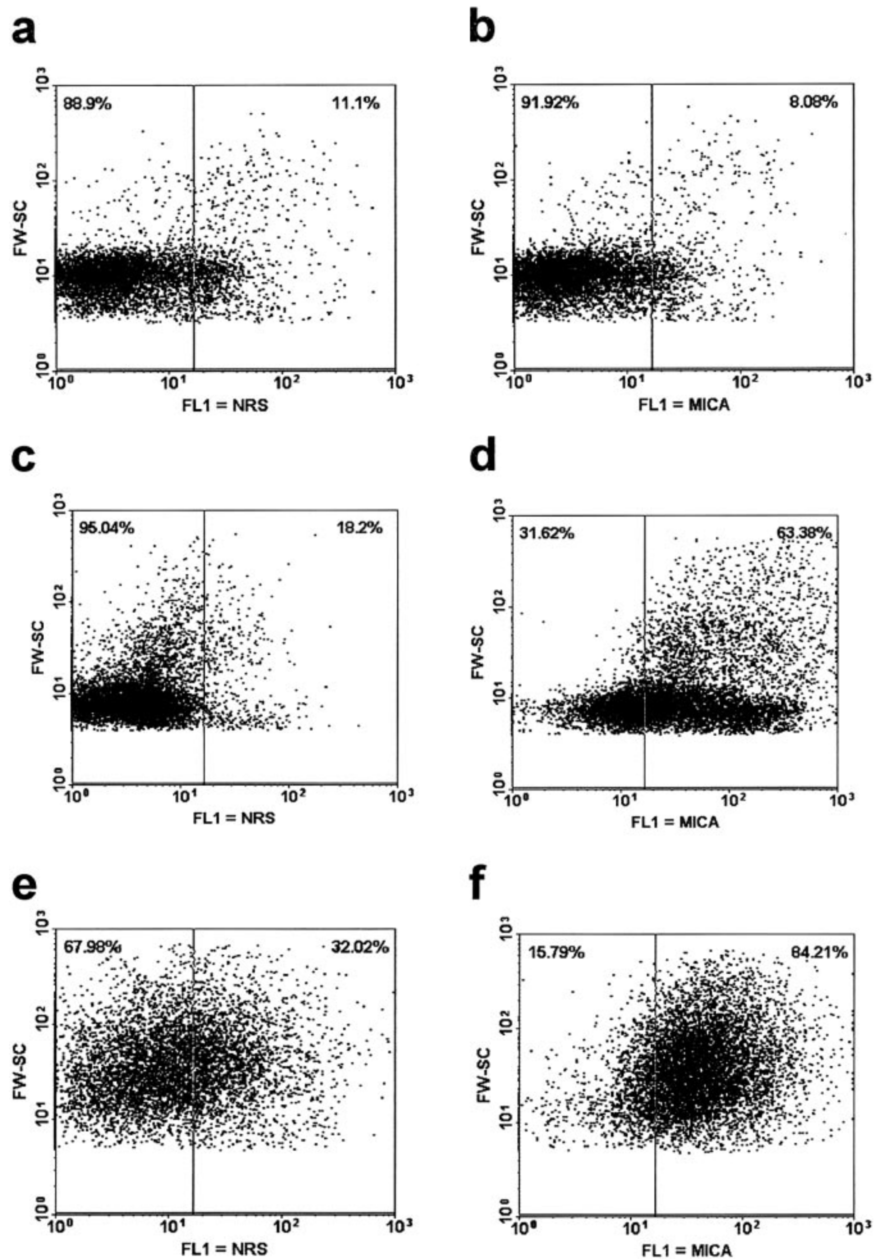


Fig. 5. Induction of surface MICA expression on PBMCs stimulated for 3 days with anti-CD3 mAb or anti-CD28 mAb + PMA. Flow cytometry of unstimulated (a and b) and anti-CD3- (c and d) and anti-CD28/PMA-stimulated PBMCs (e and f) stained with negative control serum (NRS; a, c, and e) or with anti-MICA serum #622 (b, d, and f). The data are presented in two dimensions with MICA expression on the *x*-axis and forward scatter on the *y*-axis. These data correspond to resting + activated (blasting) T lymphocytes, gated from the side scatter versus forward scatter dot plots. The percentage of cells in each panel is expressed as inset. Quadrants were established with the negative control Ab used for the flow cytometries. Isotype control-, anti-CD28-, or PMA-stimulated T cells (not shown) produced similar dot plots as unstimulated T cells (a and b). The results shown are representative of three independent experiments performed with three different blood donors.

Our results demonstrate that engagement of CD3 and CD28 surface receptors triggers MICA expression. These alternative or redundant mechanisms would probably converge to activate specific intracellular mediators and transcription factors. Experiments to address these issues are currently in progress in our laboratory.

The expression of MICA by proliferating T lymphocytes and its detection in different cell lines and tumors [1, 3, 6] suggest that MICA could be a cell proliferation sensor. These findings indicate that proliferation signals, as well as previously described stress stimuli, can activate expression of this polymorphic HLA-related alloantigen. Hence, it appears that many factors that disrupt T-cell homeostasis induce the expression of MICA, extending the previous suggestion that MICA operates not only as a cell stress sensor but also as a proliferation/activation sensor or, more generally, as a cell homeostasis sensor. Its induction during the course of an Ag-specific or allogeneic-immune response on T lymphocytes might be a signal for recognition by cytotoxic cells expressing NKG2D. This mechanism might destroy MICA-expressing T cells and might operate in conjunction with other known mechanisms to shut off immune-effector functions such as apoptosis induced by Fas engagement [20]. Accordingly, it has been shown that stress renders T-cell blasts sensitive to killing by activated syngeneic natural killer (NK) cells [21] and that normal PHA-induced lymphoblasts can be destroyed by NK cell clones in an NKG2D-dependent way [22]. However, formal proof of the involvement of MICA in this event has yet to be provided. Thus, the multiple surface receptor engagements that lead to MICA expression on activated T lymphocytes might constitute a novel mechanism of regulation of uncontrolled T-cell activation during an ongoing immune response and may reveal potential targets for immune intervention to modulate MICA expression in pathological situations.

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