Up-regulated expression of MICA on activated T lymphocytes involves Lck and Fyn kinases and signaling through MEK1/ERK, p38 MAP kinase, and calcineurin

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Abstract: Major histocompatibility complex class I-related chain (MICA) is a cell stress-regulated molecule recognized by cytotoxic cells expressing the NKG2D molecule. MICA can be induced on T cells after CD3 or CD28 engagement. Here, we investigated the intracellular pathways leading to activation-induced expression of MICA. The Src kinase inhibitor PP1 inhibited up-regulated expression of MICA on anti-CD3-stimulated T cells. Downstream signaling routes involved mitogen-activated protein kinase (MAPK) kinase (MEK)1/extracellular signal-regulated kinase (ERK), p38 MAPK, and calcineurin, as MICA expression was prevented by U0126, SB202190, cyclosporin A, and FK506. Also, Lck and Fyn as well as MEK1/ERK and p38 MAPK were found to regulate MICA expression in anti-CD28/phorbol 12-myristate 13-acetate-stimulated T cells. Expression of MICA on activated T cells involved interleukin-2-dependent signaling routes triggered by Janus tyrosine kinases/signal transducer and activators of transcription and p70^{S6} kinase, as it could be inhibited by AG490 and rapamycin. This is the first demonstration of the intracellular pathways involved in activation-induced expression of MICA, which may reveal potential targets for immune intervention to modulate MICA expression in pathological disorders. *J. Leukoc. Biol.* 73: 815–822; 2003.

Key Words: *major histocompatibility complex · signaling pathways*

INTRODUCTION

Major histocompatibility complex class I-related chain (MICA) is an human leukocyte antigen-related, codominantly expressed polymorphic gene, which encodes for a surface-expressed, nonantigen-presenting polypeptide of ~65 kDa [1–4], which exhibits a restricted pattern of tissue expression [3, 5–7]. MICA is a cell stress sensor, up-regulated by heat shock [6], oxidative stress [8], or infection with virus [9] or intracellular bacteria [10]. MICA induction triggers recognition of stressed cells by γδ T cells [10, 11], peripheral blood CD8$^+$, CD28$^-$ αβ T lymphocytes [10], and natural killer (NK) cells [12] by interaction with the cytotoxicity-activating, lectin-like NKG2D molecule [12, 13].

We have recently demonstrated that expression of MICA can be induced on peripheral blood CD4$^+$ and CD8$^+$ T lymphocytes stimulated with allogeneic cells, involving engagement of CD3 and costimulation through CD28 [14]. However, there is still no evidence about the molecular mechanisms and intracellular pathways involved in this up-regulated expression.

Full T cell activation requires the integration of signals triggered by CD3 engagement and costimulation through CD28 [15]. Proximal events after engagement of the T cell receptor (TCR)/CD3 complex are phosphorylation and activation of two Src family kinases, Lck and Fyn [15]. Cooperative integration between Lck and Fyn is required for optimal phosphorylation/activation of the ζ-associated protein-70 and the Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa [16], which triggers activation of a plethora of downstream signaling molecules [15]. Important components of these intracellular mediators are the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK), c-jun NH_2-terminal kinase (JNK), and p38. In turn, these kinases activate specific transcription factors and early immune response genes such as the interleukin (IL)-2 and IL-2 receptor (R) genes [17]. Disruption of the MAPK kinase (MEK)1/ERK pathway with specific inhibitors suppresses the proliferative response as well as the production of several cytokines by T lymphocytes [18]. TCR ligation also induces a rapid phosphorylation and activation of the JNK [19] and p38 MAPK [20].

In addition, the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin also plays an important role in T cell activation. Activation of calcineurin by a rise in cytosolic Ca^{2+} levels induced by TCR engagement induces dephosphorylation of the cytosolic transcription factor, nuclear factor of activated T cells (NFAT), which is then translocated to the nucleus, inducing transcription of early immune-response genes in conjunction with other transcription factors such as

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activated protein-1 (AP-1) [21]. The activity of calcineurin can be blocked with inhibitors such as cyclosporin A (CsA) and FK506, which bind to a group of intracellular proteins called immunophilins [22].

Conversely, costimulation through CD28 on T cells also leads to activation of the Src family protein tyrosine kinases Lck and Fyn [23, 24], which integrate signals elicited by TCR/CD3 engagement necessary to achieve full T cell activation [15]. It has been demonstrated that CD28 engagement increases ERK signaling [25], JNK activation [26, 27], and p38 MAPK activity [28], highlighting their role for integration of signals 1 and 2 during T lymphocyte activation.

A key step in T lymphocyte activation is CD3- and CD28-induced secretion of IL-2. Upon interaction with the activation-inducible, high-affinity IL-2R, this cytokine activates the Janus tyrosine kinase (Jak)/signal transducer and activator of transcription (STAT) pathway [29, 30] and other intracellular kinases such as the $p70^{S6}$ kinase [31]. Jak1 and Jak3 as well as STAT5 and STAT3 have been demonstrated to participate in IL-2-triggered intracellular activation pathways [29].

In the present study, we investigated the molecular mechanisms and intracellular pathways involved in activation-induced expression of MICA. We observed that up-regulated expression of MICA on T lymphocytes stimulated by CD3 engagement involves activation of Lck and Fyn kinases and subsequent signaling through simultaneous routes that proceed through MEK1/ERK, p38 MAPK, and calcineurin. Moreover, up-regulated expression of MICA on T lymphocytes stimulated by CD28 engagement and phorbol 12-myristate 13-acetate (PMA) also involves the Src kinases Lck and Fyn, signaling through MEK1/ERK and p38 MAPK but not calcineurin. Part of these effects operates through induction of IL-2-dependent, intracellular signaling routes triggered by the Jak/STAT pathway and activation of the $p70^{S6}$ kinase.

MATERIALS AND METHODS

Reagents

U0126, SB202190, and rapamycin were purchased from Calbiochem (La Jolla, CA). AG490 (an inhibitor of Jak3) and PP1 (an Src family kinase inhibitor) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The drugs were dissolved in dimethyl sulfoxide (DMSO) at 5, 2, 1, 25, and 10 mM, respectively. CsA was kindly provided by Novartis Argentina (Buenos Aires). FK506 (Fujisawa Ireland, Kerry) was kindly provided by Gador Argentina (Buenos Aires). PMA (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO at 25 ng/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). Anti-ERK polyclonal Ab, antiphosphorylated ERK (anti-pERK) mAb, and peroxidase-conjugated antiphosphotyrosine-specific mAb were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Peripheral blood mononuclear cell (PBMC) isolation and stimulation

PBMCs were isolated from blood donors from healthy human volunteers by Ficoll-Paque™ Plus (Amersham Biosciences, Piscataway, NJ) gradient centrifugation, washed with RPMI 1640 (Sigma Chemical Co.), and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Life Technologies, Gaithersburg, MD), sodium pyruvate, glutamine, and penicillin-streptomycin (RPMI/FCS). PBMCs were stimulated with PMA (0.5 ng/ml), anti-CD3 mAb (25 ng/ml), anti-CD28 mAb (500 ng/ml), anti-CD28 mAb (0.5 μg/ml) plus PMA (0.5 ng/ml) and were cultured for 3 days in 96-well, "U"-bottomed plates or in 24-well, flat-bottomed plates (Becton Dickinson Labware). In some experiments, PBMCs were preincubated for 30 min at 37°C with pharmacologic inhibitors of intracellular signaling molecules. In other set of experiments, inhibitors were added to the cultures at the moment of the mAb stimulation. Control experiments were performed, stimulating PBMCs with an isotype-matched, negative-control mAb [32]. Cultured cells were then used for proliferation assays 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 counts per minute (cpm) of triplicate wells ± SD. Percentages of inhibition of proliferation induced by each drug were calculated as $100 - 100 \times (cpm_{drug} - cpm_{background})/(cpm_{without\ drug} - cpm_{background})$, where "drug" represents the cpm obtained in the presence of the drug, "without drug" represents the cpm obtained in mAb-stimulated cells in the absence of drugs, and "background" represents the background ^{3}H-Thy incorporation obtained with the isotype-matched, negative-control mAb. Statistical analysis was performed applying the parametric, ordinary ANOVA test with Bonferroni's correction.

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 serum #621 was raised against a peptide corresponding to amino acid residues 140–160 of the translated sequence of MICA [1]. Their reactivity and specificity have been characterized previously [3, 5, 33].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Cells were washed three times with phosphate-buffered saline 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 (Tris-buffered saline), in the presence of a mixture of protease inhibitors (Sigma Chemical Co.) and 1 mM sodium *ortho*-vanadate. Protein concentration of lysates was measured with the Micro bicinchoninic acid kit (Pierce, Rockford, IL). Denaturing 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 Biosciences), 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 1/10,000), anti-ERK (1/3000), or anti-pERK (1/1000) Ab. Bound Ab were detected with peroxidase-labeled anti-rabbit or mouse immunoglobulin G (Bio-Rad, Hercules, CA) and chemiluminescent detection with the enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences) and Kodak BioMax films. No bands were observed in Western blots incubated with normal rabbit sera. In other experiments, PVDF membranes were incubated with peroxidase-conjugated, antiphosphotyrosine mAb PY-20 for 3 h and were developed by chemiluminiscent detection as described.

Films were analyzed with the Scion image analysis software (Scion, 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). The percentage of inhibition of MICA expression induced by the drugs was calculated as $100 - 100 \times (AU_{drug} - AU_{background})/(AU_{without\ drug} - AU_{background})$, where "drug" represents the AU obtained in the presence of each drug, "without drug" represents the AU obtained in mAb-stimulated cells in the absence of drugs, and "background" represents the background AU obtained with the isotype-matched, negative control mAb.

Flow cytometry

Cell viability after culture with different drugs and inhibitors was checked by propidium iodide (PI) exclusion and monitored by flow cytometry using an Ortho cytoron flow cytometer (Ortho, Raritan, NJ). Apoptosis was assessed by analyzing the percentage of hypodiploid cells after culture with different drugs and inhibitors, as described previously by Nicoletti et al. [34].

RESULTS

We have recently demonstrated that activation-induced expression of MICA on T lymphocytes involves CD3 or CD28 engagement [14]. We now investigated the molecular mechanisms implicated in this phenomenon. As Lck and Fyn tyrosine kinases have been demonstrated to initiate the TCR/CD3 signal-transduction pathway, we first inhibited these kinases with the pharmacologic agent PP1 [35] and analyzed the induction of MICA on anti-CD3-activated T cells. The concentrations of PP1 used in our experiments did not induce apoptosis on resting or anti-CD3-stimulated T cells, as assessed by PI staining of hypodiploid DNA content and flow cytometry (data not shown). However, when PBMCs were activated with anti-CD3 mAb in the presence of PP1, a profound inhibition of proliferation was observed (**Fig. 1a**). MICA expression was strongly inhibited (Fig. 1b), suggesting that activation of Lck and Fyn kinases is a critical, proximal event that leads to activation-induced expression of MICA on T cells. The inhibitory activity of PP1 was confirmed, as basal levels of tyrosine-phosphorylated proteins were detected in Western blots of anti-CD3-stimulated cells incubated with this drug (Fig. 1c).

As inhibition of proximal events in CD3-induced T cell activation affects MICA expression, we investigated downstream events triggered by Lck and Fyn that might participate in this phenomenon. First, we used inhibitors of the MEK1/ERK pathway (U0126; ref. [36]), the p38 MAPK pathway (SB202190; ref. [37]), and calcineurin (CsA and FK506; refs. [38, 39]). The concentrations of U0126, SB202190, CsA, and FK506 used in our experiments did not induce apoptosis on resting or anti-CD3-stimulated T cells, as assessed by PI staining of hypodiploid DNA content and flow cytometry (data not shown). The specific inhibitor U0126 completely abrogated the proliferative response of anti-CD3-stimulated T cells, and SB202190 produced inhibitions of 85–92% (**Fig. 2a**). In addition, both drugs strongly inhibited MICA expression (Fig. 2b). Densitometric analysis of Western blots indicates that U0126 induced inhibitions ranging from 78% to 100%, and SB202190 inhibited MICA expression in percentages ranging from 84% to 93%, depending on the experiment (Fig. 2b, lower panel). pERK was detected only during early stages of the culture (15 and 30 min), which was inhibited by U0126. However, pERK could not be detected after 72 h of culture (even in the absence of U0126), the time at which the cells were harvested for analysis of MICA expression (Fig. 2c). These results indicate that the MEK1/ERK and the p38 MAPK signaling pathways participate in activation-induced expression of MICA following engagement of CD3.

Inhibition of calcineurin with CsA and FK506 also induced a strong reduction of the proliferative response in anti-CD3-stimulated T cells (64–75% and 71–93% inhibition, respectively, Fig. 2a). Also, both compounds strongly inhibited MICA expression (Fig. 2b), indicating that calcineurin is also involved in the signaling pathways that lead to up-regulated expression of MICA on T lymphocytes after CD3 engagement.

Inhibition of the proliferative response observed with the different pharmacologic inhibitors used in our experiments suggests that their concentrations are sufficient to dramatically affect normal cell proliferation. Maximal inhibition of MICA

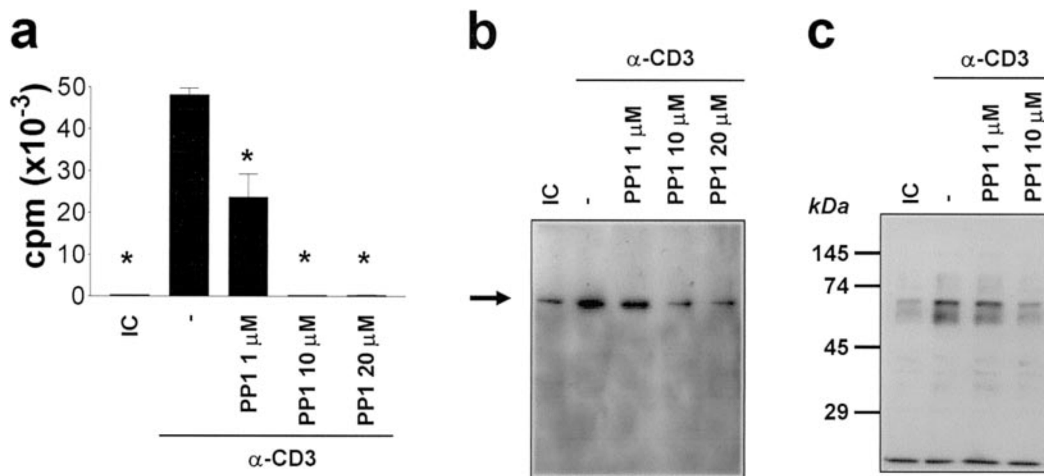

Fig. 1. Inhibition of Lck and Fyn on anti-CD3-stimulated T lymphocytes prevents up-regulated expression of MICA. PBMCs were stimulated for 72 h with anti-CD3 mAb (α-CD3) in the absence (–) or presence of 1, 10, or 20 μM Src kinase inhibitor PP1, harvested, and used for proliferation assays (a) or Western blot analysis for MICA expression (b). As negative control, PBMCs stimulated with an isotype-matched mAb (IC) were also analyzed. The arrow indicates the ~65-kDa band corresponding to MICA. In addition, the broad inhibitory effect on tyrosine phosphorylation of PP1 was assessed by Western blot using the antiphosphotyrosine mAb PY-20 in lysates from cells stimulated for 15 min with anti-CD3 mAb in the absence (–) or presence of 1 or 10 μM Src kinase inhibitor PP1 (c). Basal tyrosine phosphorylation was assessed in lysates of cells incubated with the isotype-matched, negative-control mAb (IC). Molecular weight markers are shown on the left. The results shown are representative of three independent experiments performed with different blood donors. *, $P < 0.001$, versus α-CD3 without PP1.

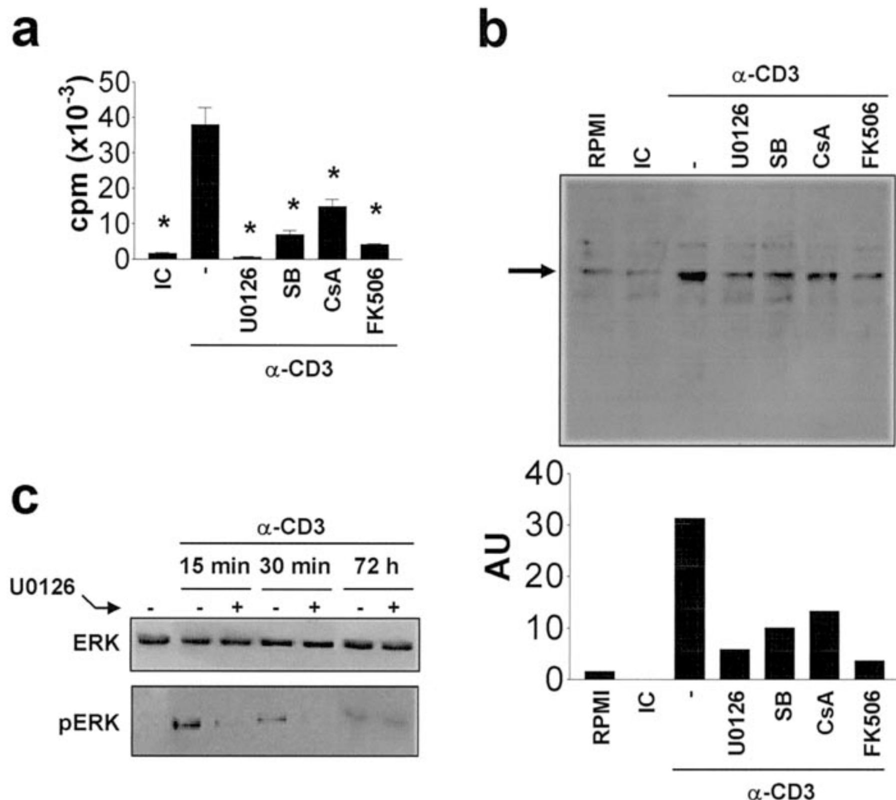

Fig. 2. Inhibition of MEK1, p38 MAPK, and calcineurin prevents up-regulated expression of MICA on anti-CD3-stimulated PBMCs. Cells were stimulated for 72 h with anti-CD3 mAb (α-CD3) in the absence (–) or presence of 20 μM MEK1 inhibitor U0126, 10 μM p38 MAPK inhibitor SB202190 (SB), or 1 μM calcineurin inhibitors CsA and FK506, harvested, and used for proliferation assessment (a) or Western blot analysis for MICA expression (b). As negative control, PBMCs stimulated with an isotype-matched mAb (IC) or PBMCs cultured for 3 days without mAb (RPMI) were also analyzed. The arrow indicates the ~65-kDa band corresponding to MICA. The intensity of each band was quantified by densitometric analysis and expressed in AU (lower panel). In addition to proliferation, efficacy of the U0126 treatment was also assessed by Western blot using anti-ERK and anti-pERK (pERK) Ab in lysates from cells stimulated for 15 min, 30 min, or 72 h with anti-CD3 mAb (α-CD3) in the absence (–) or presence (+) of 20 μM U0126 (c). Basal levels of ERK and pERK were analyzed in freshly isolated, untreated cells (lane 1). The results shown are representative of three independent experiments performed with different blood donors. *, $P < 0.001$, versus α-CD3 without pharmacologic inhibitors.

expression was observed with 1 μM U0126, CsA, and FK506 and 10 μM SB202190. However, higher concentrations of these inhibitors did not further inhibit MICA expression (**Fig. 3**). As CsA and FK506 were unable to completely inhibit MICA expression, it is likely that some calcineurin-independent, alternative routes might also signal for MICA expression on anti-CD3-stimulated T cells.

As the Src kinases Lck and Fyn, ERK, and p38 MAPK are also implicated in activation during CD28 cosignaling in T lymphocytes, we investigated their involvement in MICA expression on T lymphocytes activated with anti-CD28 mAb plus PMA (**Fig. 4**). Although only 20 μM Src kinase inhibitor PP1 diminished the proliferative response of the cells (Fig. 4a), the inhibition of up-regulated expression of MICA was already evident at 1 μM of the drug (Fig. 4b). Similarly, U0126 and SB202190 slightly affected the ^{3}H-Thy uptake (Fig. 4c), which ranged from 16% to 18% for U0126 and 28% to 34% for SB202190, depending on the experiment. However, both drugs markedly inhibited the induction of MICA expression, which ranged from 45% to 68% for U0126 and 67% to 100% for SB202190, depending on the experiment (Fig. 4d). In addition to proliferation assessment, the effects of PP1 and U0126 were confirmed, as basal levels of phosphotyrosine proteins were detected in the presence of PP1 (Fig. 4e), and low levels of pERK were detected in U0126-treated cells shortly after stimulation (15 and 30 min). However, pERK reached high levels after 72 h of stimulation, even in the presence of U0126 (Fig. 4f). The use of higher doses of U0126 and SB202190 demonstrated a dose-dependent effect on MICA expression on cells activated with anti-CD28/PMA (**Fig. 5**). Furthermore, complete inhibition of MICA expression was achieved with 50 μM U0126 (Fig. 5). Our results indicate that MICA expression induced on T cells upon engagement of CD28 involves activation of Lck and Fyn, MEK1/ERK, and p38 MAPK signaling pathways.

As expected, calcineurin, which does not participate in cosignaling after CD28 engagement [15], is not involved in anti-CD28/PMA-induced MICA expression, as CsA and FK506 slightly inhibited the proliferative response (5–10% inhibition for CsA and 4–15% inhibition for FK506, Fig. 4a) and MICA expression (11–24% inhibition for CsA and 7–13% inhibition for FK506, Fig. 4b). Ten times higher concentrations of CsA or FK506 (10 μM) did not induce higher inhibitions of MICA expression on anti-CD28/PMA-stimulated cells (data not shown).

A key event upon CD3 engagement and CD28 costimulation is the induction/secretion of IL-2 and the expression of the

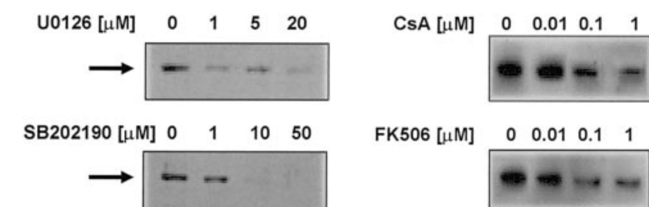

Fig. 3. Dose-dependent effect of U0126, SB202190, CsA, and FK506 on MICA expression in anti-CD3-stimulated PBMCs. Cells were stimulated for 72 h with anti-CD3 mAb (α-CD3) in the absence or presence of different micromolar concentrations of U0126, SB202190, CsA, or FK506 (indicated at the top of each panel), harvested, and used for Western blot analysis for MICA expression. The results shown are representative of three independent experiments performed with different blood donors.

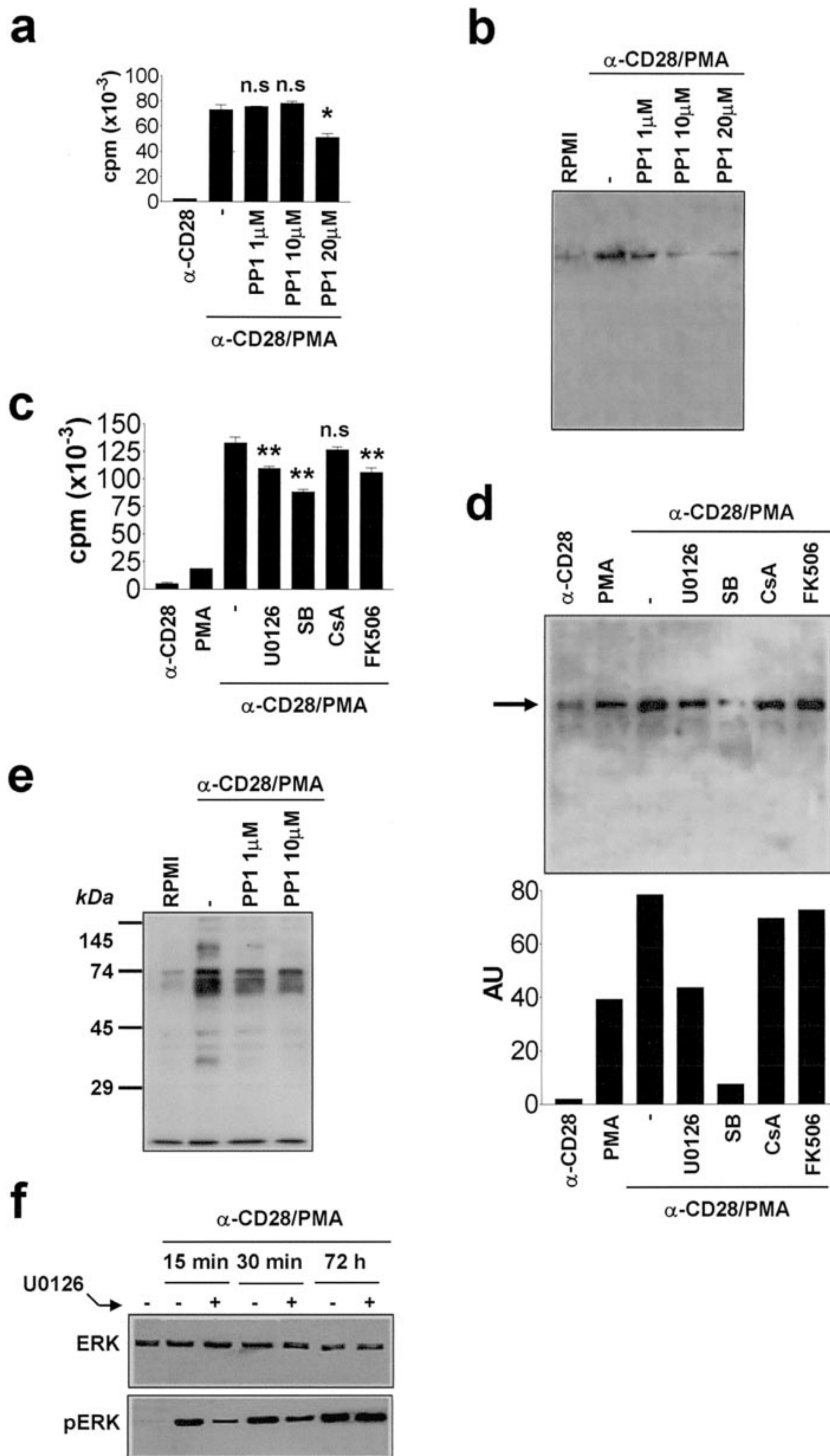

Fig. 4. Inhibition of Lck, Fyn, MEK1, and p38 MAPK but not calcineurin prevents up-regulated expression of MICA in anti-CD28/PMA-stimulated PBMCs. Cells were stimulated for 72 h with anti-CD28 mAb plus PMA (α-CD28/PMA) in the absence (–) or presence of 1, 10, and 20 μM Src kinase inhibitor PP1 (PP1, a and b) or 20 μM MEK1 inhibitor U0126, 10 μM p38 MAPK inhibitor SB202190 (SB), or 1 μM calcineurin inhibitors CsA or FK506 (c and d), harvested, and used for proliferation assessment (a and c) or Western blot analysis for MICA expression (b and d). As negative control, PBMCs cultured with medium alone (RPMI, b), anti-CD28 mAb alone (α-CD28, a–d), or PMA alone (c and d) were also analyzed. The arrow indicates the ~65-kDa band corresponding to MICA. The intensity of each band from panel d was quantified by densitometric analysis and expressed in AU (lower panel). In addition, the broad, inhibitory effect on tyrosine phosphorylation of PP1 was assessed by Western blot with the antiphosphotyrosine mAb PY-20 in lysates from cells stimulated for 15 min with α-CD28/PMA in the absence (–) or presence of 1 or 10 μM Src kinase inhibitor PP1 (e). Basal tyrosine phosphorylation was assessed in lysates of cells incubated without mAb (RPMI). Molecular weight markers are shown on the left. Also, efficacy of the U0126 treatment was assessed by Western blot with anti-ERK and anti-pERK (pERK) Ab in lysates from cells stimulated for 15 min, 30 min, or 72 h with α-CD28/PMA in the absence (–) or presence (+) of 20 μM U0126 (f). Basal levels of ERK and pERK were analyzed in freshly isolated, untreated cells (lane 1). The results shown are representative of three independent experiments performed with different blood donors. *, $P < 0.05$, versus α-CD28/PMA without PP1; **, $P < 0.001$, versus α-CD28/PMA without pharmacologic inhibitors (U0126, SB202190, CsA, or FK506).

high-affinity IL-2R [15]. Using subapoptotic concentrations of AG490 (an inhibitor of Jak3 that disrupts signaling through the Jak/STAT pathway; refs. [29, 30, 40]), we observed a strong inhibition of the proliferative response of anti-CD3- and anti-CD28/PMA-stimulated T cells (**Fig. 6a**). Similarly, the $p70^{S6}$ kinase inhibitor rapamycin [31, 41] also inhibited the ^{3}H-Thy uptake by anti-CD3- and anti-CD28/PMA-stimulated T cells (Fig. 6b). Both inhibitors induced a dose-dependent suppression of activation-induced expression of MICA on T lymphocytes upon CD3 engagement or CD28 costimulation (Fig. 6, c–e).

Therefore, expression of MICA induced by stimulation of T cells with anti-CD3 mAb involves activation of Lck and Fyn and signaling through the MEK1/ERK, p38 MAPK, and cal-

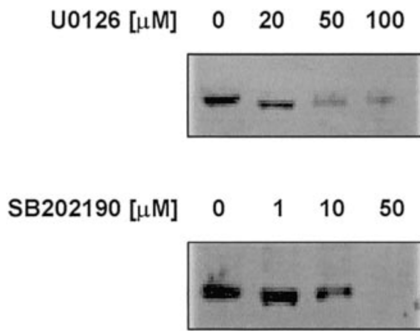

Fig. 5. Dose-dependent effect of U0126 and SB202190 on MICA expression on anti-CD28/PMA-stimulated PBMCs. Cells were stimulated for 72 h with anti-CD28/PMA in the absence or presence of different micromolar concentrations of U0126 and SB202190 (indicated at the top of each panel), harvested, and used for Western blot analysis for MICA expression. The results shown are representative of three independent experiments performed with different blood donors.

cineurin pathways. In contrast, expression of MICA on anti-CD28/PMA-stimulated T cells also involves activation of Lck and Fyn and signaling through the MEK1/ERK and the p38 MAPK pathways.

DISCUSSION

We have previously demonstrated that MICA expression on activated T cells involves engagement of CD3 and costimulation through CD28 [14]. In the present study, we investigated the intracellular mediators involved in this phenomenon.

In the presence of accessory cells, inhibition of Lck and Fyn in T cells activated by TCR/CD3 engagement strongly inhibited up-regulated expression of MICA (Fig. 1). As Lck and Fyn activate the Ras-Raf-MEK1/ERK pathway in T cells stimulated by CD3 engagement, we investigated the participation of this signaling route in MICA expression. Our results using U0126 indicate that MICA expression relies on an early,

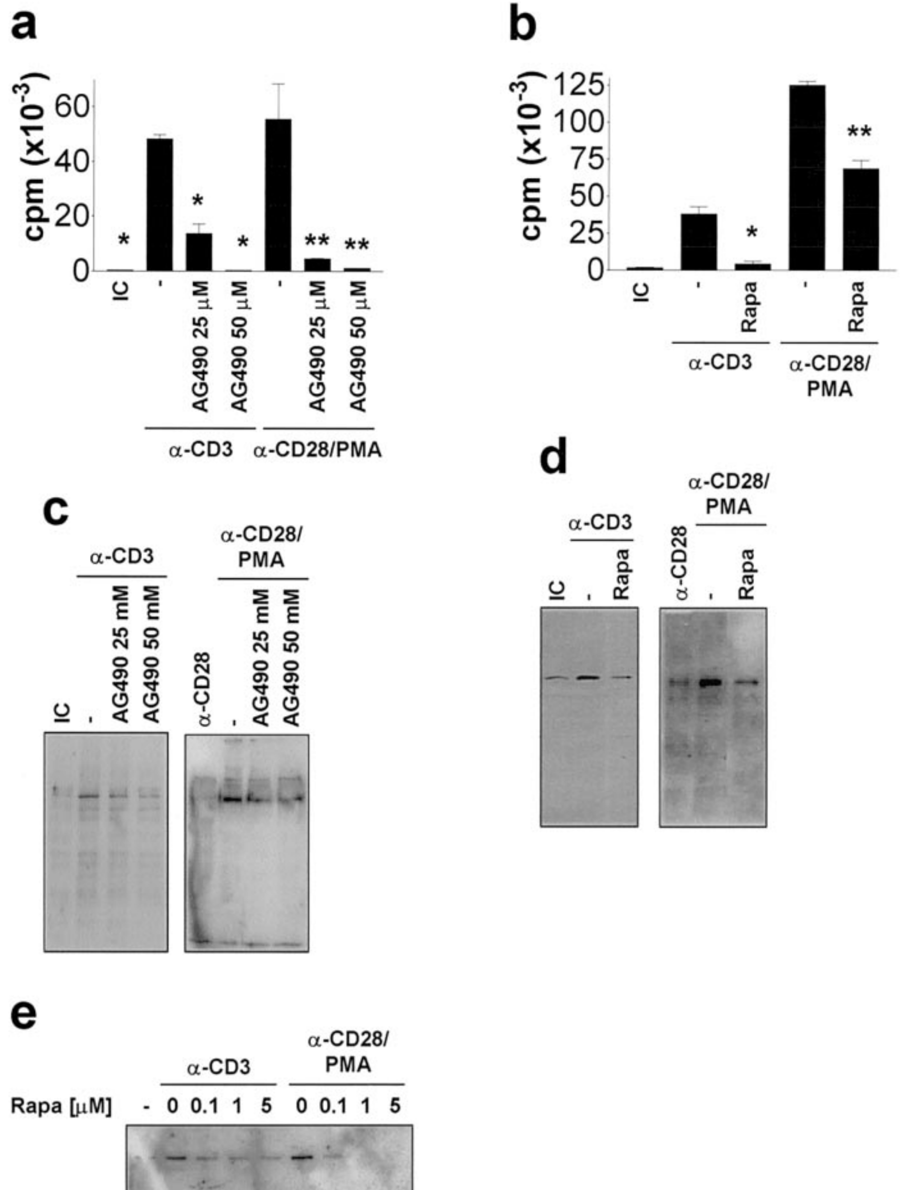

Fig. 6. Inhibition of Jak/STAT signaling and $p70^{S6}$ kinase prevents up-regulated expression of MICA in anti-CD3- and anti-CD28/PMA-stimulated PBMCs. Cells were stimulated for 72 h with anti-CD3 mAb (α-CD3) or with anti-CD28 mAb plus PMA (α-CD28/PMA) in the absence (–) or presence of 25 and 50 μM Jak inhibitor AG490 (a and c) or with 1 μM $p70^{S6}$ kinase inhibitor rapamycin (Rapa; b and d), harvested, and used for proliferation assessment (a and b) or Western blot analysis for MICA expression (c and d). In addition, the dose-dependent effect of rapamycin on MICA expression was also analyzed on PBMCs stimulated with α-CD3 or α-CD28/PMA (e). As negative control, PBMCs stimulated with an isotype-matched mAb (IC) or α-CD28 alone were also analyzed. The results shown are representative of three independent experiments performed with different blood donors. *, $P < 0.001$, versus α-CD3 without AG490 (a) or Rapa (b), and **, $P < 0.001$, versus α-CD28/PMA without AG490 (a) or Rapa (b).

functional Ras-Raf-MEK1-ERK signaling pathway. In agreement with previous observations indicating an early but not sustained ERK activity necessary for T cell activation [19, 42, 43], we observed the presence of pERK only early after stimulation (15–30 min) in anti-CD3-stimulated cells, which was critical for the induction of MICA expression (Figs. 2 and 3).

Another signaling pathway activated by Lck and Fyn is the p38 MAPK pathway [15, 20]. We confirmed the participation of this kinase in the proliferative response of anti-CD3-stimulated T cells and demonstrated that p38 MAPK is an important intracellular mediator that leads to up-regulated expression of MICA after CD3 engagement (Figs. 2 and 3).

TCR/CD3 ligation also activates the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin [21, 22]. Two different calcineurin inhibitors (CsA and FK506) inhibited the proliferative response, as well as MICA expression on anti-CD3-activated T cells (Figs. 2 and 3), confirming that this phosphatase participates in activation-induced MICA expression.

We had also demonstrated that in the presence of accessory cells, CD28 engagement induces MICA expression [14]. Here, we observed that the Lck and Fyn kinases, MEK1/ERK, and the p38 MAPK pathway, which are activated upon CD28 stimulation [23–25, 28, 44], are also involved in up-regulated expression of MICA (Fig. 4). The strong effect observed on MICA expression contrasts with the weak inhibition of the proliferative response when the MEK1/ERK or the p38 MAPK pathways were disrupted. These findings suggest that T cells have alternative and mutually compensating routes to initiate cell-cycle progression and proliferation upon CD28 engagement but that disruption of any of those routes produces a profound impact on MICA expression. The minimal effect of CsA and FK506 on the proliferation of T cells after CD28 costimulation is in agreement with previous findings about a marginal role of calcineurin in costimulation-dependent T cell activation [45]. In addition, we observed only a marginal effect of FK506 and CsA on MICA expression levels in anti-CD28-stimulated T cells.

It is known that Jak1, Jak3, and STAT5 are activated upon IL-2 interaction with its high-affinity receptor [29, 30] and that the $p70^{S6}$ kinase participates in the elicited intracellular pathways [31]. We observed that the Jak/STAT pathway and the $p70^{S6}$ kinase participate in the up-regulated expression of MICA upon CD3 engagement or CD28 costimulation (Fig. 6), most likely via autocrine/paracrine IL-2-mediated effects. The signaling routes involved in IL-2-mediated, up-regulated expression of MICA are currently under study in our laboratory.

Although we cannot rule out the possibility that the pharmacologic agents used in our experiments could affect other cellular targets that participate in MICA expression on T lymphocytes, our approach of using pharmacologic inhibitors has the advantage over cell-line transfection-based strategies in that it can be applied to normal (resting) cells. Hence, our results do not correspond to a cell line that has disregulated, intracellular metabolic pathways and high basal kinase activities. In addition, there is experimental evidence that indicates that overexpression of different kinases induces activation of intracellular pathways that do not operate during T cell-specific signal-transduction pathways under normal, physiological responses [46].

The expression of MICA induced by multiple cytoplasmic signaling routes demonstrated in this study is probably achieved through activation of specific transcription factors such as NFAT, nuclear factor-κB, and AP-1 [15, 21, 47]. We can speculate that a full set of transcription factors activated by MEK1/ERK, p38 MAPK, calcineurin, and Jak proteins is required to induce a cooperative effect that activates transcription of the MICA gene. Pharmacologic inhibition of signaling routes that activate one or some of these transcription factors should therefore affect MICA expression, as observed in this study. Hence, our results set the basis for experiments to elucidate the transcription factors involved in up-regulated expression of MICA, which are currently in progress in our laboratory.

Our results constitute the first demonstration of intracellular mediators involved in MICA expression. Up-regulated expression of MICA on T lymphocytes stimulated by CD3 engagement involves activation of Lck and Fyn kinases and subsequent signaling through simultaneous routes that proceed through MEK1/ERK, p38 MAPK, and calcineurin. Moreover, expression of MICA on T lymphocytes stimulated by CD28 engagement and PMA also involves Lck, Fyn, MEK1/ERK, and p38 MAPK but not calcineurin. Part of these effects appears to operate through induction of IL-2-dependent signaling routes triggered by the Jak/STAT pathway and activation of the $p70^{S6}$ kinase. The elucidation of the multiple, intracellular signaling pathways and mediators involved in the up-regulated expression of MICA may reveal potential targets for immune intervention to modulate MICA expression in pathological disorders.

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