

CD101 Expression and Function in Normal and Rheumatoid Arthritis-affected Human T Cells and Monocytes/Macrophages

DRAGAN V. JOVANOVIĆ, LAURENCE BOUMSELL, ARMAND BENSUSSAN, XAVIER CHEVALIER, ARTURO MANCINI, and JOHN A. DI BATTISTA

ABSTRACT. Objective. It was recently reported that CD101 surface expression discriminates potency among CD4⁺CD25⁺ FoxP3⁺ regulatory T cells in the mouse. We investigated whether CD101 may also have a role in the suppressor function of regulatory T cells in humans given that the latter population may affect the autoimmune response in patients with rheumatoid arthritis (RA).

Methods. Sorted T cells and monocyte/macrophage cell populations were analyzed by flow cytometry using conjugated antibodies specific for cell-surface markers. T cell proliferation assays were conducted by [³H]thymidine incorporation and CD8^{high} cytotoxicity measurements by Cyto-Scan-LDH cytotoxicity assays. ELISA were used to measure cytokines in cell culture supernatants and Western blotting was performed for profiling mitogen-activated protein (MAP) kinase activation using specific antiphospholipid antibodies.

Results. CD101 expression coincided with PMA-induced monocyte/leukocyte lineage differentiation. CD8^{high}CD101[−] T cells exhibited greater cytotoxic activity than CD8^{high}CD101⁺ T cells, while no difference was observed between CD4⁺CD25^{high}CD101⁺ and CD4⁺CD25^{high}CD101[−] Treg inhibitory activity through responder T cells. LPS-induced proinflammatory cytokine production and p38 MAP kinase activation were made possible by ligation of CD101 with an anti-CD101 antibody F(ab')₂ fragment.

Conclusion. These results suggested a modulatory/coregulatory function of CD101 in the human immune system, in contrast to murine models, in which CD101 surface expression discriminates potency among FoxP3⁺ regulatory T cells. Cytotoxic CD8^{high}CD101⁺ T cells were markedly less cytotoxic than CD8^{high} T cells negative for the CD101 antigen and were conspicuously downregulated in patients with RA, suggesting a possible role for CD101 expression and function in the control of certain manifestations of RA pathology. (J Rheumatol First Release Dec 15 2010; doi:10.3899/jrheum.100676)

Key Indexing Terms:

CD101
MONOCYTES/MACROPHAGES

MODULATION

T CELLS
RHEUMATOID ARTHRITIS

From the Division of Rheumatology and Clinical Immunology, and the Department of Anatomy and Cell Biology, Department of Medicine, McGill University, Montreal, Quebec, Canada; Division of Rheumatology, Hôpital Henri-Mondor, Paris V University, Paris; INSERM U841, Faculté de Médecine, Créteil; and INSERM U976, Hôpital Saint-Louis, Paris, France.

Supported in part by the Canadian Institutes for Health Research, grant number M11557.

D.V. Jovanovic, MD, PhD, Division of Rheumatology and Clinical Immunology, McGill University; L. Boumsell, MD, PhD, INSERM U841, Faculté de Médecine; A. Bensussan, PhD, INSERM U976, Hôpital Saint-Louis; X. Chevalier, MD, PhD, Division of Rheumatology, Hôpital Henri-Mondor, Paris V University; A. Mancini, MSc, Department of Anatomy and Cell Biology, Department of Medicine, McGill University; J.A. Di Battista, PhD, Division of Rheumatology and Clinical Immunology, McGill University.

Address correspondence to Dr. J.A. Di Battista, Division of Rheumatology and Clinical Immunology, Royal Victoria Hospital, 687 Pine Avenue West, Room M11-22, Montreal, Quebec H3A 1A1, Canada.
E-mail: john.dibattista@mcgill.ca

Accepted for publication November 3, 2010.

CD101 is a human cell-surface type 1 glycoprotein expressed on monocytes, granulocytes, the subpopulation of dendritic cells (DC)¹, and on mucosal T lymphocytes². In the T cell lineage, CD101 expression is positively modulated after lymphocyte activation, while freshly isolated lymphocytes and thymocytes weakly express this molecule³. Langerhans and histiocytic cells are positive for this molecule and it was suggested that CD101 might be useful in combination with other markers for the diagnosis of Langerhans cell histiocytosis⁴. CD101 is a 240-kDa disulfide-linked homodimer protein migrating under reducing conditions at 130 kDa. Sequencing shows a 1021 amino acid type I transmembrane protein containing 7 immunoglobulin type Ig-V domains with 7 glycosylation sites in the extracellular portion and a short cytoplasmic tail with several serine/threonine phosphorylation consensus sites⁵.

Ligation of the CD101 molecule on T cells with anti-

CD101 monoclonal antibody (mAb) blocks T cell receptor (TCR)/CD3-induced proliferation by inhibiting calcium flux and activation of tyrosine kinase, resulting in the suppression of interleukin 2 (IL-2) transcription^{6,7}. In contrast, anti-CD101 mAb (anti-p126) were costimulatory with sub-optimal concentrations of anti-CD3 mAb-inducing proliferation of cultured intestinal intraepithelial lymphocytes². It was also reported that after anti-CD28 and phorbol myristate acetate (PMA) stimulation, only CD28+CD101+ cells proliferate, while CD28+CD101- subsets fail to respond to these stimulations³. Unlike CD4+CD101- T cells, CD4+CD101+ lymphocytes proliferate in the presence of immobilized anti-CD3 and in the absence of costimulating cells⁸. Acting on cutaneous DC, anti-CD101 antibodies inhibited T cell proliferation through IL-10 production, suggesting an important regulatory role played by the CD101 molecule on DC during T cell activation⁹. A recent study demonstrated that the nonobese diabetic (NOD) allele of CD101 in C57BL/6 mice had 17 exonic single-nucleotide polymorphisms, resulting in 10 amino acid substitutions in the CD101 protein: the authors suggested that CD101 could be responsible for the Idd10 effect¹⁰. Fernandez, *et al*¹¹ recently reported that CD101 expression distinguished murine Treg with potent suppressor activity.

Even though 90% of human peripheral blood CD14+CD147+ cells express CD101, the function of this molecule in human monocytes/macrophages is not known. The ligand(s) and molecules associated with CD101 as well as disease relevance are ill defined. We compared the expression of CD101 on different peripheral blood cell populations of patients treated for rheumatoid arthritis (RA) and healthy donors. We tested the effect of anti-CD101 mAb on human monocyte/macrophage functions in order to understand the physiological function of this receptor. We also examined the functional differences between CD101+ and CD101- regulatory T cells and between CD101+ and CD101- CD8+ cytotoxic T cells.

MATERIALS AND METHODS

Antibodies for flow cytometry and for cell sorting. The following fluoro-chrome-conjugated antibodies were used for surface staining and cell sorting: phycoerythrin-conjugated anti-CD101 (AbD Serotec, Raleigh, NC, USA), FITC-conjugated anti-CD8, CD20, CD25, CD33, CD147, CD235a (ImmunoTools, Friesoythe, Germany), TCR V- α 24 (Immunotech, Vaudreuil-Dorion, QC, Canada), CD34 (BD Pharmingen, San Diego, CA, USA) and antigranzyme B (Abcam, Cambridge, MA, USA), antigen-presenting cell-conjugated anti-CD4, CD14, CD25 (ImmunoTools) and CD8 (BD Pharmingen), and PC5-conjugated anti-CD8, CD11c, CD123 (BD Pharmingen) and CD27 (Beckman Coulter, Mississauga, ON, Canada).

Patients, donors, and FACS analysis. Peripheral blood mononuclear cells (PBMC) consisting of lymphocytes and monocytes were isolated from buffy coats of healthy seronegative donors (Centre de Transfusion Sanguine, Cr  teil, France), and from peripheral blood of patients with RA (H  pital Henri-Mondor, Paris V University, Cr  teil, France). Five patients with active disease and fulfilling the revised classification criteria of the American College of Rheumatology for RA were included in this study,

with duration of disease of 13.3 ± 3.2 years, treated with anti-tumor necrosis factor- α (TNF- α) antibody (infliximab, 3 to 5 mg/kg) for 10.3 ± 2.9 years, corticosteroids (prednisone, 2 to 10 mg/day) for 10.3 ± 2.9 years, and methotrexate (MTX, 10 to 20 mg/week) for 3.0 ± 0.6 years. The blood donor controls were sex-matched and age-matched with the patients; they were 5–7 women, average age 46 ± 9 years. Local Ethical Committee approval was received for this study and informed consent of all participating subjects was obtained.

The samples were diluted 1:2 with phosphate buffered saline (PBS) and centrifuged over Ficoll-Paque Plus (Amersham Biosciences, Baie d'Urfe, QC, Canada). PBMC were washed 3 times in RPMI-1640 medium containing penicillin and streptomycin, 100 U/ml and 100 mg/ml, respectively (Life Technologies, Carlsbad, CA, USA), and supplemented with 2 mM L-glutamine (Life Technologies). Cell viability was determined by trypan blue exclusion. After labeling for 30 min at 4  C, the cells were washed and analyzed by flow cytometry using FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo version 6.3.4 software. Unstimulated and PMA-stimulated histiocytic lymphoma (U-937) and promyelocytic leukemia (HL-60) mast cells, and unstimulated and erythropoietin (EPO)-stimulated erythroleukemia (TF-1) cell lines were used to examine the expression of CD101 on differentiating cell lineages.

Cell purification and culture. PBMC were seeded in untreated plastic petri dishes, area per well about 28.2 cm, at a density of about 4×10^5 cells/cm², in a final volume of 5 ml. The cells were allowed to adhere to the dishes for 90 min at 37  C, in 5% CO₂, in RPMI medium not supplemented with fetal bovine serum (FBS)¹². Nonadherent cells (mainly lymphocytes) were removed by vigorous washes with PBS (3 times). Macrophages obtained in this fashion were > 91% pure, as determined by FACSCalibur analysis with anti-CD14. Cells were stimulated with lipopolysaccharide (LPS; 500 ng/ml) in the absence or presence of F(ab)₂ anti-CD101 (10 μ g/ml) for 48 h. The supernatants were collected and tested for cytokine and prostaglandin E₂ (PGE₂). For *in vitro* culture of CD4+ cells and for functional studies on T helper cells, CD4+ cell enrichment using magnetic cell sorting was done according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). These CD4+ enriched cells were then labeled with anti-CD4 antigen-presenting cell, anti-CD101 phycoerythrin, and anti-CD25 FITC monoclonal antibodies. CD4+CD25-CD101- and CD4+CD25-CD101+ as responder cells and CD4+CD25^{high}CD101- and CD4+CD25^{high}CD101+ as regulatory T cells were sorted using BD FACS Aria. PBMC were also labeled with anti-CD8 FITC-conjugated antibodies and CD8^{high}CD101- and CD8^{high}CD101+ cells were sorted for the proliferation and cytotoxicity assays.

T cell stimulation and proliferation assay. To measure cell proliferation, 5×10^4 responder cells were seeded in round-bottom 96-well plates previously coated overnight with anti-CD3 in PBS buffer. CD4+ and CD8+ T cells were stimulated for 3 days with anti-CD28 (1.5 μ g/ml) and with anti-CD101 (10 μ g/ml). T cell proliferation was determined by measuring [³H]thymidine incorporation during the last 16 h of culture, using a liquid scintillator counter (LKB, Perkin-Elmer). In some experiments, CD4+CD25^{high}CD101- or CD4+CD25^{high}CD101+ Treg were added to the culture of anti-CD3/anti-CD28-stimulated CD4 or CD8 cells, in a 1:1 ratio of responder to Treg.

Detection of cytotoxic activity and granzyme B expression. CD8^{high}CD101- and CD8^{high}CD101+ cells were isolated from the peripheral blood of healthy donors, as described. Collected cells were washed once with RPMI containing 2% human serum albumin. About 4×10^4 T cells were cocultured with target cells (K562 human erythroleukemia cell line) at 32:1 to 2:1 ratios in round-bottom tissue culture plates in 200 ml culture medium for 4 h at 37  C under 95% CO₂. Supernatants were collected and the lactate dehydrogenase (LDH) was measured using CytoScan-LDH Cytotoxicity Assay (Amersham Biosciences) according to the instructions from the manufacturer. Triton X-100 was used to determine the maximum lysis and target cells alone to determine spontaneous lysis. Percentage of lysis was calculated according to this formula: experimental

optimal density (OD) – spontaneous OD – spontaneous effector OD/(maximal OD – spontaneous target OD)¹³.

The experiment was repeated 3 times in triplicate. For intracellular granzyme B labeling, peripheral blood leukocytes were first incubated with anti-CD8 and anti-CD101 for 30 min. Cells were then resuspended in Permeafix for 30 min at room temperature before labeling with anti-granzyme B-FITC monoclonal antibody.

Cytokine analysis and intracellular killing. Measurement of IL-1 β , TNF- α , IL-10, and PGE₂ in 72 h supernatants obtained from the monocyte/macrophage stimulation assays was performed using specific ELISA kits. Acute *Listeria monocytogenes* infection in cultured monocytes/macrophages has been used as a model of *in vitro* killing assay. The modified protocol of Dileepan, *et al*¹⁴ was performed to measure killing efficacy. PBMC were plated for 4 h at a density of 10⁶ cells per well (24-well plate). Nonadherent cells were removed and monolayers were cultured for 24 h in RPMI 10% FBS, washed with RPMI without antibiotics, and activated with LPS (500 ng/ml) and interferon- γ (IFN- γ ; 100 U/ml) and anti-CD101 F(ab')₂ (10 μ g/ml) for an additional 12 h. Live *L. monocytogenes* was added (2 \times 10⁵/well) in fresh RPMI without antibiotics, LPS, and IFN- γ . The plates were centrifuged at room temperature for 10 min at 3000 rpm and incubated for 70 min at 37°C in 5% CO₂. In order to kill extracellular bacteria, the cultures were treated for an additional 5 h with gentamycin (5 μ g/ml). Triton X-100 0.05% was used to lyse the cells. The cells were plated on brain-heart infusion agar in PBS-diluted lysate for the determination of viable counts. Prior to addition of gentamycin, control wells were lysed to determine the total number of viable bacteria exposed to macrophage monolayer. Dividing the number of bacterial colonies by the number of viable macrophages at the time when *L. monocytogenes* was added normalized the bacteria's survival, expressed as relative units.

Intracellular calcium [Ca²⁺]_i measurement. For estimation of [Ca²⁺]_i, macrophages were loaded with 1 μ M Indo-1/AM in RPMI supplemented with 1% FBS for 40 min at 37°C. After 2 washes with Hanks' buffered salt solution, cells were introduced at 37°C at a rate of about 350 events/s into cytofluorometer, using blue and ultraviolet lasers. CellQuest Pro and FlowJo for Macintosh (TreeStar, Ashland, OR, USA) software was used for acquisition and analysis, respectively. Data were collected using a 2-dimensional density plot to display the Indo-1 log violet/blue ratio as a function of time. For the maximum increase in [Ca²⁺]_i, cells were treated with 2 nM ionomycin.

Pepsin digestion of monoclonal antibodies. Monoclonal antibodies against CD101 were purified from mice ascites (hybridoma BB27) obtained from our INSERM lab. F(ab')₂ from mAb was generated by pepsin digestion (ImmunoPure F(ab')₂ Preparation Kit, Pierce, Rockford, IL, USA; Manitoba Centre for Proteomics and Systems Biology, University of Manitoba, Winnipeg, Canada), according to the manufacturer's instructions. The specificity of the antibody was verified by Western blot analysis under nonreducing conditions using purified human CD101.

Western blotting. Phosphorylation of p38, p42/44, and stress-activated protein kinase/Jun-N-terminal kinase (SAP/JNK) mitogen-activated protein (MAP) kinases in monocytes/macrophages was determined using specific PhosphoPlus kits (Cell Signaling, Waverly, MA, USA) with a Phototope-horseradish peroxidase (HRP) Western blot detection system. The monocytes/macrophages were dissolved into radioimmunoprecipitation assay buffer consisting of 1% NP40, 0.5% sodium deoxycholate, and 1% sodium dodecyl sulfate, 10 μ g/ml of pepstatin, leupeptin, aprotinin, O-phenthroin, sodium-orthovanadate, and 10 mM of phenylmethylsulfonyl fluoride and dithiothreitol. Proteins (15 μ g) were separated by SDS-polyacrylamide gel electrophoresis using 10% gels under reducing conditions. Proteins were transferred electrophoretically onto Hybond-N nitrocellulose filters and blocked using SuperBlock Blocking Buffer (Pierce), and the blot incubated overnight with primary antibody, rabbit anti-phospho p42/44, p38, and SAP/JNK (dilution 1:1000; Cell Signaling, Beverly, MA, USA). The blots were incubated with goat antirabbit secondary antibody linked to HRP conjugate (1:10,000; Pierce) for 1 h, and finally, with SuperSignal Ultra chemi-

luminescence substrate (Pierce). Following incubation with the substrate, the membrane was autoradiographed and exposed to X-Omat film (Eastman Kodak, Rochester, NY, USA).

Statistical analysis. Values were expressed as the mean \pm SEM. The significance of the results was determined using the Student t test with Bonferroni posthoc correction. Significance was $p < 5\%$.

RESULTS

Expression of CD101 on PBMC and on cell lines. The disease relevance and the function of CD101 in humans and in animal models are not known. We examined the expression of CD101 on different human blood cell populations from 7 healthy donor cell samples compared with the expression of this molecule on cells from patients with RA. There was no difference in the percentage of CD4+CD101+ lymphocytes from healthy controls (6.0 \pm 1.8%) compared with CD4+CD101+ cells from patients with RA (4.5 \pm 3.1%; Figure 1A). However, significantly less ($p < 0.05$) CD101 was expressed on CD8+ RA lymphocytes (1.9 \pm 0.1%) compared with control cytotoxic/suppressor CD8+ cells (9.5 \pm 2.0%). Only a small percentage of myeloid dendritic cells (CD11c+CD33^{bright}CD14-) of controls and patients with RA (4.9 \pm 1.3% and 3.1 \pm 2.2%, respectively), plasmacytoid dendritic cells (CD123^{bright}CD14-), (0.3 \pm 0.1% and 0.8 \pm 0.5%, respectively), and natural killer cells (CD56+CD16^{bright}, 0.3 \pm 0.1% and 0.8 \pm 0.5%, respectively) were stained for CD101. B cells (CD20+) and natural killer T cells (CD4+CD8-Va24+) were not labeled with anti-CD101 antibodies (data not shown).

The vast majority of monocytes, in both controls and patients with RA (88.7 \pm 3.9% and 92.6 \pm 1.5%, respectively), were positive for CD101 (Figure 1A). We thus tested the expression of CD101 on malignant myelomonocytic cell lines THP-1, U-937, as well as erythromyelomonocytic cell line K562 in order to establish whether CD101 expression coincided with cellular differentiation. In the CD101-promyelocytic leukemia cell line (HL-60), PMA-stimulated cell differentiation was coincident with CD101 expression, while in CD101+ hystiocytic lymphoma cells (U-937) increased CD101 labeling was found after differentiation (Figure 1B). Mast cells were negative for CD101 before and after PMA stimulation. When CD101 slightly positive common progenitor TF-1 erythroleukemia cells (CD34+) were driven with EPO toward erythrocyte differentiation, the cells decreased CD34 expression and became glycophorin A (CD235a)+, while the expression of CD101 ended.

Role of CD101 in T cell proliferation and in Treg function. The signal delivered by CD101 on activated T cells by anti-CD101 antibody ligation was shown to inhibit TCR/CD3-induced T cell proliferation¹³. In this regard, it was recently reported that CD101 surface expression discriminates potency among CD4+CD25+ FoxP3+ regulatory T cells in the mouse¹¹. We investigated whether CD101 may also have a role in the suppressor function of regulatory T cells in humans, since the latter population may affect the

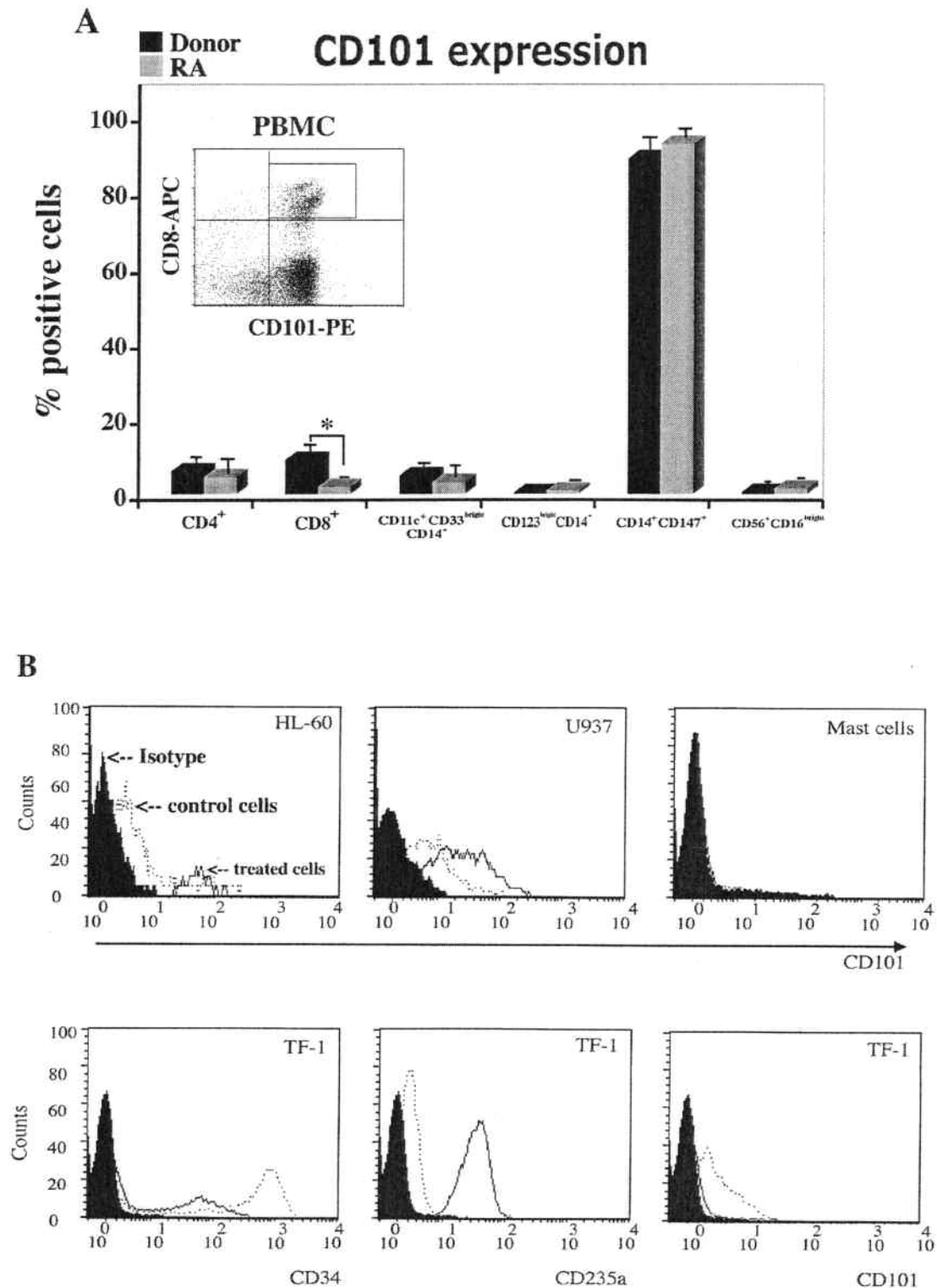


Figure 1. Expression of CD101 on peripheral blood mononuclear cells (PBMC) and on different cell lines. **A.** PBMC were isolated and sorted from peripheral blood of healthy controls ($n = 7$) and patients with RA ($n = 5$) and analyzed for CD101 expression by flow cytometry. **B.** Unstimulated and PMA-stimulated (50 ng/ml for 72 h) histiocytic lymphoma (U-937), promyelocytic leukemia (HL-60), murine mast cells, or unstimulated and EPO-stimulated (5 U/ml, for 3 days) erythroleukemia (TF-1) cell lines were used to examine the expression of CD101 on differentiating cell lineages. Cells were stained with monoclonal antibodies and analyzed by flow cytometry. * $p < 0.05$.

autoimmune response in patients with RA. Regulatory T cells from peripheral blood were separated into CD4+CD25^{high}CD101⁻ and CD4+CD25^{high}CD101⁺ subpopulations and were added to the culture of CD3/CD28-stimulated CD4+CD25⁻CD101⁻ and CD4+CD25⁻CD101⁺ as well as to the CD3/CD28-stimulated CD8^{high}CD101⁻ and CD8^{high}CD101⁺ cells (i.e., responder cells). There were no differences in the proliferative responses of CD4⁺ and CD8^{high} T lymphocyte populations after CD3/CD28 stimulation. The addition of anti-CD101 monoclonal antibodies (10 μ g/ml) to the culture of stimulated CD4⁺ responder cells induced significantly higher inhibition ($p < 0.02$) of [³H]thymidine incorporation in the CD101⁺CD4⁺ subpopulation compared with CD101⁻CD4⁺ cells (71.2% and 23.0%, respectively; Figure 2A). The response of CD4 T cells to the inhibition mediated by regulatory T cells was independent of the presence of CD101 on the cell surface of

responding T cells. In contrast to the higher suppression of CD101⁺CD4⁺ than CD101⁻CD4⁺ T cells by anti-CD101 monoclonal antibodies, this difference was not observed in CD8⁺ cell populations (Figure 2B). However, CD8⁺CD101⁻ cells incorporated significantly less [³H]thymidine when cocultured with either CD101⁻ ($p < 0.05$) or CD101⁺ ($p < 0.05$) regulatory T cells compared with CD8⁺CD101⁺ lymphocytes.

Cytotoxic activity of CD8^{high} lymphocytes. Cytotoxic activity of CD8^{high} lymphocytes was determined by measuring nonspecific-mediated lysis of K562 target cells. The percentage of lysis of target cells (mean \pm SE) was significantly higher in the CD101⁻CD8 population for all effector:target ratios (Figure 3A). The maximum lysis of K562 target cells by this population ($33.4 \pm 5.9\%$) was found for the effector:target ratio of 16:1. This difference in killing was not due to the different intracellular content of granzyme B

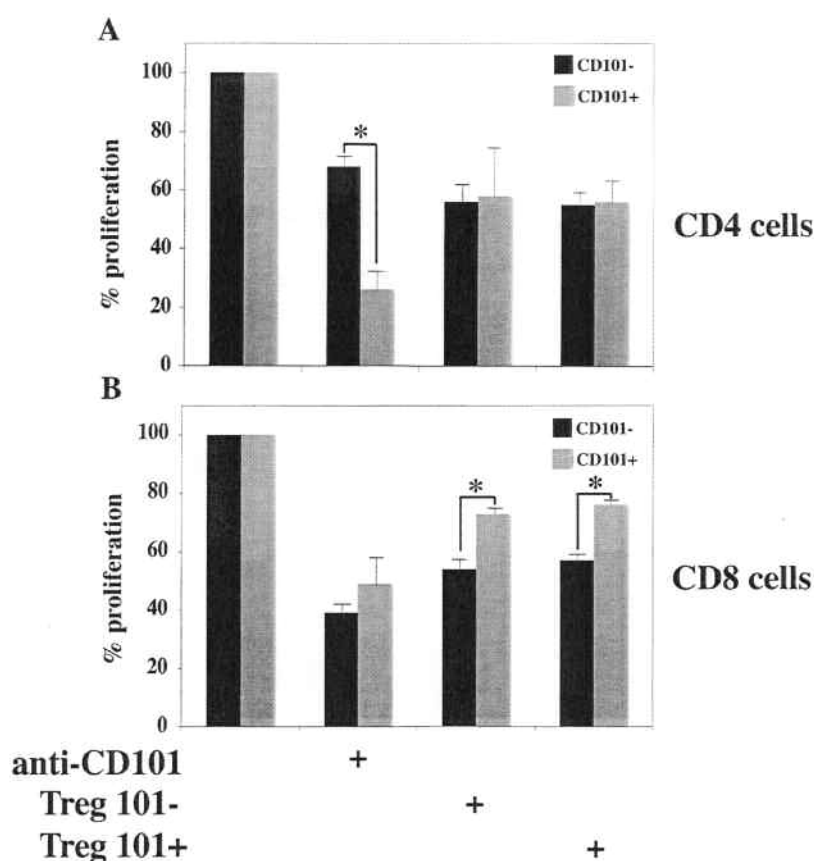


Figure 2. Effect of anti-CD101 monoclonal antibody on the proliferation of CD4 (A) and CD8 lymphocytes (B) and the inhibitory capacity of CD101⁺ and CD101⁻ Treg. These cells were sorted and used as responder cells: CD4+CD25⁻CD101⁻, CD4+CD25⁻CD101⁺, CD8^{high}CD101⁻, and CD8^{high}CD101⁺. The latter cells were cultured 3 days in plates coated with anti-CD3 stimulated for 3 days with anti-CD28 (1.5 μ g/ml), with or without anti-CD101 (10 μ g/ml; $n = 4$). In some experiments, CD4+CD25^{high}CD101⁺ or CD4+CD25^{high}CD101⁻ Treg were added to the culture of responder T cells in a 1:1 ratio of responder:Treg (A and B). T cell proliferation was determined by measurement of [³H]thymidine incorporation during the last 16 h of culture. * $p < 0.05$ ($n = 3$ determinations).

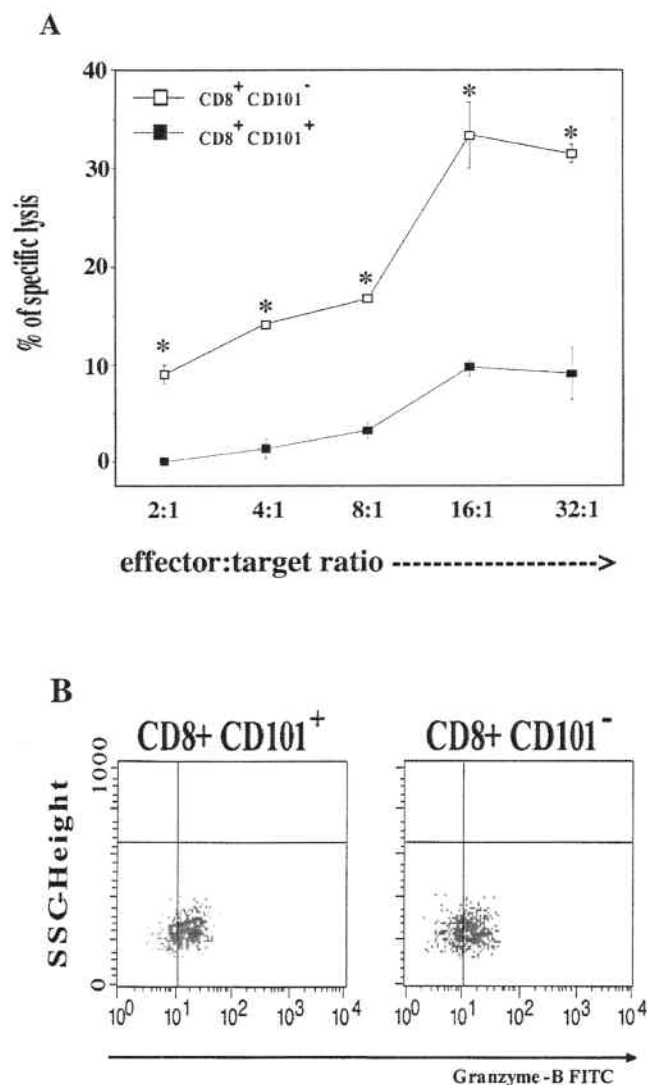


Figure 3. Cytotoxic activity and granzyme B expression (B) in CD101+ and CD101- CD8^{high} lymphocytes. A. Purified T cells were cocultured for 4 h with target cells (K562 human erythroleukemia cell line) at 32:1 to 2:1 ratios. Supernatants were collected and LDH was measured using CytoScan-LDH Cytotoxicity Assay. * $p < 0.05$ ($n = 3$ determinations). B. For intracellular granzyme B staining, permeabilized CD8^{high} T lymphocytes were labeled with antigranzyme B-FITC monoclonal antibody and analyzed by flow cytometry.

in CD101+ and CD101- cytotoxic T cells, as both cell populations were similarly labeled with monoclonal antibody specific for this enzyme ($69.7 \pm 12.7\%$ and $55.4 \pm 12.6\%$, respectively; Figure 3B).

The effect of anti-CD101 on macrophage cytokine and PGE₂ production, and on intracellular killing. Similar to T cell populations, the function of CD101 expressed on human monocytes is not known. We thus examined the effect of anti-CD101 ligation on control and LPS-stimulated monocytes by measuring 72 h production of the proinflammatory cytokines IL-1 β and TNF- α , antiinflammatory cytokine

IL-10, and the immunomodulatory eicosanoid PGE₂. In addition, the capacity for intracellular killing was measured by incubating monocytes/macrophages in the presence of the intracellular bacteria *L. monocytogenes*. To avoid the nonspecific effect of the antibodies' Fc domain ligation on monocytes by FcR, an F(ab)₂ fragment of anti-CD101 mAb was used in these experiments. Anti-CD101 was found to increase TNF- α and PGE₂ levels over untreated cells, although the differences did not reach significance. In cells exposed to LPS, the secretion of IL-1 β (13.8%) and TNF- α (18.1%) rose modestly with anti-CD101 stimulation, while IL-10 and PGE₂ were robustly and significantly upregulated by 24.6% and 44.1%, respectively (Figure 4A and 4B). The presence of anti-CD101 antibodies in the culture of LPS-IFN- γ -stimulated monocytes/macrophages decreased the survival of *L. monocytogenes* by a statistically significant $17.5 \pm 5.5\%$ ($n = 5$ determinations, $p < 0.05$; data not shown).

The effect of F(ab')₂ anti-CD101 on Ca²⁺ flux and on stress kinase activation in human monocytes/macrophages. Although ligation of CD101 on T cells inhibits anti-CD3-induced [Ca²⁺]_i transients^{6,7}, its function in human macrophages is unknown. Ligation of F(ab')₂ anti-CD101 fragments with monocytes/macrophages in culture had no effect on the [Ca²⁺]_i (Figure 5A) and did not suppress TCR/anti-CD28-induced [Ca²⁺]_i transients (data not shown).

In monocytes and macrophages, LPS is known to activate, among other pathways, stress kinases and the downstream nuclear factor transcription factor (NF)- κ B. Ligation of CD101 on monocytes/macrophages with F(ab')₂ anti-CD101 fragments had no effect on p38 MAP kinase phosphorylation, although anti-CD101 antibodies increased phosphorylation of p38 MAP kinase by 48% in LPS-stimulated monocytes/macrophages (Figure 5B). Interestingly, SAP/JNK, PI-3K, and MAP kinase pathways were unaffected by CD101 signaling (data not shown).

DISCUSSION

The leukocyte surface protein CD101 is expressed on several immune cells, such as DC, monocytes, and activated T cells^{1,6,7}. Gouttefangeas, *et al*³ reported that 29% of both CD4+ and CD8+ from the peripheral blood were stained with anti-CD101 monoclonal antibodies. In contrast, we found that only $6.0 \pm 1.8\%$ of CD4+ and $9.5 \pm 2.0\%$ of CD8+ T lymphocytes freshly isolated from the peripheral blood of healthy controls expressed this molecule. This difference may be due to the variations in expression of CD101 between donors as well as to the different affinity of antibodies used for staining.

As disease relevance and function of CD101 in humans remain unknown, we examined the expression of CD101 on Treg and CD8+ peripheral blood lymphocytes of patients with RA treated with common pharmacological algorithms.

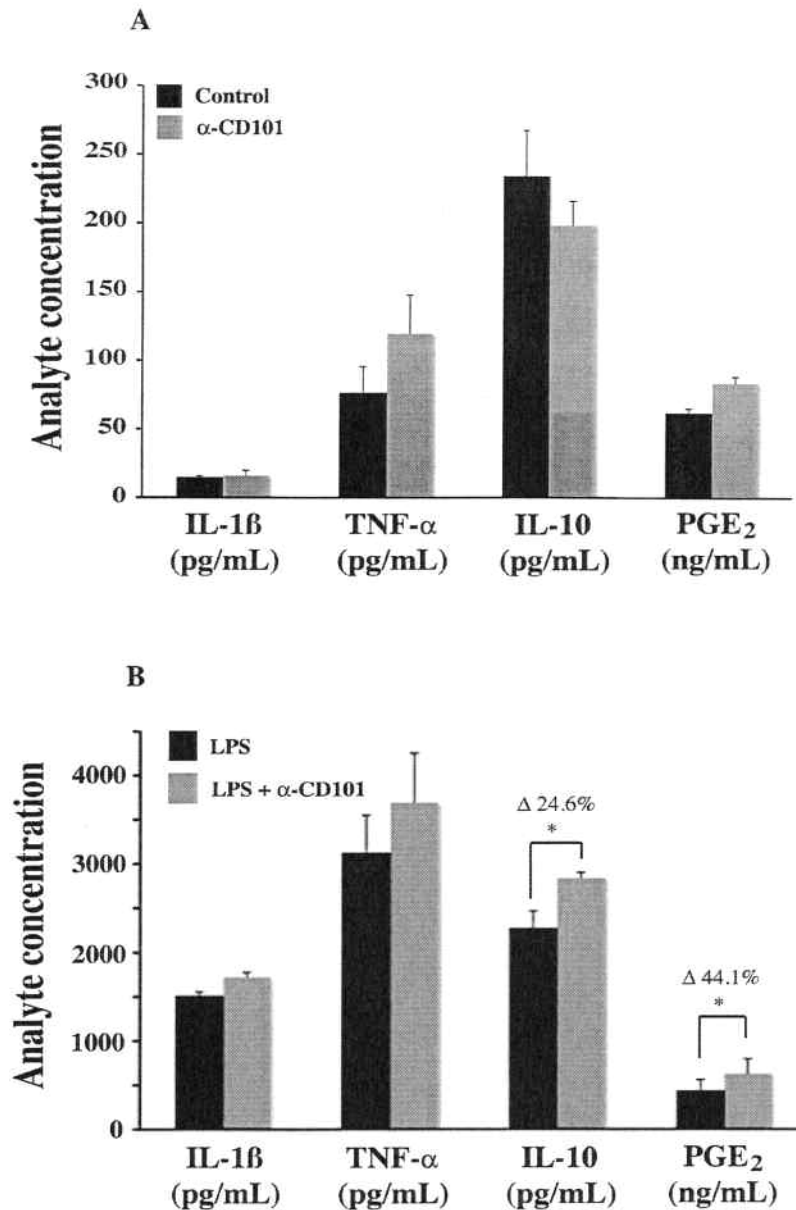


Figure 4. The effect of F(ab')₂ anti-CD101 fragments on monocyte/macrophage cytokine and PGE₂ production. Cultures were treated with vehicle (control, A) or with 500 ng/ml LPS (B) in the presence or absence of 10 μ g/ml of the monoclonal anti-CD101 F(ab')₂ fragment as indicated. Measurement of IL-1 β , TNF- α , IL-10, and PGE₂ in 72-h supernatants obtained from the monocyte/macrophage stimulation assays was performed using ELISA. *p < 0.05 (n = 5 determinations).

The number of CD4+CD25+ cells in peripheral blood from patients with RA expressing CD101 was comparable to that from healthy controls, suggesting that there is no significant alteration in CD101 expression on the circulating CD4+CD25+ Treg cell population in RA as a consequence of treatment algorithms. However, RA CD8+ cells expressed CD101 significantly less compared to control CD8+ cells and the expression was inversely correlated with CD8^{high} cytotoxicity. In this connection, depletion of CD8+ cells (low CD101 expression) in animal models of RA¹⁵ strongly reduces joint inflammation, suggesting not only

that CD101 may have a regulatory function in terms of CD8+ activity, but also that inflammatory mediators may target its expression.

In T cell lineages, the expression of CD101 is positively modulated after lymphocyte activation³, and this can explain the inhibitory effect of anti-CD101 mAb on CD4+CD101- cell proliferation. We also tested the possible role of CD101 on the function of regulatory T cells. Studies in mice have provided firm evidence for the existence of a naturally occurring population of CD4+CD25^{high} professional regulatory/suppressor T cells, which, upon *in vitro*

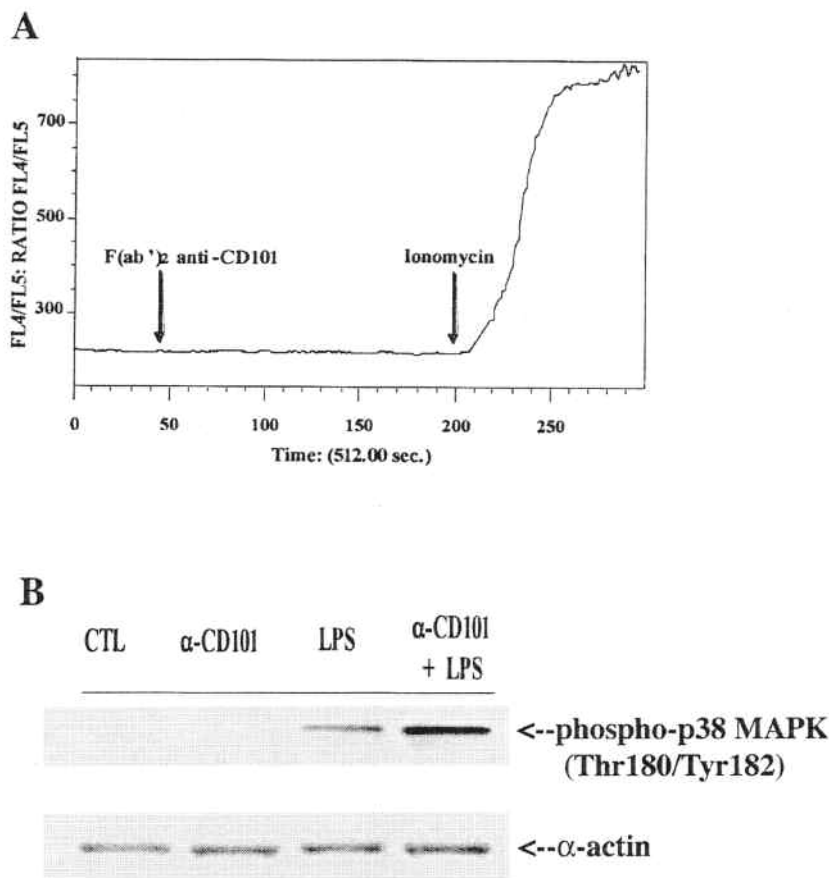


Figure 5. The effect of F(ab')₂ anti-CD101 fragments on Ca²⁺ flux (A) and p38 MAP kinase activation (B) in human macrophages. A. Macrophages were loaded with 1 μM Indo-1/AM for 40 min in RPMI medium supplemented with 1% FBS at 37°C. Cells were analyzed using blue and ultraviolet lasers in the presence or absence of anti-CD101 monoclonal antibody; the data display the Indo-1 log violet/blue ratio as a function of time. Cells treated with 2 nM ionomycin were used for the maximum increase in [Ca²⁺]_i. B. Cultures were treated with vehicle (control) or with 500 ng/ml LPS in the presence or absence of 10 μg/ml of the monoclonal anti-CD101 F(ab')₂ fragment, and cells were extracted for protein and subjected to Western blot analysis using antiphospholipid p38 MAP kinase and α-actin antibodies. CTL: control.

TCR-mediated stimulation, suppress the proliferation of effector T cells^{16,17}. We found that the suppressive capacity of CD101+ Treg did not differ from the suppressive capacity of CD101- Treg for the inhibition of both CD4+CD101- and CD4+CD101+ autologous responder T cells. These results suggested that the direct cell-to-cell contact by CD101 molecule between Treg and responder T cells was not a part of the mechanism/s by which Treg suppressed T cell function. Also it confirmed a previous report showing that CD101 was not a marker that distinguished activated from nonactivated Treg¹⁸. Contrary to our results, Fernandez, *et al*¹¹ recently demonstrated that CD101 surface expression was highly correlated with functional suppressor activity within CD4+CD25+ Treg populations both *in vitro* and *in vivo*. The authors sorted CD4+CD25+CD101^{high} and CD4+CD25+CD101^{low} cells from spleen and compared their suppressor activity in mixed lymphocyte reaction and

in a lethal graft-vs-host disease (GVHD) model. In our experiments, we measured the capacity of human CD4+CD25+CD101+ and CD4+CD25+CD101- Treg, isolated from peripheral blood, to suppress the proliferation of autologous CD4+ or CD8+ peripheral blood T cells. Although Fernandez, *et al*¹¹ found a difference in regulatory activity between CD101^{high} and CD101^{low} Treg, they did not compare CD101+ with CD101- Treg isolated from the peripheral blood of mice.

CD8+ T lymphocytes are important mediators of adaptive immunity contributing to resistance against intracellular infections with certain viral, protozoan, and bacterial pathogens¹⁹. CD8+ T cells may exert their specific effector function through the production of cytokines such as IFN-γ and TNF-α²⁰ or through a direct cytolytic mechanism²¹. In particular, CD8+ T cells can kill other cells by releasing granule contents such as perforin and granzyme²². In our

experiments, CD8+ T cells negative for CD101 were found to be significantly more effective in killing K562 target cell line compared to CD101+ CD8 lymphocytes. This difference was not due to the different intracellular content of granzyme B in these 2 cell subpopulations. The function of the CD101 molecule expressed on the cytotoxic T cells is not known and we can speculate that its presence on the surface of these cells may be attributed to their contact with the target cells or in the granzyme B release from the CD8 T cells.

Boulou, *et al*⁹ demonstrated that CD101 triggering on cutaneous DC inhibited T cell proliferation through paracrine IL-10 production, and our study indicated that ligation of CD101 on human monocytes/macrophages, in either control or LPS-stimulated conditions, modified cytokine and cyclooxygenase-2 (PGE₂) expression. We observed that the putative costimulatory effect of anti-CD101 mAb was not followed by any changes in the [Ca²⁺]_i or in other second messenger systems (e.g., [cAMP]_i, data not shown). However, ligation of the CD101 molecule augmented p38 MAP kinase phosphorylation (but not other MAP kinase pathways) in LPS-stimulated monocytes; the latter pathway is strongly associated with inflammation, autoimmunity, diabetes, and cancer, probably through the control of inflammatory mediator expression²³. A large body of evidence has shown that p38 regulates the stability of messenger RNA (mRNA) with short half-lives, implicating cytokines, chemokines, oncogenes, and other proinflammatory mediators as targets²⁴. Indeed, cyclooxygenase-2 mRNA stability is strongly regulated by p38 MAP kinase signaling²⁵.

CD101 can be considered a differentiation marker of the monocyte/leukocyte lineage. Ligation of CD101 cotransduced a p38 MAP kinase signal with downstream effects on cell proliferation, cytokine production, inflammatory mediator release, and intracellular killing. Despite our strong interest, no difference in the inhibitory function was found between CD101+ and CD101- regulatory T cells in contrast to mouse models of GVHD, where CD101 surface expression was highly correlated with functional suppressor activity within the CD4+CD25+ Treg population. However, CD8+CD101+ T cells were far less cytotoxic than CD101-naïve cytotoxic T cells, which were in fact enriched in patients with RA. In this regard, management of RA still requires continuous and aggressive immunosuppression, with the risk of untoward effects. We propose testing, by adoptive transfer of CD8+CD28-CD101+ T cell clones, a novel immunotherapy for suppressing synovitis and systemic manifestations in mouse models of RA, as a preclinical approach.

REFERENCES

1. Rivas A, Ruegg CL, Zeitung J, Laus R, Warnke R, Benike C, et al. V7, a novel leukocyte surface protein that participates in T cell activation. I. Tissue distribution and functional studies. *J Immunol* 1995;154:4423-33.
2. Russell GJ, Parker CM, Sood A, Mizoguchi E, Ebert EC, Bhan AK, et al. p126 (CDw101), a costimulatory molecule preferentially expressed on mucosal T lymphocytes. *J Immunol* 1996;157:3366-74.
3. Gouttefangeas C, Jackuot S, Meffre E, Schmid M, Bousmell L, Bensussan A. Differential proliferative responses in subset of human CD28+ cells delineated by BB27 mAb. *Int Immunol* 1994;6:423-30.
4. Boulou A, Boulland ML, Geissmann F, Fraitaig S, Andry P, Teillac D, et al. CD101 expression by Langerhans cell histiocytosis cells. *Histopathology* 2000;36:229-32.
5. Ruegg C, Rivas A, Madani ND, Zeitung J, Laus R, Engleman EG. V7, a novel leukocyte surface protein that participates in T cell activation. II. Molecular cloning and characterization of the V7 gene. *J Immunol* 1995;154:4434-44.
6. Soares LR, Tsavaler L, Rivas A, Engleman EG. V7 (CD101) ligation inhibits TCR/CD3-induced IL-2 production by blocking Ca²⁺ flux and nuclear factor of activated T cell nuclear translocation. *J Immunol* 1998;161:209-17.
7. Soares LR, Rivas A, Tsavaler L, Engleman EG. Ligation of the V7 molecule on T cells blocks energy induction through a CD28-independent mechanism. *J Immunol* 1997;159:1115-24.
8. Soares LR, Rivas A, Ruegg C, Engleman EG. Differential response of CD4+ V7+ and CD4+ V7- T cells to T cell receptor-dependent signals: CD4+ V7+ T cells are co-stimulation independent and anti-V7 antibody blocks the induction of energy by bacterial superantigen. *Eur J Immunol* 1997;27:1413-21.
9. Boulou A, Bagot M, Delaire S, Bensussan A, Bousmell L. Triggering CD101 molecule on human cutaneous dendritic cells inhibits T cell proliferation via IL-10 production. *Eur J Immunol* 2000;30:3132-9.
10. Yamaji K, Ikegami H, Fujisawa T, Noso S, Nojima K, Babaya N, et al. Evidence for Cd101 but not Fcgr1 as candidate for type 1 diabetes locus, Idd10. *Biochem Biophys Res Commun* 2005;331:536-42.
11. Fernandez I, Zeiser R, Karsunky H, Kambham N, Beilhack A, Soderstrom K, et al. CD101 surface expression discriminates potency among murine FoxP3+ regulatory T cells. *J Immunol* 2007;179:2808-14.
12. Bennett S, Por SB, Stanley ER, Breit SN. Monocyte proliferation in a cytokine-free, serum-free system. *J Immunol Methods* 1992;153:201-12.
13. Vukmanovic-Stejeic M, Vyas B, Gorak-Stolinska P, Noble A, Kemeny DM. Human Tc1 and Tc2/Tc0 CD8 T-cell clones display distinct cell surface and functional phenotypes. *Blood* 2000;95:231-40.
14. Dileepan KN, Page JC, Li Y, Stechschulte DJ. Direct activation of murine peritoneal macrophages for nitric oxide production and tumor cell killing by interferon-gamma. *J Interferon Cytokine Res* 1995;15:387-94.
15. Raposo BR, Água-Doce AM, Pereira da Silva JA, Graça L, Souto-Carneiro MM. Disease amelioration in the K/BxN mouse model of spontaneous chronic arthritis after CD8 T cell depletion. *Ann Rheum Dis* 2010;69:A66.
16. Sakaguchi S, Sakaguchi N, Shimizu J, Yamazaki S, Sakihama T, Itoh M, et al. Immunologic tolerance maintained by CD25+ CD4+ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 2001;182:18-32.
17. Bach JF. Regulatory T cells under scrutiny. *Nat Rev Immunol* 2003;3:189-98.
18. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 2004;22:531-62.

19. Harty JT, Tvinnereim AR, White DW. CD8+ T cell effector mechanisms in resistance to infection. *Annu Rev Immunol* 2000;18:275-308.
20. Guidotti LG, Chisari FV. Cytokine-mediated control of viral infections. *Virology* 2000;273:221-7.
21. Zamoyska R. CD4 and CD8: modulators of T-cell receptor recognition of antigen and of immune responses? *Curr Opin Immunol* 1998;10:82-7.
22. Barry M, Heibei JA, Pinkoski MJ, Lee SF, Moyer RW, Green DR, et al. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. *Mol Cell Biol* 2000;20:3781-94.
23. Saklatvala J. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. *Curr Opin Pharmacol* 2004;4:372-7.
24. Clark A, Dean J, Tudor C, Saklatvala J. Post-transcriptional gene regulation by MAP kinases via AU-rich elements. *Front Biosci* 2009;14:847-71.
25. Faour WH, He Y, He QW, de Ladurantaye M, Quintero M, Mancini A, et al. Prostaglandin E(2) regulates the level and stability of cyclooxygenase-2 mRNA through activation of p38 mitogen-activated protein kinase in interleukin-1 beta-treated human synovial fibroblasts. *J Biol Chem* 2001;276:31720-31.