VLA-4 integrin concentrates at the peripheral supramolecular activation complex of the immune synapse and drives T helper 1 responses

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The integrin $\alpha4\beta1$ (VLA-4) not only mediates the adhesion and transendothelial migration of leukocytes, but also provides costimulatory signals that contribute to the activation of T lymphocytes. However, the behavior of $\alpha4\beta1$ during the formation of the immune synapse is currently unknown. Here, we show that $\alpha4\beta1$ is recruited to both human and murine antigen-dependent immune synapses, when the antigen-presenting cell is a B lymphocyte or a dendritic cell, colocalizing with LFA-1 at the peripheral supramolecular activation complex. However, when conjugates are formed in the presence of anti-$\alpha4$ antibodies, VLA-4 colocalizes with the CD3-ζ chain at the center of the synapse. In addition, antibody engagement of $\alpha4$ integrin promotes polarization toward a T helper 1 (Th1) response in human *in vitro* models of $CD4^+$ T cell differentiation and naïve T cell priming by dendritic cells. The *in vivo* administration of anti-$\alpha4$ integrin antibodies also induces an immune deviation to Th1 response that dampens a Th2-driven autoimmune nephritis in Brown Norway rats. These data reveal a regulatory role of $\alpha4$ integrins on T lymphocyte-antigen presenting cell cognate immune interactions.

After the recognition of antigens (Ag) presented by dendritic cells (DCs), naïve T lymphocytes proliferate and differentiate into T helper (Th) 1 or 2 effector cells. These effector lymphocytes are characterized by distinct patterns of cytokine production and homing behavior. Th1 cells mainly produce IFN-γ and IL-2 and have a key role in the cellular immune responses. Conversely, Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and promote the humoral immune responses (1). DCs are the only Ag-presenting cells (APCs) involved in the priming of naïve Th cells and their polarization toward Th1 or Th2 differentiation. To acquire this capacity, DCs must undergo a maturation process characterized by the loss of their Ag-capturing capacity and the increase of their expression of costimulatory and adhesion molecules, including $\alpha4\beta1$ integrin (2). However, other APCs (e.g., B lymphocytes) are also involved in regulating the cytokine profiles of Th cell responses, indicating the importance of postpriming events (3).

The interaction between T cells and APCs plays an important role in directing Th cell polarization. The strength of antigenic stimulation, the duration of T cell receptor engagement, the presence of different cytokines, and the participation of distinct costimulatory molecules are critical in determining the phenotype of differentiated T cells. The cytokine IL-12, high doses of Ag, and CD28/B7–1 interaction promote Th1 differentiation, whereas an environment enriched in IL-4, low doses of Ag, and CD28/B7–2 or inducible costimulator (ICOS)/ICOS ligand participation promote Th2 responses (4).

Integrins are a large family of $\alpha\beta$ heterodimeric transmembrane proteins that mediate cell–cell and cell–extracellular matrix adhesion. Several integrins, lymphocyte function-associated (LFA-1; $\alpha_L\beta_2$), very late activation antigen-4 (VLA-4; $\alpha4\beta1$), and VLA-1 ($\alpha1\beta1$) have been involved also in the transduction of costimulatory signals in T cells (5). However, whereas the involvement of $\alpha_L\beta2$ during Ag presentation is well known, the role of $\alpha4\beta1$ has not been addressed. The $\alpha_L\beta2$ integrin mediates T cell adhesion to APCs, facilitating the formation of the immunological synapse (IS) (6). The pair $\alpha_L\beta_2$/intracellular adhesion molecule-1 (ICAM-1) forms an adhesion ring that is called the peripheral supramolecular activation complex (pSMAC), that surrounds the T cell receptor–peptide–MHC complexes localized at the central SMAC of the IS (7, 8). Several studies in mouse models revealed that $\alpha_L\beta_2$/ICAM-1 interaction could be important for driving Th1 polarization (9, 10).

The $\alpha4\beta1$ integrin is predominantly expressed on hematopoietic cells and serves as a receptor for fibronectin and vascular cell adhesion molecule 1 (VCAM-1). In addition to mediating leukocyte adhesion to endothelium and extracellular matrix, $\alpha4\beta1$ has been implicated in T cell costimulation (11–13). The dual role of $\alpha4\beta1$ as an adhesion and costimulatory molecule suggests that this integrin could be involved in the modulation of the T cell response during Ag presentation. However, the behavior of $\alpha4\beta1$ and its possible function during the establishment of an IS has not been examined. Here, we show that $\alpha4\beta1$ is recruited to the pSMAC of IS colocalizing with LFA-1 integrin. We also demonstrate the functional involvement of this integrin in the priming of T lymphocytes toward a Th1 response *in vitro* and *in vivo*.

Materials and Methods

Cells, Antibodies, and Reactives. The Jurkat-derived human T cell line $V\beta8^+$ J77cl20, the lymphoblastoid B cell line Raji, the murine Th cell clone SR.D10, the I-A^k transgenic B lymphoma cell line TAK, and the S3085B (S3) human T cell clone have been described (14). Human monocyte-derived DCs were generated as described (14) and matured with lipopolysaccharide (LPS) (10 ng/ml) (Sigma) or calcium ionophore A23187 (150 ng/ml) (Sigma) for 48 h or with tumor necrosis factor (TNF)-α (50 ng/ml) plus IL-1β (10 ng/ml) for 4 days. The phenotype of mature DCs ($CD83^{high}$ $CD86^{high}$ $CD14^{low}$) was confirmed by flow cytometry. The murine anti-human $\alpha4$ integrin mAbs HP2/1 (epitope B1) and HP2/4 (epitope B2) are mouse IgG1κ, bind with comparable affinity to $\alpha4$ integrin, and cross-react with rat $\alpha4$ integrins (15); and anti-$\alpha4$ integrin mAb HP1/7 (epitope A) have been described (16). Anti-human $\beta1$ integrin TS2/16,

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Abbreviations: Ag, antigen; DC, dendritic cell; Th, T helper; APC, Ag-presenting cell; SMAC, supramolecular activation complex; pSMAC, peripheral SMAC; VCAM-1; vascular cellular adhesion molecule 1; ICAM-1, intracellular adhesion molecule 1; IS, immunological synapse; LPS, lipopolysaccharide; TNF, tumor necrosis factor; CMAC, chloromethyl derivative of aminocoumarin; SEB, staphylococcal enterotoxin B; SEE, staphylococcal enterotoxin E; BN, Brown Norway; PMA, phorbol 12-myristate 13-acetate.

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anti-CD3 T3b, anti-α_L integrin TP1/40, anti-CD45 D3/9, anti-human CD9 VJ1/10, and anti-CD28 (CD28.2) mAbs have been described (14). Rat anti-mouse α4 PS/2 mAb was provided by T. Issekutz (Dalhousie University, Halifax, Canada), rat anti-mouse ICAM-1 was provided by D. Vestweber (University of Münster, Münster, Germany), anti-JAM-A antibody was provided by E. Dejana (Instituto FIRC di Oncologia, Milan), and rabbit anti-human CD3-ζ chain 448 was provided by B. Alarcón (Centro de Biología Molecular, Madrid).

Mouse anti-rat mAbs specific for CD45RC (FITC-OX22), CD4 (PE-W6/25), and CD45 (OX1) were purchased from Serotec (Oxford). Secondary Alexa 488- and Rhodamine red X-labeled Abs, streptavidin, and the fluorescent tracker CMAC (chloromethyl derivative of aminocoumarin) were from Molecular Probes. Poly(L -lysine) was from Sigma, and the superantigen staphylococcal enterotoxin E (SEE) was from Toxin Technology (Sarasota, FL). The human recombinant IL-2 was obtained through the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, contributed by M. Gately (Hoffmann-LaRoche, Nutley, NJ).

Cell Conjugate Assays and Immunofluorescence Microscopy. APCs were loaded for 20 min at 37°C with the fluorescent tracker CMAC (10 μM) (Molecular Probes) and incubated for 20 min at 37°C with 2 μg/ml SEE (Raji) and 1 μg/ml staphylococcal enterotoxin B (SEB) (DC) or for 16 h with 1 mg/ml conalbumin (Sigma) (TAK). Jurkat, S3, or SR.D10 (10^5 cells) were mixed with Raji (10^5 cells), DCs (5×10^4 cells), or TAK (10^5 cells), respectively, and incubated for 15 min. Cells were plated onto poly(L-lysine) (50 μg/ml) coated slides, incubated for 15 min at 37°C, and fixed in 4% formaldehyde. For immunofluorescence assays, samples were blocked with TNB (0.1 M Tris·HCl/0.15 M NaCl/0.5% blocking reagent; Boehringer Mannheim), and Fc receptors were blocked with human γ-globulin (100 μg/ml) or mouse serum (Sigma). After staining with primary mAb followed by an Alexa 488-labeled specific secondary Ab, samples were examined with a DMR photo-microscope (Leica) using Leica QFISH 1.0 software. For double staining, cells were incubated with the primary Ab followed by a goat anti-mouse rhodamine Red X, saturated with mouse serum, and incubated with the biotinylated anti-α4 HP2/1 or HP1/7 and streptavidin-Alexa Fluor 488. Series of optical sections were obtained with a Leica TCS-SP confocal scanning laser microscope. Three-dimensional reconstructions of confocal sections (distanced 0.2 μm in the vertical axis) were assembled by using the Leica confocal software.

***In Vitro* mAb-Mediated CD4$^+$ T Cell Activation.** Purified anti-CD3 T3b (0.5 μg/ml) in combination with 10 μg/ml anti-α4 (HP2/1 or HP2/4), anti-CD28 (CD28.2), or anti-CD9 (VJ1/10) were diluted in carbonate buffer (pH 9.0), and then 50 μl were added to each well of 96-well culture plates and incubated at 37°C for at least 4 h. The wells were rinsed with RPMI medium 1640 before use. Freshly isolated CD4$^+$ T cells were resuspended in complete medium at 5×10^4 cells per 200 μl and plated onto the mAb-coated wells. After 48 h, CD4$^+$ T cells were restimulated with phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) (Sigma) and calcium ionophore A23187 (1 μM) for 6 h. IL-4 and IFN-γ levels in cell culture supernatant were measured by ELISA (human IL-4 and IFN-γ Eli-Pair, Diaclone).

Isolation of Th Naïve Lymphocytes and Cocultures with Autologous DCs. Human CD4$^+$ CD45RA$^+$ CD45RO$^-$ naïve Th cells were isolated from human PBMC by using CD4/CD45RO columns kit (R & D Systems). Autologous mature DCs were preincubated with SEB (1 ng/ml) and γ-globulin. Naïve Th cells (5×10^4 cells per 200 μl) and DCs (2×10^4 cells per 200 μl) were cocultured in 96-well culture plates (Costar) in the presence of 2 μg/ml HP2/1, HP2/4, or control Ab anti-α3 integrin VJ1/6. On day 5, IL-2 (10 units/ml) was added. On day 14, Th cells were stimulated with PMA/A23187, and IL-4 and IFN-γ levels were measured as described above.

Flow Cytometry Analysis. Mature DCs were incubated with human γ-globulin (100 μg/ml) in PBS for 20 min and then with anti-α4 integrin HP2/1 or anti-CD3 T3b mAb (negative control) for 30 min. After washing, cells were incubated with an FITC-conjugated secondary Ab and analyzed in a FACScan flow cytometer (Becton Dickinson).

***In Vivo* Treatment Protocols.** Brown Norway (BN) rats were separated into two main groups. Group 1 served as a normal control, whereas group 2 animals received five s.c. injections of $HgCl_2$ (1 mg/kg body weight) over a period of 2 weeks (15). Within each group, rats were i.p. injected with vehicle (phosphate buffer, pH 7.5) or 0.5 mg of HP2/1, HP2/4, or OX1 (anti-rat CD45) mAbs on days 0, 8, and 13. At days 0, 6, 9, 13, 15, and 23, rats ($n = 6$) were bled and killed, and their spleens were removed. Blood was either heparinized for flow cytometry studies or allowed to clot to obtain serum.

Serum samples were tested for IFN-γ levels by ELISA (R & D Systems). Spleen mononuclear cell suspensions were isolated and stimulated with PMA (50 ng/ml) and ionomycin (Sigma) (150 ng/ml) for 24 h. IL-4 levels in cell culture supernatants were assayed by ELISA (OpEIA rat IL-4 set, Pharmingen).

Results

α4β1 Integrin Concentrates at T Cell–APC Immune Contacts. The α4β1 integrin has not only been involved in cellular adhesion phenomena, but also in providing costimulatory signals during T cell activation (11, 13). We assessed the subcellular localization of this β1 integrin during the interaction of T lymphocytes with APCs. The human B cell line Raji is able to present the SEE superantigen to J77 Vβ8$^+$ Jurkat T cells rendering functional IS (14). Whereas α4β1 integrin was evenly distributed on cell conjugates formed in the absence of SEE, it concentrated at IS of conjugates formed in the presence of superantigen (Fig. 1 *A* and *B*). As expected, CD3 and LFA-1 were also localized at the IS in a SEE-specific manner, whereas CD45 remained homogeneously distributed. The localization of α4 integrin subunit in primary human DC–T cell conjugates was also determined. α4 integrin relocalized at the cell–cell contact area of conjugates between mature monocyte-derived DCs SEB-pulsed with S3 SEB-specific primary T cells (Fig. 1*C*). As expected, LFA-1 showed a similar behavior.

To determine whether the localization of α4β1 integrin at the IS depends on the presence of its ligands at the APC, we first analyzed the expression of VCAM-1 in these cell–cell conjugates. Flow cytometry analyses revealed that Raji cells and monocyte-derived DCs do not express VCAM-1 on the surface (data not shown). Next, because cell–cell conjugates were generated in the presence of serum, we tested the possibility that plasma fibronectin could be adsorbed to the cell surface and mediates α4β1 redistribution to cell–cell contact. However, the presence of fibronectin on Raji-J77 conjugates was undetectable by immunostaining (data not shown). To completely rule out the presence of fibronectin, conjugates were formed under serum-free conditions. In these conditions, VLA-4 was also concentrated at the IS (data not shown).

To extend our observations to a peptide-specific model of Ag presentation, we used a murine transgenic T lymphocyte cell line specific for a peptide of conalbumin presented by I-A^k transgenic B cell line (14). We observed that α4 integrin was also localized at the contact area between peptide-specific T-B cell conjugates.

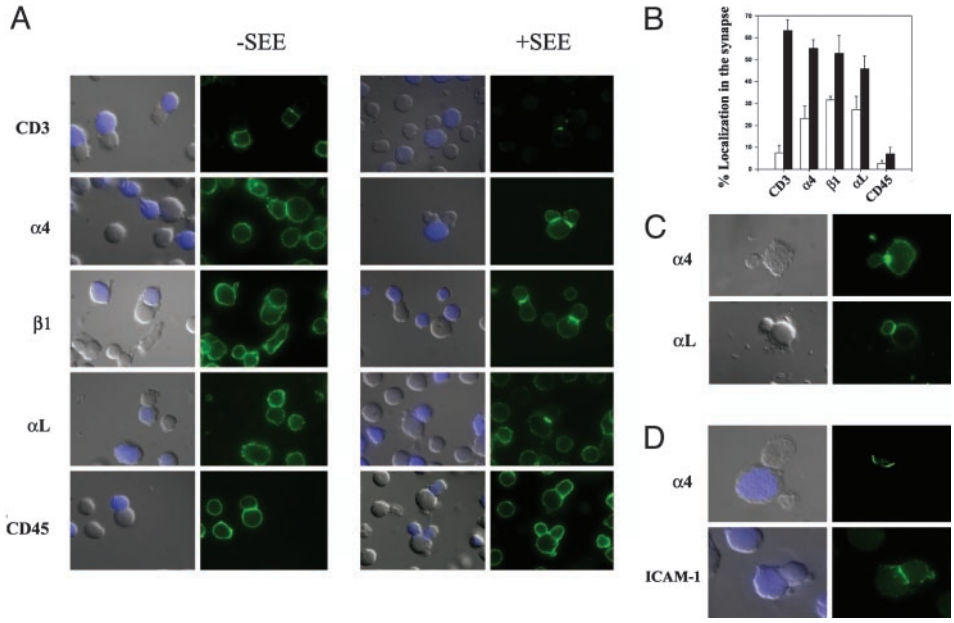

Fig. 1. $\alpha4\beta1$ integrin concentrates at the T cell–APC immune contacts. (*A*) Raji B cells and J77 T cells were conjugated in the presence (*Right*) or absence (*Left*) of SEE and stained for CD3, CD45, and $\alpha4$, $\beta1$, or α_L integrin chains (green). Differential interference contrast (DIC) pictures merged with blue fluorescence images from CMAC-loaded Raji cells are shown. (*B*) Percentage of cell conjugates in which CD3, CD45, and $\alpha4$, $\beta1$, or α_L integrins were relocalized at the T cell–APC contact area in the presence (filled bars) or absence (open bars) of SEE. At least 100 conjugates from two independent experiments were analyzed. Results correspond to the arithmetic mean ± SD. (*C*) Human DCs and the nontransformed human T cell clone S3 were allowed to conjugate in the presence of SEB, adhered to poly(L-lysine)-coated coverslips, fixed, and stained (green) for α_L and $\alpha4$ integrins. Corresponding DIC images are shown. (*D*) Mouse TAK B cells were incubated with conalbumin and conjugated with SR.D10 T cells fixed and stained (green) for ICAM-1 and $\alpha4$ integrin. DIC pictures merged with blue CMAC-loaded TAK cells are shown.

As expected, ICAM-1 showed a similar subcellular distribution (Fig. 1*D*).

$\alpha4$ Is Localized at the pSMAC Ring of the IS. The formation of an IS between a T cell and an APC is characterized by the clustering of the T cell receptor/CD3 in the central SMAC complex surrounded by a ring of $\beta2$ integrin LFA-1 (pSMAC) (7). To topographically map $\alpha4$ within the IS, double staining for $\alpha4$ and IS markers on Raji-J77 cell conjugates generated in the presence of SEE was performed. Double staining of α_L and $\alpha4$ integrins revealed that both integrins colocalized at pSMAC. CD3 molecules concentrated at the central cluster, which is surrounded by the peripheral ring where $\alpha4$ and $\beta1$ integrin chains colocalized. In contrast, CD45 was evenly distributed at the plasma membrane of both T lymphocytes and APCs (Fig. 2*A*).

To assess the role of $\alpha4\beta1$ integrin during the formation of T cell–APC conjugates, Raji and J77 cells were preincubated with two different anti-$\alpha4$ antibodies, HP2/1 against epitope B1 or HP2/4, which recognizes epitope B2, before conjugate formation. Neither HP2/1 nor HP2/4 blocked the formation of T

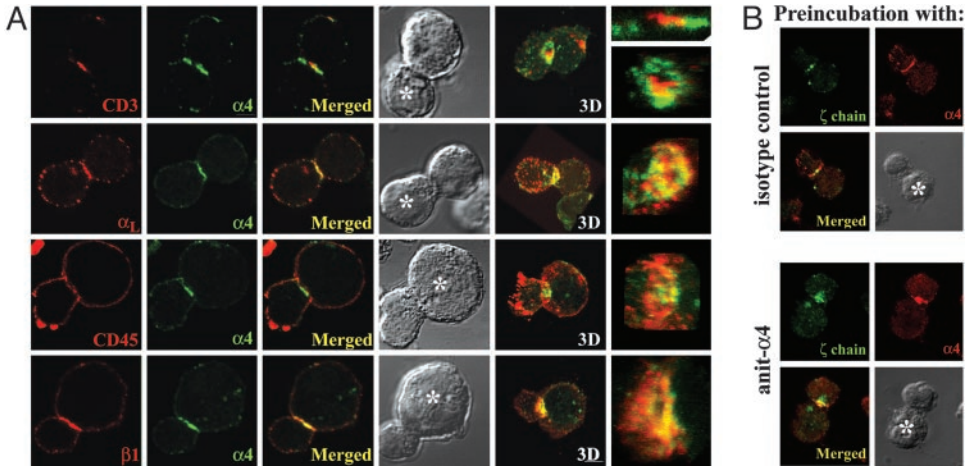

Fig. 2. $\alpha4\beta1$ segregates to the pSMAC of the IS. (*A*) J77-Raji cell conjugates were generated in presence of SEE and double-stained for $\alpha4$, $\beta1$, and α_L integrin chains, CD3, or CD45. One representative confocal section, the DIC image, a merged 3D reconstruction, and a high magnification of IS area are shown. (*B*) Raji and J77 cells were incubated with 10 μg/ml HP2/1, HP2/4, or an isotype control (VJ1/10) for 10 min. Then, Raji and J77 cells were mixed in the presence of SEE and double-stained for CD3-ζ chain and VLA-4 epitope A by using biotinylated HP1/7 mAb. One representative confocal section and the DIC image are shown. Asterisks indicate APCs.

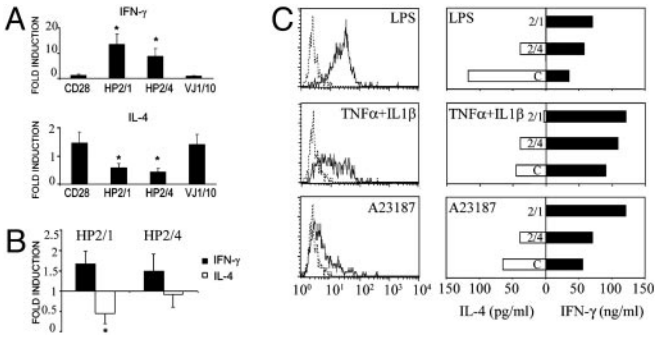

Fig. 3. Engagement of α4 integrin *in vitro* promotes Th1 cell polarization. (*A*) $CD4^+$ cells were activated with immobilized CD3 in the presence or absence of anti-CD28 (CD28.2), anti-α4 (HP2/1, HP2/4), or isotype control anti-CD9 (VJ1/10). After 48 h, T cells were restimulated with PMA/A23187. IL-4 and IFN-γ in the supernatant were measured by ELISA. Results correspond to fold induction of IFN-γ and IL-4 levels in the presence of anti-α4 mAbs, CD28, or VJ1/10 with respect to the control in the presence of CD3 alone. The arithmetic mean ± SEM is shown. *, $P < 0.01$ using the Mann–Whitney *U* test. (*B*) SEB-loaded mature DCs were cocultured with naïve Th cells in the absence or presence of HP2/1 or HP2/4 mAb. After 14 days, T lymphocytes were restimulated, and IL-4 and IFN-γ in the supernatant were measured as above. Results correspond to fold induction of IFN-γ and IL-4 levels in the presence of anti-α4 mAbs with respect to the control in the absence of Ab. The arithmetic mean ± SEM is shown. *, $P < 0.01$ using Student's *t* test. (*C*) Human monocyte-derived DCs induced to mature with LPS or ionophore A23187 for 48 h or with TNF-α plus IL-1β for 4 days were analyzed for the surface expression of α4 integrin (black line) by flow cytometry analysis. The dotted line corresponds to the negative control. Differentially matured DCs were cocultured with naïve T lymphocytes as in *B*. IL-4 and IFN-γ in the supernatant were measured by ELISA. Results correspond to one representative experiment out of three.

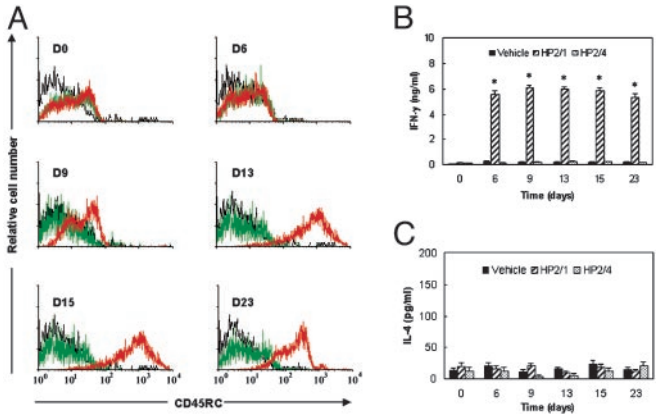

Fig. 4. Effect of anti-α4 mAbs administration on the Th1/Th2 balance in rats. (*A*) Kinetics of the expression (days 0–23, D0–D23) of CD45RC on $CD4^+$ cells in BN rats receiving vehicle (black line), HP2/1 mAb (red line), or HP2/4 mAb (green line). Histograms are representative of six different experiments performed. (*B* and *C*) Levels of IFN-γ in serum and IL-4 in different culture supernatants from PMA/ionomycin-stimulated splenocytes in rats treated as in *A*. Results are expressed as the arithmetic mean ± SD. *, $P < 0.001$ using Student's *t* test.

cell–APC conjugates or prevented the clustering of CD3-ζ chain, PKCθ, and MHC class II molecules to the IS (data not shown). Interestingly, when these conjugates were stained with mAb HP1/7, which recognizes a spatially separated epitope A on the α4 chain of VLA-4 (16), we found that, although the clustering of VLA-4 was not significantly inhibited, pretreatment with anti-VLA-4 antibodies interfered with the localization of VLA-4 at the pSMAC. Confocal microscopy analysis revealed that, under these conditions, VLA-4 colocalized with CD3- ζ chain, forming an atypical pattern of distribution within the IS (Fig. 2*B*). A quantitative estimation showed that this atypical synapse was found in 11 of 14 conjugates preincubated with HP2/1 and 14 of 14 conjugates preincubated with HP2/4, as compared with 3 of 14 cell conjugates preincubated with the isotype control.

Engagement of α4 Integrin Promotes *in Vitro* Th1 Cell Polarization. To ascertain the functional consequences of VLA-4 engagement during T cell–APC contacts, freshly isolated $CD4^+$ T cells were treated with a suboptimal concentration of immobilized CD3 in the presence or absence of coimmobilized anti-CD28, anti-α4 integrin (HP2/1 and HP2/4), or an isotype control (VJ1/10). Supernatants were collected 48 h after activation and assayed for IL-4 and IFN-γ. Anti-α4 mAb promoted IFN-γ secretion and inhibited IL-4 production (Fig. 3*A*), favoring a Th1 environment.

To determine whether anti-α4 antibodies are able to induce Th1 differentiation in naïve T lymphocytes, we used an *in vitro* model of naïve T lymphocyte differentiation by autologous DCs. After maturation with TNF-α plus IL-1β, human monocyte-derived DCs were incubated with SEB (1 ng/ml) and cocultured with autologous naïve T lymphocytes either in the presence or the absence of mAb against the different epitopes of α4. Then, the pattern of cytokine secretion of primed lymphocytes was analyzed. Interestingly, ligation of α4 with the HP2/1 mAb, which recognizes the B1 epitope of α4 (16), fully abrogated the differentiation of Th2 cells, as reflected by high levels of IFN-γ and a very low synthesis of IL-4. α4 engagement by the HP2/4 mAb, which recognizes the B2 epitope of α4 integrin (16), also promotes IFN-γ secretion; however, it did not induce a significant effect on IL-4 synthesis (Fig. 3*B*). The isotype control anti-α3 integrin VJ1/6 did not exert any effect on cytokine levels (data not shown).

Both T lymphocytes and mature DCs expressed α4β1 integrin. Thus, to discriminate whether the effects of anti-α4 mAb were exerted on DCs or T lymphocytes, we analyzed the induction of α4β1 expression by several DC maturation conditions. We found that, whereas LPS induced high levels of α4β1 on DCs, other stimuli, such as TNF-α plus IL-1β or calcium ionophore A23187, induced medium and low levels of α4β1 expression, respectively (Fig. 3*C*). Remarkably, HP2/1 mAb promoted IFN-γ and inhibited IL-4 secretion independently of the levels of α4β1 expression on DCs, suggesting that the effect of HP2/1 was exerted mainly on T lymphocytes (Fig. 3*C*).

α4 Regulates Th1 Differentiation *in Vivo*. To further assess the role of the α4 integrin in the regulation of the Th1/Th2 balance, we investigated the *in vivo* effect of anti-α4 integrin mAb administration on Th cell subsets in rats. Anti-α4 mAbs HP2/1 and HP2/4 recognized the rat α4 subunit by both biochemical and flow cytometry analyses (ref. 15 and data not shown). In BN rats, the expression of MRC-OX22 (high molecular mass isoform CD45RC) defines subsets of $CD4^+$ T cells with different functions; $CD45RC^{high}CD4^+$ cells produce IL-2 and IFN-γ, but little IL-4 (Th1), whereas $CD45RC^{low}CD4^+$ cells provide B cell help, producing more IL-4 with lower IL-2 and IFN-γ (Th2) (19, 20). BN rats had a basal higher percentage of $CD4/CD45RC^{low}$ (Th2) cells than $CD4/CD45RC^{high}$ (Th1) cells (Fig. 4). Administration of the anti-α4 mAb HP2/1 induced a shift toward a Th1-phenotype determined as the increase in the $CD4/CD45RC^{high}$ cells, starting at day 6 and lasting up to day 23. In contrast, rats treated with either the anti-α4 mAb HP2/4, or the control mAb (OX1) showed not significant changes in the $CD4/CD45RC^{low}$ (Th2) staining profile (Fig. 4*A* and data not shown). In addition to the Th1 profile in T cells, a clear-cut increment in IFN-γ secretion after HP2/1 treatment was found (Fig. 4*B*), whereas no significant changes in IL-4 production were observed (Fig. 4*C*).

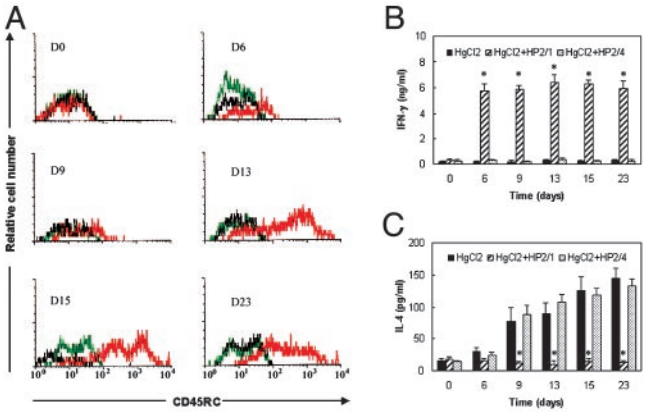

Fig. 5. Effect of anti-α4 mAbs administration on the Th1/Th2 balance in Th2-driven autoimmune disease. (*A*) Kinetics of the expression (days 0–23, D0–D23) of CD45RC Ag on $CD4^+$ cells was measured by flow cytometry in BN rats receiving $HgCl_2$ alone (black line), $HgCl_2$ and HP2/1 mAb (red line), or $HgCl_2$ and HP2/4 mAb (green line), as described in *Materials and Methods*. Histograms are representative of six different experiments. (*B* and *C*) Levels of IFN-γ in serum and IL-4 in culture supernatants of PMA/ionomycin-stimulated splenocytes in rats treated as in *A*. Results are expressed as the arithmetic mean ± SD. *, $P < 0.001$ using Student's *t* test.

α4 Engagement Dampens a Th2-Driven Autoimmune Disease. Next, we assessed whether anti-α4-induced Th1 response could have a therapeutic effect on Th2-mediated immune diseases. Repeated exposure to $HgCl_2$ induces an autoreactive Th2 cell subset-inducing polyclonal B cell activation (21, 22), with development of an autoimmune syndrome characterized by synthesis of antiglomerular basement membrane (GBM) Abs (23), with glomerular linear deposits of IgG, proteinuria, and interstitial nephritis (22, 24, 25). The administration of HP2/1, but not HP2/4, mAb to $HgCl_2$-treated rats blocked anti-GBM antibody production, preventing glomerular deposits and proteinuria (15, 26). These effects were exerted independently of the blockade on cell adhesion and glomerular leukocyte infiltration, which are caused by both HP2/1 and HP2/4 anti-α4 integrin (15). As occurred with healthy rats, administration of HP2/1 in $HgCl_2$-treated rats induced an increment in $CD4/CD45RC^{high}$ (Th1) cells (Fig. 5). Moreover, the progressive increment in IL-4 levels induced by $HgCl_2$ was completely abrogated by treatment with HP2/1 anti-α4 mAb, which increased the levels of IFN-γ. In contrast, these effects were not observed by treatment with anti-α4 mAb HP2/4 or with control mAb OX1 (Fig. 5 and data not shown).

Together, these data support the role of α4 integrins in the regulation of Th1/Th2 balance, likely by exerting a regulatory role at the initial phases of T cell–APC cognate interaction.

Discussion

The present work reports the localization of α4β1 integrin in both human and murine Ag-dependent IS of T lymphocytes with B cells or DCs as APCs. It is well known that α4β1 integrin is essential for leukocyte adhesion to the extracellular matrix or to endothelium. However, the specific role of α4β1 integrin in T cell–APC interaction has not been previously addressed. Herein, we report the recruitment of α4β1 to the pSMAC of the IS in a similar manner as $\alpha_L\beta2$. However, whereas $\alpha_L\beta2$ has a well established ligand on APCs (ICAM-1), the classic ligands described for α4β1,VCAM-1, and fibronectin are not expressed by these cellular types. Recently, junctional adhesion molecules (JAMs) have been described as counterreceptors for VLA-4 and LFA-1 integrins (17, 18), although in Raji-J77 conjugates, the JAM-A molecule is not significantly concentrated at the contact zone (M.M. and F.S.-M., unpublished data). Further investigations will be necessary to conclude whether members from the JAM family act as ligands of VLA-4 during T cell–APC interaction. On the other hand, the pattern of organization of α4β1 integrin at the cell–cell interface could be driven either by the binding of the integrin to a putative but as yet unknown ligand or in a ligand-independent manner involving the lateral association with other molecules present at the pSMAC. In this regard, it has been described that α4β1 integrin form complexes with tetraspanins (27). However, α4β1 and CD81 tetraspanin are apparently segregated to different domains of the IS, as CD81 is localized at the central SMAC (14). It is important to highlight that CD81 has been also involved in the regulation of Th1/Th2 balance (28, 29). Finally, the localization of α4β1 within the IS could be caused by its association with cytoskeleton. In this context, it has been described that the cytoplasmic tail of α4β1 associates to the signaling adapter molecule paxillin (30).

It is well known that costimulatory molecules, besides their role in mediating T lymphocyte activation, are involved in Th cell differentiation (4). It is therefore conceivable that regulatory signals generated by α4β1 at the pSMAC could modify Th1/Th2 balance. Hence, it has been described that stimulation of $CD8^+$ $CD28^-$ T cells with anti-CD3 and fibronectin induces a Th1-like response (31). On the other hand, blockade of fibronectin–α4β1 integrin interaction by the synthetic CS-1 peptide depressed intragraft expression of Th1-type cytokines in recipient animals conditioned with CD4 depleting mAb (32). Here we provide experimental evidence that integrin α4β1 induces a Th1 deviation both *in vitro* and *in vivo*. Our data show that α4 integrin chain engagement by the mAb HP2/1, which recognizes the B1 epitope in both human and rat α4 integrin, promotes a Th1 immune response in both *in vitro* models of $CD4^+$ activation and naïve T cell priming by DCs, as well as in rats. However, the mAb HP2/4, which recognizes the B2 epitope of the human and rat α4 and has the same isotype as HP2/1 (15), also induces Th1 deviation on CD4 T cells *in vitro*, but does not induce a significant effect on Th1/Th2 balance *in vivo*. It is feasible that other biological properties, such as the distinct α4-mediated signaling intensity, Fc binding to APC, or others may account for the differential *in vivo* behavior of anti-α4 HP2/1 vs. anti-α4 HP2/4. The differential effect of the *in vivo* administration of these mAbs, allows us to dissect the two main effects of anti-α4 mAb on Th cells, namely inhibition of adhesion and Th1/Th2 polarization. The mAb HP2/4 induces a blockade of α4β1/VCAM-1 interaction at similar extent as HP2/1 (16), but it nevertheless does not induce an immune Th1 deviation *in vivo*. Although both antibodies act as antagonist molecules in the adhesion of VLA-4 to its ligands, HP2/1 and HP2/4 neither block the formation of T cell–APC conjugates nor prevent the clustering of CD3 and MHC class II molecules to the IS (data not shown). Interestingly, anti-VLA-4 antibodies modify the topography of α4 at the IS, but it still remains unknown how the subtle changes on IS organization caused by anti-α4 antibodies are translated in cell signaling leading to Th1 polarization.

Over the last few years, the balance of Th1- and Th2-type response has been postulated to be an important mechanism determining resistance or susceptibility to autoimmunity (33). Studies on IL-4 and IFN-γ synthesis profiles in BN rats have indicated that autoreactive $CD4^+$Th2 cells seem to be implicated in the susceptibility to Hg administration (34). Thus, $HgCl_2$ induces a self-limiting autoimmune disorder characterized by enhanced secretion of IL-4, polyclonal B cell activation, synthesis of autoantibodies, widespread tissue injury, and heavy proteinuria. The spontaneous resolution of the autoimmune response in BN rats is associated with enhanced expression of both IFN-γ and IL-12. The protective effect of both anti-α4 mAb against infiltration of renal interstitium has been reported in the $HgCl_2$ model of nephritis. HP2/1 mAb, but not HP2/4 mAb, abolished

the anti-GBM antibody synthesis and glomerular deposits (15). The data presented herein strongly suggest that the differential effect of the HP2/1 mAb may reside in its capacity of altering Th1/Th2 polarization. These data further support that $CD4^+$ Th1 cells, as well as the high levels of IFN-γ induced by HP2/1 mAb, seem to have protective effect in the development of this autoimmune disease. Therefore, an α4-based therapy might be a valuable tool against Th2 immune diseases. In this regard, it has been reported that intrapulmonary blockade of α4 with the PS/2 mAb inhibits all signs of lung inflammation, IL-4 and IL-5 release, and airway hyperresponsiveness in a mouse model of asthma (35). These effects might indicate that PS/2 has properties similar to those of HP2/1 in altering the Th1/Th2 balance.

Therapy with anti-α4 integrin mAb has been found to be very effective in different inflammatory conditions, such as experimental allergic encephalitis, adjuvant-induced arthritis, spontaneous colitis, peritonitis, and autoimmune diabetes (36). Furthermore, a humanized monoclonal antibody against α4 has been recently used in clinical trials of patients with multiple sclerosis and Crohn's disease (37, 38). The complexity of α4 integrin activity, in both cellular trafficking and Th polarization, is reflected in a recent report (39) using a small-molecule VLA-4 antagonist to regulate relapsing experimental autoimmune encephalomyelitis (EAE). When the VLA-4 antagonist is administrated before clinical disease onset or at the peak of acute disease, it causes protection of mice from EAE, but leads to severe disease exacerbation upon treatment removal. Interestingly, mice undergoing short-term treatment with VLA-4 antagonist displayed an enhanced IFN-γ secretion. Therefore, it appears that continuous small-molecule treatment may be effective in EAE by preventing cell trafficking; however, the treatment seems to facilitate activation of Th1 resulting in a significant rebound upon cessation of treatment. Collectively, our data together with previous reports indicate that, although the mechanism of action of anti-α4 mAb has been primarily attributed to their capacity to interfere with leukocyte adhesion, the additional effect of some of these anti-α4 antibodies in modulating T cell priming and Th1/Th2 balance should be taken into account.

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