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Chemokines Fail to Up-Regulate β_1 Integrin-Dependent Adhesion in Human Th2 T Lymphocytes[1]

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Th1 and Th2 cells are functionally distinct subsets of CD4$^+$ T lymphocytes whose tissue-specific homing to sites of inflammation is regulated in part by the differential expression of P- and E-selectin ligands and selected chemokine receptors. Here we investigated the expression and function of β_1 integrins in Th1 and Th2 cells polarized in vitro. Th1 lymphocytes adhere transiently to the extracellular matrix ligands laminin 1 and fibronectin in response to chemokines such as RANTES and stromal cell-derived factor-1, and this process is paralleled by the activation of the Rac1 GTPase and by a rapid burst of actin polymerization. Selective inhibitors of phosphoinositide-3 kinase prevent efficiently all of the above processes, whereas the protein kinase C inhibitor bisindolylmaleimide prevents chemokine-induced adhesion without affecting Rac1 activation and actin polymerization. Notably, chemokine-induced adhesion to β_1 integrin ligands is markedly reduced in Th2 cells. Such a defect cannot be explained by a reduced sensitivity to chemokine stimulation in this T cell subset, nor by a defective activation of the signaling cascade involving phosphoinositide-3 kinase, Rac1, and actin turnover, as all these processes are activated at comparable levels by chemokines in the two subsets. We propose that reduced β_1 integrin-mediated adhesion in Th2 cells may restrain their ability to invade and/or reside in sites of chronic inflammation, which are characterized by thickening of basement membranes and extensive fibrosis, requiring efficient interaction with organized extracellular matrices. *The Journal of Immunology,* **2000, 164: 3292–3300.**

Progression of leukocytes from the bloodstream into lymphoid or peripheral tissues occurs by a defined series of activation and adhesion steps, which culminate in the acquisition of directional motility by the extravasating leukocytes (1, 2). Chemokines have been placed at the center stage in directing leukocyte extravasation (3, 4). Accumulating evidence suggests that most chemokines are able to promote the conversion of leukocytes from a weakly adherent, selectin-dependent phenotype to a firmly adherent, integrin-dependent phenotype during leukocyte extravasation, implying that these cytokines are capable of up-regulating integrin function by an "inside-out" activation mechanism (5–10). This contention is supported by in vivo findings showing that integrin-dependent leukocyte extravasation is sensitive to pertussis toxin treatment (implying a role of a $G_{\alpha i/q}$ protein-coupled receptor in the process) (11) and that mice carrying targeted deletions in nonredundant chemokine or chemokine-receptor genes display leukocytosis and impaired leukocyte transmigration into sites of inflammation (12–15).

A large number of chemokines are capable of up-regulating integrin expression and function in leukocytes belonging to various lineages (5–10). This functional up-regulation is typically transient and dose-dependent, in some cases showing a bimodal pattern that results in the inhibition of basal integrin function at high nanomolar concentration of the chemokine (6), as expected for a stimulus that is associated with cell movement rather than with static adhesion. Further, chemokines such as monocyte chemotactic protein-1 (MCP-1),[5] RANTES, and IL-8 induce preferential activation of selected integrin heterodimers within a given leukocyte lineage subpopulation (8, 10, 16), indicating that integrin subfamilies may have a differential sensitivity and/or may require alternative activation pathways even within the same cell population.

While differences in chemokine-induced adhesion can be accounted for by variable chemokine receptor expression or lineage-specific behaviors of integrins, the molecular steps involved in the process downstream of chemokine receptor occupancy are less well defined. This issue is particularly relevant for sublineages of T lymphocytes, as they express a largely overlapping profile of chemokine receptors and have been shown to possess unique recirculation patterns, in some cases associated with pathological conditions characterized by acute or chronic inflammatory responses. Th1 and Th2 cells are functionally distinct subsets of CD4$^+$ T lymphocytes orchestrating polarized versions of the immune response. IFN-γ-secreting Th1 cells regulate phagocyte-dependent immune responses, whereas Th2 cells, producing IL-4 and

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[5] Abbreviations used in this paper: MCP-1, monocyte chemotactic protein-1; SDF-1, stromal cell-derived factor-1; PI-3 kinase, phosphoinositide-3 kinase; BIM, bisindolylmaleimide; WM, wortmannin; LY, LY294002; CCD, cytochalasin D; ECM, extracellular matrix; PKC, protein kinase C; PKB, protein kinase B; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; RT, room temperature; KRH, Krebs-Ringer-HEPES; PAK, p21-activated kinase.

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IL-5, promote IgE production and eosinophil function (17). Despite their common lineage origin, Th1 and Th2 cells show clearly distinct homing behaviors (for a review see Ref. 18). While Th1 cells migrate and/or reside preferentially into sites of chronic inflammation, characterized by extensive fibrosis and thickening of the basement membrane, Th2 cells show preferential migration into sites of acute inflammation, such as allergic responses, typically associated with vascular changes such as vasodilation and increased capillary permeability. Tissue-specific homing of Th1 and Th2 cells to sites of inflammation is currently thought to be regulated by differential expression of P- and E-selectin ligands (19) and selected chemokine receptors in the two lymphocyte subsets (20–26). However, given the complexity of the extravasation process and the multiplicity of the molecular steps involved, it is likely that additional features confer selected recirculation patterns to Th1 and Th2 lymphocytes.

In this study, we investigated the expression and function of integrins in the two sublineages of Th lymphocytes. We found that β_1 integrin-mediated adhesion to extracellular matrix (ECM) proteins in response to chemokines is markedly reduced in Th2 cells, suggesting a developmentally regulated uncoupling of chemokine receptor-generated signals leading to β_1 integrin activation. The molecular step(s) responsible for defective Th2 adhesion in response to chemokines appears to lie downstream of phosphoinositide-3 kinase (PI-3 kinase) activation and actin polymerization or in a complementary signaling pathway required for β_1 integrin up-regulation.

Materials and Methods

Abs and reagents

The human recombinant chemokines RANTES and stromal cell-derived factor-1 (SDF-1) were obtained from R&D Systems (Minneapolis, MN). PMA, cytochalasin D (CCD), wortmannin (WM), LY294002 (LY), and bisindolylmaleimide (BIM) were purchased from Calbiochem (La Jolla, CA). Monoclonal Ab TS1.22 (anti-CD11a) was kindly provided by T. A. Springer (Harvard Medical School, Boston, MA); mAb anti-integrin α_3 chain (VLA-3), anti-α_4 chain (VLA-4), and anti-α6 chain (VLA-6) were purchased from AMAC (Westbrook, ME). FITC-conjugated goat anti-mouse IgG was obtained from Zymed Laboratories (South San Francisco, CA). Recombinant human ICAM-1, fused to the amino terminal region of the Ig-binding subunit of *Staphylococcus aureus* protein A (zz-ICAM-1) was generously provided by A. Randi (Glaxo-Wellcome, Stevenage, U.K.). Fura-2 AM, fibronectin, FITC-conjugated phalloidin, and BSA were obtained from Sigma (St. Louis, MO). Laminin-1 was purchased from Life Technologies (Grand Island, NY). The neutralizing anti-cytokine mAbs were purchased from PharMingen (San Diego, CA). IL-12 was obtained from Hoffmann-LaRoche (Nutley, NJ). IFN-γ was kindly provided by Hoffman-LaRoche AG (Basel, Switzerland). Fluo-3AM was purchased from Molecular Probes (Eugene, OR). Anti-human Vav and anti-phosphotyrosine (4G10) mAbs were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-phosphotyrosine (PY99) mAb was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of polarized human Th lymphocytes

Human neonatal leukocytes were isolated from freshly collected, heparinized, neonatal blood by Ficoll-Hypaque density gradient centrifugation. Polarized Th cell lines were generated by stimulation with 2 μg/ml PHA in the presence of various combinations of cytokines and neutralizing anti-cytokine Abs, as previously described (26). Briefly, Th1 cells were generated by addition of 5 ng/ml IL-12 and 200 ng/ml neutralizing anti-IL-4 Ab. Th2 cells were generated by addition of 10 ng/ml IL-4 and 2 μg/ml neutralizing anti-IL-12 Abs 17F7 and 20C2. Cells were cultured in complete RPMI 1640 medium supplemented with 5% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin. On day 3, the cultures were washed and expanded in complete medium with the addition of 100 U/ml IL-2.

Intracellular staining for cytokines

After 10 days of culture, Th1 and Th2 cells were washed, collected, and restimulated with 50 ng/ml PMA and 1 μg/ml ionomycin for 4 h. Then, 10 μg/ml of brefeldin A were added for the last 2 h of culture. Cells were then fixed with 4% paraformaldehyde, permeabilized with saponin, and stained with FITC-labeled anti-IFN-γ and PE-labeled anti-IL-4 Abs (PharMingen). Samples were analyzed by flow cytometry with a FACScan (Becton Dickinson, Mountain View, CA).

Evaluation of integrin expression by immunofluorescence

Phenotypic analysis was performed by staining 1×10^6 cells for 30 min on ice with saturating concentrations of mAbs specific for different surface markers in PBS containing 1% FBS. Cells were then washed and incubated for 30 min on ice with 10 μg/ml of FITC-conjugated goat anti-mouse IgG. After washing, cells were resuspended in PBS and analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). α_6 integrin antigenic sites were determined after calibration of the flow cytometer by using fluorescein-labeled microspheres (Flow Cytometry Standards, Research Triangle Park, NC; No. 824) as previously described (27).

Analysis of intracellular calcium mobilization

Th1 and Th2 cells were washed twice in Krebs-Ringer-HEPES (KRH) medium, counted, and resuspended at 5×10^6 cells/ml. The fluorescent Ca^{2+} indicator acetoxymethyl-fura 2 (2 μM) was added to the cells, followed by a 60-min incubation at 37°C on a shaking waterbath. Fluorescence measurements were conducted in a Perkin-Elmer LS50 B spectrofluorometer (Norwalk, CT) under continuous stirring. Calibration of the fluorescent signal was conducted as described previously in detail (27).

Actin polymerization assay

F-actin was quantitated by staining with FITC-conjugated phalloidin. Briefly, 1×10^6 cells were resuspended in RPMI 1640 medium with 10% FBS and pretreated at 37°C for 30 min with or without the following drugs: 10 μM CCD, 200 nM WM, 50 μM LY, or 2 μM BIM. Cells were stimulated with chemokines (100 ng/ml) or PMA (100 nM) at 37°C for the indicated time points. Incubation was stopped by adding three volumes of 3.7% paraformaldehyde at room temperature (RT) for 10 min, followed by washing with PBS and permeabilization on ice for 2 min with 0.1% Triton-HEPES. Thereafter, cells were stained with FITC-phalloidin (2 μg/ml) at RT for 30 min, washed with PBS, and analyzed by flow cytometry with a FACScan.

Cell adhesion assay

Ninety-six-well ELISA plates (Linbro/Titertek, Huntsville, AL) were coated overnight at 4°C with 10 ng/ml of laminin 1, fibronectin, or BSA in PBS, followed by blockade with 2% BSA at 37°C for 1 h. Recombinant zz-ICAM-1 was adsorbed at 2 μg/ml onto plates precoated for 2 h at RT with 20 mg/ml human IgG (Sandoglobulin, Sandoz S.A., Basel, Switzerland) in PBS. Because the "zz" tag binds to Igs, the precoating with human IgGs allows a correct orientation of bound ICAM-1. Thereafter, plates were washed with PBS blocked with 2% BSA, and 1×10^5 cells were added to triplicate wells with or without chemoattractants (100 ng/ml) or PMA (100 nM). For competitive inhibition experiments, cells were preincubated with serial dilutions of function-blocking anti-α_6 Ab for 20 min on ice and washed before addition to ligand-coated plates. In some experiments, cells were pretreated at 37°C for 30 min with or without the following drugs: WM (200 nM), LY (50 μM), or BIM (2 μM). Plates were centrifuged for 2 min at 800 rpm to allow rapid cell sedimentation and were incubated for the indicated time periods at 37°C. Nonadherent cells were removed by washing four times with medium; adherent cells were fixed with 3.7% paraformaldehyde at RT for 10 min, washed with PBS, and stained with 0.5% crystal violet at RT for 10 min. Plates were washed four times with water, crystal violet was then extracted by the addition of 1% SDS, and adsorbance at 540 nm was measured in an ELISA reader. After subtraction of the background binding, as assessed with BSA-coated control wells, specific binding was calculated as percentage of total input cells.

Immunoprecipitation and Western blot

Cells (1.5×10^7 cells/sample) were either left untreated or stimulated for 2 min at 37°C with chemokines (RANTES, 200 ng/ml; or SDF-1, 200 ng/ml) or with 50 μg/ml of anti-CD3 (TR66), mAb plus goat anti-mouse IgG Ab. Cells were then lysed in 2× Lysis buffer (100 mM Tris, pH 8, 300 mM NaCl, 2 mM EDTA, 2 mM EGTA, pH 8, 2% Nonidet P-40, 2 nM PMSF, 20 mM NaF, 2 mM Na_3VO_4, 2 μg/ml aeupeptin, 2 μg/ml apoprotinin) for 10 min on ice. For immunoprecipitation, protein G-Sepharose was preincubated with anti-human Vav Ab, washed twice in PBS, incubated with lysates at 4°C overnight, and eluted in reducing sample buffer. Samples were analyzed by SDS-PAGE and transferred onto nitrocellulose. The membrane was blocked in 5% milk-PBS, incubated with 4 μg/ml of

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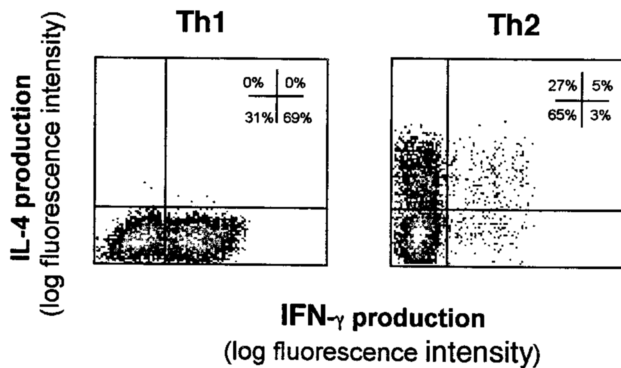

FIGURE 1. Cytofluorometric evaluation of single-cell IFN-γ and IL-4 production by Th1 and Th2 cells generated in vitro. Th1 and Th2 cells generated as described in *Materials and Methods* were harvested and restimulated with PMA and ionomycin. The intracellular production of IL-4 and IFN-γ was analyzed by flow cytometry. Data are shown as dot plot cytograms, and the fraction of cells located in each quadrant is indicated in the inset. Results are representative of >15 separate experiments.

anti-human Vav or with a combination of two anti-phosphotyrosine Abs (Py99, 0.2 μg/ml; and 4G10, 1 μg/ml) followed by an incubation with an appropriate HRP-conjugated Ab. Peroxidase reaction was developed using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

GST-p21-activated kinase (PAK) pull-down assay

The sequence encoding the amino-terminal 252 aa of rat PAKa was amplified using the primers GCT CTA GAA ATG TCA AAT AAC GGC TTA GAC and CTC TAA GCT TGA TCT CCT CAT CAG ACA TTT TAC. The PCR fragment was digested with *Xba*I and *Hin*dIII and cloned into pGEX KG. pXJ40HA wild-type PAKa was a kind gift from L. Lim (UCLA, Los Angeles, CA). To prepare the purified GST-PAK fusion protein, one *Escherichia coli* colony was inoculated and grown for 16 h at 37°C under agitation in 2× YTA (16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 100 μg/L ampicillin). The culture was diluted 1:100 in fresh, prewarmed 2× YTA and grown by monitoring OD at 590 nm. Then, 100 mM isopropyl-β-D-thiogalactoside was added to a final concentration of 0.1 mM, and the culture was grown for additional 2 h. The induced cells were then centrifuged for 10 min at 4°C at 7700 × *g* and resuspended in 50 μl ice-cold PBS per ml of culture. Cells were disrupted by sonication. Triton X-100 was added to a final concentration of 1%, and samples were incubated for 30 min at 4°C. Cells were centrifuged for 10 min at 4°C and 12,000 × *g,* and the supernatant was saved and stored in aliquots at −80°C. To purify the expressed protein, to 1 ml of the sonicate 40 μl of in PBS equilibrated glutathione Sepharose (4B; Pharmacia, Upsala, Sweden) was added, and samples were incubated with gentle agitation for 30 min at RT. The suspension was centrifuged at 500 × *g* for 3 min, and the pellet washed three times with PBS. For pull-down assays, 25 × 10^6 cells in 0.5 ml PBS were stimulated with 100 nM chemokines or 10 μg/ml anti-CD3 mAb for 30 s at 37°C. Then, 0.5 ml ice-cold 2× lysis buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 2 mM sodium orthovanadate, 100 mM NaF, 2 mM PMSF, 20 mM $MgCl_2$, 2% Triton X-100, 1 mM DTT, 20 μg/ml leupeptin and aprotinin) was added, and lysates were vortexed and were immediately cleared for 1 min at 13,000 rpm. The cleared lysates were added to 40 μl GST-PAK beads and incubated for 5 min at 4°C under agitation. The GST-PAK beads were then quickly washed twice with wash buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 2 mM DTT), and bound proteins were immediately eluted with sample buffer (2% SDS, 0.005% bromophenol blue, 10% glycerol, 50 mM Tris, pH 6.8). A standard SDS-PAGE was performed, and proteins were transferred to a nitrocellulose membrane (Amersham). GST-PAK-bound Rac1 was identified by immunoblotting with an anti-Rac1 mAb (Transduction Laboratories, Lexington, KY).

Results

Heterogeneous expression of β_1 and β_2 integrins on Th1 and Th2 cells

Following their expansion in the presence of polarizing chemokines, Th1 and Th2 cells were defined phenotypically by their reciprocal expression of IFN-γ and IL-4, respectively (Fig. 1). A cytofluorometric analysis of surface-expressed integrins in polarized Th cell subsets revealed that the overall levels of β_2 integrins are higher in Th1 cells, while surface β_1 integrins appear to be comparatively similar in the two subsets, as judged by staining with Abs recognizing the common β subunits of the various integrin subfamilies. Conversely, the composition of β_1 integrin heterodimers expressed at the cell surface is qualitatively heterogeneous in the two subsets: the $\alpha_4\beta_1$ integrin, a receptor for fibronectin and VCAM-1, is more highly expressed on Th2 cells, while the $\alpha_6\beta_1$ integrin, the major laminin receptor, displays consistently higher expression levels in Th1 cells (Fig. 2). The surface density of the α_6 subunit in particular was found to range between 20,000 and 25,000 sites/cell and between 45,000 and 55,000 sites/cells in Th2 and Th1 lymphocytes, respectively. The observed differences in surface expression of $\alpha_6\beta_1$ in the two subsets are consistent with the finding that the steady-state levels of the mRNA coding for the α_6 subunit are markedly up-regulated by IL-12 treatment in Th1 cells (28). The $\alpha_3\beta_1$ and $\alpha_5\beta_1$ receptors were found to be equally expressed in the two subsets, while the β_4 integrin subunit, which in selected cell types dimerizes with the α_6 subunit to yield a receptor for laminin V, was not expressed in either cell subpopulation.

Chemokine-induced adhesion to fibronectin and laminin is defective in Th2 cells

To assess the functional properties of integrins expressed by Th1 and Th2 cells, we performed adhesion assays on immobilized integrin ligands under static conditions. Fig. 3*A* shows that Th1, but not Th2, lymphocytes display chemokine-inducible adhesion to fibronectin and laminin I in response to both RANTES and SDF-1. Such inducible adhesion reaches a plateau at 15 min poststimulation and is typically transient, returning to prestimulation levels within 45–60 min (Fig. 3*B*). The defective response observed in Th2 cells can be bypassed using phorbol ester stimulation, which induces a long-lasting up-regulation of integrin avidity, or by performing the adhesion assay in the presence of 1 mM Mn^{2+}, a condition known to promote an allosteric transition in integrins' extracellular domains resulting in a persistent increase in ligand affinity (29). Competitive inhibition experiments using function-blocking Abs specific for the various integrin α subunits confirmed that the adhesion of both cell subsets to fibronectin is cooperatively mediated by the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ heterodimers (not shown), while adhesion to laminin is entirely dependent on the $\alpha_6\beta_1$ integrin (see below). Experiments performed to assess β_2 integrin function in the two subsets, using immobilized ICAM-1 as a ligand, showed a high baseline adhesion by both subsets, which could be only marginally up-regulated by chemokine or phorbol ester treatment (not shown). These findings demonstrate that intrinsic structural or functional alterations of integrins are unlikely to account for the reduced adhesion observed in Th2 cells in response to chemokines and suggest that a molecular step involved in the signaling pathway linking chemokine receptor stimulation to functional up-regulation of β_1 integrins is defective in the latter cell population.

Comparative analysis of $\alpha_6\beta_1$ receptor function in Th1 and Th2 cells

To assess whether the observed difference in $\alpha_6\beta_1$ surface expression levels is responsible for the variable adhesion efficiency of the

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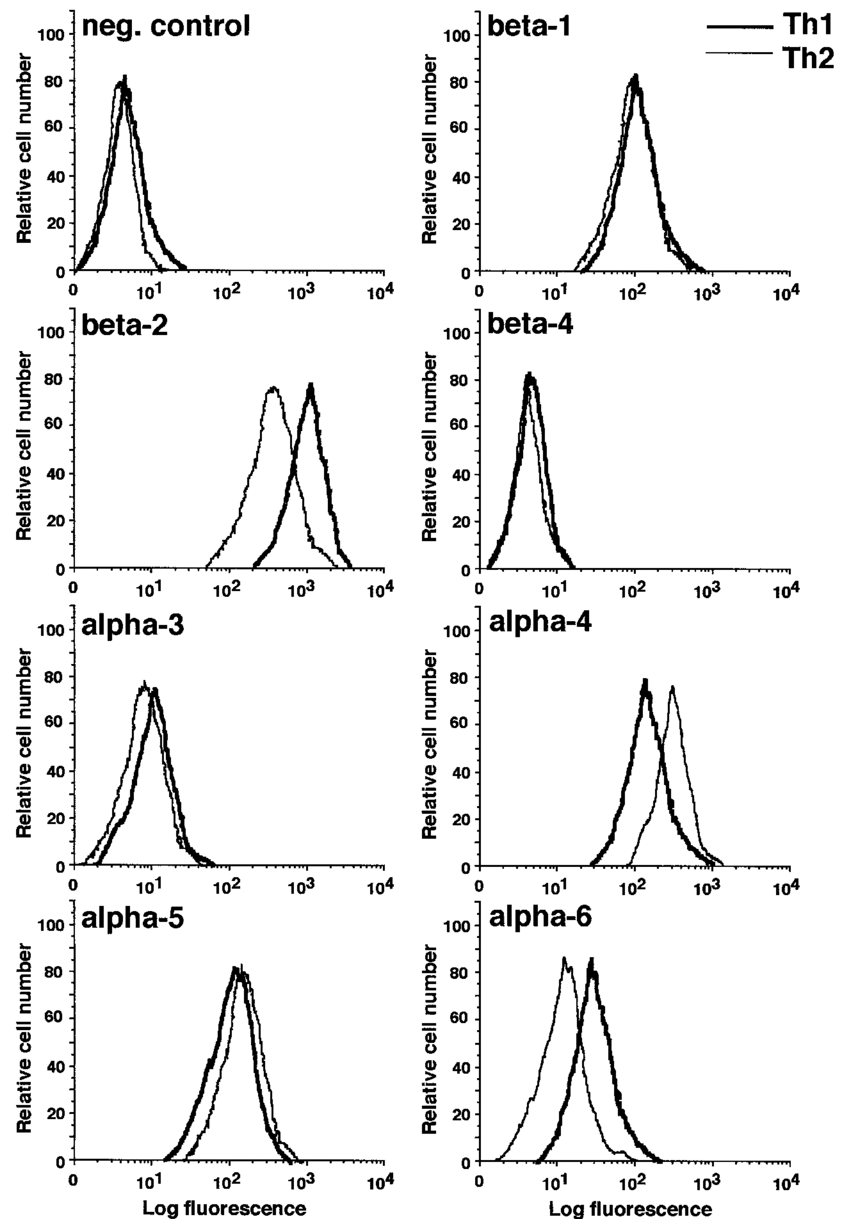

FIGURE 2. Expression of integrins in polarized Th cell subsets. Shown are representative FACS profiles of Th1 (thick lines) or Th2 (thin lines) lymphocytes stained with saturating concentrations of mAbs specific for the indicated integrin subunits, followed by incubation with FITC-conjugated goat anti-mouse IgG. Negative controls were conducted using isotype-matched irrelevant primary mAb.

two lymphocyte subsets, we performed competitive inhibition experiments using serial dilutions of a function-blocking anti-α_6 mAb. Fig. 4*A* shows a titration curve generated to determine the saturating concentration of the anti-α_6 mAb in the two subsets. At 0.1 μg/ml of Ab, virtually all surface-expressed $\alpha_6\beta_1$ integrin is saturated in Th2 cells, while ~50% of the $\alpha_6\beta_1$ receptors expressed on Th1 cells are still unsaturated at this mAb concentration. Based on the estimated receptor density reported above for the two cell subsets, the number of $\alpha_6\beta_1$ sites available for laminin binding in Th1 cells, in the presence of 0.1 μg/ml anti-α_6 Ab, should approximate the total number of receptors expressed by untreated Th2 cells. The experiment shown in Fig. 4*B* demonstrates that in the presence of 0.1 μg/ml anti-α_6 mAb, the adhesion efficiency of SDF-1-stimulated Th1 cells is still significantly higher compared with Th2 cells in the absence of Ab (24 ± 2% vs 7 ± 3%, respectively). Thus, the defective adhesion to laminin displayed by chemokine-stimulated Th2 cells cannot be explained by a lower surface expression of the related $\alpha_6\beta_1$ integrin receptor. Of note, chemokine-induced adhesion to laminin of Th1 cells is completely abolished by as little as 1 μg/ml anti-α_6 mAb, further indicating that this is the only functional laminin receptor expressed by this cell subset.

Analysis of chemokine-driven intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) responses in Th1 and Th2 cells

A simple explanation for the observed differences in the adhesive response of Th1 and Th2 cells following chemokine stimulation is that Th2 cells are less responsive to the chemokines under study at the receptor level due to lower receptor density and/or sensitivity. As one of the earliest functional responses to chemokines is a rise in free $[Ca^{2+}]_i$ levels resulting from phospholipase C activation and breakdown of membrane phosphoinositides, we analyzed such response in the two lymphocyte subsets using RANTES and SDF-1 at nanomolar concentrations. Fig. 5 shows that indeed Th2 cells display a less pronounced elevation of free $[Ca^{2+}]_i$ in response to RANTES when compared with Th1 cells. This is consistent with previous reports showing that Th1 cells do express higher levels of selected receptors for RANTES, such as CCR1

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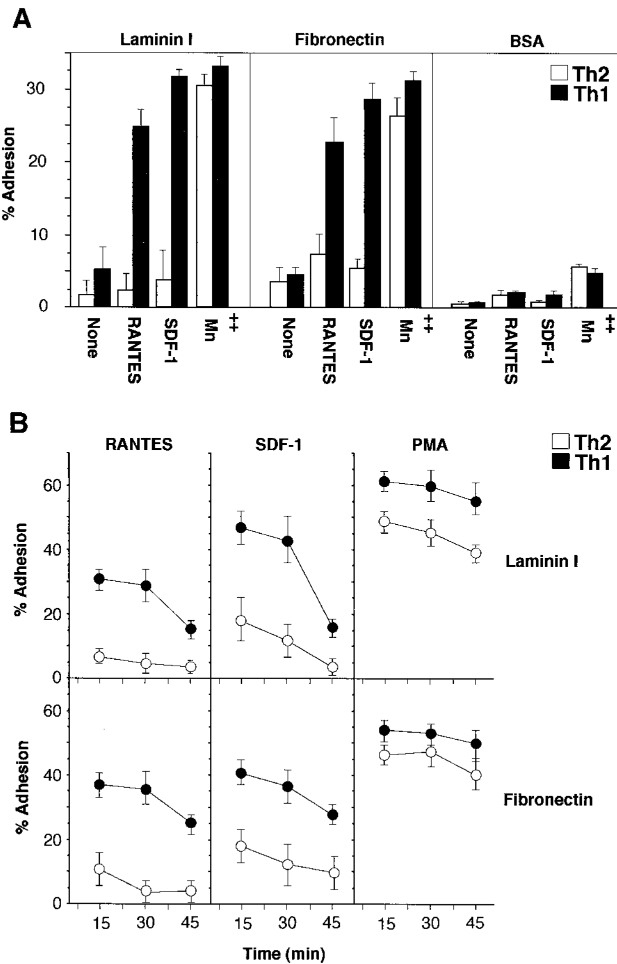

FIGURE 3. Chemokine-induced adhesion of Th1 and Th2 cells to β_1 integrin ligands. *A,* Th1 (■) and Th2 (□) cells were plated onto BSA-, laminin-, or fibronectin-coated wells in the presence of RANTES, SDF-1 (100 ng/ml), or Mn^{2+} (1 mM) and incubated for 30 min at 37°C, followed by removal of nonadherent cells and quantitation of adhesion as described in *Materials and Methods*. Data are expressed as percentage adhesion over the total input cells and are the mean ± SD of 10 separate experiments. *B,* Adhesion was performed as described in *A*, but cells were left in culture for the indicated time-points before removal of nonadherent cells and quantitation of specific adhesion. Results are the mean ± SD of three independent experiments.

(28) and CCR5, and respond preferentially to RANTES in chemotactic migration assays. In contrast, the elevation of free $[Ca^{2+}]_i$ induced by SDF-1 is virtually identical in the two subsets, despite their differential response to this chemokine in the static adhesion assays reported in Fig. 3.

Signaling pathways involved in up-regulating β_1 integrin-dependent adhesion in Th1 cells following chemokine stimulation

To investigate the mechanisms underlying the observed variability in chemokine-induced β_1 integrin functional up-regulation, we performed chemokine- or phorbol ester-driven adhesion assays in Th1 cells in the presence of selected enzymatic inhibitors. Interestingly, chemokine-induced adhesion to β_1 integrin ligands is markedly inhibited in Th1 cells by both PI-3 kinase and protein kinase C (PKC) inhibitors (Fig. 6), suggesting that parallel signaling pathways, involving PI-3 kinase and PKC activation, are triggered by the chemokines under study and are both required for the regulated adhesion of Th1 cells to β_1 integrin ligands. Persistent activation of PKC by the irreversible agonist PMA is sufficient per se, independently of PI-3 kinase activation, in driving β_1 integrin functional up-regulation, as judged by its resistance to PI-3 kinase inhibitors and by its complete inhibition with the selective PKC inhibitor BIM.

Chemokine-induced actin polymerization and analysis of the signaling pathways involved

The results described above indicate that the molecular step(s) responsible for the reduced adhesive response to chemokines of Th2 cells lies downstream of receptor occupancy, phospholipase C activation, and second messenger generation, at least if one considers the dissociated functional response of Th2 lymphocytes to SDF-1 by comparing the induction of free $[Ca^{2+}]_i$ elevations vs β_1 integrin-mediated adhesion. Moreover, our findings show that two major enzymatic pathways triggered by chemokines, namely PI-3 kinase and PKC, are required to up-regulate β_1 integrin function in Th1 cells. Among the requirements for inducible integrin-mediated adhesion are an increased rate of actin polymerization and an extensive reorganization of the actin-based cytoskeleton, both of which are under the control of small GTPases of the Rho family and are largely abrogated by CCD pretreatment (30–32). Therefore, we set out to investigate whether chemokines indeed promote net actin polymerization in Th1 and Th2 cells, and the potential mechanisms involved in such response. Fig. 7*A* shows that following chemokine stimulation both cell subsets undergo a rapid burst of actin polymerization, which peaks at 30 s to 1 min and subsides to baseline levels within 5–10 min poststimulation. Such response is completely abrogated by pretreatment of the cells with CCD, which binds irreversibly to free actin monomers and selectively blocks the ATP-dependent actin polymerization process. Similar to the $[Ca^{2+}]_i$ response reported above, Th2 cells displayed a delayed and quantitatively reduced actin polymerization response upon RANTES stimulation, whereas the two cell subsets showed an identical response when stimulated with the α chemokine SDF-1. Both subsets responded to PMA treatment with a moderate but persistent increase of actin polymerization. To analyze the enzymatic pathways involved in such response, we pretreated the two subsets with selected inhibitors before chemokine or phorbol ester stimulation. Fig. 7*B* shows that the two chemokines and PMA display a reciprocal pattern of inhibition: the burst of actin polymerization induced by both chemokines is substantially inhibited by the PI-3 kinase inhibitors WM and LY, but is unaffected by the selective PCK inhibitor BIM. Conversely, PMA-induced actin polymerization is resistant to both PI-3 kinase inhibitors but is completely abrogated by BIM. These findings demonstrate that the PI-3 kinase-dependent burst of actin polymerization induced by chemokines appears to be equally rapid and efficient in both Th1 and Th2 lymphocytes and parallels the chemokines' ability to generate second messengers following receptor occupancy.

Analysis of selected intracellular pathways potentially involved in chemokine-induced adhesion to ECM proteins in Th1 and Th2 cells

To clarify the signaling mechanisms responsible for the observed phenomena, we investigated the expression and functional activation of a number of molecular intermediates reportedly involved in promoting net actin polymerization and increased integrin-mediated adhesion upon exposure to chemokines in the two T lymphocyte subsets. As a positive control in the assay, we used solid phase-bound anti-CD3 Abs, as most of the signal trasduction pathways analyzed are known to be triggered by such treatment in T

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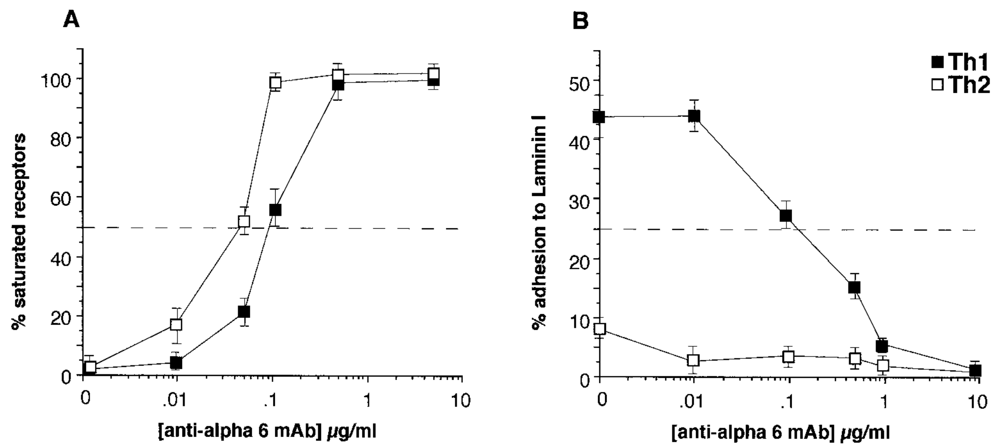

FIGURE 4. Titration of the expression and function of $\alpha_6\beta_1$ integrin by Th1 and Th2 cells using function-blocking anti-α_6 mAb. *A*, Th1 (■) and Th2 (□) cells were stained with the indicated concentration of anti-α_6 mAb, followed by incubation with a FITC-conjugated goat anti-mouse IgG and analysis with a flow cytometer. Data are expressed as percentage saturated receptors, where 100% represents the mean fluorescence value (arbitrary units) observed at saturating concentrations of the mAb. Results are representative of three separate experiments. *B*, Th1 and Th2 cells were preincubated with the indicated concentrations of function-blocking anti-α_6 mAb before their addition to laminin-coated wells and evaluation of SDF-1-induced adhesion as described in Fig. 3.

lymphocytes. To indirectly assess the functional activation of PI-3 kinase, we analyzed the phosphorylation levels of protein kinase B (PKB)/Akt, a known downstream effector of PI-3 kinase (33). Fig. 8 shows that a fraction of PKB/Akt undergoes rapid phosphorylation in both subsets following chemokine stimulation, indicating that PI-3 kinase becomes activated by both chemokines. This is further suggested by the observation that chemokine-induced PKB/Akt phosphorylation was completely abrogated by PI-3 kinase inhibitors (not shown). Comparable levels of PKB/Akt phosphorylation were induced in both subsets by SDF-1, whereas RANTES consistently induced higher PKB/Akt phosphorylation levels in Th1 as opposed to Th2 lymphocytes, again confirming the existence of a higher sensitivity of the former T cell subset to RANTES. Activation of the Rho-like GTPase Rac1 has been proposed to link PI-3 kinase activation to actin polymerization, adhesion, and chemotaxis in several cell types. To assess whether Rac1 undergoes GDP to GTP exchange following chemokine stimulation, we used an agarose matrix-immobilized GST-PAK fusion protein in pull-down assays, which specifically identify the GTP-bound fraction of Rac1 (34). Such assays demonstrate that a small but significant fraction of endogenous Rac1 binds to the effector protein PAK rapidly after chemokine stimulation. Such binding was observed at comparable levels in the two subsets and was completely abrogated by pretreatment of the cells with PI-3 kinase inhibitors (not shown). Among the exchange factors known to promote the conversion of GDP for GTP in Rho-like GTPases, Tiam-1 and Vav have been reported to be preferentially active on Rac1 (35, 36) and to be expressed in lymphoid cells. Fig. 8 shows that indeed both proteins are expressed at comparable levels in Th1 and Th2 cells. However, unlike immobilized anti-CD3 Abs, neither RANTES nor SDF-1 induce detectable phosphorylation of Vav on tyrosine residues, an event which appears to be required for the acquisition of full catalytic activity by this exchange factor (37). These findings indicate that a major signaling pathway involved in receptor-mediated actin polymerization and cell motility is activated at comparable levels in both Th1 and Th2 lymphocytes following chemokine stimulation, despite the differential ability of the two subsets to up-regulate β_1 integrin adhesion in response to chemokines.

Discussion

In this work, we report that human Th1 and Th2 lymphocytes differ substantially in their adhesive response following chemokine stimulation. Th2 cells fail to functionally up-regulate β_1 integrins and to adhere to laminin and fibronectin upon being treated with chemokines such as SDF-1, MCP-1, and RANTES. Such a defect, which can be bypassed by phorbol ester stimulation or by incubation of Th2 cells in a Mn^{2+}-containing buffer, does not appear to

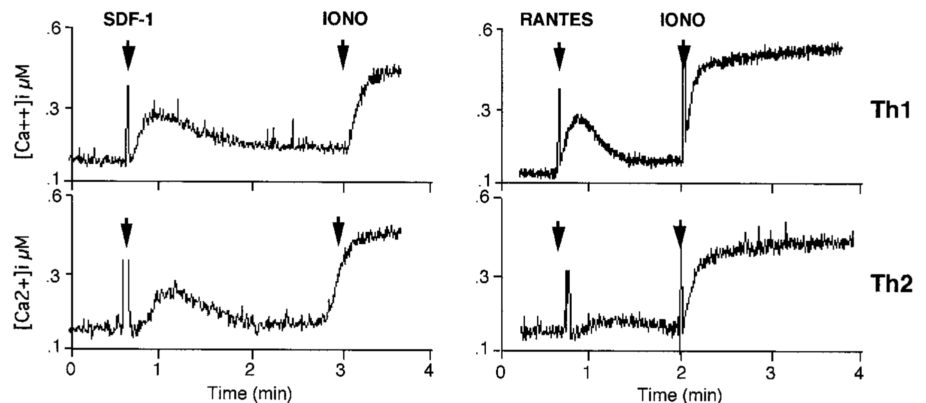

FIGURE 5. Analysis of chemokine-induced $[Ca^{2+}]_i$ variations in Th1 and Th2 cells. Th1 and Th2 cells were loaded with the fluorescent Ca^{2+} indicator fura-2 AM (emission wavelength, 495 nm) and analyzed in real time by spectrofluorometry before and after the addition of 100 ng/ml of the indicated chemokines. Time of addition of the chemokine and of 0.5 μM ionomycin (loading control) are indicated by arrows. Assessment of $[Ca^{2+}]_i$ was performed as described in *Materials and Methods*. Results are representative of three separate experiments.

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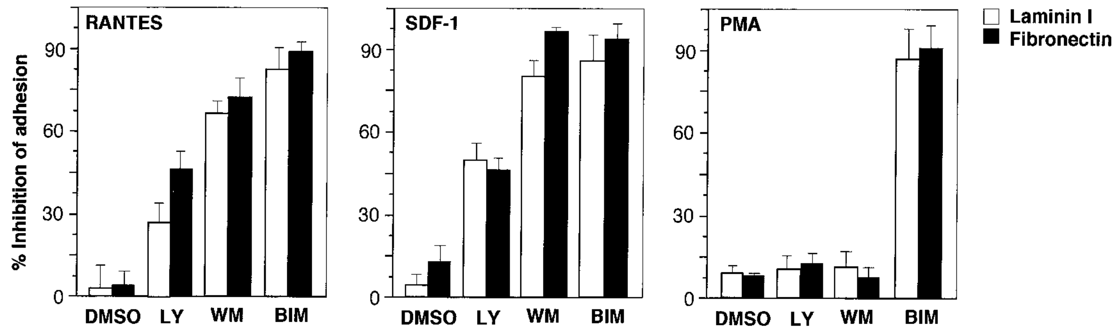

FIGURE 6. Pharmacological inhibiton of β_1 integrin-dependent adhesion in Th1 lymphocytes. Th1 cells were pretreated with WM (200 nM) LY (50 μM), or BIM (2 μM) for 30 min at 37°C before plating onto laminin (□)- or fibronectin (■)-coated wells and addition of chemokines (100 ng/ml) or PMA (50 nM) in a 30-min static adhesion assay, as described in Fig. 3. Data are expressed as percent inhibition of agonist-induced adhesion over untreated cells. Results are the mean ± SD of two separate experiments performed in triplicate.

be caused by reduced surface expression of integrins, or by a defect in proximal signals originated from chemokine receptors following ligand engagement.

Despite their demonstrated role in supporting lymphocyte transendothelial migration, integrins have not been extensively analyzed phenotypically or functionally in polarized subsets of Th cells. As expected for T lymphocytes cultured for extended periods in the presence of mitogens, we found that both Th1 and Th2 cells express high surface levels of integrins. However, β_2 integrins appear to be expressed at higher levels in Th1 cells, while β_1 integrins are expressed at comparable levels in the two subsets. On a more qualitative basis, Th1 cells express higher surface levels of the α_6 integrin subunit, which, as previously reported by our group, appears to be a transcriptional target of the Th1-polarizing cytokine IL-12 (28). In contrast, Th2 cells express higher levels of the α_4 integrin subunit. Competitive inhibition experiments demonstrated that in our experimental model adhesion to laminin appeared to be exclusively mediated by the $\alpha_6\beta_1$ receptor, while adhesion to fibronectin was cooperatively mediated by $\alpha_4\beta_1$and $\alpha_5\beta_1$ integrins.

The aim of this work was to evaluate the ability of chemokines to functionally up-regulate β_1 integrins in polarized T helper cells.

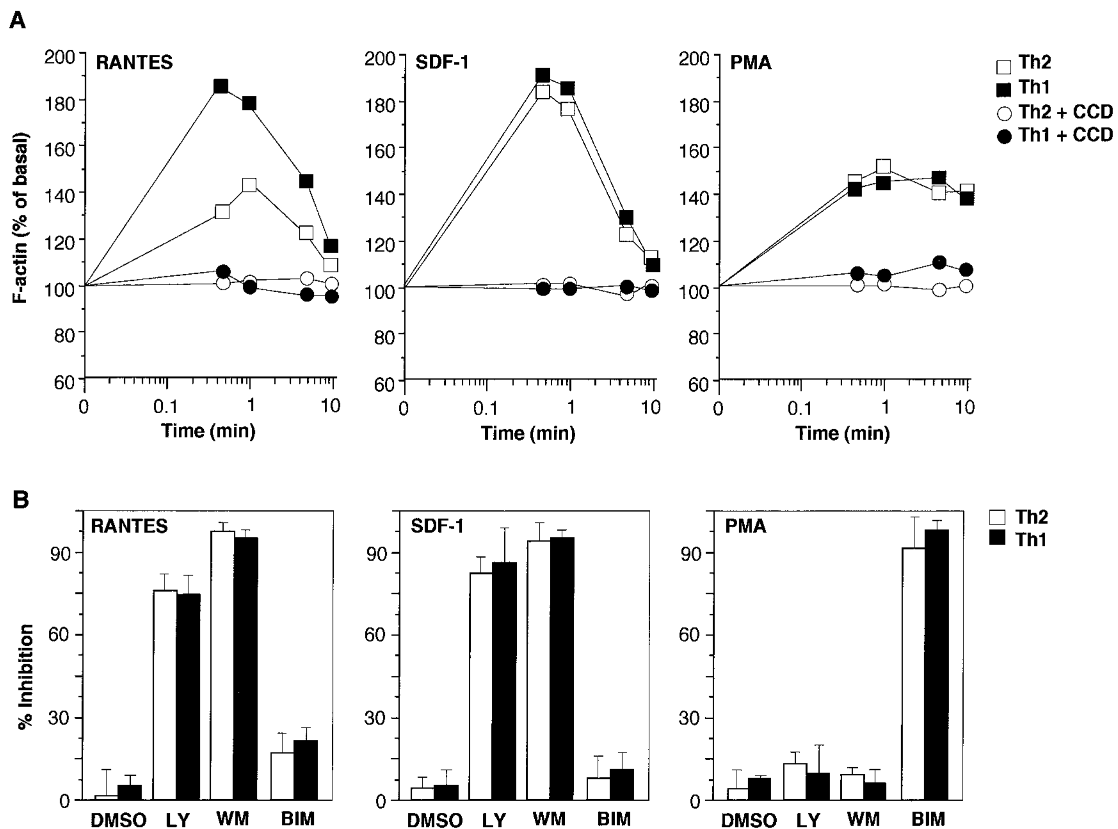

FIGURE 7. Chemokine-induced actin polymerization in Th1 and Th2 cells. *A,* Kinetics of chemokine- or PMA-induced actin polymerization in Th1 (filled symbols) and Th2 (open syimbols) cells. Cells were pretreated with medium in the presence or absence of 10 μM CCD for 20 min at 37°C, followed by the addition of chemokines (100 ng/ml) or PMA (50 nM) for the indicated time-points. The amount of F-actin was quantitated as described in *Materials and Methods*. *B,* Th1 and Th2 cells were pretreated with the indicated pharmacological inhibitors for 30 min at 37°C before evaluation of chemokine-induced actin polymerization, as indicated in *A*. Data are expressed as percent inhibition of chemokine-induced actin polymerization over untreated cells, evaluated at 1 min following chemokine stimulation. Results are the mean ± SD of three separate experiments.

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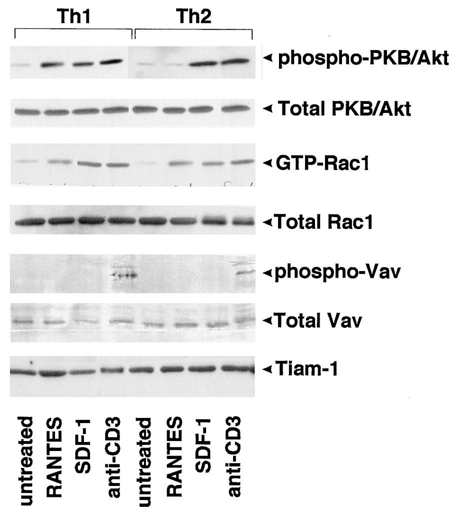

FIGURE 8. Biochemical characterization of signaling intermediates involved in chemokine-induced actin polymerization and β_1 integrin-dependent adhesion. Th1 and Th2 cells were stimulated with control medium, the indicated chemokines (100 nM for 1 min at 37°C), or immobilized anti-CD3 mAb (10 μg/ml for 10 min at 37°C). Cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting for the indicated proteins as described in *Materials and Methods*. GTP-Rac1 was detected by immunoblotting of the eluate of GST-PAK columns with anti-Rac1 Ab. Phospho-Vav was analyzed by immunoblotting of anti-Vav immunoprecipitates with anti-phosphotyrosine mAb. Results are representative of three separate experiments.

The most significant finding reported here is that in Th2 but not Th1 lymphocytes, the early response induced by chemokines, in terms of phospholipase C activation and Ca^{2+} influx, is functionally uncoupled from the activation of integrin-mediated adhesion. The experiments performed using known activators of integrin-dependent adhesion, such as phorbol esters or buffers containing Mn^{2+} ions, suggest that integrins are indeed competent to engage their specific ligands in Th2 lymphocytes and that one or more molecular intermediates linking chemokine responses to integrin functional up-regulation are defective in these cells when compared with Th1 cells. In search of such intermediates, we explored selected intracellular pathways that are known to be involved in transducing membrane receptor-generated signals required for cytoskeletal reorganization, adhesion, and motility in most lymphoid and nonlymphoid cells. One such pathway involves the activation of PI-3 kinase by G protein-coupled receptors, including chemokine receptors, and the subsequent activation of subfamilies of Rho-like GTPases. Two lines of evidence in this work support the conclusion that PI-3 kinase is efficiently activated by chemokines at comparable levels in Th1 and Th2 cells: first, we observed comparable levels of phosphorylation of PKB/Akt, a known downstream effector of PI-3 kinase, in both Th1 and Th2 cells upon exposure to SDF-1, a chemokine that promotes comparable chemotattractant responses in the two subsets; second, selective inhibitors of PI-3 kinase abolish the burst of actin polymerization induced by SDF-1 in both lymphocyte subsets. Furthermore, using a sensitive biochemical assay, we provide for the first time evidence for the rapid activation of Rac1 by chemokines, which is compatible with the role played by this Rho-like GTPase in supporting cell adhesion and motility. The activation of Rac1 appears to lie downstream of PI-3 kinase activation and is likely to be primarily responsible for the burst of actin polymerization observed in both T cell subsets. Indeed, both the exchange of GDP for GTP in Rac1 and the observed burst in actin polymerization are efficiently blocked by selective PI-3 kinase inhibitors, and dominant negative Rac1 mutants have been shown by others to prevent actin polymerization induced by various agonists (38–40). Other members of the Rho family, such as Cdc42, have been reported to be involved in chemokine-induced morphologic changes in myeloid cells, suggesting that Rho GTPases are key regulators of chemoattractant responses in multiple cell types (41, 42). Several recent reports have highlighted a potential mechanism linking PI-3 kinase activation to the accelerated excange of GTP for GDP in Rho-like GTPases by showing that Vav, a specific exchange factor for Rac1, binds to and is directly controlled by substrates and products of PI 3-kinase (37). Interestingly, these reports show that the product phosphatidylinositol- 3,4,5-trisphosphate enhances phosphorylation and full catalytic activation of Vav by srk-family kinases such as Lck (37). Our findings demonstrate that Vav and Tiam-1, two major exchange factors for Rac1, are expressed at comparable levels in Th1 and Th2 cells. However, we could not detect any tyrosine-phosphorylated Vav in either T cell subset upon short-term exposure to chemokines, suggesting that Vav is unlikely to be fully activated by chemokine stimulation in T lymphocytes. Collectively, our findings suggest that the reduced activation of β_1 integrin-dependent adhesion observed in Th2 cells exposed to chemokines cannot be explained by a defective activation of PI-3 kinase, Rac-1, and the related processes leading to a burst of actin polymerization, which in several models is required to support integrin-mediated adhesion, spreading, and motility.

A complementary signaling pathway that impacts on chemokine-induced adhesion involves the activation of the small GTPase RhoA and the functionally interdependent enzymatic activities of phospholipase D and PKC (43). Several reports have indicated that RhoA activity is required for agonist-induced β_1 and β_2 integrin activation in lymphoid cells (7, 30). Likewise, a large body of experimental work has shown that PKC activation plays a central role in integrin function and integrin-cytoskeletal interactions (44, 45). Our findings indicate that the selective inhibition of PKC in Th1 cells abolishes β_1 integrin-dependent adhesion in response to chemokines and, as expected, to phorbol esters. Interestingly, the activity of PKC does not seem to be required for chemokine-induced actin polymerization. This suggests that two independent signaling pathways, one involving PKC and the other requiring PI-3 kinase activity, are both required for the observed adhesion to ECM ligand upon chemokine stimulation and may be differentially regulated in Th1 vs Th2 cells. Although our findings do not provide a mechanistic explanation for the markedly reduced fucntional activation of β_1 integrins in Th2 cells exposed to chemokines, they demonstrate that the observed differences are not due to a defective activation of a signaling pathway involving PI-3 kinase, the small GTPase Rac1, and other intracellular regulators of actin polymerization. Conversely, the possibility exists that the variable efficiency of chemokine-induced β_1 integrin function in the two subsets might reside in the differential expression and functional regulation of selected isoforms of PKC expressed by lymphoid cells and/or by regulators and effectors of the RhoA GTPase. In support of this conclusion, recent reports (46) have demonstrated the involvement of selected PKC isoforms, such as the atypical PKC-ζ, in chemokine-induced adhesion to integrin ligands. In apparent contrast with this hypothesis, we show here that Th2 cells respond efficiently to PMA in terms of integrin-dependent adhesion. However, being phorbol esters metabolically stable and devoid of selectivity as PKC activators, the possibility exists that subtle differences in the expression and regulation of selected PKC isoforms or their specific substrates may be responsible for the observed behavior of β_1 integrins in chemokine-stimulated Th2 lymphocytes.

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In conclusion, our data indicate that in addition to the demonstrated differential expression of chemokine receptors and selectin ligands, Th1 and Th2 cells show different requirements for β_1 integrin functional activation upon exposure to chemokines. If confirmed by appropriate experimental models in vivo, these findings may provide an additional mechanistic clue to the understanding of the markedly different homing behavior of Th1 vs Th2 cells. Reduced β_1 integrin-mediated adhesion in Th2 cells could restrain their ability to invade and/or reside in sites of chronic inflammation, which are characterized by thickening of basement membranes and extensive fibrosis, requiring efficient interaction with organized ECMs. In contrast, such limitations may not affect margination of Th2 cells into areas of acute inflammation, in which a host of soluble mediators, by increasing microvascular permeability, reduce the selectivity of lymphocyte transmigration.

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