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α1β1 Integrin+ and Regulatory Foxp3+ T Cells Constitute Two Functionally Distinct Human CD4+ T Cell Subsets Oppositely Modulated by TNFα Blockade

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The expression of the collagen receptor α1β1 integrin (VLA-1) on CD4+ T cells is largely restricted to CCR7−CD45RO+ cells that localize to inflamed tissues. Moreover, neutralizing α1 integrin, in vivo, has been shown to compromise cell-mediated immunity. Our current study shows that the expression of VLA-1 on human CD4+ T cells is restricted to conventional effectors. In contrast, Foxp3+ T regulatory cells (Tregs) do not express this receptor. Moreover, Foxp3 or VLA-1 expression remained a mutually exclusive event in CD4+ T cells even upon polyclonal anti-CD3-induced activation. Because TNFα blockade ameliorates certain T cell-dependent autoimmune disorders in humans, we investigated, in vitro, whether neutralizing TNFα affected the balance between the proinflammatory VLA-1+ effectors and the counteracting Tregs. We found that anti-CD3 stimulation of freshly isolated PBL from healthy individuals, coupled with continuous TNFα blockade, inhibited the typical activation-dependent generation of CD4+ VLA-1+ Th1 cells. In contrast, it augmented the outgrowth of VLA-1neg/dimCD25high and Foxp3+ CD4+ T cells. Indeed, repeated anti-CD3 stimulation coupled with TNFα blockade generated CD4+ T cell lines enriched for VLA-1−Foxp3+ Tregs. Importantly, these CD4+ T cells displayed potent suppressive functions toward autologous CD4+ PBL, including the suppression of the activation-dependent induction of VLA-1+ effectors. Thus, we propose a novel mechanism by which anti-TNFα therapy may restore self-tolerance, by shifting the balance between VLA-1+ effectors and Foxp3+ Tregs, during immune activation, in favor of the latter suppressor cell population. The Journal of Immunology, 2007, 178: 201–210.

A ctivation of T cells results in the expression of a programmed set of activation-related genes that determine its eventual effector function and tissue tropism. The period following activation has traditionally been divided into early (6–24 h) and late (5–7 days) phases. Classical molecular markers of early activation include CD154, CD69, and CD25, whereas the late stages of activation are characterized by the induction on the cell surface membrane of late, e.g., HLA-DR, and very late Ags, e.g., VLA-1 and VLA-2 (1).

Interestingly, some of these molecular markers of T cell activation are also constitutively expressed on functionally distinct subsets of memory T cells. For example, the high-affinity IL-2R, CD25, is stably expressed in a unique subset of CD4+ T cells, the naturally occurring T regulatory cells (Tregs) (2, 3). In a similar manner, the collagen receptor VLA-1 (CD49a; α1 integrin) that enhances the tissue migration potential of effector T cells (4) is expressed at homeostasis, primarily on the cell surface of a subset of CCR7− T effector memory (TEM) cells (5). In addition, in the course of influenza infection in mice, VLA-1 is expressed on the majority of influenza-specific CD8+ CTL in the lungs. After the resolution of infection, it continues to be stably expressed on the memory anti-influenza CTL retained in the lungs. Accordingly, treatment with anti-α1 integrin mAbs reduces secondary immunity to influenza (6).

The functional importance of VLA-1+ T cells also pertains to tissue-residing autoreactive TEM. For instance, the T cells infiltrating the target tissues in many chronic Th1-mediated inflammatory disorders in humans, including rheumatoid arthritis (RA), contain a large percentage of VLA-1+ TEM cells (7–9). Furthermore, in animal models of type I inflammation, including delayed-type hypersensitivity, experimental arthritis, and graft-versus-host disease, treatment with function-blocking mAbs to α1 integrin (or deleting this gene) suppresses inflammation (10–12).

In a previous study (5), we showed that within freshly isolated human PBL, the CD4+ VLA-1+ TEM only rarely coexpress CD25. Thus, we hypothesize that VLA-1 expression may be restricted to effector-type T cells, whereas the differentiation programs of Tregs may exclude its expression. Because the number of cell surface markers that can unambiguously sort activated conventional effector T cells from Tregs is very limited (13, 14), it was of interest to study whether VLA-1 is indeed such a marker. We used flow cytometry to identify putative human Tregs, based on the intracellular expression of Foxp3, considered the “master control gene” for their development (15, 16).

In this study, we show that the expression of VLA-1 and Foxp3 in CD4+ T cells is mutually exclusive. Thus, conventional VLA-1+ effectors and VLA-1−Foxp3+ Tregs formed two distinct populations.
that could be clearly sorted by single-cell FACS analysis and functional properties. This separation was maintained within resting PBL, freshly isolated synovial fluid lymphocytes (SFL), and even following anti-CD3 (OKT3) stimulation of PBL or SFL.

In addition, given the clinical efficacy of anti-TNFα therapy in Th1 cell-dependent human autoimmunity (17) and prompted by our clinical observations that infliximab (humanized mAb to TNFα) treatment decreases the percentage of circulating VLA-1+ T cells with a regulatory-type phenotype, we studied how TNF-α blockade affects the growth and function of VLA-1+ T cells compared with Tregs. Thus, we used an experimental system in which endogenous TNFα produced during the stimulation of human PBL with OKT3 was continuously neutralized with infliximab. We found that although TNFα blockade inhibited the typical activation-dependent induction of VLA-1+ Th1 effectors, it augmented the expansion of CD4+ T cells with a regulatory-type phenotype VLA-1+CD25highFoxp3+. Accordingly, infliximab-treated CD4+ T cells enriched, for this latter regulatory population, displayed an enhanced capacity to exert contact-dependent suppression toward autologous CD4+ PBL.

Reagents
Recombinant human IL-2 (rhIL-2) was purchased from Boehringer Mannheim. The humanized chimeric mAb to TNFα, infliximab, was obtained from Centocor. The pooled human Ig (hIg) preparation was purchased from Omrix Biotechnologies. rhTNFα was purchased from BioSource International.

Monoclonal Abs
The anti-human Foxp3 mAb (PCH101) staining kit was purchased from eBioscience. The mouse mAbs 1B3.1 (IgA) directed against the I domain of the α chain, α integrin (18), TS2/7 (IgG1) directed against a membrane proximal, non-I domain region of the α chain, and OKT3 (IgG1) were all purified from supernatants of the respective hybridomas. The PE-conjugated mouse mAb to human α chain integrin (SR84; CD49a) was purchased from BD Biosciences Pharmingen. The AGF1.1 mAb (mouse IgG2b), directed against the I domain of the α chain, integrin, was obtained from BiogenIdec. The following fluorescent-conjugated mouse mAbs directed against CD3 (FITC or PerCP), CD4 (FITC, PE-Cy5, or allophycocyanin), HLA-DR (FITC), and CD25 (PE or allophycocyanin) as well as their appropriate isotype control fluorochrome-conjugated mAbs were all purchased from BD Biosciences Pharmingen. The purified negative control mAb TEP1-15 (IgA isotype, antiphosphoryl choline), and the FITC-conjugated polyclonal goat anti-mouse IgA and IgG Abs (for indirect staining) were obtained from Sigma-Aldrich.

FACS analysis
The immunostaining and subsequent flow cytometric analysis of human lymphocytes was conducted as previously described (5). Briefly, cells were first treated with purified hlg (Sigma-Aldrich) and subsequently incubated with saturating concentrations of the indicated fluorescein-conjugated mAb for 15 min at room temperature (18–26°C). In some experiments the purified IB3.1 mAb (IgA) was used in the initial incubation step, and then the

Materials and Methods
Human subjects
This study was approved by the Institutional Ethics Committee at the Chaim Sheba Medical Center (Tel Hashomer, Israel). Blood samples were obtained from healthy blood donors. Synovial fluid samples from RA and JIA patients were obtained from patients treated at the Adult and Pediatric Rheumatology Clinic at the Chaim Sheba Medical Center, and was only drawn when clinically indicated.
cells were thoroughly washed and incubated for 15 min at room temperature with FITC-polyclonal goat anti-mouse IgA in staining buffer containing 5% goat serum. The cells were analyzed on a FACS Calibur using the CellQuest software (both from BD Biosciences). Viable lymphocytes were defined by their forward scatter/side scatter characteristics and propidium iodide (Sigma-Aldrich) exclusion.

Cytokine detection
For intracellular cytokine detection, the T cells were activated with 20 ng/ml PMA and 0.8 μM ionomycin (Sigma-Aldrich) in the presence of 2 μg/ml monensin (GolgiStop; BD Biosciences) for 5 h. Subsequently, the cells were harvested, fixed, and permeabilized using the Cytofix Cytoperm Plus kit, and then stained with anti-IFN-γ-allophycocyanin, IL-2-PE, and CD4 FITC (all from BD Biosciences). Alternatively, supernatants were collected, and IL-2, IL-4, IL-5, IL-10, IFNγ, and TNFα levels were measured using the human Th1/Th2 cytometric bead array kit from BD Biosciences, in accordance with the manufacturer’s instructions.

Cell isolation and T cell subset separation
PBL and SFL were isolated by density gradient centrifugation on Histopaque 1077 (Sigma-Aldrich). The VLA-1+ and VLA-1− cell populations were separated by first staining the PBL with TS2/7 mAbs and positively selecting the VLA-1+ fraction using goat anti-mouse IgG MicroBeads and the MACS cell separation system (Miltenyi Biotec). The CD4+CD25+ and CD25− populations were purified using the CD25+ Regulatory T Cell Isolation kit, and CD4+ T cells were isolated with anti-CD4 MicroBeads (all from Miltenyi Biotec). The cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Life Technologies) and maintained at 37°C in a humidified 5% CO2 incubator. The cells were typically plated at 2 × 10^6 cells/well in 24-well plates (Costar; Corning) and stimulated with 1 μg/ml OKT3 mAb. Three days later, the cultures were supplemented with 100 IU of rHuIL-2, every 48 h. Infliximab or hIg (both at 50 ng/ml) was added into the medium, at the initiation of the cultures and with every medium change (every 48–72 h). Alternately, when indicated, the cocultures were initially activated by PMA and ionomycin (5 μg/ml; BD Biosciences) for 10 min (37°C) with constant shaking. Subsequently, the CFSE was quenched with 50% FBS for 1 min, and the cells were washed twice with large volumes of medium. The cells were then fixed and permeabilized for intracellular cytokine detection. Viable lymphocytes were selected using 5% goat serum. The cells were analyzed on a FACSCalibur using the CellQuest software (both from BD Biosciences). To generate short-term CD4+ T cell lines, freshly purified PBL were stimulated as above and cultured with or without infliximab for 8 days. Subsequently, the CD4+ T cells were isolated and restimulated with irradiated autologous PBL and OKT3, then treated with infliximab or not, as appropriate, and cultured for an additional 8 days.

Proliferation assay
T cell proliferation was assessed, as previously described (5), using FACS-based analysis of serial hashing of the vital dye CFSE. Briefly, up to 1 × 10^6 cells suspended in 1 ml of PBS and then pulsed with 2.5 μM CFSE (Molecular Probes) for 10 min (37°C) with constant shaking. Subsequently, the CFSE was quenched with 50% FBS for 1 min, and the cells were washed twice with large volumes of medium. The cells were then stimulated and cultured, as indicated, and at the end of culture were analyzed for CFSE dilution.

Suppression assay
The various T cell populations were tested, as previously described (14), for their ability to suppress the proliferation of restituting autologous CD4+ T cells responders (Resp) following OKT3 activation. Briefly, CFSE-labeled Resp at 1 × 10^6/well were plated into 96-well plates (Costar; Corning) and activated in the presence of graded amounts of suppressors (Supp). The cells were cultured for 5 days, harvested, and CD4+ Resp were analyzed for divisions. Equal numbers of live-gate CFSE-labeled Resp (~5000 events) were usually acquired for analysis. In certain experiments, the Supp were prevented from directly contacting the Resp by a 0.4-μm pore size Transwell membrane (Costar; Corning). The proliferation index was calculated by dividing the percentage of proliferation of Resp in a particular culture by the percentage of proliferation in the Resp-alone (control) culture.

Statistical analysis
The p values were calculated by either paired Student’s t test with logarithmic transformation or Wilcoxon’s signed rank test as appropriate. Values of p < 0.05 were considered significant.

Results
VLA-1 and Foxp3 expression are mutually exclusive events in both circulating and synovium-infiltrating human CD4+ T cells
In a previous study, we observed that circulating CD4+ VLA-1+ T cells, a population highly enriched for Foxp3+, were usually CD25+ at homeostasis (5). Because CD25 is constitutively expressed on Tregs, we hypothesized that VLA-1 expression may further distinguish conventional CD4+ T cells from Tregs. To identify putative CD4+ Tregs, we used surface CD25 expression coupled with intracellular Foxp3 expression, as detected by the PCH101 mAb (19).

Thus, the CD4+ T cell lineage in freshly isolated PBL from healthy donors was analyzed by flow cytometry for the surface expression of VLA-1 and CD25, as well as intracellular Foxp3 (Fig. 1 A). The combined data analysis from 15 consecutive healthy blood donors revealed that Foxp3+ cells were restricted to the VLA-1+ fraction of the CD4+ T cell lineage (mean ± SD, 4.6 ± 1.0% (n = 15)) compared with the circulating CD4+ VLA-1− T cells that did not express this transcription factor (0.5 ± 0.2%, p < 0.001; Fig. 1 D, left bar graph).

Similarly, upon the separation of freshly isolated PBL into VLA-1+ and VLA-1− fractions, we found that the CD4+ VLA-1+ fraction was significantly enriched for CD25+ (4.5%; 5.2 ± 0.9% (n = 5); Fig. 1 B) and Foxp3+ T cells (4.7%; 4.9 ± 1.0%). In contrast, the purified VLA-1− fraction was highly depleted of...
Next, we asked whether VLA-1 expression could also, reliably, sort between Tregs and activated effector CD4+ T cells from inflamed synovial fluids of RA and JIA patients. As shown in Fig. 1C, depicting a SFL sample from a typical RA patient, 16% of the inflamed synovial fluids of RA and JIA patients. As shown in Fig. 1C, depicting a SFL sample from a typical RA patient, 16% of the inflammatory synovial fluids obtained from healthy subjects further confirmed that the VLA-1+ population. In contrast, the HLA-DR molecule, a classical marker of T cell activation, was generally coexpressed with VLA-1. Furthermore, the combined analysis of inflammatory synovial fluids obtained from 18 RA and JIA patients showed that the VLA-1+ subset (17.4% ± 5.2% vs 2.2% ± 1.2%, p < 0.001 (n = 8); Fig. 2B). Notably, many of the activated VLA-1+ T cells expressed the canonical activation marker CD25, as opposed to the continued absence of Foxp3 expression (Fig. 2A, left lower dot plot).

Given that in vivo the VLA-1+ T cells are typically enriched for IFNγ+ cells (6), we now asked whether the CD4+ VLA-1+ T cells induced ex vivo by OKT3 stimulation also represent a cell population skewed toward the Th1 phenotype. The analysis of the expression of surface VLA-1 and intracellular IFNγ, as shown in a representative experiment (Fig. 2C, upper dot plot), revealed that a larger fraction of the VLA-1+ cells produced IFNγ (13 of 20 = 65%) compared to the VLA-1− cells, of which only a minority was IFNγ+ (12 of 80 = 15%). As seen, it was also clear that not all of the IFNγ+ cells are necessarily VLA-1+. Additionally, the combined data from five independent experiments confirmed that VLA-1+ cells are significantly enriched for Th1 cells compared with VLA-1− cells (61.5 ± 5.3% vs 14.8 ± 4.8%, p < 0.01). Yet again, VLA-1 and Foxp3 expression remained distinct (Fig. 2C, lower dot plot). Thus, even after polyclonal CD4+ T cell activation, VLA-1 expression primarily marks Th1 effectors, while consistently excluding Foxp3+ cells.
Foxp3+ Tregs remain VLA-1dim upon activation when exerting their suppressive function

To further determine whether Foxp3+CD4+ T cells indeed remain VLA-1- when functioning as Supp, CD4+CD25+ and CD25- T cells were isolated from PBL using the MACS Regulatory T Cell Isolation kit. Thus, we obtained a CD25+ fraction, highly enriched for Foxp3+ cells, and a CD25- fraction almost depleted of Foxp3+ cells (Fig. 3A). Next, to verify that this purified CD25+ Foxp3-enriched population indeed displayed suppressor activity, we cocultured the CFSE-labeled CD25+ Resp either with unlabeled CD4+CD25+ Supp or CD25- T cells (as control Supp), at equal numbers. On day 5 of culture, the cells were collected and analyzed for divisions (Fig. 3A, lower panel). As can be seen, addition of CD25+ Supp strongly inhibited proliferation. Moreover, analyzing the proliferation of CD25- Resp in the presence of serial 2-fold dilutions of autologous CD25+ Supp further revealed their efficient suppressor capacity (Fig. 3B). In addition, we separately analyzed the purified CD25+ or CD25- CD4+ T cells for VLA-1 expression and activation-induced proliferation. We found that the CD4+CD25+ cells displayed profound anergy and did not express VLA-1 compared with the CD25- cells (1.9% vs 24% total VLA-1+ events; Fig. 3C). Furthermore, the flow cytometric analysis of cocultures containing CFSE-labeled CD25+ Resp in the presence of serial 2-fold dilutions of CD25+ Supp (Fig. 3D; Resp:Supp ratio 2 and 8) showed that the CD25+ Supp generally retained their VLA-1neg/dim phenotype (Fig. 3D, right panel, circled). In contrast, a significant fraction of the unlabeled CD25- T cells (~35%) expressed high levels of VLA-1 under similar conditions (Fig. 3D, left panel, circled). Together these data further suggest that Tregs are, basically, excluded from α4 integrin expression and, importantly, this feature is sustained while they exert, in vitro, activation-dependent suppression of adjacent conventional CD4+ T cells.

Neutralizing TNFα during polyclonal CD4+ T cell activation inhibits the generation of VLA-1+ effectors

In RA patients responding favorably to therapy with infliximab, there is a statistically significant reduction in the numbers of circulating VLA-1+ T cells (I. Bank, manuscript in preparation). This observation motivated us to study in vitro how blocking TNFα affects the activation-dependent development of VLA-1+CD4+ T.

Therefore, freshly isolated PBL from healthy blood donors were activated with OKT3 and pulsed with either infliximab or pooled hIg (both at 50 µg/ml). Thereafter, the cultures were analyzed on days 3, 5, and 8 for the expression of VLA-1 on CD4+ T cells. We found that in the control cultures, as predicted, the initially low percentage of VLA-1+ T cells (3.5 ± 1.0%, n = 4) gradually increased in the first 8 days of culture. However, infliximab treatment inhibited this typical increase, by 50% on average, at all measured time points compared to the hIg-pulsed cultures (Fig. 4A; significant p values obtained for all time points).

Furthermore, we performed similar experiments with CFSE-labeled PBL designed to track VLA-1 and CD25 expression (day 8) primarily on activated dividing cells (Fig. 4B). As shown in this representative experiment (Fig. 4B, upper panel), the effect of infliximab on VLA-1 expression was particularly evident within the
activated dividing CD4⁺ T cells. Notably, the majority of the dividing cells, regardless of TNFα blockade, was equally activated to express the canonical activation marker CD25 (Fig. 4B, lower panel). Moreover, the pooled data from the repeated independent experiments in 15 healthy blood donors confirmed that TNFα blockade significantly inhibited the expansion of VLA-1⁺ cells among the dividing CD4⁺ T cells (16.1 ± 5.1% vs 7.9 ± 2.5% (n = 15), p < 0.01).

In addition, we performed analogous experiments with CFSE-labeled VLA-1⁺ and VLA-1⁻ T cells directly isolated from fresh PBL. We found that infliximab reduced the activation-dependent development of VLA-1⁺ cells into VLA-1⁻ effectors. Conversely, TNFα blockade did not significantly affect the stable expression of high levels of α1 integrin on the surface of the dividing, likewise activated, pre-existing VLA-1⁺ CD4⁺ T cells (data not shown).

Next, we studied the effect of infliximab on the Th1 cytokine milieu. Thus, we used the human Th1/Th2 cytometric bead array kit (BD Biosciences) to analyze the cell culture supernatants (days 4 and 5) for the secretion of TNFα, IFNγ, IL-4, IL-5, IL-10, and IL-2. We found that infliximab, apart from effectively neutralizing all of the free TNFα in the medium, also significantly diminished the concentration of IFNγ in the supernatants (4138 ± 1145 pg/ml vs 1613 ± 438 pg/ml (n = 5), p < 0.01; Fig. 4C). The effects on the secretion of the other four cytokines analyzed were inconsistent.

Thus, the data indicate that polyclonal activation of CD4⁺ T cells in a milieu depleted of TNFα reduces the induction of VLA-1⁺ cells as well as IFNγ secretion.

**TNFα blockade enhances the expansion of CD4⁺ T cells with a regulatory phenotype: VLA-1⁻/dimCD25⁺/highFoxp3⁺**

We next investigated how TNFα blockade affected the expansion of the counteracting subset of VLA-1⁻ Foxp3⁺ Tregs. To address this question, we followed infliximab-treated culture (Inflix-TC) or untreated (control (con)-TC) CD4⁺ T cell lines generated by two rounds of stimulation with OKT3 (as described in Materials and Methods). The cells were analyzed every 4 days by flow cytometry for CD25, Foxp3, and VLA-1 expression. The data presented in Fig. 5 were obtained from day 16 cultures, 8 days following the second activation, a time point when the phenotypic differences between the two conditions became most apparent and, moreover, were less likely to represent transient activation-dependent phenomena.

The FACS analysis, as shown in Fig. 5A (upper panel), revealed that infliximab induced a shift in the percentage of VLA-1⁺/dimCD25⁺ (effector-type) vs CD25⁺/highCD4⁺ VLA-1⁻ (regulatory-type) cells in favor of the latter population. For example, in this particular experiment, TNFα blockade reduced the T effector (Fig. 5A, lower box) to Treg ratio (Fig. 5A, upper box) from 3.2 to 0.6. This phenomenon, as shown in the lower panel of Fig. 5A, was directly correlated with a significant increase in the percentage of Foxp3⁻/CD25⁺ T cells in the Inflix-TC culture. Indeed, the compiled data from multiple experiments (Fig. 5B) confirmed that Inflix-TCs contained significantly more CD25⁺/high (16.5 ± 2% vs 6.1 ± 1.4% (n = 6), p = 0.02) and Foxp3⁻ cells (11.9 ± 3.2% vs 4.1 ± 1.2%, p = 0.01) compared with the con-TCs. Moreover, as we have already found for shorter-term cultures (day 8), significantly less VLA-1⁺ cells were induced in Inflix-TC (day 16) compared with control TC lines (8.9 ± 1.6% vs 22.2 ± 1.7%, p = 0.02). Relevantly, the Inflix-TC were also highly lacking in IL-2⁺ and/or IFNγ⁺ cells compared with the con-TC (Fig. 5C).

Thus, at the same time that repeated TCR triggering coupled with TNFα blockade inhibits the generation of VLA-1⁺ and IFN-γ⁺ effectors, it also augments the expansion of CD25⁺/highFoxp3⁺ Tregs.

**TNFα blockade during ex vivo TCR activation does not induce de novo generation of Foxp3⁺ Tregs from CD25⁻ Foxp3⁻ T cells**

It is postulated that the majority of the natural CD25⁺ CD4⁺ Tregs are generated in the thymus, as a functionally distinct T cell lineage, and that the transcription factor FOXP3 controls their development (13). However, recent studies, in vivo in mice (20) and in vitro in humans (21) raise the possibility that extrathymic Foxp3⁺ Tregs can be generated in the periphery from conventional CD25⁻.
precursors (22). In this context, it was essential to determine whether the mechanism of action of TNFα/H9251 blockade included the de novo induction of Foxp3+ Tregs from Foxp3+ conventional T cell precursors.

Thus, we asked whether activation in the presence of infliximab induces Foxp3 expression in selected T cell populations that lack Foxp3+ Tregs at the outset: 1) freshly isolated VLA-1+ PBL (see Fig. 1B); and 2) freshly isolated PBL effectively purged of the Foxp3+ Tregs using anti-CD25 mAb (Fig. 6A). Of note, in the following set of experiments, to enable optimal ex vivo expansion of Tregs, as recently reviewed by Bluestone and colleagues (23), the cultures were stimulated with plastic-bound OKT3 and soluble anti-CD28.

As seen in Fig. 6B, we found that TCR activation coupled with TNFα blockade supported an increase in the number of CD4+ Foxp3+ Tregs in cultures derived from VLA-1− PBL (Fig. 6B, upper panel). In contrast, in the cultures derived from VLA-1− PBL, where CD4+Foxp3+ cells were initially very rare, TNFα blockade did not result in induction of new Foxp3+ Tregs (Fig. 6B, lower panel). Moreover, we found that when CD25+ PBL, CD25− PBL, or unselected PBL were activated along with TNFα blockade, a relative expansion of Foxp3+ T cells was only detected within CD25+ PBL or unselected PBL (Fig. 6C). In contrast, we did not detect de novo induction of Foxp3+CD4+ T cells in the cultures originating from CD25−Foxp3− PBL, regardless of TNFα blockade (Fig. 6C, middle panel). These findings were uniform in all subjects studied (n = 5). Thus, TNFα blockade, during ex vivo T cells activation, augments the preferential expansion of preformed Foxp3+ Tregs, but does not support the de novo induction of Foxp3+ Tregs.
CD4+ Inflix-TC lines display effective suppressor functions compared with con-TC

We next asked whether the enrichment for cells with a regulatory phenotype, observed in the CD4+ Inflix-TC lines, is actually associated with an enhanced functional suppressor capacity. To test this hypothesis, CD4+ con-TC and Inflix-TC (Supp) generated from the PBL of four different healthy subjects were cocultured with CFSE-labeled autologous resting PBL at various ratios and stimulated with immobilized-OKT3. First, we analyzed the CFSE-labeled CD4+ T cells (Resp), on day 5 for divisions. We found that the Inflix-TC strongly inhibited the proliferation of the Resp. In contrast, the con-TC did not possess meaningful suppressive functions (Fig. 7A).

In addition, we analyzed VLA-1 expression on the CFSE-labeled Resp in the cocultures with a Resp:Supp ratio of 2 (day 7 cocultures). As shown in Fig. 7B, VLA-1 expression on Resp was significantly inhibited in the presence of Inflix-TC (4 ± 3.2%, n = 4) compared to con-TC (13.8 ± 6.7%, p = 0.04) or Resp alone (16.3 ± 5%, p < 0.01). Furthermore, in parallel experiments, we also found that the physical separation of Inflix-TC from Resp by a Transwell membrane significantly abrogated their suppressive function (data not shown).

Thus, polyclonal activation of CD4+ T cells coupled with TNFα blockade augments the expansion of potent Foxp3+ Supp that can further suppress the activation-induced expansion of new VLA-1+ effectors.

Exogenous TNFα further inhibits the expansion of Foxp3-expressing Tregs

In a recent study (24), published during the final preparation of this manuscript, it was shown that overnight incubation of freshly isolated Tregs with TNFα, at doses of 50 ng/ml and above, inhibits their suppressor function and Foxp3 expression. Consequently, it was of interest to study how treatment with similar doses of exogenous TNFα affects the expansion of Tregs. Thus, optimally activated CFSE-labeled CD25+ PBL (Foxp3+ Tregs enriched) and CD25+ PBL (Foxp3+ Tregs depleted) were either pulsed with TNFα (50 ng/ml; every 48 h), infliximab (50 µg/ml), or left untreated. Eight days after activation, the CD4+ T cells in the various cultures were analyzed for divisions and Foxp3 expression (Fig. 8A). We found that exogenous TNFα directly reduced the percentage of Foxp3+ cells within the dividing CD25+ PBL when compared with anti-TNFα-pulsed cultures (51.6 ± 7.6% vs 13.4 ± 3.5% (n = 4), p < 0.01; Fig. 8B) or neutral conditions (32.1 ± 8.9%, p = 0.02).

In agreement with the findings of Lipsky and colleagues (24), we also found that the TNFR2 (p75) was the major TNFα receptor expressed on both T effectors and Tregs, and its expression was particularly increased in the CD25+Foxp3+ subset (data not shown). Thus, along with our earlier observation that blocking endogenous TNFα enhances the expansion of Foxp3+ Tregs, the present data further suggest that TNFα is directly detrimental to the expansion of Foxp3+ Tregs, presumably via TNFR2-dependent signaling.

Discussion

In this study, we show for the first time that VLA-1+CD4+ T cells are clearly distinct phenotypically and functionally from Foxp3+ Tregs. Thus, the expression of VLA-1 distinguishes a large subset of Th1 effectors from Tregs both at homeostasis as well as during ongoing immune responses. Moreover, we show that continuous TNFα blockade, during the in vitro expansion of activated PBL, inhibits the development of VLA-1+ and Th1-type effectors, while augmenting the expansion of VLA-1+CD25+Foxp3+ Tregs.

Previous studies have identified CD4+ T cells expressing the α1 integrin as effector and memory T cells that play an essential role in normal adaptive immunity (4–8, 10–12). The expression of the α1 integrin on Ag naive T cells is induced a few days following Ag priming. Through VLA-1-collagen interactions, these cells are
thought to acquire an enhanced capacity to migrate into extralymphatic tissues and effect inflammation and/or protective immunity (4–6). A fraction of the VLA-1+ effectors are later retained in tissues as long-lived TIL (6). Conversely, multiple studies have identified CD4+ T cells that constitutively express the high-affinity IL-2R α-chain (CD25) and mature in the thymus as naturally occurring Supp, indispensable for the maintenance of self-tolerance (28, 29); similarly circulating Tregs in humans have been identified as a subset of CD4+ T cells enriched for Th1-type effectors, excluding Tregs. This finding, when taken along with the observation that treating mice with anti-α4 integrin mAbs inhibits T cell-mediated tissue inflammation (10–12), leads us to speculate that the efficacy of the anti-α4 integrin therapy is related to the fact that it specifically targets T effectors, whereas conspicuously sparing Tregs.

The identification of VLA-1 as an activation molecule that is expressed on a subset of TIL, but excludes Tregs, is of particular significance since there is broad interest in the identification of cell surface Ags that can sort Tregs from T effectors. In this regard, molecules constitutively expressed on Tregs, e.g., CD25, CTLA-4, or glucocorticoid-induced TNFR family-related gene (GITR), are also induced following activation on conventional CD4+ T cells, and thus cannot be used to unambiguously sort Tregs from activated T effectors (13, 25). Consequently, the definite identification of Tregs has to rely primarily on the detection of intracellular expression of the transcription factor Foxp3 (26) that also controls expression of the high-affinity IL-2R α-chain (CD25) and mature in the thymus as naturally occurring Supp, indispensable for the maintenance of self-tolerance (28, 29); similarly circulating Tregs in humans have been identified as a subset of CD4+ T cells enriched for Th1-type effectors, excluding Tregs. This finding, when taken along with the observation that treating mice with anti-α4 integrin mAbs inhibits T cell-mediated tissue inflammation (10–12), leads us to speculate that the efficacy of the anti-α4 integrin therapy is related to the fact that it specifically targets T effectors, whereas conspicuously sparing Tregs.

In this context, we show that at homeostasis in the PBL of healthy individuals, the subset of CD4+ T cells expressing VLA-1 is distinct from that expressing intracellular Foxp3, and that this separation is maintained following the polyclonal activation of unselected PBL. Even more importantly, VLA-1 expression on CD4+ T cells isolated from inflamed joint fluids of arthritis patients, containing many in vivo-activated CD25+ T effectors and Tregs, clearly identified a population vastly devoid of Foxp3 Supp. This novel finding may facilitate future studies that require the separation of viable Tregs from effector T cells within mixed populations of T cells residing at sites of inflammation.

Interestingly, Sallusto and colleagues (14) showed in a recent study that coexpression of CD27 on CD25+ CD4+ T cells in SFL from JIA patients identifies them as Tregs and not T effectors (14). We also found that many Foxp3+ T cells express CD27, as opposed to VLA-1 (data not shown). However, as their data also show, CD27 is also expressed on a large fraction of the CD25+ CD4+ T cells. Moreover, upon the ex vivo activation of CD25+ CD27- PBL, a large fraction of the cells retain CD27 expression, even at 5 days after activation. In contrast, our data show that VLA-1 unequivocally marks a subset of conventional T cells, devoid of Tregs, under all circumstances.

Previous studies have indicated that VLA-1+ T cells are rarely found in lymphoid tissues both in mice and humans and are accordingly usually CCR7- (5, 6). In contrast, lack of VLA-1 expression appears to be a hallmark of several functionally important subsets of CCR7+ T cells that use CCR7 to home into lymph nodes (LN), e.g., Ag naïve T cells and central memory T cells (27). Relevantly, recent studies in mice indicate that homing of Tregs into LN is essential for the maintenance of peripheral self-tolerance (28, 29); similarly circulating Tregs in humans have been reported to be frequently CCR7+ (30, 31). Therefore, it may be reasoned that the cellular programs of Tregs provide the means to maintain an enhanced capacity to home into LN, including the repression of α4 integrin expression. Yet, it is unknown whether VLA-1 expression directly interferes with LN occupancy.

Next, it is shown that continuous TNFα blockade produces opposite effects on the expansion and/or functions of VLA-1+ T effector vs Foxp3+ Tregs. In detailed experiments, we show that the activation-dependent differentiation of resting PBL into VLA-1+ and IFNγ and IL-2-producing T effectors are all significantly reduced in the presence of infliximab. These latter findings are also supported by our preliminary observation that a favorable clinical response to infliximab therapy in RA patients is associated with a decrease in the percentage of circulating CD4+ VLA-1+ T cells (data not shown). Moreover, these results are both consistent with and complement recent studies in TNFR2-/mice suggesting that TNFα, mostly through TNFR2-dependent activation of the NF-κB pathway, is essential for the proliferation, survival, and Th1 polarization of naïve CD8+ T cells (32, 33). Conversely, under similar conditions, continuous TNFα blockade significantly augmented the number and suppressor function of preformed Foxp3+ Tregs. This phenomenon was observed following short (and longer)-term expansion of unselected PBL, as well as CD25+ PBL. Moreover, during the expansion of CD25+ PBL, a population highly enriched at the outset for Foxp3+ Tregs, treatment with exogenous TNFs specifically reduced the expansion of Foxp3+ cells compared with control cultures, and particularly when compared with infliximab-treated cultures. These results are in agreement with clinical studies in RA patients and in NOD mice showing that in vivo TNFα blockade increased the number of circulating CD25high T cells and restored their in vitro suppressor function (34, 35).

In addition, we found that most ex vivo-activated T cells, including CD25highFoxp3+ cells, express TNFR2 and not TNFR1 (data not shown). Therefore, although our study did not directly address the mechanisms involved in the dichotomous effects of TNFα blockade on the VLA-1+ and FoxP3+ subsets, it is tempting to hypothesize that TNFR2-mediated signaling plays a role in this regard. This idea is further supported by a very recent study (24) showing that overnight incubation of freshly isolated CD25high Tregs with either TNFα or an agonistic mAb to TNFR2 inhibits their Foxp3 expression and suppressor function. Interestingly, other members of the TNFR superfamily (e.g., GITR and OX40) also inhibit the suppressor function of Tregs (36, 37). Thus, we envision that activation of the NF-κB pathway, the principal pathway activated by TNFR superfamily members (38), may lead to different outcomes in Tregs vs T effectors.

Importantly, the observed increase in VLA-1+ Foxp3+ cells seen in cultures generated by prolonged TNFα blockade was also coupled to augmented in vitro suppressor function. Thus, we show that the Inflix-TC (enriched for VLA-1+ Foxp3+ and IFN-γ- cells), as opposed to con-TC (enriched for VLA-1+ Foxp3- and IFN-γ- cells) could efficiently suppress in a contact-dependent manner the proliferation of resting autologous CD4+ T cells and their development into VLA-1+ T effectors. Our experiments also lend support to the view that the expression of Foxp3 in human T cells is restricted, as it is in mice (26), to the Treg lineage. In our hands, potent activation of CD25+ Foxp3+ T cells with plastic-bound OKT3, anti-CD28, and exogenous rIL-2 did not induce de novo expression of Foxp3, even in the presence of infliximab. It is important to note, as revealed by the manufacturer, that the PCH101 mAb recognizes an epitope near the amino terminus of Foxp3 also present in the other human Foxp3 splice variant (Δexon2), and thus very likely identifies all relevant Foxp3+ cells. Obviously, our findings do not exclude the possibility that in vivo TNFα blockade may enhance de novo production of Tregs. In this regard, studies in NOD mice show that
neonatal treatment with anti-TNFα increases the number of CD4+ CD25+ T cells in the thymus and spleen, whereas TNFα treatment decreases their amount (35).

In conclusion, the detailed studies described in this manuscript are the first to demonstrate that VLA-1 expression in CD4+ T cells is restricted to T effectors, but uniformly excludes Foxp3+ Tregs. Moreover, we have shown that TNFα blockade inhibits the in vitro expansion of human CD4+ VLA-1+ T effectors, while simultaneously augmenting Tregs, a finding that advances our understanding of how TNFα shapes the adaptive T cell response. Our study also suggests that the ex vivo expansion of Tregs for cellular therapy of certain autoimmune disorders could be facilitated by neutralizing TNFα during the ex vivo expansion protocol.

Disclosures
The authors have no financial conflict of interest.

References