In vitro assessment of the effects of vedolizumab binding on peripheral blood lymphocytes

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Vedolizumab (VDZ) is a humanized monoclonal antibody in development for the treatment of inflammatory bowel disease. VDZ binds to the $\alpha_4\beta_7$ integrin complex and inhibits its binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1), thus preventing lymphocyte extravasation to gut mucosal tissues. To understand whether VDZ has additional effects that may affect its overall safety as a therapeutic molecule, we examined other potential actions of VDZ. In vitro assays with human peripheral blood lymphocytes demonstrated that VDZ fails to elicit cytotoxicity, lymphocyte activation, and cytokine production from memory T lymphocytes and does not interfere with the suppressive ability of regulatory T cells. Furthermore, we demonstrated that VDZ induces internalization of $\alpha_4\beta_7$ and that the integrin is rapidly re-expressed and fully functional after VDZ withdrawal. These studies provide insight into the mechanisms underlying the observed safety profile of VDZ in clinical trials.

Introduction

Vedolizumab (VDZ) is a humanized immunoglobin (Ig)G$_1$ monoclonal antibody (mAb) currently in development for the treatment of ulcerative colitis and Crohn's disease. It acts by targeting the $\alpha_4\beta_7$ integrin and inhibiting its ability to bind mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on the gastrointestinal endothelium,[1] thus inhibiting T-cell migration into the gut. The safety and efficacy of VDZ [previous humanized versions were known as LDP-02, MLN02, and MLN0002; for simplicity, all versions of Millennium's investigational humanized antibodies with the epitopic specificity of the murine version of VDZ (ACT-1) mAb[2] will be referred to herein as "vedolizumab"] have been studied in Phase 2[3-5] and Phase 3 [6-9] trials. In clinical studies, VDZ was not associated with depletion of lymphocyte subsets and did not cause lymphocytosis.[3-5] Complete blood counts in normal volunteers and patients with inflammatory bowel disease have not shown increases in circulating lymphocytes, monocytes, eosinophils, or basophils after VDZ administration.[3-5]

During the construction of VDZ, point mutations were made to the Fc receptor (FcR) γ binding motif (ELLGGP), exchanging Leu[239] and Gly[241] with Ala to reduce binding to the FcR.[10] The observed clinical pharmacology and safety profiles are consistent with both Fc modifications, but it is nonetheless important to confirm that VDZ has no function other than that of inhibiting $\alpha_4\beta_7$ binding to its ligand.

Antibody-dependent cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are potential consequences associated with IgG$_1$ binding to FcR and complement components, respectively. Under normal circumstances, these processes aid in the removal of infectious organisms and infected or damaged cells; however, in the case of VDZ, these properties could potentially lead to depletion of $\alpha_4\beta_7$-expressing cells or alterations in cellular function. Lysis of target cells, particularly memory T lymphocytes, can lead to a prolonged absence of cellular activity, which may be disadvantageous if adverse events occur in response to a therapeutic. Additionally, it has been previously demonstrated that some integrins are internalized after binding to ligands or antibodies.[11,12] Theoretically, VDZ-$\alpha_4\beta_7$ may also be internalized, which could lead to various biological consequences such as cytokine release and cellular activation. Therefore, the objective of this study was to characterize the effect of VDZ on peripheral blood cells by examining Fc
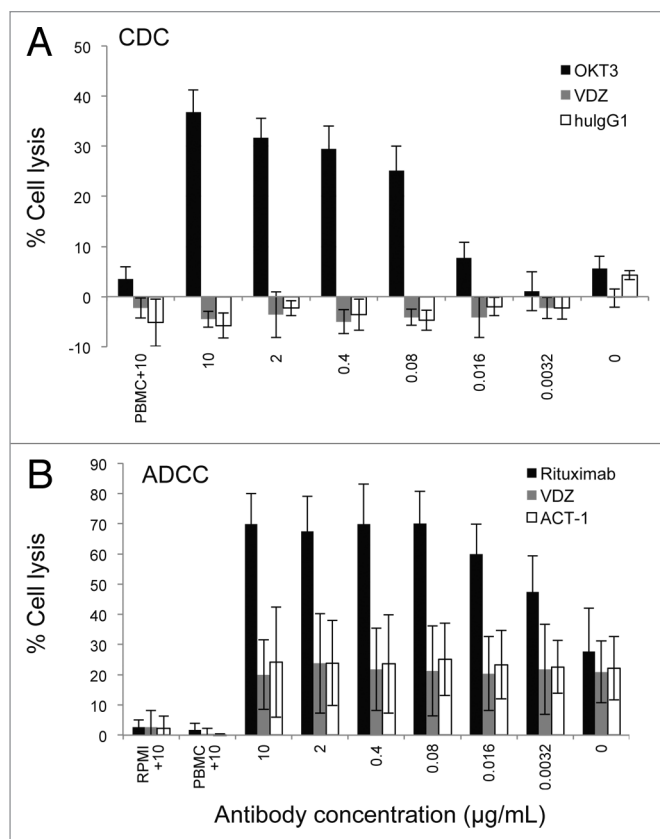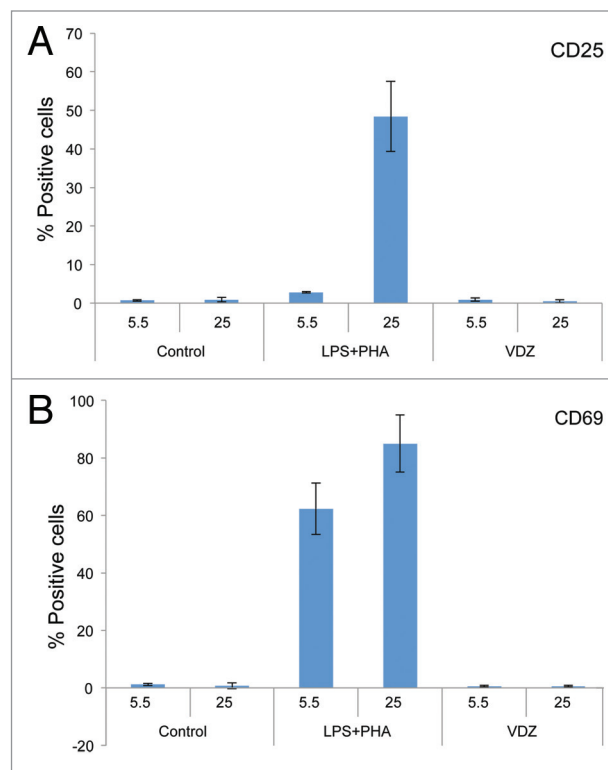
Figure 1. VDZ does not affect CDC or ADCC. (**A**) PBMCs were incubated with increasing concentrations of VDZ, OKT3, or human IgG₁ in the presence of rabbit complement. Spontaneous lysis of the cells in the presence of 10 μg/mL of each antibody in the absence of complement is shown (n = 4). (**B**) CD20⁺ α₄β₇⁺ RPMI8866 cells were incubated with increasing concentrations of either VDZ, rituximab (anti-CD20), or ACT-1 (the murine precursor of VDZ) in the presence of PBMCs. Spontaneous lysis of the cells in the presence of 10 μg/mL of each antibody in the absence of either effectors (PBMCs) or targets (RPMI8866) is shown (n = 3). Results are representative of 3 experiments.

Figure 2. VDZ does not induce the expression of the activation markers CD25 or CD69 in T cells. Peripheral blood was incubated in the presence of PBS (control), LPS + PHA, or VDZ and examined at 5.5 h and 25 h for expression of either (**A**) CD25 or (**B**) CD69 by flow cytometry (n = 4). Results are representative of 1 of 3 experiments.

effector functions, cellular activation, and internalization of the α₄β₇ integrin complex.

Results

Vedolizumab binding to leukocyte α₄β₇ integrin does not elicit Fc-mediated functions. CDC and ADCC are common Fc-mediated, cytotoxic mechanisms of action for therapeutic IgG₁ mAbs, exemplified by the anti-CD3 OKT3 and the anti-CD20 rituximab, respectively.[13,14] VDZ was engineered to contain 2 amino acid changes (Leu[239] and Gly[241] to Ala) in the FcR binding region of the heavy chain to eliminate these binding sites. We compared potential CDC activity of VDZ in human peripheral blood mononuclear cells (PBMCs) with that of OKT3 in vitro. No CDC was observed in the presence of VDZ or IgG₁ isotype control at a concentration as high as 10 μg/mL (**Fig. 1A**), a concentration that was approximately 20-fold greater than that needed to saturate binding of VDZ to human whole blood cells. In contrast, OKT3 induced CDC in PBMCs in a

dose-dependent manner, with maximal lysis occurring at 10 μg/mL. These results suggest that the in vivo activity of VDZ does not involve CDC.

We also compared potential ADCC activity of VDZ with that of anti-CD20 rituximab in RPMI8866 cells, which stably express high levels of the α₄β₇ integrin and CD20 (data not shown). Relative to the cells treated with IgG₁ isotype control, there was no ADCC activity observed in the presence of VDZ at a concentration as high as 10 μg/mL (**Fig. 1B**), a concentration that was approximately 100-fold greater than that needed to saturate binding of VDZ to RPMI8866. In contrast, rituximab induced ADCC in RPMI8866 cells in a dose-dependent manner, with maximal lysis occurring at 0.08 μg/mL. These results suggest that VDZ does not induce ADCC activity.

Vedolizumab binding to leukocyte α₄β₇ integrin does not elicit agonist activity. It is important to understand whether the binding and internalization of VDZ leads to cellular activation and cytokine release, given that antibody-induced cytokine release can have significant clinical effects.[15] We therefore examined the ability of VDZ to activate T lymphocytes and induce cytokine production. VDZ binding to human whole blood does not affect expression of the T-lymphocyte early activation marker CD69 or late activation marker CD25 (**Fig. 2**). In contrast, stimulation with lipopolysaccharide (LPS) or phytohemagglutinin (PHA) did induce upregulation of CD69 and CD25 on T lymphocytes.

Table 1. In vitro cytokine production (ng/mL)*

Time post treatment, h	Treatment	IFN-γ	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-12 (p70)	IL-17	TNF
5.5	PBS	2.2 ± 3.5	0.2 ± 2.0	1.8 ± 2.0	1.0 ± 0.4	7.0 ± 1.8	30 ± 50	0.8 ± 0.3	6.9 ± 6.0	5.5 ± 6.0
	VDZ	3.0 ± 5.0	0.2 ± 2.3	1.9 ± 2.3	1.4 ± 1.1	9.0 ± 8.2	29 ± 36	1.1 ± 0.9	7.4 ± 9.2	7.2 ± 9.7
	LPS	39 ± 13	118 ± 10	19 ± 10	9.2 ± 3.2	25517 ± 9614	5755 ± 1903	1.9 ± 1.3	56 ± 31	660 ± 211
24	PBS	2.8 ± 4.4	0.2 ± 2.6	3.9 ± 2.6	1.0 ± 0.3	112 ± 191	422 ± 685.4	1.1 ± 0.8	27 ± 34	15 ± 14
	VDZ	3.6 ± 6.2	0.2 ± 3.3	3.6 ± 3.3	0.9 ± 0.4	77 ± 122	126 ± 124	1.4 ± 1.4	31 ± 37	22 ± 25
	LPS	4345 ± 2963	3403 ± 30	50 ± 30	17 ± 1.8	49258 ± 9606	2292 ± 3632	4.0 ± 2.9	96 ± 83	1408 ± 517

LPS, lipopolysaccharide; IL, interleukin; PBS, phosphate-buffered saline; TNF, tumor necrosis factor; VDZ, vedolizumab; IFN, interferon. *n, 4.
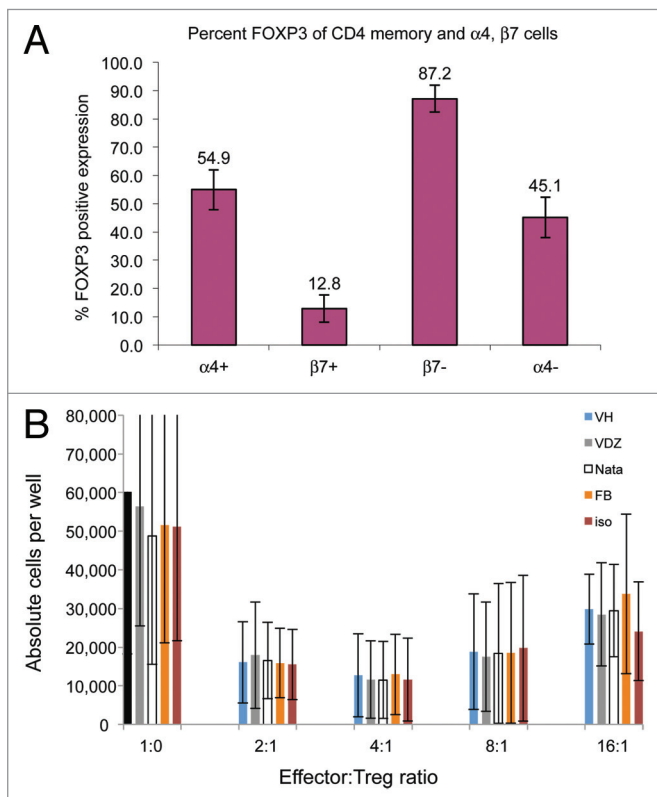
Figure 3. VDZ does not affect suppressor activity of $\alpha_4^+\beta_7^+$ Treg cells. (**A**) FoxP3+ Treg cells were examined for the expression of α_4 and β_7 by flow cytometry, demonstrating the FoxP3+ Treg population constitutes only approximately 13% of the CD4+ CD45RO β_7^+ memory population. Results are representative of one of 3 experiments. (**B**) Suppressor activity of purified Treg cells was assessed after pre-incubation with vehicle, VDZ, natalizumab, FIB504, or isotype control, demonstrating a lack of effect of VDZ on suppressor cells. Results are from 3 donors.

To examine whether VDZ induces cytokine release, PBMCs were incubated with VDZ, LPS, or phosphate-buffered saline (PBS) for 5.5 h or 24 h, and cultures were examined for the presence of cytokines. VDZ binding to leukocytes did not elicit cytokine production, including interferon-γ (IFN-γ), tumor necrosis factor (TNF), or interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-12 (p70), and IL-17 (**Table 1**). In contrast, LPS elicited early cytokine responses that persisted and accumulated over 24 h.

Effect of vedolizumab binding on regulatory T cells. Regulatory T (Treg) cells play a crucial role in maintaining mucosal immune homeostasis, suggesting that Treg cell dysfunction could be a contributing factor in the pathogenesis of human inflammatory bowel disease. For this reason, the effect of VDZ on the suppressive activity of human Treg cells was investigated. To determine if any fraction of the $\alpha_4^+\beta_7^+$ cells in the periphery were Treg cells, PBMCs from healthy human volunteers were stained with fluorescently labeled anti-α_4 and anti-β_7 antibodies and analyzed by flow cytometry. Treg cells (as defined by gating on the CD4+ CD45RO$_+$ $\alpha_4^+\beta_7^+$ and subsequent gating on FoxP3) constituted approximately 13% of the total β_7^+ CD4+ cell population residing in peripheral blood (**Fig. 3A**).

The presence of Treg cells in the $\alpha_4^+\beta_7^+$ population suggests a potential for VDZ to perturb the regulatory balance in the gastrointestinal mucosa. To address this, 3 different $\alpha_4\beta_7$ antagonists (VDZ, natalizumab, and the anti-β_7 mAb FIB504) were examined for their ability to affect the suppressive activity of this Treg cell subpopulation in vitro. There was no consistent effect of VDZ, natalizumab, or FIB504 on the suppressive activity of highly purified human $\alpha_4^+\beta_7^+$ CD4+ CD25+ CD127^{low} FoxP3+ Treg cells compared with that of vehicle or an isotype control antibody. These data indicate that VDZ does not affect the suppressive activity of human Treg cells (**Fig. 3B**).

The $\alpha_4\beta_7$-vedolizumab complex is internalized by CD4+ memory lymphocytes. It has been previously demonstrated that antibody binding to integrins induces internalization of the integrin-antibody complex.[12] VDZ binds specifically to the $\alpha_4^+\beta_7$ integrin with high affinity; it does not bind to other integrin heterodimers containing α_4 or β_7 chains.[1] We therefore investigated whether the VDZ-$\alpha_4\beta_7$ surface complex is internalized. $\alpha_4\beta_7^+$ cells were incubated at 37 °C with fluorescently labeled VDZ and examined using microscopy, revealing the classic punctate staining pattern indicative of internalization into endosomes.[16,17] In contrast, the staining pattern after incubation at 4 °C indicated membrane staining without collection into endosomes (**Fig. 4A**). To verify the internalization of the complexes, an acid-stripping flow cytometric assay was used. Alexa 647-labeled VDZ was incubated with CD4+ CD45RO+ memory lymphocytes at
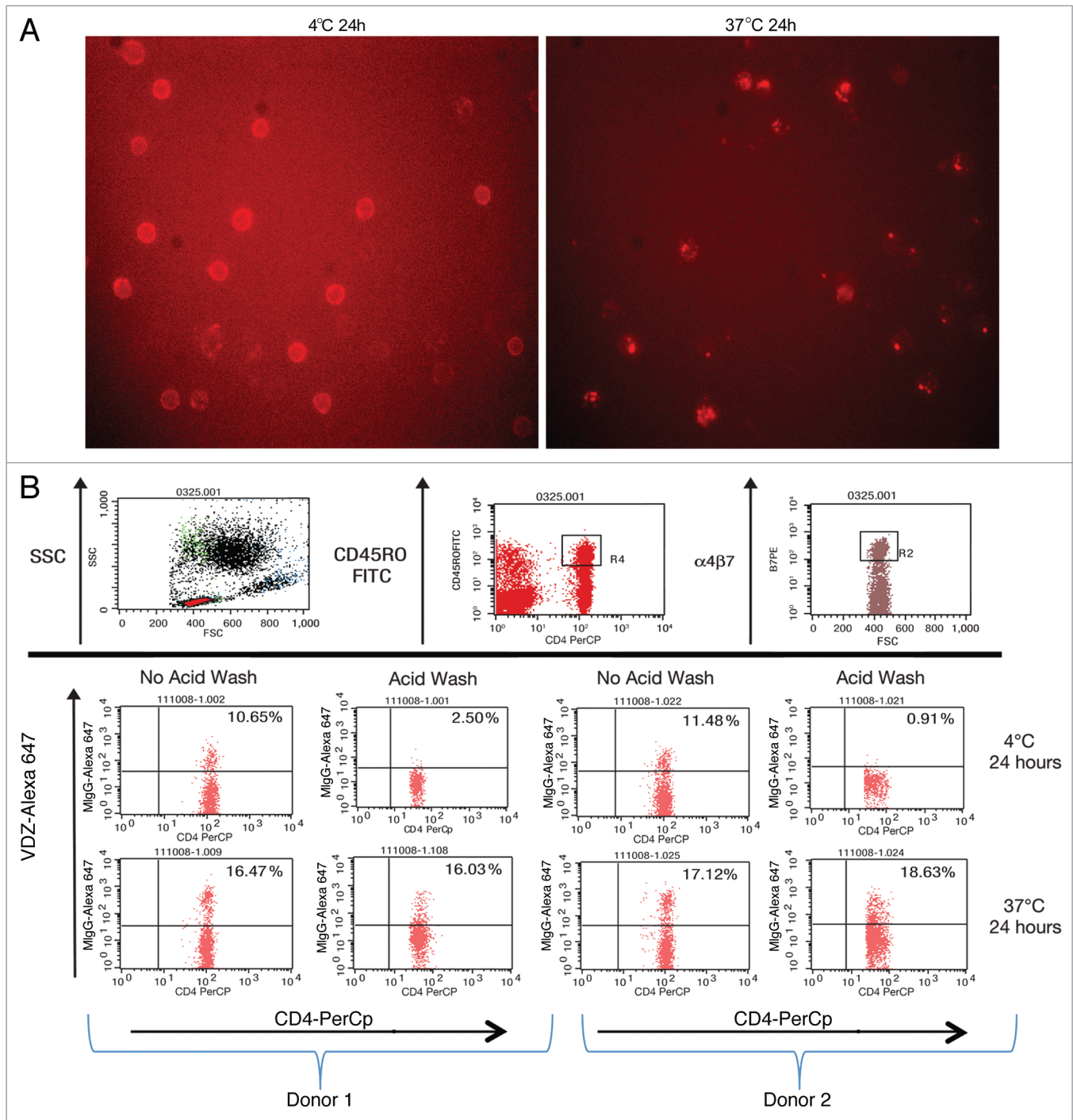
Figure 4. The $\alpha_4\beta_7$ integrin is internalized after VDZ binding. (**A**) Peripheral blood T lymphocytes were stained with VDZ–Alexa 647 and visualized by fluorescence microscopy. An intracellular punctate staining pattern is typical of internalization into endosomes, whereas a ringed pattern indicates surface staining. (**B**) PBMC from 2 representative donors were stained with VDZ-Alexa 647, incubated, and then subjected to acid wash to remove extracellular antibody. For the flow cytometery, cells were gated first on the lymphocyte side scatter (SSC) gate, followed by gating on the CD4+ CD45RO high memory T-cell population. The CD4+ CD45RO high population was subsequently examined for binding to labeled VDZ.

37 °C for 24 h, then washed to remove surface-bound antibody. Alexa 647-positive staining after the wash indicated the receptors were internalized by CD4+ CD45RO+ memory lymphocytes (**Fig. 4B**). In contrast, cells incubated at 4 °C showed no staining after acid wash. The internalization was target specific and was not a result of nonspecific pinocytosis of labeled antibody, as mouse IgG-Alexa 647 was not internalized by CD4+ memory lymphocytes (**Fig. 5**). A time course for internalization
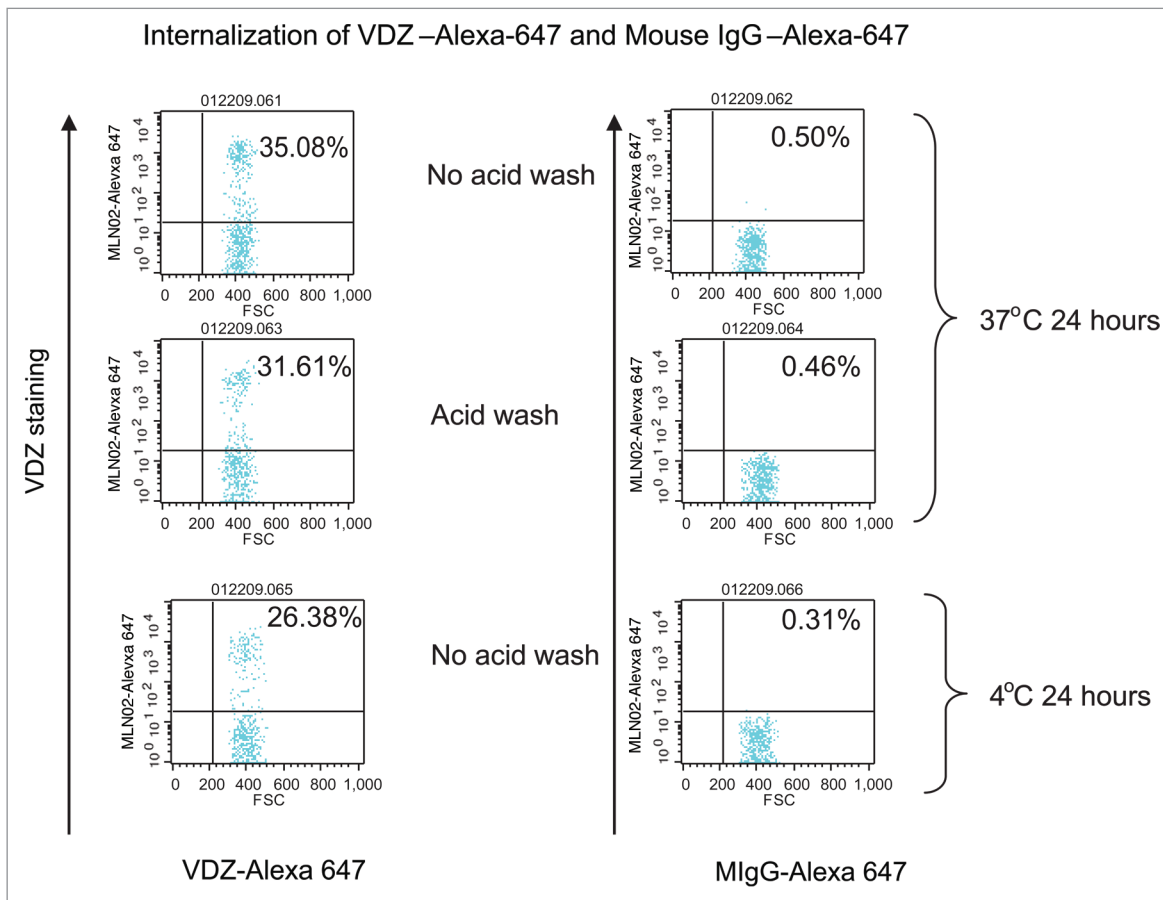
Figure 5. Internalization of Alexa-647 labeled antibodies occurred only with VDZ and not with mouse immunoglobulin (mIgG), indicating the internalization is specific for VDZ. Cells were incubated with either Alexa-647 labeled mIgG control or VDZ for 24 h at 37 °C. Cells were gated initially on forward scatter FCS/SSC followed by CD4 and CD45RO to obtain the CD4+ CD45RO+ memory T cells.

showed that within 3.5 h, 50% of $\alpha_4\beta_7$ receptors were internalized, and within 24 h, 100% of the receptors were internalized (**Fig. 6A**).

Basal levels of $\alpha_4\beta_7$ integrin expression on CD4+ memory lymphocytes are restored. A question that remained was whether the $\alpha_4\beta_7$ heterodimer returns to the cell surface after removal of VDZ. To address this, cells that had been treated with VDZ and had internalized the VDZ-$\alpha_4\beta_7$ complex were washed extensively and incubated at 37 °C for 4 d. Re-expression of the $\alpha_4\beta_7$ integrin on the cell surface was monitored on days 0, 1, and 4 by flow cytometry. On average, 54% (n = 3) of the basal level of $\alpha_4\beta_7$ surface expression was restored within 24 h and 83% (n = 3) was restored within 96 h, relative to cells that had not been pretreated with VDZ (**Fig. 6B**). Treatment with monensin indicated that the recovery of receptors was dependent on intracellular transport through the Golgi (**Fig. 6C**).[18]

Newly expressed $\alpha_4\beta_7$ receptors bind to MAdCAM-1. The function of $\alpha_4\beta_7$ integrin is to facilitate adherence of $\alpha_4\beta_7$-expressing cells to surface-bound MAdCAM-1. Because of the processing of the cells through the VDZ treatment, $\alpha_4\beta_7$ internalization, and $\alpha_4\beta_7$ re-expression, however, the adherence assay was, too variable to demonstrate functional binding of

MAdCAM-1 to the newly expressed receptor. Therefore, it was important to correlate the ability of newly expressed $\alpha_4\beta_7$ to bind MAdCAM-1. To accomplish this, both RPMI8866 and purified CD4+ CD45RO+ memory T cells were tested for adherence and MADCAM-1 binding in the presence of VDZ. The 50% inhibitory concentration (IC_{50}) for each cell type and the goodness of fit were compared between the 2 assays. IC_{50} values between MAdCAM-1-mFc binding and adhesion assays were within 2-fold in RPMI8866 cells (binding: 28.19 ng/mL; adhesion: 51.85 ng/mL; **Fig. 7A**) and in CD4+ memory T lymphocytes from PBMCs (binding: 20.65 ng/mL; adhesion: 33.89 ng/mL; **Fig. 7A**). Furthermore, MAdCAM-1 binding and adhesion were well correlated in both cell types (RPMI8866 cells, r^2 = 0.9377; CD4+ memory T lymphocytes, r^2 = 0.9071; **Fig. 7A**).

Since binding to soluble MAdCAM-1-mFc was comparable to adhesion, the functional capacity of newly expressed $\alpha_4\beta_7$ was examined using soluble MAdCAM-1-mFc binding to the newly expressed cell surface $\alpha_4\beta_7$. The amount of MAdCAM-1 bound to $\alpha_4\beta_7$ receptors increased from day 1 to day 4, as demonstrated by an increase in the amount of VDZ required to inhibit MADCAM-1-mFc binding to the newly expressed integrin complex (IC_{50}: 2 ng/mL and 22 ng/mL for day 1 and day 4, respectively; **Fig. 7B**). These results are consistent with

the reappearance of $\alpha_4\beta_7$ on the surface of the cells and their functional binding to soluble MAdCAM-1. Taken together, these results demonstrate that the $\alpha_4\beta_7$ that is re-expressed after VDZ-$\alpha_4\beta_7$ complex internalization is functional.

Discussion

mAbs are an important part of the physician's armamentarium for treatment of many autoimmune and oncologic diseases. The safety and pharmacology of these drugs is dependent on both the primary and secondary effector properties of each antibody,[19] so it is important to fully characterize the effector properties of the antibody during development. VDZ is a humanized IgG$_1$ antibody in development for the treatment of ulcerative colitis and Crohn disease.[4,5] To date, VDZ has been shown to be tolerable and to provide benefit in Phase 2 and Phase 3 clinical trials.[3-9] The safety and pharmacology of therapeutic antibodies is dependent on both the primary and secondary effector properties of each antibody,[19] and it is thus important to fully characterize these properties.

To investigate activities that could lead to potential safety concerns, we investigated if VDZ is stimulatory or has Fc-mediated activity. Here, we demonstrated that VDZ does not induce CDC or ADCC activity. Additionally, VDZ does not activate T lymphocytes or stimulate the production of cytokines from PBMCs, including the pro-inflammatory cytokines IL-1β, IL-6, and TNF. These data are consistent with results observed in clinical trials. In early Phase 1 studies, there were no observed changes in serum levels of TNF, IL-2, IL-1, or IFN-γ in patients dosed with VDZ (unpublished results). Along with clinical data in the literature,[3-9] the results reported here suggest that the induction of cytokines after VDZ treatment is unlikely.

Treg cells are of particular interest because they are postulated to provide inhibitory activity that may decrease inflammation in ulcerative colitis and Crohn's disease patients.[20] There was no consistent effect of VDZ or 2 other $\alpha_4\beta_7$ antagonists on the suppressive activity of highly purified human $\alpha_4\beta_7^+$ CD4$^+$ CD25$^+$ CD127^{low} FoxP3$^+$ Treg cells (**Fig. 3B**), indicating that VDZ does not affect the suppressive activity of human Treg cells in the interstitial matrix of stroma.

We demonstrated that VDZ-$\alpha_4\beta_7$ complexes are rapidly and completely internalized within 24 h of VDZ exposure. This effect is reversible in that, after removal of VDZ, cells will replace functional $\alpha_4\beta_7$ on their surface within 24–48 h. These results suggest that the effect of VDZ binding to cells is that of inhibiting the binding of $\alpha_4\beta_7^+$ cells to ligands, particularly MAdCAM-1, and does not include Fc-mediated effects. These data indicate that inhibition of $\alpha_4\beta_7$ function by VDZ in vivo
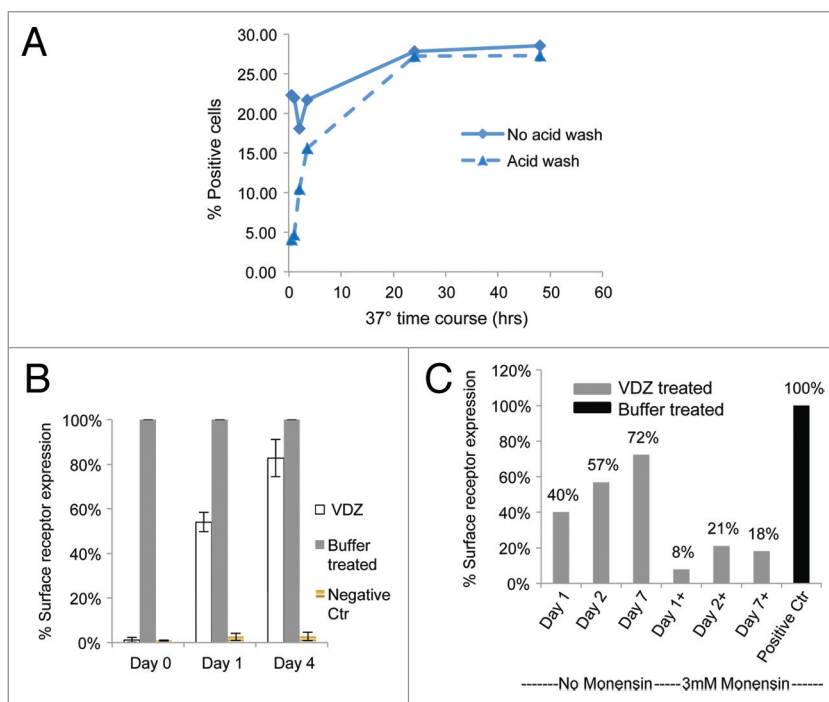

Figure 6. $\alpha_4\beta_7$ expression is restored after removal of VDZ in a Golgi-dependent manner. Whole blood was incubated with VDZ, washed, and cultured in the presence or absence of the Golgi inhibitor monensin, and $\alpha_4\beta_7$ expression was assessed by flow cytometry. (**A**) Time course of receptor internalization. After 24 h, VDZ internalization was reached a plateau. (**B**) $\alpha_4\beta_7$ expression returned after 1 d and is nearly complete after 4 d of culture in the absence of VDZ. A negative control of fluorescently labeled mouse Ig was included to show percentage of cells with fluorescent background staining. (**C**) The re-expression of $\alpha_4\beta_7$ was inhibited by co-incubation with monensin.

includes a direct interference with MAdCAM-1 binding and decreased surface expression of $\alpha_4\beta_7$ through internalization.

Notably, cells that have internalized the complexes are still viable and able to re-express functional $\alpha_4\beta_7$ after removal of VDZ. These data suggest that the immune system has the potential to return to its previous state upon VDZ withdrawal, which could restore protective activity, as well as pathogenic inflammation. Taken together, these results provide molecular and cellular bases to explain key aspects of VDZ's tolerability profile in clinical trials to date.

Materials and Methods

Reagents. MAdCAM-1-mFc fusion protein, VDZ, and Alexa 647–labeled VDZ were available in-house. Cy5 goat anti-mouse IgG (H$^+$L) (Cat # 115-175-062) was purchased from Jackson ImmunoResearch. CD4-PerCP (Cat # 550631), CD45RO-FITC (Cat # 555492), and mouse IgG$_1$–Alexa 647 (Cat # 557732) were purchased from BD Biosciences. Sucrose (Cat # S7903), monensin (Cat # M5273), and bovine serum albumin (BSA) fraction V (Cat # A3294) were obtained from Sigma-Aldrich. Fetal bovine serum (Cat # SH30071.02) was obtained from Thermo Fisher Scientific. Dulbecco's PBS without Ca^{2+} and Mg^{2+} (Cat # 14190), RPMI-1640 (Cat # 22400), DMEM high glucose-no phosphates medium (Cat #11971), penicillin-streptomycin (Cat # 15070),
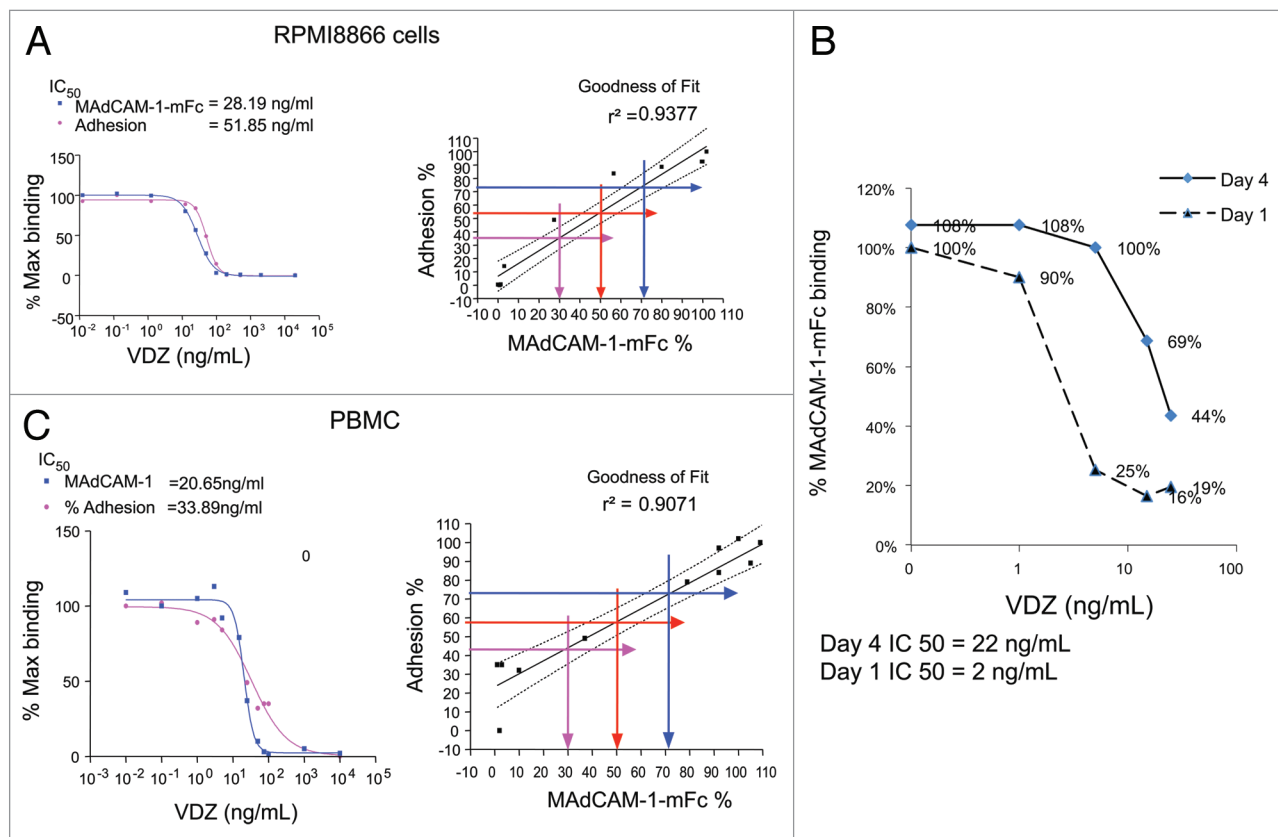
Figure 7. The re-expressed $\alpha_4\beta_7$ integrin is functional. (**A**) The binding of soluble MAdCAM-1-mFc to RPMI8866 cells and PBMC (gated on the CD4+CD45RO+ population) strongly correlated with adherence of the cells to plate-bound MAdCAM-1-Fc, demonstrating the use of soluble MAd-CAM-1 as a surrogate for functional adhesion. (**B**) MAdCAM-1-Fc was incubated with whole blood 1 and 4 d after initiation of the recovery of the $\alpha_4\beta_7$ complex in the presence of increasing concentrations of VDZ, demonstrating the ability of the newly expressed receptor to bind MAdCAM-1-Fc.

and L-glutamine (Cat # 25030) were obtained from Invitrogen. Red blood cell (RBC) lysis buffer (Cat # 555899) was obtained from BD Biosciences. Alamar Blue® (Cat # 00-100) was from Trek Diagnostic System. The $\alpha_4\beta_7$-stably expressing human B-cell lymphoma cell line RPMI8866 was a kind gift from Dr David Erle (San Francisco, CA). Culture medium for RPMI8866 cells consisted of RPMI-1640 medium supplemented with 1% penicillin/streptomycin and 1% L-glutamine and 10% US-defined fetal bovine serum. The mouse mAb ACT-1 used was an in-house reagent.

Cytotoxicity assays. Briefly, CDC was measured by incubating VDZ or control antibodies with $\alpha_4\beta_7$-expressing target cells in the presence of rabbit complement at 37 °C. OKT3 (anti-CD3) and normal human IgG$_1$ were used as positive and negative controls, respectively. Spontaneous release was demonstrated in the absence of added complement (PBMC-10) but in the presence of the maximum concentration of antibodies. To measure ADCC, $\alpha_4\beta_7$-expressing target cells were incubated with VDZ or control antibodies, followed by incubation with PBMCs. The anti-CD20 antibody rituximab was used as a positive control, and the murine ACT-1 parental antibody for VDZ as a negative control. Spontaneous release was determined by incubation of both target (RPMI) and effector cells (PBMCs) individually in the presence of the maximum concentration

of antibodies. Cellular toxicity for both the CDC and ADCC assays was measured using the colorimetric CytoTox 96 assay (Promega Cat # TB163), which quantitatively measures lactate dehydrogenase, a stable cytosolic enzyme that is released upon cell lysis. Visible wavelength absorbance data were collected using a standard 96-well plate reader. Assays were performed per the manufacturer's instructions.

Isolation of peripheral blood mononuclear cells and CD4+ memory T lymphocytes. Human whole blood was collected in sodium heparin tubes and mixed 1:1 with Dulbecco's PBS without Ca^{2+} and Mg^{2+} and layered onto Ficoll-Hypaque solution (GE Healthcare, Cat # 17-1440-02). The mononuclear cells at the plasma-Ficoll interface were collected based on the manufacturer's instruction manual. CD4+ memory T lymphocytes were purified using immunomagnetic bead depletion based on the manufacturer's instructions (Miltenyi Biotech, Cat # 130-091-893).

Internalization of $\alpha_4\beta_7$ receptors on CD4+ memory T lymphocytes. Purified CD4+ memory T lymphocytes or PBMCs were incubated with VDZ–Alexa 647 or mouse IgG-Alexa 647 at 4 °C or 37 °C for 24 h. Internalization was inhibited with 0.45 M sucrose in control samples. Cells were then washed with an acidic solution (0.5 M NaCl and 0.2 M acetic acid) to remove the external fluorescence. The intensity of VDZ-Alexa

647 on CD4^{+} memory T lymphocytes was measured using flow cytometry.

Cytokine production and cellular activation. Blood was collected in heparin tubes, diluted 1:5 with assay buffer, distributed into endotoxin-free tubes, and stimulated with 5 μg/mL LPS or PHA in the presence or absence of 400 μg/mL VDZ. Production of the following cytokines was measured by enzyme-linked immunosorbent assay (ELISA): IFN-γ, TNF, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12 (p70), and IL-17 by Pierce Biotechnology (Thermo Fisher). Cells were examined for the presence of activation markers CD25 (BD Cat # 555434) and CD69 (BD Cat # 555560) using flow cytometry.

Time course for $\alpha_4\beta_7$ re-expression. Whole blood was incubated with 500 ng/mL VDZ at 37 °C for 24 h and extensively washed to remove unbound antibody. The samples were subsequently incubated at 37 °C with or without 3 μM monensin. $\alpha_4\beta_7$ expression was measured on days 1, 4, and 7 by staining with VDZ-Alexa 647 or mouse IgG-Alexa 647 and visualized by flow cytometry after RBC lysis.

$\alpha_4\beta_7$ binding ability. Whole blood was incubated with 1–1000 ng/mL VDZ at 37 °C for 24 h, washed thoroughly, and cultured. On day 1 and day 4, cells were stained with MAdCAM-1-mFc and Cy5 anti-mouse antibody. Mouse serum was added to absorb free Cy5 anti-mouse antibody before staining with anti-CD45RO and anti-CD4 and RBCs lysis. Staining was visualized by flow cytometry.

To assess IC_{50}, whole blood cells that had internalized and then re-expressed receptors were incubated with 3 μg/mL MAd-CAM-1-mFc and 4 mM $MnCl_2$ in the presence or absence of VDZ, washed, then stained with 10 μg/mL Cy5 anti-mouse. For lymphocyte samples, mouse serum was added to absorb free Cy5 anti-mouse antibody prior to staining with anti-CD45RO and anti-CD4. For RPMI8866 cells (0.2×10^6 cells/sample), 1 mM $MnCl_2$ was included in the assay buffer.

$\alpha_4\beta_7$ adhesion ability. Ninety-six-well ELISA plates were coated with 3 μg/mL MAdCAM-1-mFc at 4 °C overnight, washed, and blocked with 0.5% BSA in PBS at 37 °C for 1 h. Purified CD4^{+} memory T lymphocytes or RPMI8866 cells (0.2×10^6/well) were pre-incubated with VDZ and added to the plate, incubated at 37 °C for 1 h, and washed. The bound CD4^{+} memory T lymphocytes were detached using 5 mM EDTA/PBS and were analyzed by flow cytometry. The bound RPMI8866 cells were incubated with 20% Alamar Blue at 37 °C for 3 h and were read at 530 nm/590 nm on a fluorescence plate reader (Molecular Devices). The percentage of bound cells vs. antibody concentration was plotted using GraphPad Prism Version 4; IC_{50} values were determined using nonlinear regression curve fits.

Flow cytometry. Samples were analyzed with a FACSCalibur™ instrument and CellQuest™ Pro software (BD Biosciences). 2000 events were collected in the CD4^{+} memory lymphocyte gate, as determined by FSC vs. SSC and CD4^{+}/CD45RO^{+} staining. The percentage of $\alpha_4\beta_7$-positive cells was examined in the CD4^{+} memory lymphocyte gate. Alexa 647-labeled mIgG was used as a negative control.

All procedures utilizing human blood were collected under policies approved by an institutional review board. All samples were deidentified.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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