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RESEARCH PAPER

A novel multi-parameter assay to dissect the pharmacological effects of different modes of integrin αLβ2 inhibition in whole blood

Karl Welzenbach[1], Riccardo V Mancuso[2], Stephan Krähenbühl[2] and Gabriele Weitz-Schmidt[1,3*]

[1]*Novartis Pharma AG, Novartis Institutes of Biomedical Research, Basel, Switzerland* [2]*Division of Clinical Pharmacology and Toxicology, University Hospital, Basel, Switzerland*

Correspondence
Dr Gabriele Weitz-Schmidt, AlloCyte Pharmaceuticals AG, Hochbergerstrasse 60C, CH-4057 Basel, Switzerland.
E-mail: gabriele.weitz@allocyte-pharma.com
* AlloCyte Pharmaceuticals AG, Basel, Switzerland

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BACKGROUND AND PURPOSE
The integrin αLβ2 plays central roles in leukocyte adhesion and T cell activation, rendering αLβ2 an attractive therapeutic target. Compounds with different modes of αLβ2 inhibition are in development, currently. Consequently, there is a foreseeable need for bedside assays, which allow assessment of the different effects of diverse types of αLβ2 inhibitors in the peripheral blood of treated patients.

EXPERIMENTAL APPROACH
Here, we describe a flow cytometry-based technology that simultaneously quantitates αLβ2 conformational change upon inhibitor binding, αLβ2 expression and T cell activation at the single-cell level in human blood. Two classes of allosteric low MW inhibitors, designated α I and α/β I allosteric αLβ2 inhibitors, were investigated. The first application revealed intriguing inhibitor class-specific profiles.

KEY RESULTS
Half-maximal inhibition of T cell activation was associated with 80% epitope loss induced by α I allosteric inhibitors and with 40% epitope gain induced by α/β I allosteric inhibitors. This differential establishes that inhibitor-induced αLβ2 epitope changes do not directly predict the effect on T cell activation. Moreover, we show here for the first time that α/β I allosteric inhibitors, in contrast to α I allosteric inhibitors, provoked partial downmodulation of αLβ2, revealing a novel property of this inhibitor class.

CONCLUSIONS AND IMPLICATIONS
The multi-parameter whole blood αLβ2 assay described here may enable therapeutic monitoring of αLβ2 inhibitors in patients' blood. The assay dissects differential effect profiles of different classes of αLβ2 inhibitors.

Abbreviations
CsA, cyclosporin A; ICAM-1, intercellular adhesion molecule-1; I domain, inserted domain; LFA-1, lymphocyte function-associated antigen-1; mAbs, monoclonal antibodies; PE, phycoerythrin; PerCp, peridinin–chlorophyll–protein complex; TCR, T cell receptor

Tables of Links

TARGETS
CD28
Catalytic receptors
Integrin αLβ2 (LFA-1)

LIGANDS
CsA, cyclosporin A
ICAM-1, intercellular adhesion molecule-1
Pravastatin

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

The integrin αLβ2 [also known as lymphocyte function-associated antigen-1 (LFA-1) or CD11a/CD18] is an α/β heterodimeric receptor belonging to the integrin superfamily of cell surface adhesion molecules and is expressed exclusively on leukocytes. The main ligand of αLβ2 is intercellular adhesion molecule-1 (ICAM-1) (CD54), which is up-regulated on both leukocytes and endothelial cells by different pro-inflammatory stimuli (Tan, 2012).

The integrin αLβ2 has at least two major functions: firstly, as an adhesion molecule, αLβ2 mediates leukocyte extravasation out of the blood stream into inflamed tissue, and secondly, as a costimulatory receptor, αLβ2 is involved in lymphocyte activation and proliferation during immune responses (Tan, 2012). Moreover, there is recent evidence that αLβ2 engagement may play an important role in directing the differentiation of naïve T cells (Verma *et al.*, 2012; Verhagen and Wraith, 2014).

These central roles of αLβ2 in the immune system require tight control. Normally, αLβ2 resides on the cell surface in an inactive state. Intracellular inside–out signalling, typically induced by chemokine receptor or T cell receptor (TCR) engagement, is required to convert αLβ2 from its inactive state to an active, ligand binding state (Hogg *et al.*, 2011). The activity of αLβ2 can also be modulated extracellularly by divalent cations such as Mg^{2+} or Mn^{2+} (Li *et al.*, 2013). During the activation process, αLβ2 and its ligand binding domain [the so-called inserted (I) domain] undergo remarkable conformational changes, which can be detected via conformation-sensitive monoclonal antibodies (mAbs) (Weitz-Schmidt *et al.*, 2011). Upon ligand binding, αLβ2 transduces signals back into the cell (outside–in signalling), triggering subsequent cellular responses depending on the cell type involved (Hogg *et al.*, 2011).

αLβ2 has been recognized as an important therapeutic target in autoimmune diseases and transplant rejection (Ford and Larsen, 2009; Suchard *et al.*, 2010; Reisman *et al.*, 2011; Kitchens *et al.*, 2012; Sheppard *et al.*, 2014). To date, several classes of low MW αLβ2 inhibitors have been identified and characterized (Giblin and Lemieux, 2006; Zhong *et al.*, 2012; Kollmann *et al.*, 2014). Currently, the most advanced small molecule αLβ2 inhibitor is in phase III clinical trials as a topical treatment for dry eye syndrome (Sheppard *et al.*, 2014).

According to their mode of action, low MW αLβ2 inhibitors can be grouped into two major classes. The first class has been designated α I allosteric inhibitors. These inhibitors act via the αL chain of αLβ2 by binding to an allosteric site within the α I domain, thereby stabilizing the bent, low-affinity conformation of αLβ2. α I allosteric inhibitors are known to be highly selective for αLβ2 (Shimaoka and Springer, 2003a). In contrast, the second group of inhibitors, designated α/β I allosteric inhibitors, act via the β2 chain of αLβ2, thereby perturbing an important interface between the β2 subunit and the α subunit of αLβ2 (Shimaoka and Springer, 2003a). Intriguingly, upon binding of α/β I allosteric inhibitors, the α I domain (ligand binding domain) of the α chain of αLβ2 remains in an inactive state, while the rest of αLβ2 adapts an extended pseudo-liganded conformational state, as shown by the exposure of several activation epitopes. This conformation of αLβ2 has been shown to be 'semi-active' by mediating αLβ2/ICAM-1-dependent rolling adhesion but not firm adhesion of leukocytes (Salas *et al.*, 2004). α/β I allosteric inhibitors have been demonstrated to also target other β2 integrins such as αMβ2 and αXβ2 (Shimaoka and Springer, 2003a).

Given these fundamental differences in the modes of action of αLβ2 inhibitors, it is highly desirable to establish a methodology that allows the assessment, in patients' whole blood, of the effects of these inhibitors in terms of receptor interactions and immune cell functions. Here, we established a multi-parameter flow cytometry assay that simultaneously measures inhibitor interaction with αLβ2 by epitope change, αLβ2 surface expression and T cell activation at the single-cell level in human whole blood. The assay was validated using αLβ2 inhibitors of both the α I allosteric and α/β I allosteric classes.

Methods

Preparation of test compounds

The α I allosteric inhibitor LFA878 and the α/β I allosteric inhibitor XVA143 (also referred to as Roche compound #5) (Shimaoka *et al.*, 2003b) were dissolved in DMSO at 10 mM and serially pre-diluted in DMSO to avoid precipitation, before they were added to human whole blood from healthy volunteers or before performing final dilution steps in buffer. All samples contained identical DMSO concentrations. The final DMSO concentration in the samples was kept at ≤1% and did not alter the cell viability as indicated by light scattering properties of the samples.

Human blood αLβ2 epitope gain or epitope loss assays

The interaction of α I allosteric and α/β I allosteric αLβ2 inhibitors with αLβ2 were detected by measuring the binding of the conformation-sensitive anti-αL (CD11a) mAb R7.1 and anti-β2 (CD18) mAb MEM48 respectively. Blood samples from healthy volunteers were obtained from the Blood Donation Center at the University Hospital of Basel and the Novartis Medical Center, Basel. Blood was drawn according the institutional regulations accepted by the local Ethics Committee, which includes informed consent by all volunteers that the blood or blood constituents can be used for scientific purposes after anonymization. All blood samples were destroyed upon completion of analysis. Blood samples were heparinized with sodium heparin (B. Braun Medical AG, Switzerland; 100 $U \cdot mL^{-1}$). Blood aliquots (198 μL) were mixed with the compound solution or DMSO (2 μL) and incubated for 30–60 min at room temperature (RT). The compound-containing blood samples (90 μL) were transferred to 96-deep-well plates (2 mL, polypropylene, conical bottom, BD Biosciences, Switzerland). The FITC-conjugated mAb R7.1 or mAb MEM48 were added at final concentrations of 1–3 $\mu g \cdot mL^{-1}$. After 25 min staining at RT, erythrocytes were lysed with FACS lysing solution (BD Biosciences). Samples were centrifuged at 200× *g* for 5 min, and pellets were washed twice in PBS, pH 7.4 containing 0.5% BSA (Sigma-Aldrich, Switzerland) and resuspended in 150 μL of the same buffer. Bound antibodies were detected by flow cytometry (FACSCalibur, Becton & Dickinson, BD) gating the major leukocyte populations according to their light scatter properties. In each sample, 10 000 lymphocytes were counted. Mean fluorescence intensities were calculated using the CellQuest software (BD). In some experiments, mAb MEM48 binding to human $CD3^+$ lymphocytes was quantified by using FITC-conjugated mAb MEM48 and peridinin–chlorophyll–protein complex-conjugated (PerCp) anti-CD3. IC_{50} and EC_{50} values were determined by using the dose response curve fitting tool of ORIGIN V 7.0 (OriginLab Corporation).

Mg^{2+} effect on T cell activation in human blood

The anti-CD3 mAb OKT3 (purified in-house from hybridoma supernatants, if not otherwise indicated) or an isotype antibody control (IgG2a) in PBS, pH 8, was adsorbed onto 96-well microtiter plates (Maxisorb, Nunc, USA) (0.01–30 $\mu g \cdot mL^{-1}$, 100 μL per well) at 4°C, overnight. The plates were washed twice and blocked with PBS, pH 8, containing 0.5% BSA for 1 h at 37°C. After this incubation and washing steps, PBS, pH 7.4, with or without 4 mM $MgCl_2$ (if not otherwise indicated) was added to each well (50 μL per well) followed by the transfer of heparinized human blood (50 μL per well). After 22 h incubation in a cell culture incubator (37°C and 5% CO_2), CD69 expression on human $CD2^+CD4^+$ lymphocytes was analysed in three individually activated blood samples (referred to as technical replicates) by flow cytometry using phycoerythrin-conjugated (PE) anti-CD69 mAb, FITC-conjugated anti-CD2 mAb and PerCp-conjugated anti-CD4 mAb. CD69 expression on $CD3^+$ lymphocytes was analysed using PE-conjugated anti-CD69 mAb and PerCp-conjugated anti-CD3 mAb.

Simultaneous assessment of αLβ2 expression, αLβ2 inhibitor-induced epitope changes and T cell activation in human blood

The anti-CD3 mAb OKT3 (purified in-house from hybridoma supernatants) in PBS, pH 8 (1 $\mu g \cdot mL^{-1}$) – or alternatively a combination of anti-CD3 mAb OKT3 (0.1 $\mu g \cdot mL^{-1}$) and anti-CD28 mAb (clone 15E8, 1 $\mu g \cdot mL^{-1}$) in PBS, pH 8 – were immobilized on 96-well microtiter plates at 4°C, overnight. The plates were washed and blocked as described above. Heparinized human blood (1 mL) was added to wells of 2 mL 96-deep-well plates (polypropylene, conical bottom, BD Biosciences) and supplemented with test compounds (2 μL) or DMSO (2 μL). After an incubation step of 1 h at room temperature, the blood samples were transferred to the anti-CD3 or anti-CD3/anti-CD28 coated microtiter plates (50 μL per well) containing 4 mM $MgCl_2$ in PBS, pH 7.4 (50 μL per well) or PBS alone (50 μL per well) respectively. The plates were incubated for 22 h at 37°C. Following this incubation step, four individually activated blood samples were combined and 200 μL of the pooled blood samples transferred to 2 mL 96-deep-well plates. Leukocytes in the blood cultures were stained simultaneously with FITC-conjugated mAb R7.1 (1.5 μL) or FITC-conjugated mAb MEM48 (1 $\mu g \cdot mL^{-1}$), PE-conjugated anti-CD69 (2.5 μL), PerCp-conjugated anti-CD3 mAb (1.3 μL) and ALEXA Fluor 647-conjugated anti-αL (CD11a) mAb TS2/4 (1 μL) for 20 min at RT. Erythrocytes were lysed with FACS lysing solution (1.4 mL). After 10 min lysis, the plates were centrifuged (250× *g*) at RT for 6–7 min. Samples were washed once with PBS containing 0.5% BSA, and bound mAbs were analysed by flow cytometry. For all calculations, the compound concentration added to undiluted whole blood samples was used. Six to seven different concentrations per compound were tested to generate concentration response curves.

Data analysis

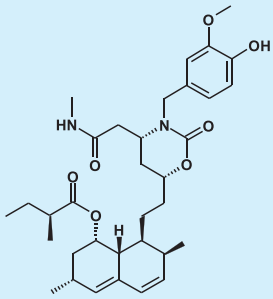
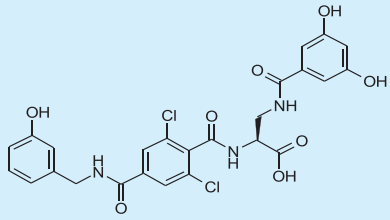
All values are expressed as the mean ± SD of three determinations, unless otherwise stated. For statistical analysis the Mann-Whitney test algorithm (GraphPad Prism V6.04), paired t-test (GraphPad Prism V6.04) and one-way ANOVA and Tukey-Kramer multiple comparison test were used as indicated. $P < 0.05$ was considered statistically significant. Data analyses were conducted using ORIGIN V7.0 (OriginLab Corporation).

Materials

FITC-conjugated anti-human αL (CD11a) mAb R7.1 was obtained from Biosource, Camarillo, CA, USA. FITC-conjugated anti-human β2 (CD18) mAb IB4 was purchased from Ancell Corp., USA. FITC-conjugated anti-human β2 (CD18) mAb MEM48 or FITC-conjugated anti-human CD2 mAb (clone MEM65) and all isotype controls (IgG1 and IgG2a) were obtained from Immunotools, Germany. PE-labelled anti-human CD69 mAb (clone L78) and PerCp-conjugated anti-human CD3 mAb (clone UCHT1) were purchased from BD Biosciences. Anti-human CD28 mAb (clone 15E8) was a kind gift of Prof. L. Aarden, Sanguin Inc., Netherlands. Hybridoma cell lines producing anti-human αL (CD11a) mAb TS2/4.1.1 (TS2/4) or anti-human CD3 mAb OKT3 were obtained from the American Type Culture Collection (USA).

Table 1

Chemical structure of αLβ inhibitors LFA878 and XVA143, mode of action and activity in αLβ2-dependent binding assays

Parameter	LFA878	XVA143
Chemical structure		
Mode of action	α I allosteric	α/β I allosteric
αL/β2/ICAM-1: IC_{50} (μM)	0.050 ± 0.01*	0.020 ± 0.008*
HUT78/ICAM-1: IC_{50} (μM)	0.280 ± 0.15*	0.005 ± 0.004*

*Values were taken from Welzenbach *et al.*, 2002 and Weitz-Schmidt *et al.*, 2004. Results were generated in a cell-free ELISA-type binding assay measuring the interaction of immobilized αLβ2 with recombinant ICAM-1 (αLβ2/ICAM-1) and in a cell-based assay quantifying the adhesion of HUT78 cells to immobilized ICAM-1 (HUT78/ICAM-1).

Production and purification of mAbs were conducted by standard protocols. TS2/4 was conjugated with ALEXA Fluor 647 using an antibody labelling kit (Life Technologies, Switzerland) and following manufacturer's instructions. LEAF™ purified anti-human CD3 mAb OKT3 was purchased from Biolegend, San Diego, CA. Anti-human IgG was purchased from Sigma-Aldrich. The α I allosteric inhibitor LFA878 and the α/ß I allosteric inhibitor XVA143 were synthesized and supplied by Novartis, Switzerland. Pravastatin was purchased from Sigma-Aldrich. Cyclosporin A (CsA) was supplied by Novartis.

Results

αLβ2 inhibitors investigated

The well-established allosteric αLβ2 inhibitors LFA878 and XVA143 were selected for the present study as representatives for the α I allosteric and α/β I allosteric inhibitor classes respectively. LFA878 is a statin-derived and XVA143 a peptidomimetic αLβ2 inhibitor (Welzenbach *et al.*, 2002; Weitz-Schmidt *et al.*, 2004). The chemical structures of these αLβ2 inhibitors and their biological activity in cell-free and cell-based αLβ2-dependent binding assays are shown in Table 1.

Conformational change is a sensitive marker of inhibitor binding to αLβ2 in whole blood

The development of the whole blood flow cytometry assay described here required the stepwise optimization of several read-outs. Firstly, we developed a method to measure the interaction of α I or α/β I allosteric inhibitors with αLβ2. This method was based on the prior observation that binding of allosteric inhibitors to αLβ2 induces epitope changes, detectable by conformation-sensitive mAbs (Welzenbach *et al.*, 2002; Woska *et al.*, 2003; Weitz-Schmidt *et al.*, 2004).

For the measurement of α I allosteric inhibitor binding to αLβ2, the anti-αL chain mAb R7.1 was selected. mAb R7.1 binds to a region involving the C-terminal linker of the αLβ2 I domain located on the αL chain and the β propeller located on the β2 chain of αLβ2 (Weitz-Schmidt *et al.*, 2011). All α I allosteric inhibitors (of diverse chemical scaffolds) investigated to date with this antibody, that is, BIRT377, lovastatin, LFA703, LFA451 and LFA878, consistently induced R7.1 epitope loss (Welzenbach *et al.*, 2002; Shimaoka *et al.*, 2003b; Weitz-Schmidt *et al.*, 2004). The sensitivity of the R7.1 epitope to α I allosteric inhibition can be considered to be established for the entire class of inhibitors. In agreement with the earlier studies we found that LFA878 reduced the binding of mAb R7.1 to αLβ2 in a concentration-dependent manner in whole blood cultures (Figure 1A). In comparison, XVA143 did not affect the R7.1 epitope, indicating the specificity of the read-out for the compound's mode of action (Figure 1A).

For the quantification of α/β I allosteric inhibitor interactions with αLβ2 we identified the anti-β2 chain mAbs MEM48 and MEM148 as the best suited antibodies (see Supporting Information Table S1 for all mAbs investigated). mAb MEM48 binds equally well to resting and activated αLβ2 (Lu *et al.*, 2001) whereas MEM148 is known to bind to activated but not resting αLβ2 (Tang *et al.*, 2005). To validate the MEM48 epitope as a 'reporter' epitope for the α/β I allosteric class of αLβ2 inhibitors, we investigated the effect of three different α/β I allosteric inhibitors. We were able demonstrate that all three inhibitors assessed induced a MEM48 epitope gain by > 2 fold in whole blood. The results obtained for the α/β I allosteric inhibitor XVA143 are shown here. XVA143 increased the binding of mAb

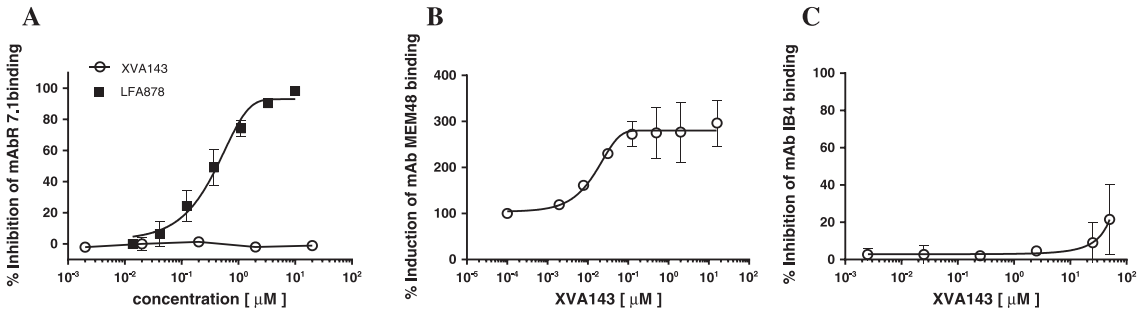


Figure 1

Effect of αLβ2 inhibitors LFA878 and XVA143 on mAbs R7.1, MEM48 and IB4 binding to leukocytes in human whole blood. The binding of mAb R7.1 (A) or mAb MEM48 (B) and mAb IB4 (C) to blood lymphocyte, monocyte and granulocyte subsets in presence of indicated inhibitor concentrations was individually quantified by flow cytometry as described. Each point represents the mean value ± SD of these measurements. Representative experiments out of three independent experiments are shown.

MEM48 to αLβ2 by ≥ 2.5 fold (Figure 1B) whereas the α I allosteric inhibitor LFA878 did not modulate the β2 chain specific antibody (data not shown). We also attempted to measure the α/β I allosteric inhibitor interaction by monitoring the epitope of the anti-β2 chain mAb IB4. In the presence of cations, that is, Mg^{2+} and Mn^{2+}, the expression of the IB4 epitope had been shown to be attenuated by α/β I allosteric inhibitors (Welzenbach *et al.*, 2002). However, in blood cultures the binding of mAb IB4 to αLβ2 was not altered by XVA143 (Figure 1C), even if the blood was supplemented with Mn^{2+} (data not shown). This result indicated that mAb IB4 was not suitable to assess the interaction of α/β I allosteric inhibitors with αLβ2 in human whole blood. Taken together, these results establish that conformational changes reported by R7.1 and MEM48 reliably detect interactions of α I allosteric or α/β allosteric inhibitors with αLβ2 respectively. As the respective epitope changes were observed consistently with different inhibitors of either class, it can be assumed that these changes are inhibitor class-specific rather than compound-specific.

Detection of αLβ2-dependent T cell activation in human blood cultures

In a second step, we developed a procedure permitting the detection of αLβ2 -dependent T cell activation by flow cytometry in whole blood. Regarding the selection of stimuli, we focused on activating principles known to trigger αLβ2-dependent T cell activation (Weitz-Schmidt *et al.*, 2001; Kuschei *et al.*, 2011). T cells in human whole blood were exposed to immobilized anti-CD3 mAb OKT3 (aCD3), triggering signal 1 via the TCR. TCR engagement also activates αLβ2 (via the inside–out pathway), which upon binding to its ligand ICAM-1 (expressed by neighbouring leukocytes) provide costimulatory signal 2 to the T cells (Leitner *et al.*, 2010). T cell activation was quantified in the present study by the up-regulation of the CD69 receptor on CD^{3+} T cells. CD69 is an established T cell activation marker and has been used as a surrogate for T cell activation and proliferation in several previous studies (e.g. González-Amaro *et al.*, 2013). However, under the initially used conditions, the blood samples tended to coagulate; immobilized aCD3 induced the internalization of the CD3 antigen (preventing accurate measurement of the $CD3^+$ T cell population), and CD69 up-regulation was only marginal and barely reproducible (data not shown). In consequence, we optimized assay conditions by diluting the blood samples 1:1 in PBS and investigating CD69 expression of $CD2^+CD4^+$ T helper cells (a subpopulation of T cells) instead of $CD3^+$ T cells. Furthermore, to enhance the CD69 signal, aCD3-stimulated blood was supplemented with $MgCl_2$. We hypothesized that Mg^{2+} would strengthen αLβ2/ICAM-1-mediated cell–cell interactions, thereby boosting αLβ2-mediated costimulatory signalling. Indeed, we found that the addition of Mg^{2+} significantly augmented CD69 expression on $CD2^+CD4^+$ T cells. Further, the degree of CD69 expression was dependent on both the concentration of immobilized aCD3 and the concentration of Mg^{2+} (Figure 2A and B). In contrast, an immobilized non-related antibody control failed to activate T cells in the presence and absence of $MgCl_2$ (Figure 2A). The combination of 1 μg·mL^{-1} immobilized aCD3 OKT3 and 2 mM $MgCl_2$ was found to be optimal for T cell activation in 1:1 diluted blood cultures as assessed by CD69 expression. Under these conditions, the CD3 antigen was still detectable and suitable to reliably quantify the $CD3^+$ T cell subpopulation. On average, a 9.9 ± 3.9% of the $CD3^+$ cells expressed CD69 upon aCD3/Mg^{2+} stimulation as compared with 1.2 ± 0.7% in the absence of stimulus (Figure 3C). The relatively low proportion of $CD3^+$ cells that can be activated in whole blood, as compared with isolated peripheral blood mononuclear cells in medium, is in line with earlier observations (Hoffmeister *et al.*, 2003). Both LFA878 and XVA143 at 10 μM potently inhibited aCD3/$MgCl_2$-induced CD69 up-regulation on $CD3^+$ T cells (Figure 3). This finding provided the first evidence that CD69 up-regulation is sensitive to αLβ2 inhibition under the conditions applied.

Combined assessment of αLβ2 conformational change, αLβ2 expression and αLβ2-mediated T cell activation in human blood cultures in the presence or absence of inhibitors

The methods established for the measurement of compound interactions with αLβ2 were combined with the method for the detection of αLβ2-dependent CD69 up-regulation. Moreover, as a third read-out, the quantification

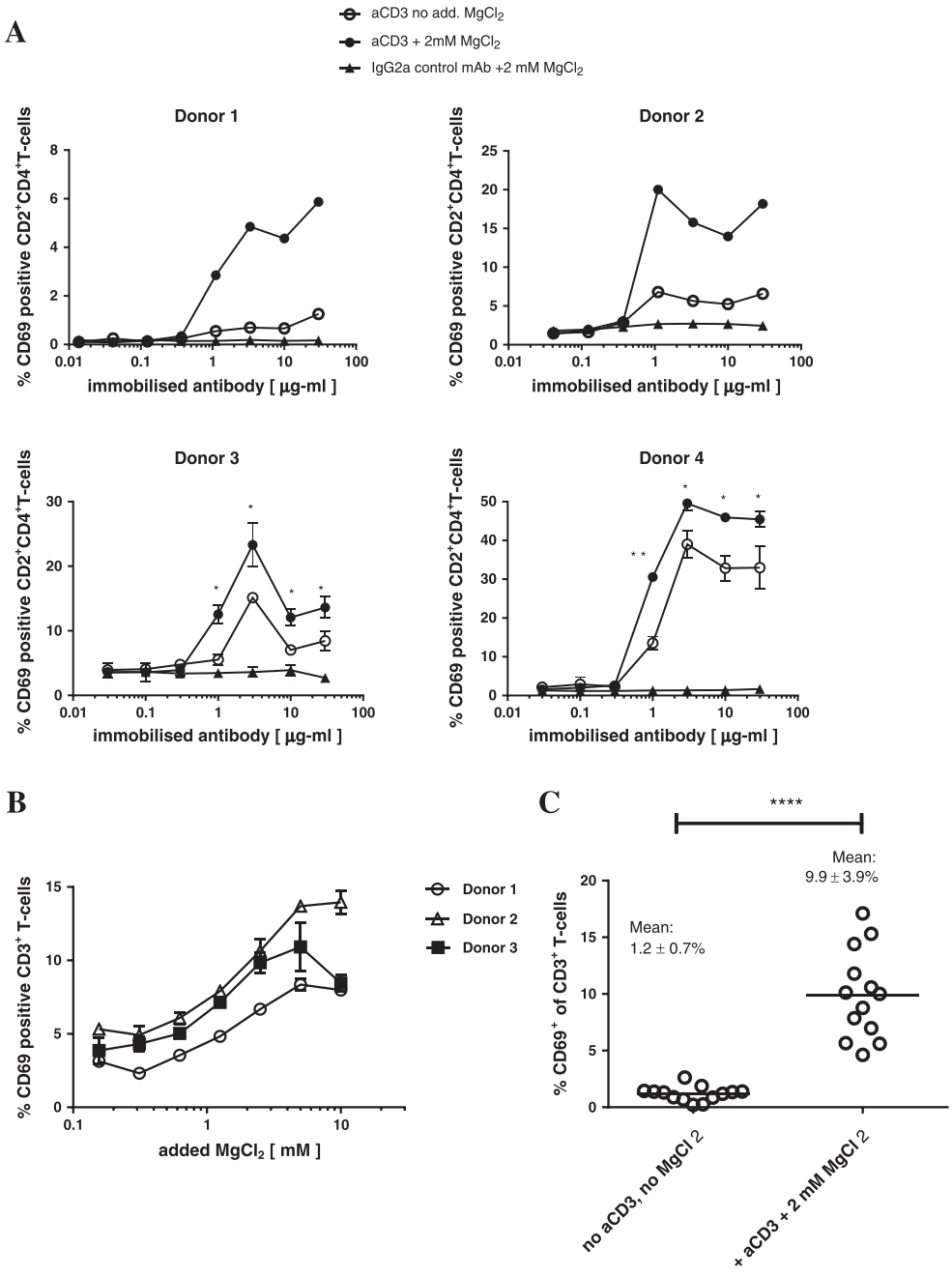


Figure 2

Mg^{2+} augments aCD3-induced CD69 up-regulation on T cells in human blood cultures. (A) Blood cultures with or without added $MgCl_2$ were stimulated with increasing concentrations of immobilized anti-CD3 mAb OKT3 (aCD3) (a commercially available mAb was used in case of donors 3 and 4) or a non-related antibody control (IgG2a) for 22 h. CD69 up-regulation on blood $CD2^+CD4^+$ T cells was quantified by flow cytometry as described. Single values generated from pooled technical triplicates are shown for donors 1 and 2 while mean values ± SD of three technical triplicates are shown for donors 3 and 4. * $P < 0.05$, ** $P < 0.01$; significant difference between groups aCD3 and aCD3 with added $MgCl_2$ from donors 3 and 4; paired *t*-test. (B) Blood cultures supplemented with $MgCl_2$ at indicated concentrations were stimulated with immobilized aCD3 (OKT3, 1 $\mu g \cdot mL^{-1}$; a commercially available mAb was used in case of donor 3) for 22 h. CD69 up-regulation on $CD3^+$ T cells was quantified by flow cytometry as described. Mean values ± SD of three individually activated samples of three donors are shown. (C) Frequency of $CD69^+CD3^+$ T cells in blood cultures after overnight incubation in presence or absence of immobilized aCD3 (OKT3, 1 $\mu g \cdot mL^{-1}$) and 2 mM $MgCl_2$. Each circle represents the mean percentage of $CD69^+CD3^+$ T cells of three technical replicates. Data shown are means± SD from seven independent experiments using a total of 14 healthy blood donors. *$P < 0.05$, significant difference between groups aCD3 and aCD3 with added $MgCl_2$; Mann–Whitney test.

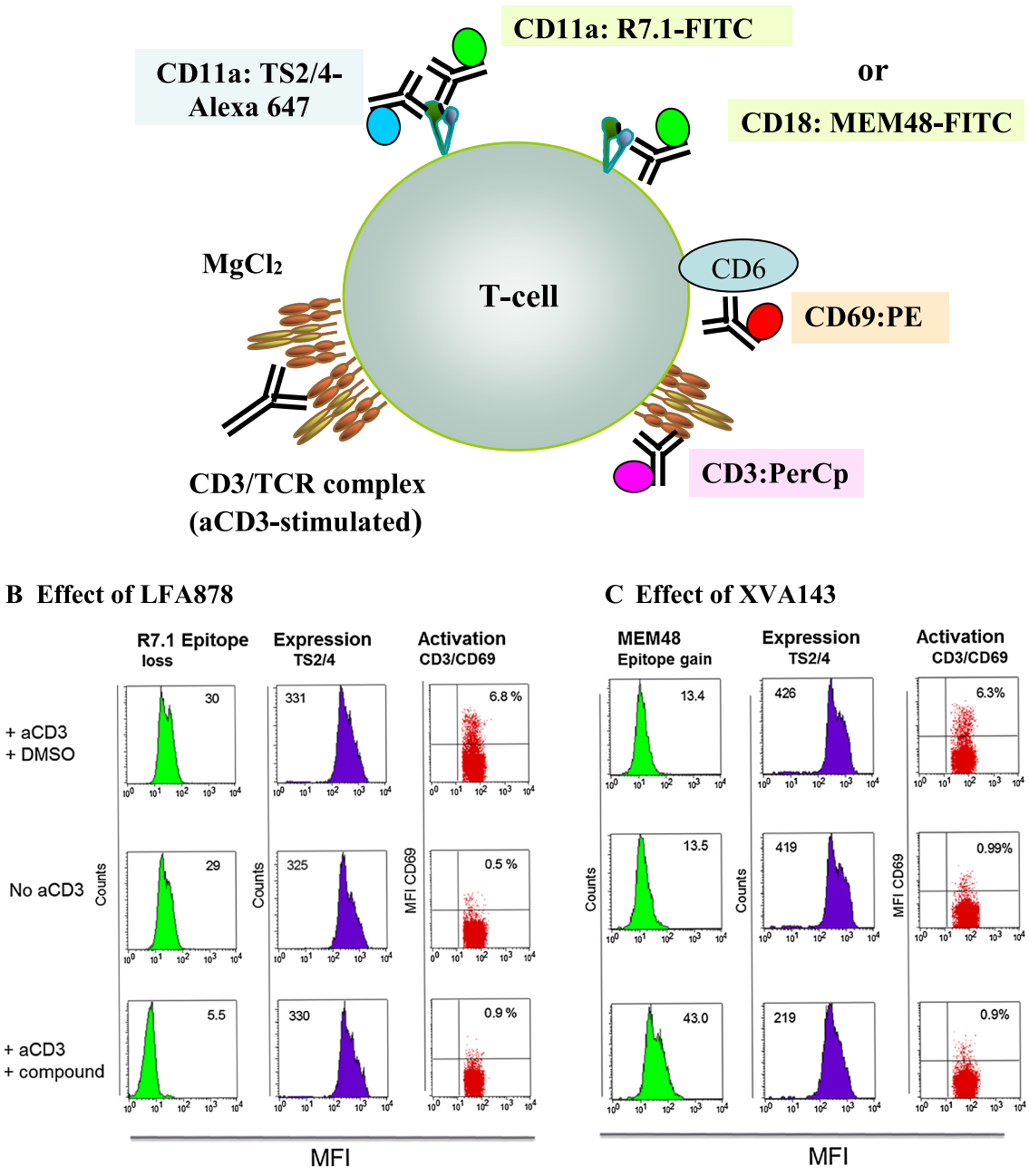


Figure 3

Multi-parameter human whole blood flow cytometry assay. (A) Schematic drawing of assay concept: the assay quantifies simultaneously αLβ2 epitope loss (detected by FITC-labelled mAb R7.1) and epitope gain (detected by FITC-labelled mAb MEM48) induced by small molecule α I or α/β I allosteric inhibitors, respectively, αLβ2 surface expression (detected by Alexa 647-labelled mAb TS2/4) and CD69 expression (detected by PE-labelled anti-CD69 mAb) on T cells (detected by PerCp-labelled anti-CD3 mAb) in blood cultures activated via immobilized anti-CD3 mAb OKT3 (aCD3) plus $MgCl_2$ by flow cytometry. (B) Simultaneous assessment of αLβ2 epitope change, αLβ2 expression and T cell activation in presence of LFA878 (10 μM) and (C) XVA143 (2 μM) and solvent control DMSO (0.2%) in blood cultures as described. Numbers inserted into the histograms indicate either median fluorescence intensities (MFIs) or percentage of $CD69^+CD3^+$ T cells. Results from one experiment out of more than three independent experiments are shown.

of αLβ2 expression was introduced. αLβ2 surface expression was investigated by quantifying the binding of mAb TS2/4 to αLβ2 expressed on $CD3^+$ T cells. This mAb detects the intact α/β heterodimer of αLβ2 and has been previously shown to bind to a region of αLβ2 unaffected by the presence of α I and α/β I allosteric inhibitors (Welzenbach

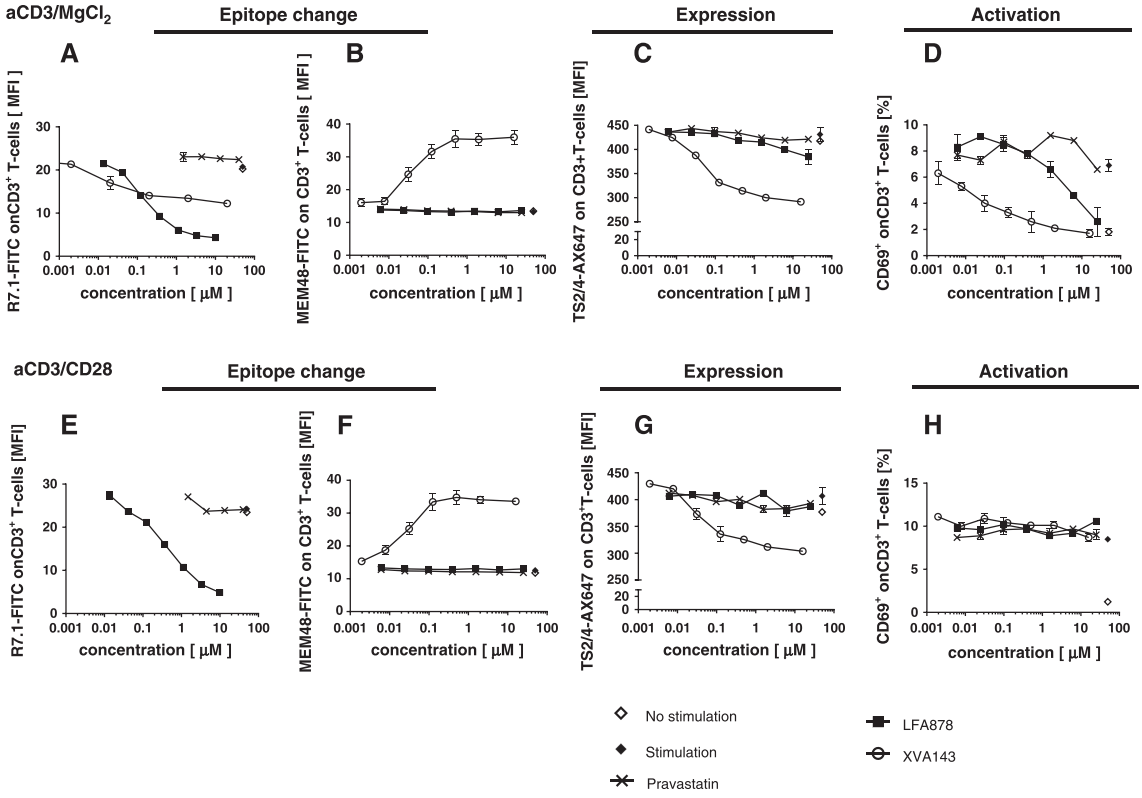


Figure 4

Simultaneous assessment of compound-induced αLβ2 epitope, αLβ2 expression and CD69 up-regulation in presence of LFA878, XVA143 or pravastatin in activated human blood. Human whole blood was pre-incubated for 1 h with the compounds at indicated concentrations. After incubation, the blood samples were diluted 1:1 with PBS and activated via aCD3/$MgCl_2$ (A–D) or aCD3/aCD28 (without $MgCl_2$) (E–H), respectively, for 22 h. The binding of anti-αLβ2 mAbs R7.1 or MEM48 (epitope change) and TS2/4 (expression) as well as the binding of anti-CD69 mAb (activation) to $CD3^+$ T cells was quantified by flow cytometry as described under the Methods section. Four individually activated blood samples per donor were pooled. From this pool, two samples were independently stained and measured. Data shown are mean values ± SD of these two samples. SD is shown to indicate range of data. Stimulation: activated blood in presence of solvent control (0.2% DMSO); no stimulation: resting blood in presence of solvent control (0.2% DMSO). One representative experiment out of more than three independent experiments is shown.

et al., 2002). The principle of the final αLβ2 multi-parameter flow cytometry blood test used in this study is illustrated in Figure 3A.

The assay was utilized to simultaneously assess the effect of LFA878 and XVA143 on αLβ2 conformation, αLβ2 expression and aCD3/$MgCl_2$-induced CD69 expression. For the first time, we demonstrated at the single-cell level that LFA878 affects the R7.1 but not the MEM48 epitope, marginally reduced αLβ2 expression at high concentrations, and inhibited CD69 expression on T cells with an IC_{50} value of 2.6 ± 1.7 µM (Figure 4A–D, Table 2). In contrast, the 3-hydroxy-3-methylglutaryl-coenzyme-A reductase inhibitor pravastatin did not modify any of the parameters assessed (Figure 4A–D, Table 2). This cholesterol-lowering drug is structurally related to the statin-derived αLβ2 inhibitor LFA878 but does not inhibit αLβ2 (Weitz-Schmidt *et al.*, 2001).

On the other hand, binding of XVA143 to αLβ2 led to an increased exposure of the MEM48 epitope and surprisingly at the same time partially reduced the binding of both, mAb TS2/4 and mAb R7.1, indicating an effect of the compound on αLβ2 surface expression (Figure 4A and C). Moreover, XVA143 potently inhibited CD69 expression with an IC_{50} value of 0.049 ± 0.016 µM (Figure 4D, Table 2).

The XVA143-induced downmodulation of αLβ2 measured in the multi-parameter assay after 22 h of exposure was highly reproducible (Figure 5) and was confirmed with two other inhibitors of the α/β I allosteric class (data not shown). However, the effect did not become evident in experiments involving XVA143 exposure times of less than 1 h, for example, as it was the case in the αLβ2 MEM48 epitope alteration assay shown in Figure 1A. This indicates that the reduction of αLβ2 surface expression by XVA143 is dependent on mechanisms requiring exposure times longer than 1 h. The exact nature of these mechanisms remains to be elucidated.

Next, we investigated the effect of the compounds on $CD3^+$ T cells in blood cultures activated with aCD3/aCD28. CD28 is a co-receptor on T cells providing costimulatory signalling independent from the αLβ2 pathway (Leitner *et al.*, 2010; Kuschei *et al.*, 2011). As expected, XVA143 and LFA878 failed to block aCD3/aCD28-induced CD69 up-regulation (Figure 4H, Table 2). The effects of the αLβ2 inhibitors on R7.1 and MEM48 epitope expression as well as the effect on αLβ2 surface expression were similar in

Table 2

Activity profile of LFA878, XVA143, pravastatin and CsA in activated human blood

Compounds	Epitope change		αLβ2 expression	Suppression of CD69 up-regulation	
	aCD3/Mg^{2+} or aCD3/aC28			aCD3/Mg^{2+}	aCD3/aCD28
	mAb R7.1 IC_{50} (μM)	mAb MEM48 EC_{50} (μM)	mAbTS2/4 IC_{50} (μM)	aCD69 mAb IC_{50} (μM)	aCD69 mAb IC_{50} (μM)
LFA878	0.5 ± 0.2*	>10#	>10#	2.6 ± 1.7*	>10#
XVA143	>50#	0.031± 0.007*	30 ± 4% ↓ at 10 μM#	0.049 ± 0.016*	>10#
Pravastatin	>40#	>40#	>10#	>40#	>40#
CsA	>10#	>10#	>10#	0.8 ± 0.26*	35% ↓ at 10 μM#

All data were generated in aCD3/Mg^{2+} or aCD3/aC28 activated human blood using the multi-parameter flow cytometry assay as described.
*Mean value ± SD of more than three independent experiments.
#Value represents the highest concentration tested, representative result of more than three independent experiments shown.

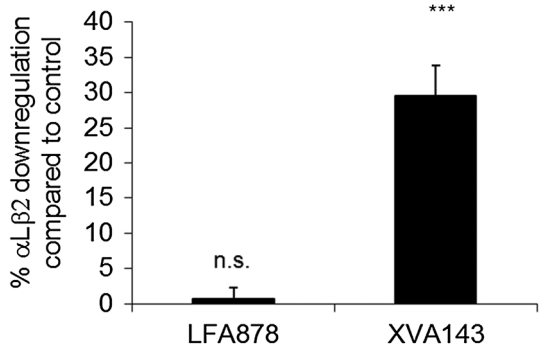


Figure 5

Effect of inhibitors on αLβ2 surface expression. Human whole blood was supplemented with LFA878 (10 μM) or XVA143 (10 μM), pre-incubated for 1 h, diluted 1:1 with PBS and then stimulated for 22 h with aCD3/$MgCl_2$. Integrin αLβ2 expression on $CD3^+$ T cells was quantified by assessing the binding of mAb TS2/4. Data shown are mean values ± SD from six (LFA878) and eight (XVA143) independent experiments, that is, six and eight blood donors respectively. *** $P < 0.001$, significantly different from control; one-way ANOVA with the Tukey–Kramer multiple comparison test.; n.s., not significantly different from control.

aCD3/aCD28 and aCD3/Mg^{2+} stimulated blood (Figure 4). This demonstrates that the level of epitope alteration and the effect on αLβ2 surface expression are independent of the stimulus utilized to activate the blood samples. It needs to be noted here that under conditions of aCD3/aCD28 activation, levels of R7.1 epitope change was measured using α I allosteric inhibitors only (Figure 4E).

Correlation of inhibitor-induced αLβ2 epitope changes with CD69 up-regulation in blood cultures

When correlating compound-induced epitope changes with aCD3/$MgCl_2$-induced CD69 up-regulation at the single-cell level, another interesting differential feature of α I allosteric and α/β I allosteric inhibitors became evident. More than 80% epitope loss was associated with half-maximal inhibition of CD69 expression in presence of LFA878 (Figure 6). In contrast, less than 40% epitope gain induced by XVA143 was associated with half-maximal inhibition of CD69 expression (Figure 6). The ratio of half-maximal inhibition of CD69 expression to half-maximal epitope change was calculated to be 5.1 for LFA878 and 1.1 for XVA143 respectively (Table 2). These differential epitope change /T cell response relationships were also observed when inhibitor-induced αLβ2 epitope changes were directly correlated to aCD3-induced T cell proliferation (rather than $MgCl_2$-induced CD69 up-regulation) in whole blood (Supporting Information Fig. S6).

Effect of CsA in the multi-parameter assay

Although the development of the present multi-parameter assay was tailored to characterize αLβ2 inhibitors, we were interested to assess in the test system, therapeutically used immunosuppressive drugs with targets different from αLβ2. Thus, we compared the profile of the αLβ2 inhibitors with the profile of the immunosuppressant cyclosporin A (CsA). CsA is a calcineurin inhibitor modulating TCR-mediated activation of T cells (signal 1), as reviewed recently (Azzi *et al.*, 2013). CsA did not affect αLβ2 conformation as monitored by mAb R7.1 and mAb MEM48 binding and did not impede αLβ2 surface expression as assessed by mAb TS2/4 binding (Table 2). As expected from its mechanism of action, CsA inhibited aCD3/$MgCl_2$-stimulated T cell activation with an IC_{50} value of 0.8 ± 0.26 μM (Table 2). Moreover, as observed for αLβ2 inhibitors, T cell activation was largely unaffected by CsA in blood cultures stimulated by aCD3/aCD28 (Table 2). These data establish that the assay methodology can be extended to immunomodulatory modalities of different mechanisms of action, such as CsA.

Discussion

With several classes of αLβ2 inhibitors in development, there is a clear need for methodologies that allow the correlation of compound/αLβ2 interactions with therapeutically relevant, downstream biological effects. Preferably, this assessment should be performed in blood taken from treated patients, enabling *ex vivo* bedside monitoring.

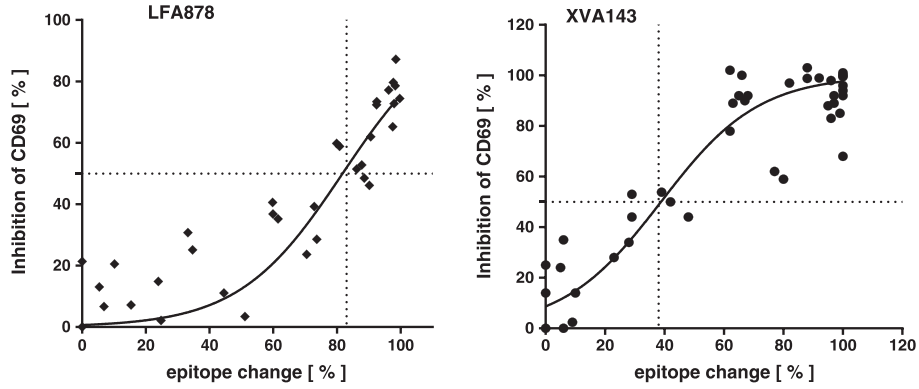


Figure 6

Correlation of αLβ2 receptor occupancy with T cell activation. Following treatment with LFA878 or XVA143, αLβ2 occupancy (as measured by mAb R7.1 or MEM48 epitope alteration) was correlated with aCD3/$MgCl_2$-induced CD69 up-regulation on T cells in human blood. Raw data from three independent experiments (three donors) are shown.

At the core of the methodology described here lies the realization that inhibitor-induced epitope changes can be used to indirectly detect integrin/inhibitor interactions in whole blood, with the nature of epitope alteration depending on the molecular mode of action. Epitope monitoring has been applied in the past to analyse the binding of low MW antagonists to the integrin receptor αIIb/β3 (GPIIb/IIIa) *in vitro* and *ex vivo* in blood samples from treated patients (Quinn *et al.*, 2000). Furthermore, it has been applied to α I allosteric αLβ2 inhibitors (but not α/β I allosteric inhibitors) *in vitro* and in preclinical animal studies (Woska *et al.*, 2003; Weitz-Schmidt *et al.*, 2004). These epitope alteration assays have in common that they quantify epitopes, which are shielded upon compound binding to the integrin, that is, which are lost.

We used the experimental knowledge gained from the earlier studies to establish *in vitro* assays of human whole blood which allowed the detection of the interactions of different inhibitor classes with the integrin αLβ2 and, at the same time, to visualize their mode of action by monitoring inhibitor-specific αLβ2 conformational changes. For the first time, we introduced compound-induced epitope *gain,* rather than epitope *loss*, as a method to assess inhibitor interaction. Moreover, the methodology is the first to apply the principle of epitope monitoring to inhibitors, which block the same integrin by different modes of action. Our study establishes the correlation between α I allosteric and α/β I allosteric inhibitor concentrations and R7.1 and MEM48 epitope changes respectively. However, the study does not measure inhibitor binding to integrin αLβ2, that is, receptor occupancy, directly and does not correlate this direct binding with respective epitope changes. This will be addressed by future investigations.

Unexpectedly, the extension of the whole blood epitope monitoring assays to the assessment of the effect of αLβ2 inhibition on immune cell function at the single-cell level posed significant methodological hurdles. These difficulties were resolved by increasing the Mg^{2+} concentration of the blood cultures beyond the normal physiological levels of 0.5 to 1 mM. The addition of Mg^{2+} finally enabled us to reproducibly quantify αLβ2-dependent T cell activation (as assessed by CD69 up-regulation) on T cells in blood. It also allowed to complete the αLβ2 multi-parameter flow cytometry assay by combining the epitope change measurements with T cell activation and αLβ2 surface expression measurements. Our observations in blood cultures further substantiate the physiological and clinical relevance of the regulatory function of Mg^{2+} within the immune system. Mg^{2+} is not only crucial for the maintenance of active conformations of immune receptors such as the integrin αLβ2 but it is also essential in the regulation of lipid-derived and phosphoinositide-derived second messengers as well as various transporters and ion channels involved in the immune response (Brandao *et al.*, 2013). Interestingly, tissue injury has been reported to result in a local increase in extracellular Mg^{2+} most likely due to leakage from damaged tissue (intracellular Mg^{2+} concentrations vary from 15 to 25 mM) (Grzesiak and Pierschbacher, 1995). Based on this finding, it has been postulated that increased Mg^{2+} levels serve to stimulate leukocyte migration into wounds (Stewart *et al.*, 1996). Here, we provide evidence in a physiological relevant environment that increased Mg^{2+} may also facilitate T cell activation driven by αLβ2-mediated co-stimulation at sites of inflammation.

The first application of the newly established whole blood flow cytometry methodology yielded interesting and surprising results. One such finding related to the differential effect of the inhibitors on the surface expression of αLβ2 itself. αLβ2 inhibitors with an α/β I allosteric mode of action reduced αLβ2 surface expression, in contrast to α I allosteric inhibitors. Reduced expression of αLβ2 has not been reported before in the presence of low MW inhibitors. In contrast, down-regulation of αLβ2 is a well-known phenomenon with anti-α Lβ2 antibodies (Gottlieb *et al.*, 2002; Clarke *et al.*, 2004; Coffey *et al.*, 2004). For instance, the anti-human αLβ2 mAb efalizumab (originally approved for the indication of plaque psoriasis but withdrawn from markets in 2009 following four cases of progressive multifocal leukoencephalopathy; Seminara and Gelfand, 2010) has been shown to induce almost complete internalization of αLβ2 in treated patients, thought to be due to αLβ2 cross-linking by the antibody (Gottlieb *et al.*, 2002; Coffey *et al.*, 2004). The α/β I allosteric inhibitor-induced downmodulation of αLβ2 was partial only and may be caused by other mechanisms. It is intriguing to

speculate that the extended, semi-active conformation stabilized by α/β I allosteric inhibitors (but not by α I allosteric αLβ2 inhibitors) may influence the dynamics of αLβ2 receptor recycling to the cell surface. This hypothesis is supported by the recent finding that an extended conformation of αLβ2, sharing several properties with the α/β I allosteric inhibitor-induced conformation of the integrin, is internalized and that the conformational state of LFA-1 directly affects LFA-1 recycling and turnover (Stanley *et al.*, 2012). Further investigations are required to elucidate the mechanisms of α/β I allosteric inhibitor-induced downmodulation of αLβ2 surface expression.

Additional interesting differences between α I allosteric and α/β I allosteric inhibitors became evident when levels of epitope changes were correlated with effects on T cell activation at the single-cell level. We found that more than 80% R7.1 epitope loss induced by α I allosteric inhibitors was associated with half-maximal inhibition of T cell activation. In contrast, an almost linear correlation between MEM48 epitope gain and blockade of T cell activation was observed for α/β I allosteric inhibitors. This remarkable difference may be explained by the fact that α I and α/β I allosteric inhibitors act via different αLβ2 subunits. α/β I allosteric inhibitors bind to the β2 chain of αLβ2. As this subunit is responsible for signal transduction into the cell, a direct relationship between compound-induced MEM48 epitope alteration and inhibitory function is not surprising (Hogg *et al.*, 2011). The absence of such relationship in the case of α I allosteric inhibitors may reflect the fact that signals from the α chain need to be conveyed to the β2 chain before they translate into functional effects. Another explanation could be that the α I and α/β allosteric inhibitors stabilize αLβ2 in different conformational and activity states (bent vs. extended and inactive vs. semi-active). These different states could translate into different thresholds for downstream effects. The detailed molecular mechanisms at receptor and intracellular pathway levels leading to these differential effect profiles remain to be elucidated.

Intriguingly in this context, treatment of psoriasis patients with efalizumab indicated a similar relationship between the degree of efalizumab binding and clinical efficacy as has been observed for low MW α I allosteric inhibitors between the degree of R7.1 epitope change and T cell activation *in vitro* in whole blood. Doses of efalizumab resulting in partial saturation of the receptor did not result in significant decreases in T cell infiltration, nor significant improvement of skin conditions as assessed by the Psoriasis Area and Severity Index score (Gottlieb *et al.*, 2000; Gottlieb *et al.*, 2002). In contrast, doses that resulted in high (>75%) levels of αLβ2 saturation led to a significant decrease in T cell infiltration and a decrease of the Psoriasis Area and Severity Index score by approximately 45% (Gottlieb *et al.*, 2000; Gottlieb *et al.*, 2002). The fact that efalizumab binds to the α chain of αLβ2 (as α I allosteric inhibitors do) may explain the similarity of the correlations observed with these two modalities.

Taken together, these data demonstrate that the degree of R7.1 and MEM48 epitope change induced by α I allosteric and α/β I allosteric inhibitors does not directly predict the magnitude of effect on immune cell function. In consequence, there is a need to establish the relationship between epitope change and biological response for each inhibitor class and for each epitope monitored individually. The assay methodology described here allowed us to achieve that.

Conclusion

The flow cytometry-based technology described here allows, for the first time, to simultaneously assess and correlate, at the single-cell level, inhibitor-specific αLβ2 conformational change, αLβ2 expression and T cell activation in human whole blood. The format, robustness and sensitivity of the assay indicate that it may be suitable for bedside monitoring of newly developed αLβ2 inhibitors. Further, the assay allowed us to identify unexpected effects of one inhibitor class not exhibited by the other class of inhibitor. This indicates that the assay may also be used as an investigational methodology to assess molecular pathways differentially affected by αLβ2 inhibitors with different modes of action. Initial data using CsA indicate that the methodology may be extended to immunomodulatory modalities of mechanisms not related to αLβ2, thus widening the context of therapeutic guidance.

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Author contributions

K. W. and R.V. M. performed the research work. K. W., G. W.-S. and S. K. contributed to the conception of the work. All authors contributed to the analysis of the data. G. W.-S. and K. W. prepared the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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Table S1 List of commercially available anti-β2 (CD18) mAbs tested for the establishment of the α/βI allosteric αLβ2 inhibitor occupancy assay. MEM48 and MEM148 were found to be the best suited antibodies to quantify the interaction of this inhibitor class with αLβ2.

Figure S6 *Correlation of inhibitor-induced αLβ2 epitope change with T cell proliferation in whole blood*. Following treatment with the LFA878 (αI allosteric inhibitor) or XVA143 (α/βI allosteric inhibitor), αLβ2 epitope alteration (as measured by mAb R7.1 or MEM48 binding, see method section of manuscript) was correlated with aCD3-induced T cell proliferation in human blood (see method described below). Raw values of three independent experiments (three donors) are shown.