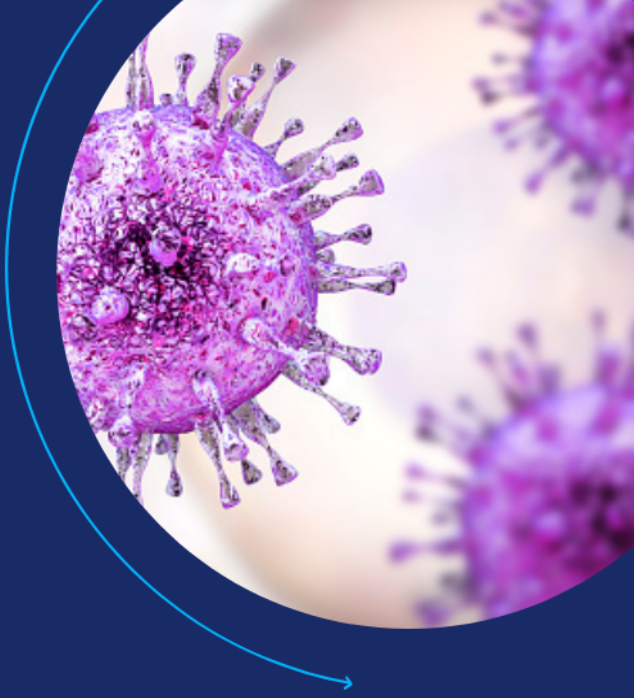
Putting SARS-CoV-2 where it belongs: Identification of potential therapeutic targets with single cell analysis



Respiratory failure is the most common adverse outcome for COVID-19 patients, but scientists and doctors are still searching for answers to the clinical complications of SARS-CoV-2 infection arising in other organs in the body, including the metabolic, cardiac, neurological, and gastrointestinal systems. Moreover, studies of SARS-CoV-2 infection mechanisms have relied on ACE2-overexpressing cells, masking the potential role of other factors in viral entry. Thus, there is an urgent need to create novel models using human disease-relevant cells to study SARS-CoV-2 biology and to facilitate drug screening.

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Differential regulation of monocyte cytokine release by αV and β_2 integrins that bind CD23

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Summary

The human soluble CD23 (sCD23) protein displays highly pleiotropic cytokine-like activity. Monocytic cells express the sCD23-binding integrins αVβ$_3$, αVβ$_5$, αMβ$_2$ and αXβ$_2$, but it is unclear which of these four integrins most acutely regulates sCD23-driven cytokine release. The hypothesis that ligation of different sCD23-binding integrins promoted release of distinct subsets of cytokines was tested. Lipopolysaccharide (LPS) and sCD23 promoted release of distinct groups of cytokines from the THP-1 model cell line. The sCD23-driven cytokine release signature was characterized by elevated amounts of RANTES (CCL5) and a striking increase in interleukin-8 (IL-8; CXCL8) secretion, but little release of macrophage inflammatory protein 1β (MIP-1β; CCL4). Antibodies to αVβ$_3$ or αXβ$_2$ both promoted IL-8 release, consistent with the sCD23-driven pattern, but both also evoked strong MIP-1β secretion; simultaneous ligation of these two integrins further increased cytokine secretion but did not alter the pattern of cytokine output. In both model cell lines and primary tissue, integrin-mediated cytokine release was more pronounced in immature monocyte cells than in mature cells. The capacity of anti-integrin monoclonal antibodies to elicit a cytokine release response is epitope-dependent and also reflects the differentiation state of the cell. Although a pattern of cytokine release identical to that provoked by sCD23 could not be elicited with any individual anti-integrin monoclonal antibody, αXβ$_2$ and αVβ$_3$ appear to regulate IL-8 release, a hallmark feature of sCD23-driven cytokine secretion, more acutely than αMβ$_2$ or αVβ$_5$.

Keywords: CD23; cytokine release; integrins

Introduction

Human CD23 is a 45 000 dalton molecular weight type II transmembrane glycoprotein of the C-type lectin family that expresses a range of biological activities in the membrane-bound and freely soluble forms.[1–3] As a membrane protein, CD23 functions as the low-affinity receptor for IgE[4] and can form cell–cell contacts with CD21,[5,6] leading to homotypic adhesion of activated B lymphocytes.[7,8] Data from CD23$^{-/-}$ mice are consistent with the interpretation that CD23 is a negative regulator of IgE synthesis by B cells.[9–11] Membrane-bound CD23 is released from cells by the action of metalloproteases,[12] and the family of soluble CD23 (sCD23) species released have pleiotropic cytokine-like activities.[13] For example, in the B-cell compartment, binding of sCD23 to CD21 promotes survival of centrocytes,[14] and sCD23 also inhibits apoptosis of B-cell precursors via an interaction with the αVβ$_5$ integrin.[15,16]

Abbreviations: db-cAMP, dibutyryl-cyclic adenosine monophosphate; ERK, extracellular-regulated kinase; FAK, focal adhesion kinase; Fn, fibronectin; GM-CSF, granulocyte–macrophage colony-stimulating factor; IL-8, interleukin-8 (CXCL8); LPS, lipopolysaccharide; mAb, monoclonal antibody; M-CSF, macrophage colony-stimulating factor; MIP-1α, macrophage inflammatory protein-1α (CCL3); MIP-1β, macrophage inflammatory protein-1β (CCL4); PBMC, peripheral blood mononuclear cells; Pyk2, proline-rich tyrosine kinase-2; RANTES, regulated upon activation, normal T-cell expressed and secreted (CCL5); sCD23, soluble CD23; TNF-α, tumour necrosis factor-α; VEGF, vascular endothelial growth factor; Vn, vitronectin.

Human monocytic cells have been reported to bind CD23 using two families of integrins. The $\alpha M\beta_2$ (CD11b-CD18) and $\alpha X\beta_2$ (CD11c-CD18) integrins have been identified as CD23 receptors[17] as has the $\alpha V\beta_3$ integrin,[18] and ligation of these cell surface glycoproteins leads to cytokine release.[19,20] It is therefore unsurprising that CD23 should be implicated as a mediator in inflammatory disease and, indeed, elevated levels of sCD23 are found in patients with a range of autoimmune inflammatory disorders including Sjögren's syndrome,[21] systemic lupus erythematosus and rheumatoid arthritis.[22–24] Moreover, $CD23^{-/-}$ mice show a delayed onset of collagen-induced arthritis and a reduced level of overall joint pathology and, in murine and rat models, administration of anti-CD23 antibody can ameliorate the onset of collagen-induced arthritis.[25,26] Nuclear magnetic resonance[27] and X-ray crystallographic studies[28] have revealed the structures of the derCD23 protein, a fragment of CD23 generated naturally by cleavage by the *Der p* 1 protease of the house dust mite *Dermatophagoides pterronysinus*,[29] and a 25 000 molecular weight sCD23 fragment, respectively. The globular lectin head domain of CD23 contains eight β strands and two α helices and there is pronounced division of acidic and basic residues on opposites faces of the head domain, and these are thought to facilitate oligomerization to yield trimeric membrane-associated CD23. The interaction surfaces for IgE and CD21 are distinct and the structure also shows a lack of acidic residues in the C-terminal region of murine CD23 that explains why murine CD23 does not bind to murine CD21.[27,28] The interaction sites for MHC class II[30] and integrins,[15] although not formally mapped by the structure, are located outside the lectin head domain.

Integrins are a large family of heterodimeric transmembrane cell surface glycoproteins that are traditionally viewed as cell adhesion molecules. Each integrin comprises one of 18α and 8β subunits to form one of 24 known heterodimers. In most models of integrin function, the heterodimer exists in an equilibrium between two forms; one form where the integrin can be thought of as folded over on itself, occluding the ligand binding site, and a second form where the structure is fully extended, rendering the ligand binding site available.[31] The classical example of integrin binding to matrix ligands is to the arg-gly-asp (RGD) tripeptide motif.[32] This has been studied in detail in the $\alpha V\beta_3$ integrin and the ligand binding site is formed by juxtaposition of the α and β subunits so that the peptide arg is secured in a deep pocket in the α subunit and the asp by a cleft on the β subunit; the gly lies in a ridge between the two subunits.[33,34] However, it is not clear whether adhesion via the RGD site triggers cytokine release nor, indeed, that this would be desirable. Our previous studies of sCD23 in pre-B-cell survival models illustrate that the $\alpha V\beta_5$ integrin captures CD23 by recognition of a region containing an arg-lys-cys (RKC) motif and that the integrin uses a site on the β subunit to achieve this binding.[15] This suggests a model whereby CD23 binds appropriate integrin β chains to initiate signalling leading to, for example, cytokine release in monocytes.

Monocytic cells express all four CD23-binding integrins to differing extents depending on their state of differentiation or previous history of stimulation. Given the potential role of sCD23 in a range of autoimmune inflammatory conditions,[21–26] it is clearly important to determine which integrin family or individual isoform stimulates cytokine release to the greatest extent and, therefore, presents the most attractive target for therapeutic intervention. The possibility that different integrins could exert inhibitory effects on cytokine release is also worthy of consideration. To address these questions, monoclonal antibodies directed to specific αV or β_2 integrin isoforms were used individually to stimulate monocytes and the cytokine release output was assessed by use of cytokine arrays and ELISA.

Materials and methods

Materials

The THP1 and U937 cells were from laboratory stocks. Normal human bone marrow and $CD14^+$ peripheral blood mononuclear cells (PBMC) were obtained from Lonza Biologicals (Slough, UK). Tissue culture supplies and NuPage pre-cast gels were from Invitrogen (Paisley, UK). The human Cartesian Array II assay and ELISA for regulated upon activation, normal T-cell expressed, and secreted (RANTES) and macrophage inflammatory protein 1β (MIP-1β) were purchased from Biosource (Paisley, UK), via Invitrogen, and the ELISA systems for tumour necrosis factor-α (TNF-α) were from R&D Systems (Abingdon, UK), who also supplied recombinant sCD23 protein. CD23-derived peptides were obtained from Mimotopes Inc (Melbourne, Australia), and the SuperSignal Pico Western substrate was obtained from Pierce Inc. (Rockford, IL). The monoclonal antibodies (mAbs) used in this study are summarized in Table 1.

Tissue culture and cell stimulation

THP1 and U937 cells were propagated in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM fresh glutamine and 1% (volume/volume) antibiotics (penicillin and streptomycin), in a 95% O_2/5% CO_2 humid atmosphere. For isolation of monocyte precursors, aliquots of bone marrow were stained for lymphocyte markers and the unstained, negatively selected fraction was collected for stimulation and analysis using a FACSAria instrument (BD Biosciences, San Jose, CA). For cytokine release assays, cells were harvested, washed thrice in

Table 1. Antibodies used in integrin stimulation experiments

Integrin bound	Antibody clone*
αV	AMF7
αV	LM142
$\alpha V\beta_3$	23C6
$\alpha V\beta_3$	LM609
$\alpha V\beta_5$	P1F6
$\alpha V\beta_5$	15F11
β_2	PAH9
β_2	MEM48
$\alpha M\beta_2$	44
$\alpha M\beta_2$	ICO-GMI
$\alpha X\beta_2$	3.9

*All antibodies are mouse monoclonal and were purchased from Millipore, Temecula, CA.

OptiMEM and then suspended in OptiMEM (Invitrogen) supplemented with 2 mM glutamine and 1% (volume/volume) antibiotics at 5×10^6/ml. Cells were then stimulated with appropriate antibodies (at 0·5–10 μg/ml), sCD23 (0·1–1·0 μg/ml) or with CD23-derived peptides (0·1–20 μg/ml) and cultured for 24–72 hr at 37°. Determinations were made in triplicate and data are presented as the mean plus standard deviation; an asterisk indicates a value of $P < 0{\cdot}05$ as determined by Student's *t*-test for the parameters being compared. Supernatants were harvested, centrifuged to pellet cells and insoluble debris and assessed for cytokine levels by ELISA or cytokine array. For differentiation experiments, monocytes grown in OptiMEM were treated with dibutyryl-cAMP (db-cAMP, 100 μM), macrophage colony-stimulating factor (M-CSF; 5 ng/ml) or granulocyte–macrophage colony-stimulating factor (GM-CSF; 2 ng/ml) for 4 days before analysis by flow cytometry or assay of cytokine release.

Flow cytometry and Western blotting

For flow cytometric analysis, 100-μl aliquots of cells (5×10^6/ml) were stained with the mAb for individual integrins for 30–60 min on ice before washing in PBS; if required, a fluorophore-conjugated secondary reagent was added and a further 30–60 minutes of incubation was conducted before washing and analysis. Appropriate isotype controls were included. Data were collected from a minimum of 10^4 cells using a FACScan instrument (BD Biosciences) and analysed using CellQuest software (BD Biosciences).

Results

Soluble CD23 promotes cytokine release

Human monocytes release cytokines following stimulation by a range of stimuli. Other groups have demonstrated that exposure of human PBMC to sCD23 promoted TNF-α release, via ligation of the $\alpha V\beta_3$ integrin,[18] and other cytokines via ligation of β_2 integrins.[17,35] Figure 1(a) illustrates that normal PBMC released TNF-α following stimulation with lipopolysaccharide (LPS) or sCD23 but not when treated with the extracellular matrix proteins vitronectin (Vn) or fibronectin (Fn), which are additional ligands for $\alpha V\beta_3$ and $\alpha V\beta_5$. However, these cells expressed high levels of three of the four integrins that are known to bind sCD23; namely $\alpha V\beta_3$, $\alpha V\beta_5$ and $\alpha X\beta_2$ (Fig. 1b). Therefore, it is not clear which of the four possible sCD23-binding integrins would be responsible for acute regulation of release of one or more discrete cytokines or groups of cytokines (Fig. 1c), or whether these integrins generate synergistic or mutually inhibitory signals. To test the broad hypothesis that individual sCD23-binding integrins differentially regulate acute cytokine release from monocytic cells, an antibody array approach was employed to determine the qualitative patterns of cytokine release from THP-1 cells following stimulation with antibodies directed against individual sCD23-binding integrin isoforms (Fig. 1c). The general principle of the assay is shown in Supplementary material, Fig. S1A and the patterns of pairs of anti-cytokine antibodies printed on the array are shown in Supplementary material, Fig. S1B.

Antibodies to different integrins promote release of distinct patterns of cytokines

The pattern of release of cytokines driven by sCD23 in monocytic cells is complex and may reflect the fact that up to four distinct sCD23 binding integrins can be ligated on the same cell, with each potentially giving rise to a distinct effect on cytokine synthesis and release. THP-1 cells plainly released some cytokines constitutively [notably RANTES, interleukin-4 (IL-4) and IL12-p40] but, in general terms, this was modulated by treatment of the cells with LPS, which strikingly promoted MIP-1β release, but also elevated secretion of other cytokines, while addition of IgG1, which will occupy high-affinity FcγR1 receptors on THP-1 cells, did not provoke significant cytokine release (Fig. 2b). The characteristic pattern of sCD23-driven cytokine release from monocytic cells (Fig. 2c), compared with unstimulated controls (Fig. 2b), comprised a striking rise in IL-8 release, a further increase in RANTES release and increases in synthesis and release of vascular endothelial growth factor (VEGF), MIP-5, IL-6 receptor and a modest effect on MIP-1β release (though this was considerably lower than that seen with LPS stimulation). Treatment of THP-1 cells with the sCD23-derived long peptide (LP), which binds with high affinity to αV integrins, promoted generalized cytokine release from the cells and was not assessed further; a peptide (#58) derived from a different part of the sCD23 protein that lacks the RKC motif was without effect (Fig. 2c).

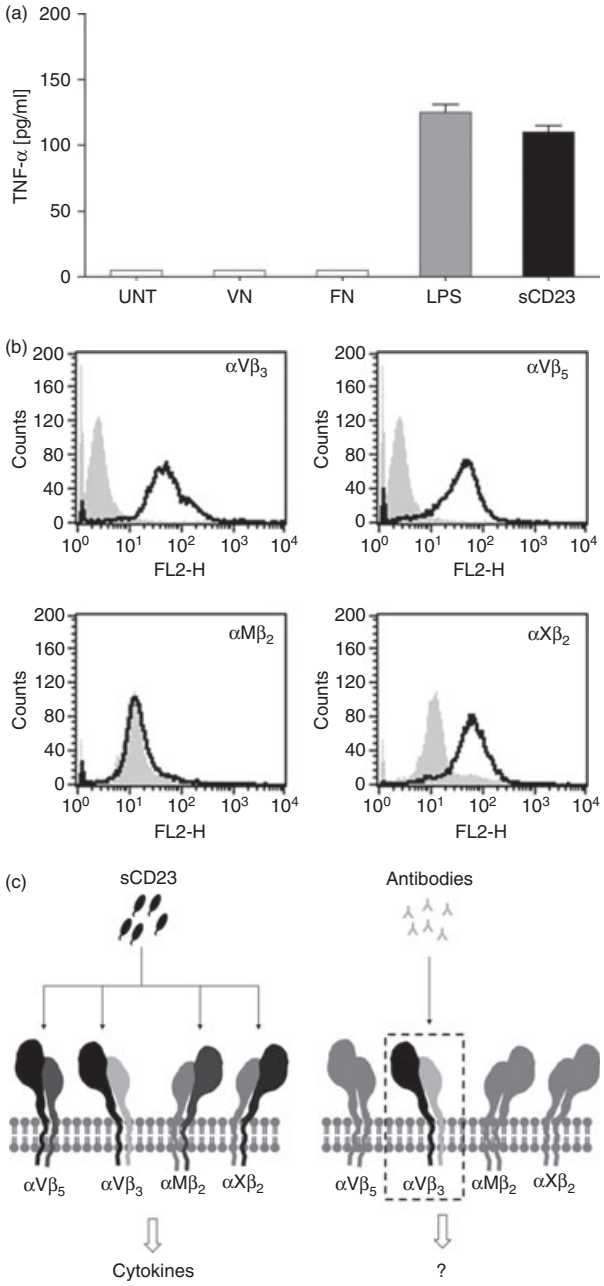

Figure 1. CD23 promotes cytokine release. (a) Human peripheral blood monocytes were cultured with no stimulus, vitronectin (Vn), fibronectin (Fn), lipopolysaccharide (LPS; grey bar) or soluble CD23 (sCD23; black bar) and tumour necrosis factor-α (TNF-α) release was measured by ELISA. Data are presented as the mean plus standard deviation of triplicate determinations and the experiment illustrated is one representative of at least three independent experiments. (b) Histograms for staining of the four indicated sCD23-binding integrins on THP-1 cells (black lines) compared with isotype-matched controls (grey shaded area). (c) Cartoon illustrating the capacity of sCD23 to bind to one of four integrins on monocyte cell surfaces and the potential for using monoclonal antibodies to individual integrins to probe their functions.

Biochemical data from both murine and human monocyte models indicate that the β_2 integrins $\alpha M\beta_2$ and $\alpha X\beta_2$ bind sCD23 and regulate cytokine release.[17,35] Treatment of THP-1 cells with the MEM48 mAb that recognizes all β_2 integrins gave a pattern of cytokine release that was very close to that observed in untreated cells (Fig. 2d). The clone 44 reagent that binds assembled $\alpha M\beta_2$ integrins promoted a more generalized release of cytokines from the treated cells but, with the exception of a slightly enhanced signal for IL-8, this pattern was again broadly similar to that found for unstimulated cells. By contrast, the HC1.1 reagent, directed to $\alpha X\beta_2$ heterodimers, provoked a different pattern of release. In this case, there was a striking increase in IL-8 and cytotoxic T-lymphocyte

(a)

	a	b	c	d	e	f	g
1	Apol/Fas	Leptin	RANTES	ICAM-1	IL-2	IL-7	Pos* (***)
2	CTLA	MIP-1α	TGF-β	VCAM-1	IL-3	IL-8	Pos*
3	Eotaxin	MIP-1β	IFN-γ	VEGF	IL-4	IL-10	Neg**
4	GM-CSF	MIP-4	TNF-α	IL-1α	IL-5	IL-12p40	Neg**
5	EGF	MIP-5	TNF-RI	IL-1β	IL-6	IL-15	Pos*
6	IP-10	MMP3	TNF-RII	IL-1RA	IL-6R	IL-17	Pos*

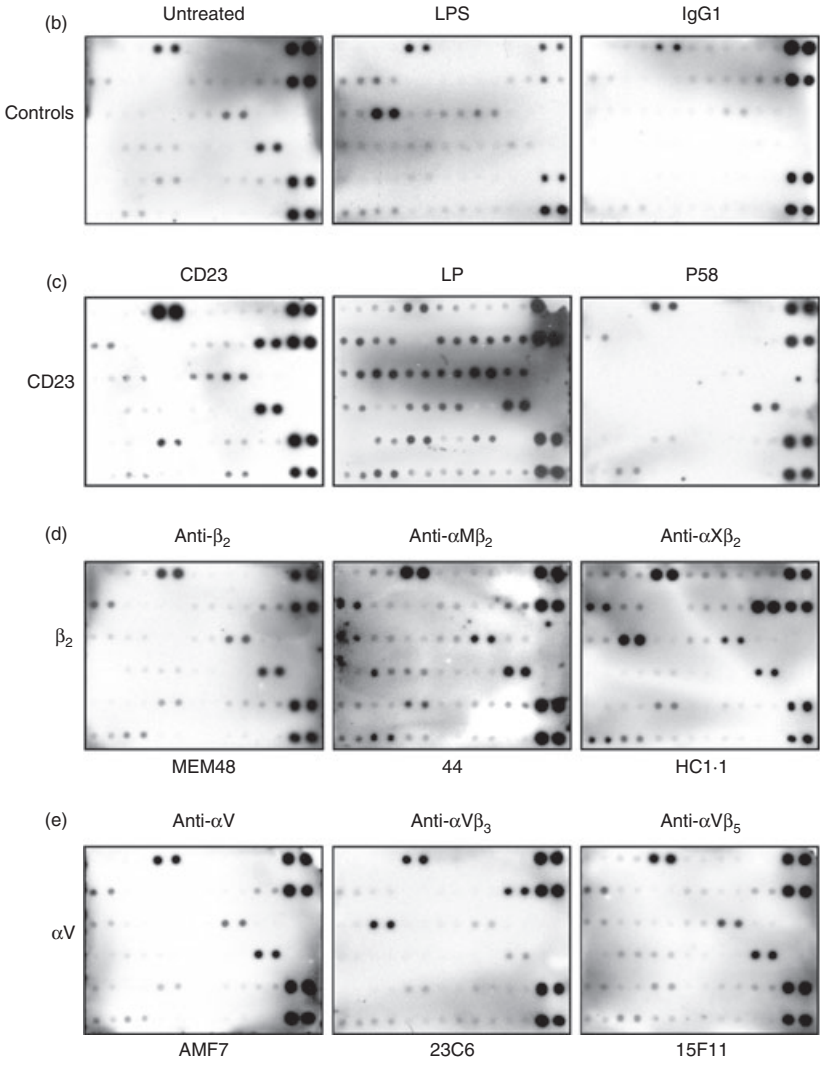

Figure 2. Anti-integrin monoclonal antibodies promote the release of distinct sets of cytokines. THP-1 cells were treated overnight with the indicated stimuli (all used at 5 μg/ml), culture supernatants were collected and applied to Cartesian II cytokine arrays and capture of cytokines was visualized by enhanced chemiluminescence. (a) Layout of anti-cytokine monoclonal antibodies on the array for reference. (b) Patterns of secretion for untreated cells and those exposed to IgG1 or lipopolysaccharide (LPS); (c) patterns for THP-1 cells stimulated with soluble CD23 (sCD23) or the CD23-derived, αV integrin binding long peptide (LP) and the negative control peptide #58. (d) Secretion patterns for THP-1 cells stimulated with β_2 integrin-directed reagents, with MEM48 binding all β_2 integrins and clones 44 and HC1.1 recognizing assembled $\alpha M\beta_2$ and $\alpha X\beta_2$ heterodimers, respectively. (e) An equivalent analysis of αV integrins, where AMF7 ligates all αV-containing integrins and 23C6 and 15F11 bind $\alpha V\beta_3$ and $\alpha V\beta_5$ heterodimers, respectively.

antigen (CTLA) in the culture supernatants, which was partly consistent with the sCD23-driven signature of cytokine release, but there was also a pronounced release of MIP-1β that was not noted with sCD23 treatment; MIP-5 levels were also reduced relative to MIP-1α levels (Fig. 2d). A similar analysis of the effect of mAbs binding to αV integrins showed that the AMF7 reagent that bound all αV integrins was without notable effect on the cells (Fig. 2e). The 23C6 anti-$\alpha V\beta_3$ reagent promoted a strong increase in both IL-8 and MIP-1β release but had no effect on CTLA output; stimulation with this mAb caused a generalized reduction in release of other cytokines, most notably IL-12p40 and IL-4, which are constitutively released by THP-1 cells. Finally, the 15F11 anti-$\alpha V\beta_5$ antibody yielded a pattern of release that was broadly similar to untreated cells, and there was no notable increase in IL-8 or MIP-1β release. The 15F11 did not cause a reduction in release of IL-12p40 or IL-4 (Fig. 2e).

The data from the array analyses are consistent with the interpretation that no single sCD23-binding integrin

appeared to regulate the sCD23-driven cytokine release signature independently of its three partners. In general terms, both $\alpha V\beta_3$ and $\alpha X\beta_2$ appeared to regulate IL-8 release acutely, whereas $\alpha V\beta_5$ could have a role in inhibiting MIP-1β synthesis and/or release. There does not appear to be a hierarchy either between or within sCD23-binding integrin families with respect to control of cytokine release.

Patterns of monocyte cytokine release are epitope and differentiation state-dependent

Integrins are best understood in terms of their adhesion-like activities, characterized by binding to linear sequences such as RGD in matrix proteins.[32] However, it is increasingly clear that other ligands that lack RGD sequences bind integrins, and many such ligands use stretches of basic residues to bind target integrins. Examples include the binding of HIV-TAT to $\alpha V\beta_5$,[36] association of the snake venom jararhagin with the I-domain of $\alpha_2\beta_1$ via an RKKH motif,[37] the interaction of the angiogenic factor CCN1 with $\alpha M\beta_2$ that is dependent on a pair of adjacent lysines,[38] and the binding of the γC fragment of fibrinogen to $\alpha IIb\beta_3$ which is also dependent on two pairs of lysine groups.[38] Our own data demonstrate that sCD23 interacted with $\alpha V\beta_5$ using a basic motif (RKC) to bind the integrin at a site that did not recognize RGD sequences.[15] Therefore, anti-integrin antibodies directed to distinct epitopes on the four integrins, including mAbs that either inhibited or failed to impede adhesion-dependent activities of the target integrins, were tested for effects on cytokine release. The responses were assessed in ELISA of supernatants from THP-1 cells, representative of an immature monocyte, and U937 cells, representative of a more differentiated macrophage-type cell.

In all cases, none of IgG1, Vn or soluble RGDS tetrapeptide provoked release of IL-8, MIP-1β or RANTES to any degree greater than that found in supernatants of untreated cells (Fig. 3a,b). For $\alpha V\beta_5$ integrins, both the P1F6 and 15F11 reagents promoted release of IL-8 and MIP-1β from THP-1 cells, though the P1F6 reagent, which inhibits RGD-mediated functions of $\alpha V\beta_5$, is by far the more effective stimulus (Fig. 3a). Neither antibody had any effect on RANTES release. By contrast, however, anti-$\alpha V\beta_5$-specific mAbs failed to drive release of either IL-8 or MIP-1β from the more mature U937 cell line (Fig. 3b). As expected, and consistent with the data from THP-1 cells, there was no effect on release of RANTES from U937 cells (Fig. 3b, black bars). For the $\alpha V\beta_3$-directed mAbs, only the 23C6 reagent promoted release of IL-8 and MIP-1β from THP-1 cells; the LM609 mAb had no effect (Fig. 3a,b). Neither reagent promoted RANTES release in THP-1 or U937 cells, and both were ineffective in promoting IL-8 or MIP-1β release in the latter cell line. The 23C6 reagent did, however, retain the capacity to elicit MIP-1β release from U937 cells. The AMF7 and LM142 anti-αV mAbs showed stimulatory effects on IL-8 and MIP-1β release in THP-1 cells, but generally not in U937 cells (Fig. 3a,b).

A similar analysis was performed using β_2 integrin-directed reagents, with a broadly comparable outcome. Hence, the anti-$\alpha M\beta_2$ reagent, clone 44, promoted a modest release of IL-8 and MIP-1β in the THP-1 cell line model, but was without significant stimulatory effect in the U937 system (Fig. 3a,b). The MEM48 pan anti-β_2 reagent did not stimulate cytokine release. Clone 3.9, an anti-$\alpha X\beta_2$ heterodimer antibody (Fig. 3a,b), stimulated significant release of IL-8, MIP-1β and, to a lesser extent, RANTES from the immature THP-1 cells but, with the exception of a small effect on IL-8 release, did not promote cytokine release from U937 cells. The difference in cytokine response between cell lines could not be attributed to differences in integrin expression levels as THP1 and U937 cells expressed similar levels of both the αV and β_2 integrin heterodimers studied (Fig. S2).

The data in Fig. 3(a,b) are based on cell line models and it is important to validate the data from such systems in primary tissue. To this end, bone marrow monocyte precursors and PBMC were assessed for their patterns of responsiveness to ligation with anti-integrin mAbs (Fig. 3c). Bone marrow monocytes and PBMC showed striking differences in expression of the sCD23-binding integrins (Fig. 3c). Bone marrow monocytes expressed $\alpha X\beta_2$ and $\alpha V\beta_3$ in moderate amounts and were weakly positive for $\alpha M\beta_2$; the cells were negative for $\alpha V\beta_5$. The PBMC expressed all four integrins, with greatly increased levels of $\alpha X\beta_2$ and $\alpha V\beta_3$, clear positivity for $\alpha M\beta_2$ and robust expression of $\alpha V\beta_5$ (Fig. 3c). Bone marrow monocytes were treated with different anti-integrin mAbs and the patterns of cytokine release were determined. None of the stimuli used, including LPS, promoted IL-8 release (data not shown), but there was a clear and robust effect on release of MIP-1β, RANTES and TNF-α. Antibodies directed to $\alpha X\beta_2$ and to $\alpha V\beta_3$ promoted significant release of all three cytokines, whereas antibodies directed to $\alpha M\beta_2$ (ICO-GMI) or $\alpha V\beta_5$ (P1F6) failed to induce cytokine release (Fig. 3c). Ligation of $\alpha X\beta_2$ on PBMC with clone 3.9 mAb promoted cytokine release, albeit to lower levels than noted with bone marrow monocytic cells, but treatment with anti-$\alpha V\beta_3$ mAbs did not drive TNF-α release. Cross-linking of $\alpha M\beta_2$ stimulated TNF-α release from PBMCs (Fig. 3c). However, none of the anti-integrin mAbs could provoke IL-8 (data not shown) or RANTES secretion from PBMC (Fig. 3c), a result that is consistent with the observations from cell lines representative of immature and mature monocytes. Finally, THP1 cells were treated with db-cAMP to induce differentiation and the effects on integrin expression and responsiveness were assessed (Fig. 3d). The db-cAMP caused a minor increase in expression of $\alpha M\beta_2$ and $\alpha V\beta_5$ in THP-1 cells and a more pronounced elevation in levels of $\alpha X\beta_2$;

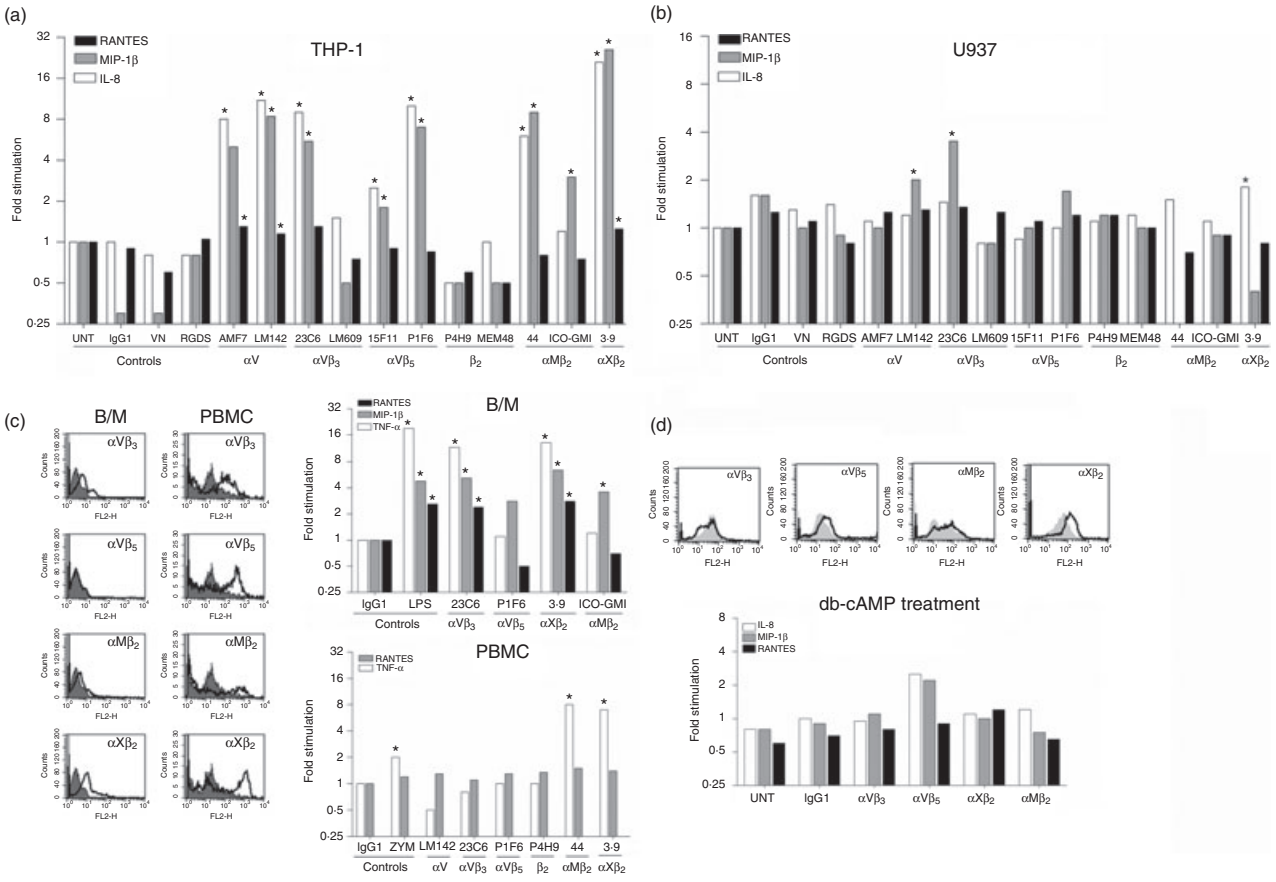

Figure 3. Monoclonal antibody-driven cytokine release is epitope and differentiation state-dependent. (a) Release of cytokines from THP-1 cells following stimulation with the indicated αV or β_2 integrin-directed monoclonal anitbodies. (b) The same analysis for U937 cells. In (a) and (b) the black bars indicate the production of regulated upon activation, normal T-cell expressed and secreted (RANTES), the grey bars indicate the release of macrophage inflammatory protein 1β (MIP-1β) and the white bars indicate the release of interleukin-8 (IL-8). In all instances, the data are presented as fold-stimulation of cytokine release relative to untreated cells. Controls include untreated cells (UNT) and cells that were treated with RGDS peptide (RGDS), isotype control immunoglobulin (IgG1) or vitronectin (Vn). For the αV integrin family, the P1F6 and 15F11 antibodies bind to different epitopes on the $\alpha V\beta_5$ heterodimer, and 23C6 and LM609 bind to distinct epitopes on the $\alpha V\beta_3$ heterodimer; LM142 and AMF7 antibodies bind to distinct epitopes on the αV integrin subunit. For the β_2 integrin family, clone 3.9 antibody binds the $\alpha X\beta_2$ heterodimer, ICO-GMI and clone 44 antibodies bind to distinct epitopes on the $\alpha M\beta_2$ heterodimer, and MEM48 and P4H9 antibodies bind to different epitopes on the β_2 integrin subunit. The data are representative of three independent experiments performed with triplicate determinations for levels of each cytokine. (c) Staining of the indicated four integrins in monocytes derived from bone marrow (B/M) or blood (peripheral blood mononuclear cells; PBMC) and the effect of different stimuli on release of the indicated cytokines from bone marrow monocytic cells and PBMC data. In all instances, the data are presented as fold-stimulation of cytokine release relative to untreated cells. All data are representative of two independent experiments. Controls were cells treated with isotype control immunoglobulin (IgG1), lipopolysaccharide (LPS) or zymosan (Zym). For the β_2 integrin family, ICO-GMI and clone 44 antibodies recognize the $\alpha M\beta_2$ integrin, clone 3.9 antibody recognizes the $\alpha X\beta_2$ integrin (black bars), and the P4H9 antibody binds to the β_2 integrin subunit. For the αV integrin family, P1F6 antibody binds the $\alpha V\beta_5$ heterodimer, 23C6 antibody binds the $\alpha V\beta_3$ heterodimer (grey bars), and LM142 antibody binds to the αV integrin subunit. (d) Histograms showing expression of the four indicated integrins before (grey shaded area) and after (black lines) treatment of THP-1 cells with db-cAMP and the effect of ligating individual integrins on release of RANTES, IL-8 and MIP-1β; IgG1 is the control for addition of stimulatory monoclonal antibodies. The data are reported as fold-stimulation relative to untreated THP-1 cells that were not exposed to db-cAMP, and are representative of three independent experiments. Student's *t*-test was used to determine statistical significance, $^*P < 0{\cdot}05$.

$\alpha V\beta_3$ levels were unchanged (Fig. 3d). Treatment with db-cAMP mediated a slight diminution of mAb-driven release of RANTES from differentiated THP-1 cells relative to control, untreated cells, but a striking increase in IL-8 and MIP-1β release was stimulated by cross-linking of $\alpha V\beta_5$ in the db-cAMP-treated THP-1 cells (Fig. 3d). Hence, although db-cAMP treatment elevated levels of $\alpha X\beta_2$ at the cell surface, there was no elevation of cytokine release triggered by this integrin, but rather the cells became more sensitive to $\alpha V\beta_5$-driven cytokine production. Pre-treatment of the

cells with M-CSF or GM-CSF did not lead to alterations in integrin expression or sensitivity to ligation relative to untreated controls (data not shown).

Anti-$\alpha V\beta_3$ mAb promotes intracellular signalling and cytokine release from monocytic cells

Stimulation of human monocytes with sCD23 provoked release of TNF-α via an interaction with the $\alpha V\beta_3$ integrin.[18] However, the LM609 antibody directed to the $\alpha V\beta_3$ heterodimer[39] failed to block this response,[18] and LM609 also failed to induce a noticeable release of cytokines in the models described in this report. By contrast, the 23C6 mAb provoked both a modest increase in RANTES release from THP-1 cells, and a far more robust and dose-dependent increase in release of MIP-1β and IL-8 from the cells compared with untreated controls. None of Vn, an IgG1 isotype control, or the RGDS tetrapeptide caused any release of cytokine greater than that observed for untreated control cells (Fig. 4a). Release of RANTES driven by LPS, 23C6 or by an anti-$\alpha X\beta_2$ mAb (clone 3.9) was sensitive to both actinomycin D and cycloheximide pre-treatment, whereas IL-8 and MIP-1β release was sensitive only to actinomycin D (Fig. 4b). Treatment of THP-1 cells with the anti-$\alpha X\beta_3$ clone 3.9 mAb or the 23C6 anti-$\alpha V\beta_3$ reagent induced a similar dose-dependent and time-dependent phosphorylation of extracellular signal-regulated kinase (ERK) (data not shown). LPS-driven release of IL-8 and MIP-1β was not significantly reduced by U0126 pre-treatment (Fig. 4c), but release of these cytokines from THP-1 cells stimulated with anti-$\alpha V\beta_3$ or anti-$\alpha M\beta_2$ mAbs was significantly reduced by U0126-mediated inhibition of MEK. Spontaneous and stimulated release of RANTES was sensitive to inhibition of ERK by

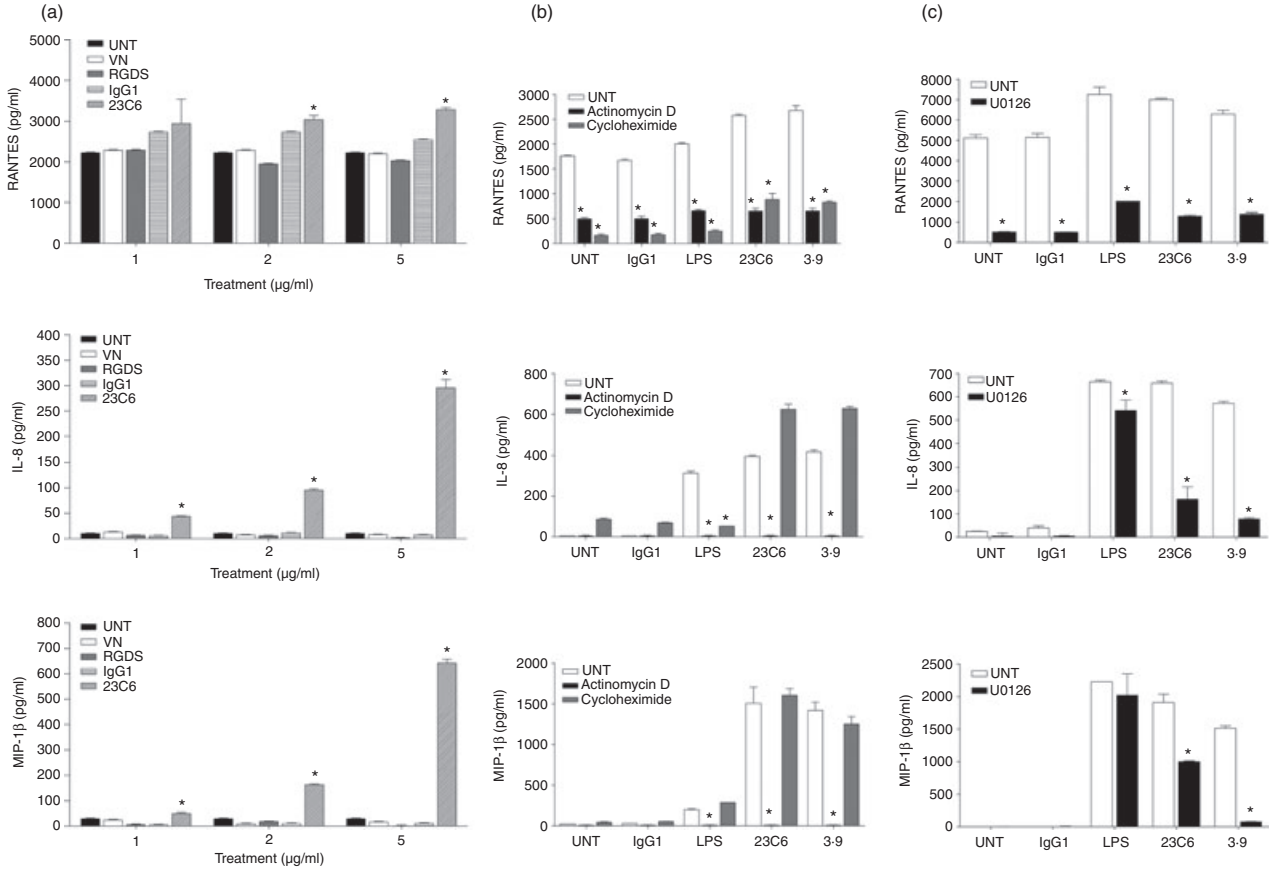

Figure 4. Antibodies against assembled $\alpha V\beta_3$ and $\alpha X\beta_2$ heterodimers provoke robust signalling and cytokine release responses in THP-1 cells. (a) THP-1 cells were cultured overnight in medium alone (black bars), or in the presence of the indicated concentrations of vitronectin (Vn; white bars), RGDS tetrapeptide (middle grey bars), IgG1 (horizontal lined bars) or anti-$\alpha V\beta_3$ monoclonal antibody (clones 23C6, diagonal lined bars) and the levels of regulated upon activation, normal T-cell expressed and secreted (RANTES), interleukin-8 (IL-8) and macrophage inflammatory protein 1β (MIP-1β) released into culture supernatant measured by ELISA. (b) Effect on secretion of the indicated chemokines of IgG1, lipopolysaccharide (LPS), or monoclonal antibodies 23C6 or clone 3.9 in combination with actinomycin D (black bars), cycloheximide (grey bars) or no additional treatment (white bars). (c) Comparison of the impact of treatment of THP-1 cells with U0126 (black bars) with untreated cells (white bars) on release of the indicated cytokines following stimulation with LPS or monoclonal antibodies directed to $\alpha V\beta_3$ or $\alpha X\beta_2$. The data are representative of three independent experiments. Student's *t*-test was used to determine statistical significance, $^*P < 0{\cdot}05$.

U0126 (Fig. 4c). These data indicate that certain anti-integrin mAbs promote cytokine release from THP-1 cells and that this release is dependent at least in part on signals delivered via the ERK pathway.

Cooperation between CD23-binding integrin families

Ligation of CD23-binding integrins with mAbs directed to individual integrin isoforms failed to induce a pattern of secretion of cytokines that matched the pattern produced by stimulation with sCD23 itself. We therefore assessed the ability of mAbs directed to two different integrin isoforms to modulate patterns of cytokine release. In brief, the effect of anti-$\alpha V\beta_3$ ligation on cytokine release could not be modified, either positively or negatively, by mAbs to other αV integrins, or by mAbs to β_2 integrins (data not shown). Similarly, ligation of $\alpha X\beta_2$ led to cytokine release patterns that were not appreciably altered by co-stimulation with anti-$\alpha V\beta_5$ or anti-pan αV reagents or by mAbs to other β_2 integrins (data not illustrated). However, a synergistic effect was noted with mAbs directed against the assembled $\alpha X\beta_2$ and $\alpha V\beta_3$ integrins (Fig. 5). Hence, the levels of release of RANTES, IL-8 and MIP-1β stimulated by a fixed dose of anti-$\alpha V\beta_3$ mAb were elevated by co-stimulation with increasing concentrations of anti-$\alpha X\beta_2$ mAb (Fig. 5a). A similar outcome was observed using a fixed $\alpha X\beta_2$ mAb concentration and increasing doses of anti-$\alpha V\beta_3$ (Fig. 5b). The data suggest that these mAbs, that are most effective in promoting cytokine secretion from THP-1 cells, are able to cooperate to promote higher levels of cytokine release.

Discussion

The data of this report demonstrate that stimulation of integrins that bind sCD23 promotes release of cytokines from human monocytic cells. The dominant feature of the cytokine release signature driven by sCD23 itself comprises a pronounced elevation in IL-8 secretion, a modest rise in RANTES release and no secretion of MIP-1β. Ligation of individual integrins did not mimic this cytokine release pattern, though stimulation of $\alpha X\beta_2$ or $\alpha V\beta_3$ promoted release of IL-8 and RANTES, consistent with sCD23-driven release, but also enhanced MIP-1β secretion. Stimulation of $\alpha M\beta_2$ and $\alpha V\beta_5$ integrins did not promote release of cytokines similar to those released following sCD23 treatment of the cells. Triggering of cytokine release via integrins was dependent on both the epitope recognized by the mAb and the state of differentiation of the target cell; less mature cells released higher levels of cytokine.

The broad patterns of cytokine release from CD23-stimulated monocytes noted in this report are generally consistent with those of other investigators assessing secretion of individual cytokines. Hence, in initial studies, sCD23 stimulation of monocytes was demonstrated to

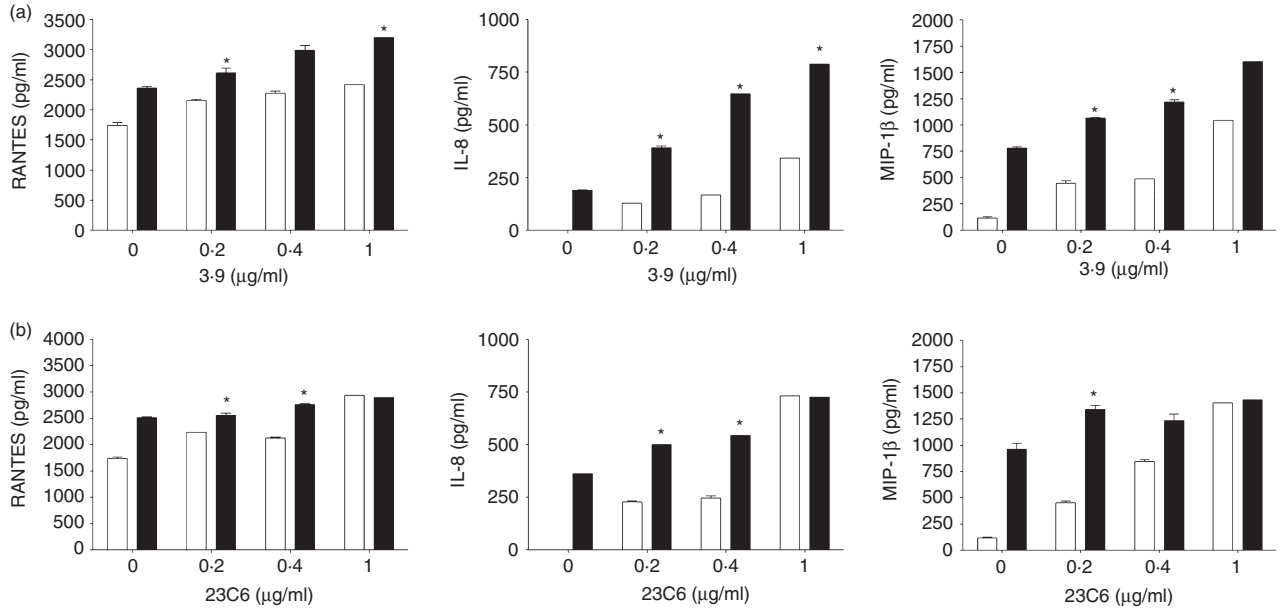

Figure 5. $\alpha V\beta_3$ and $\alpha X\beta_2$ integrins cooperate to enhance cytokine release. THP-1 cells were stimulated with a combination of the anti-$\alpha_V\beta_3$ monoclonal antibody (23C6) and the anti-$\alpha X\beta_2$ monoclonal antibody (clone 3.9) and cytokine secretion measured by ELISA. In (a), the white bars indicate cytokine release induced by increasing concentrations of $\alpha X\beta_2$ clone 3.9 alone, while the black bars show the effect of increasing concentrations of 3.9 with the addition of 0·4 μg/ml of $\alpha V\beta_3$ antibody, 23C6. (b) The reciprocal experiment, with THP-1 cells being exposed to increasing doses of anti-$\alpha V\beta_3$ (the 23C6 reagent) with (black bars) or without (white bars) a fixed concentration of anti-$\alpha X\beta_2$ (clone 3.9; 0·4 μg/ml). All data are representative of two independent experiments conducted in triplicate. Student's *t*-test was used to determine statistical significance, $^*P < 0{\cdot}05$.

promote release of IL-1β, IL-8, TNF-α and GM-CSF, but not IL-10, IL-12 or transforming growth factor-β (TGF-β)[40]; the data of Fig. 2 in this report show a prominent elevation of IL-8 secretion and an equally consistent absence of TGF-β release. Other groups using sCD23 fusion proteins and anti-β_2 integrin antibodies showed strong release of IL-1β,[19] MIP-1α and MIP-1β.[20] In our study, we noted a strong MIP-1β release when targeting the $\alpha X\beta_2$ and a less pronounced secretion when $\alpha M\beta_2$ was ligated, in keeping with previous findings.[20] However, we did not note a significant release of MIP-1α. This may reflect either an intrinsic property of the THP-1 cell line, or might be related to the epitopes recognized by the different antibodies used in the two studies.

The principle that is consistent in all the above studies is that sCD23 triggers release of pro-inflammatory cytokines and chemokines from monocytic cells and so could be considered to lie 'upstream' of the effects of these inflammatory mediators and to be closer to an initiating stimulus in inflammatory states. Indeed, many studies report findings of increased levels of sCD23 in autoimmune inflammatory disorders, with elevated levels being noted in juvenile and adult rheumatoid arthritis in both blood and synovial fluid, and particularly high levels being found in patients with disease flares. Soluble CD23 is also found in the saliva of Sjögren's syndrome patients[41,42] and in the plasma of patients with systemic lupus erythematosus,[41,42] though in the case of systemic lupus erythematosus the effect of sCD23 is likely to be mediated via its interaction with CD21 on autoimmune B cells rather than via integrins on monocytic cells.[43] The finding of high sCD23 levels in such syndromes has made both sCD23 protein itself and its various receptors attractive targets for therapeutic intervention. This aspiration is supported by data from rodent systems where anti-CD23 mAbs have been shown to both prevent initial and ameliorate existing arthritic disease,[25,26] and by the success of Lumiliximab, a humanized macaque anti-CD23 antibody, in treatment of B chronic lymphocytic leukaemia,[44] a disease characterized by strikingly high plasma sCD23 levels.[45] A different strategy, employing a CD23-binding peptide identified by phage display technology, also shows promise in preventing onset of adjuvant-induced arthritis and reducing severity of established disease in rats.[46] The identification of $\alpha V\beta_3$ as an sCD23 receptor linked to TNF-α release in human monocytes[18] suggested that antibodies to this integrin might be useful in autoimmune inflammatory disease.[47] The Etaracizumab mAb (Abergrin, Vitaxin),[48,49] a humanized form of the LM609 anti-$\alpha V\beta_3$ reagent, was shown to be potent in inhibiting angiogenesis.[50,51] However, Etaracizumab was also assessed in psoriatic arthritis but was not found to have a therapeutic effect and this is potentially explained by the fact that the parent LM609 mAb does not inhibit sCD23-driven TNF-α release from monocytes,[18] a finding that implies that the mAb does not influence the site on the integrin responsible for control of cytokine release. Our data that showed LM609 did not induce cytokine production from either THP-1 or U937 cells (Fig. 3) were also in agreement with this suggestion. Etaracizumab retains significant promise, however, and is currently in trials for therapy of metastatic melanoma.[52]

It is important to bear in mind that most previous studies on integrin function have been performed in adherent cells. The possibility of an alternative mode of integrin signalling illustrated by sCD23 is particularly interesting in the context of haematopoietic cells, including monocytes, which are non-adherent cells, but nonetheless express a wide range of integrins, and are the precursors of a number of adherent, terminally differentiated cells, such as macrophages and osteoclasts. The differentiation of monocytes into adherent counterparts is the result of paracrine or autocrine signalling in response to cytokines, such as those released by the interaction of sCD23 with integrins. Therefore, it is possible that the chronic stimulation of integrin signalling by sCD23 might also induce the differentiation of precursors into terminally differentiated cells associated with many of the diseases in which elevated sCD23 is a hallmark. These issues merit further study.

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Disclosures

The authors have no competing conflicts of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Detection of cytokine release by cytokine arrays.

Figure S2. Expression of integrins on THP-1 and U937 cells.

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