EXTENDED REPORT

The rs1143679 (R77H) lupus associated variant of ITGAM (CD11b) impairs complement receptor 3 mediated functions in human monocytes

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ABSTRACT

Objectives The rs1143679 variant of ITGAM, encoding the R77H variant of CD11b (part of complement receptor 3, CR3), is among the strongest genetic susceptibility effects in human systemic lupus erythematosus (SLE). The authors aimed to demonstrate R77H function in ex-vivo human cells.

Methods Monocytes/monocyte-derived macrophages from healthy volunteers homozygous for either wild type (WT) or 77H CD11b were studied. The genotype-specific expression of CD11b, and CD11b activation using conformation-specific antibodies were measured. Genotype-specific differences in iC3b-mediated phagocytosis, adhesion to a range of ligands and the secretion of cytokines following CR3 ligation were studied. The functionality of R77H was confirmed by replicating findings in COS7 cells expressing variant-specific CD11b.

Results No genotype-specific difference in CD11b expression or in the expression of CD11b activation epitopes was observed. A 31% reduction was observed in the phagocytosis of iC3b opsonised sheep erythrocytes (sRBCiC3b) by 77H cells (p=0.003) and reduced adhesion to a range of ligands: notably a 24% reduction in adhesion to iC3b (p=0.014). In transfected COS7 cells, a 42% reduction was observed in phagocytosis by CD11b (77H)-expressing cells (p=0.004). A significant inhibition was seen in the release of Toll-like receptor 7/8-induced pro-inflammatory cytokines from WT monocytes when CR3 was pre-engaged using sRBCiC3b, but no inhibition in 77H monocytes resulting in a significant difference between genotypes (interleukin (IL)-1β p=0.030; IL-6 p=0.029; tumour necrosis factor alpha p=0.027).

Conclusions The R77H variant impairs a broad range of CR3 effector functions in human monocytes. This study discusses how perturbation of this pathway may predispose to SLE.

METHODS

Reagents

Culture media, sera and salt solutions and secondary antibodies were from Invitrogen (Life Technologies, Paisley, UK); rhM-CSF was from Peprotech (London, UK); anti-CD11b, anti-CD14 and control antibodies were from ebioscience (Hatfield, UK); polyclonal antisheep erythrocyte IgM was from Cedarlane (Burlington, Ontario, Canada). Human CD11b and CD18 in the pRKS vector was a gift of Emmanuelle Caron, Imperial College, London. The R77H mutation was introduced using a Stratagene QuikChange site-directed mutagenesis kit (Agilent Technologies, Stockport, UK). Protein ligands were from Calbiochem, Merck-Millipore, London, UK (iC3b), R&D Systems, Abingdon, UK (ICAM-1) and Enzyme Research Laboratories Swansea, UK (fibrinogen). Human DC-SIGN was a gift of Dan Mitchell, University of Warwick.
Total RNA was extracted from $2 \times 10^6$ cells using an RNeasy kit. Quantitative real-time PCR was performed using an ABI 7300 real-time PCR system. The relative quantification was based on the comparative Ct method, with four housekeeping gene controls, and normalised to one randomly selected homoygous donor, using DataAssist software (Applied Biosystems). For primer details see supplementary text (available online only).
RESULTS

CD11b expression

We analysed CD11b cell-surface expression (using anti-CD11b antibody ICRF44) in 10 WT and 10 77H homozygous volunteers (figure 1). The neutrophils were identified by forward/side-scatter profile, and monocytes by side-scatter low/CD14 high profile. Even accounting for an outlier in the 77H group with very high CD11b expression, there was no genotype-specific difference in expression by comparison of mean fluorescent intensities (p=0.33 for neutrophils, p=0.25 for monocytes). PMA activation resulted in an increase in CD11b cell-surface expression but no difference between genotype groups (p=0.42 for neutrophils, p=0.97 for monocytes).

To confirm that the rs1143679 genotype was not influencing gene expression we performed quantitative PCR in an additional 14 WT and nine WT/77H heterozygous volunteers. There was no significance difference in the relative expression of ITGAM messenger RNA (p=0.35 for unselected peripheral blood mononuclear cells, p=0.13 for monocytes; figure 2).

CD11b activation/conformation

During receptor activation CD11b undergoes conformational change that can be reported using antibody CBRM1/5, which only binds the headpiece of CD11b in the active high affinity state.\(^{15}\) In 10 WT and 10 77H homozygous volunteers, we only observed (as expected) very low level CBRM1/5 epitope expression on unstimulated neutrophils and none on unstimulated monocytes (data not shown). PMA stimulation induced a dramatic increase in the expression of the CBRM1/5 epitope in both cell populations (figure 3), but with no genotype-specific

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Figure 1  Cell-surface expression of CD11b quantified by flow cytometry. Data are presented for neutrophils and monocytes in the resting state and after 10 min stimulation with 200 nM phorbol myristate acetate. Mean fluorescence intensity (MFI) is presented. There are no significant differences between groups. ICRF, anti-CD11b antibodies.

Figure 2  Relative expression of ITGAM mRNA in wild-type and heterozygous volunteers. There are no significant differences between groups. (A) Monocytes. (B) Peripheral blood mononuclear cells (PBMC).
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Phagocytosis
Phagocytic assay was performed using samples from seven WT and seven 77H homozygous volunteers. A mean of 258 (SD 164) macrophages per volunteer were counted, on a minimum of two coverslips. We observed no genotype-specific difference in the association of sRBCiC3b, but there was a significant reduction in phagocytosis by 77H cells quantified by phagocytic index (WT 129.3±25.1, 77H 91.6±18.8, p=0.012) or percentage phagocytosis (WT 38.9±4.6%, 77H 26.9±4.5%, p=0.005) (figure 4).

To confirm that it was the 77H variant specifically that impaired phagocytosis, we replicated this assay in COS7 cells transfected with WT or 77H variant ITGAM. In five independent experiments a mean of 91 (SD 39) COS7 cells was counted from a minimum of two coverslips. Reduced phagocytosis by CD11b (77H)-expressing cells was again seen (phagocytic index: WT 90.8±14.4, 77H 58.3±6.0, p=0.015; percentage phagocytosis: WT 18.7±0.6%, 77H 12.9±0.6%, p=0.004; figure 4). No phagocytosis was seen using sRBCIgM.

Adhesion
Using 11 fresh 77H monocyte samples and 11 WT samples we observed that 77H monocytes adhered less to iC3b (595 o.d: WT 0.192±0.014, 77H 0.145±0.014, p=0.014). We also saw lower adhesion to DC-SIGN (595 o.d: WT 0.18±0.014, 77H 0.14±0.011, p=0.017), fibrinogen (595 o.d: WT 0.18±0.015, 77H 0.14±0.012, p=0.012) and ICAM-1 (595 o.d: WT 0.175±0.015, 77H 0.142±0.009, p=0.04; figure 5). We saw no difference in adhesion to plates coated with anti-CD11b ICRF44. We saw no genotype-specific difference in the binding of fresh neutrophils (resting or PMA activated) to any ligand (data not shown).

Cytokine secretion
We measured the secretion of interleukin (IL)-1β, IL-6, tumour necrosis factor alpha (TNFα) and IL-10 from ex-vivo monocytes from 13 WT and 13 77H homozygous volunteers. CR3 stimulation was achieved using sRBCiC3b with sRBCIgM as control stimulation. Overnight stimulation of monocytes with sRBCiC3b alone triggered a minor rise in the secretion of IL-1β, IL-6 and TNFα that was less than 1% of the level observed following TLR7/8 stimulation. Overnight stimulation with sRBCIgM was not significantly different from that seen with sRBCIgM only.

To evaluate whether CR3 signalling inhibited TLR7/8 responses we compared R848 (TLR7/8 agonist) stimulated monocytes that had been preincubated with sRBCiC3b with monocytes that were preincubated with control sRBCIgM (figure 6). The overnight secretion of pro-inflammatory cytokines by WT monocytes was significantly lower in the presence of CR3 activation (mean reduction in cytokine (sRBCIgM/R848 stimulation minus sRBCiC3b/R848 stimulation): IL-1β −577±190 pg/ml, p=0.010; IL-6 −3230±1341 pg/ml, p=0.033; TNFα −2623±891 pg/ml, p=0.012). In 77H homozygous monocytes only a modest non-significant change was seen (IL-1β −130±100 pg/ml, p=0.219; IL-6 −167±570 pg/ml, p=0.774; TNFα −557±397 pg/ml, p=0.186). A direct comparison demonstrated significant genotype-specific differences in the absolute level of cytokine inhibition (IL-1β p=0.030; IL-6 p=0.029; TNFα p=0.027). No changes in IL-10 secretion were observed in either group.

DISCUSSION
We have presented an analysis of CD11b/CR3 expression and function in genotyped ex-vivo cells, demonstrating a range of impaired effector functions in cells carrying the rs1143679 polymorphism. We confirmed specific functionality of the encoded R77H variant by replicating impaired phagocytosis in genetically modified cell lines.
We observed no genotype-specific difference in the expression of CD11b by monocytes or neutrophils; an important observation when interpreting subsequent functional studies. Neither did we see genotype-specific differences in the expression of CD11b activation epitopes, even in the presence of PMA, which activates CR3 through an ‘inside-out’ signalling mechanism. We conclude that the R77H polymorphism does not impair CD11b inside-out signalling or the conformational changes required for receptor activation.

We observed a reduction in the phagocytosis of iC3b opsonised targets by ex-vivo monocyte-derived macrophages. This was observed despite the fact these cells may co-express complement receptor 4 (CR4), which could participate in phagocytosis and act as a source of random variability in this assay. We confirmed that impaired phagocytosis was an R77H-specific effect in variant-specific ITGAM-transfected COS7 cells, which lack phagocytic receptors but are functionally capable of phagocytosis when receptors are introduced. We also demonstrated a reduction in the adhesiveness of 77H monocytes, most notably to iC3b. Cell adhesion is not simply a function of the affinity of integrin to its ligand, but involves post-receptor-binding events that regulate receptor avidity and initiate cell spreading. Phagocytosis and adhesion can be considered different manifestations of the same underlying process. Taken together our results suggest defective ‘outside-in’ integrin signalling.

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**Figure 4** Phagocytosis of iC3b opsonised sheep erythrocytes (sRBC$_{iC3b}$) by ex-vivo monocyte-derived macrophages (A) and transfected COS7 cells (B). Significant differences in phagocytic index and percentage of phagocytosis are observed, but no significant difference in association index. WT, wild-type.

**Figure 5** Adhesion of ex-vivo monocytes to ligand-coated plates. Adhesion of 77H erythrocytes expressed as a percentage of the wild-type (WT) monocyte adhesion in paired assays. Significant genotype-specific differences in the adhesion to all four CR3 ligands was observed but no difference in adhesion to anti-CD11b (ICRF) antibodies.

**Figure 6** The reduction in Toll-like receptor (TLR)7/8-induced cytokine release seen when monocyte CR3 is pre-engaged using iC3b opsonised sheep erythrocytes (sRBC$_{iC3b}$). The response of wild-type (WT) (shaded) and 77H cells (unshaded) is shown with p values showing the significant difference in TLR7/8 response between CD11b genotypes. IL, interleukin; TNFα, tumour necrosis factor alpha.
Syk activation with subsequent degradation of TLR-induced one mechanism for the cross-inhibition of TLR signalling is by perhaps activation via the controversial IgM receptor.\(^29\) Proving this hypothesis that because of receptor redundancy a generalised defect in the SLE specificity of the \(rs1143679\) genetic association.\(^6\)

We demonstrated genotype-specific differences in monocyte cytokine responses, with signalling through CR3 inhibiting TLR7/8 mediated pro-inflammatory cytokine release. The current literature reports that certain anti-CD11b monoclonal antibodies, or soluble mediators (particularly\(\alpha\)CD28), induce pro-inflammatory cytokine release (including IL-1β or TNFα), either just with CR3 ligation, or as a synergistic effect with lipopolysaccharide (TLR4) activation.\(^23\) 24 On the other hand, stimulating monocytes with sRBC\(_{iC3b}\) has been reported to illicit a primarily anti-inflammatory response, with a downregulation of IL-12 and an upregulation of IL-10.\(^25\)–\(^27\) This paradox is resolved by suggesting that CR3 signalling is influenced by ligand avidity: tonic, high-avidity CR3 activation producing a transient rise in pro-inflammatory cytokine production followed by prolonged inhibition of interferon-\(\gamma\) and TLR2 and TLR4 signalling.\(^28\)

The approach we (and others) take of activating CR3 through sRBC\(_{iC3b}\) has advantages and disadvantages. The correctly orientated (thioester anchored) iC3b on sRBC is likely to trigger physiological CR3 avidity activation, but the stimulus is potentially contaminated with unconverted C3b, C1q and other factors carried over from human serum used during the opsonisation process. In addition, there is the probability of CR4 activation and an upregulation of IL-10.\(^25\)–\(^27\) This paradox is resolved by suggesting that CR3 signalling is influenced by ligand avidity: tonic, high-avidity CR3 activation producing a transient rise in pro-inflammatory cytokine production followed by prolonged inhibition of interferon-\(\gamma\) and TLR2 and TLR4 signalling.\(^28\)

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The cross-talk between CR3 and TLR has also been reported elsewhere. Activation of TLR2, TLR3 and TLR4 has been associated with a greater pro-inflammatory response in agammocyte mice and ex-vivo mouse macrophages than in WT mice or cells.\(^29\) To our knowledge we are the first to demonstrate that this inhibitory effect extends to TLR7/8. It has been proposed that one mechanism for the cross-inhibition of TLR signalling is by Syk activation with subsequent degradation of TLR-induced MyD88 and TRIF.\(^30\) As TLR7/8 also acts via the MyD88 pathway it seems intuitive that CR3 inhibits TLR7/8-induced cytokine release.

How could an under-functioning variant of CR3 increase susceptibility to SLE? Defective phagocytosis itself is clearly an important mechanism in SLE. Fc-receptor gene variants (including the monocyte/macrophage genes \(FCGR2A\) and \(FCGR3A\)) that impair ligand affinity and phagocytosis are associated with SLE, while defective complement-mediated phagocytosis is also recognised as a feature of this disease.\(^31\) 32 The ‘waste-disposal’ hypothesis proposes that impaired uptake of apoptotic cells is a key element of SLE pathogenesis, and defective uptake of apoptotic cells by lupus macrophages having been observed directly, but it is debatable whether CR3 plays a key role in this process.\(^33\)–\(^36\) Impaired clearance of iC3b-containing immune complexes is another attractive disease mechanism, because immune complex deposition underlies much of the tissue damage in SLE.\(^37\)–\(^39\)

Our finding that CR3 ligation inhibits TLR7/8 signalling in a genotype-dependent manner is particularly interesting in the context of SLE. TLR7/8 is activated by single-stranded RNA: a context of key lupus auto-antigens such as Sm and RNP. These, in addition to synthetic TLR7/8 agonists, can activate monocytes, myeloid and plasmacytoid dendritic cells and B cells.\(^38\) 39 It has been noted that many lupus auto-antigens co-ligate both Fc receptors and TLR7/8, potentially providing pro-inflammatory signals that initiate and perpetuate autoimmunity.\(^40\) It is possible that CR3, co-ligated in the context of iC3b-containing immune complexes, provides an important immunoregulatory ‘non-danger’ signal. In the presence of the T77H polymorphism this signal may be impaired.

We deliberately adopted a ‘broad-brush’ approach to demonstrate a range of T77H effects rather than focussing on any individual mechanism in depth. We focused on monocytes because of their frequency in peripheral blood (allowing a range of assays from a limited blood source) and because fresh monocytes express relatively low levels of CR4.\(^41\) We recognise, however, that other cell types may be of great relevance in SLE, in particular CR3-expressing dendritic cell or B-cell subsets. We assume the T77H CR3 is generally under-functioning in all these cell types, but this will require experimental confirmation.

**Contributors.** BR designed, performed and analysed the research and wrote the manuscript; BGF designed, performed and analysed the research and critically reviewed the manuscript; ALR performed the research; GT contributed to study design and performed the research; GS contributed to data interpretation and critically reviewed the manuscript; TS recruited and genotyped volunteers; TJV designed and interpreted the research and critically reviewed the manuscript.

**Acknowledgements** The authors acknowledge the support of Gail Clement, Karolina Zlobecka and Ayrun Nessa in assisting with the recruitment of volunteers and the technical support of Katrin Weiss.

**Funding** The Twins UK National Institute for Health Research (NIHR) biorepository is funded by the Wellcome Trust, Europeans Community’s Seventh Framework Programme (FP7/2007-2013) and ENGAGE project grant agreement (HEALTH-F4-2007-201413). The Department of Health via the NIHR comprehensive Biomedical Research Centre award to Guy’s and St Thomas’ NHS Foundation Trust in partnership with King’s College London provided support for the Twins UK cohort and funding for flow cytometry. Original genotyping was performed by the Wellcome Trust Sanger Institute, in support of the National Eye Institute via an NIH/CIDR genotyping project. All other costs were met by an Arthritis Research UK clinician scientist fellowship (18544) awarded to BF on April 18, 2022 by guest. Protected by copyright.
Basic and translational research

Correction notice This article has been corrected since it was published Online First.

REFERENCES
SUPPLEMENTARY METHODS

Quantitative RT-PCR primers

ITGAM
5′-TCCAAGAGAACGCAAGGGGCT-3′
5′-CAGGGACAGGCCCAAGGACA-3′;

RPL27 (housekeeping gene 1)
5′-ATCGCCAAGAGATCAAAGATAA-3′
5′-TCTGAAGACATCCTTATTGACG-3′

OAZ1 (housekeeping gene 2)
5′-GGATCCTCAATAGCCACTGC-3′
5′-TACAGCAGTGGAGGGAGACC-3′

GAPDH (housekeeping gene 3)
5′-CATGAGAAGTATGACAACAGCCT-3′
5′-AGTCCTTCCACGATACCAAAGT-3′

SRP14 (housekeeping gene 4)
5′-CAGATGGCTTATTCAAACCTCCT-3′
5′-ATGCCCTTTACTGTGCTGCT-3′.

ΔCt-levels were calculated in triplicates

Preparation of Opsonised Sheep Erythrocytes

Sheep erythrocytes (sRBCs) were washed and resuspended in Gelatin Veronal Buffer (Sigma) and coated in rabbit anti-sheep erythrocyte IgM (Cedarlane) for 1 hour on a rotator
wheel at room temperature. For the additional opsonisation with iC3b the IgM coated sRBC were further incubated in 10% C5-depleted human serum for 20 minutes at 37°C before washing and resuspending in serum-free RPMI medium. The presence of high concentration iC3b using this technique was confirmed by flow cytometry (using anti-human iC3b primary antibodies (AbD Serotec) – data not shown).

For COS7 phagocytic assay sRBC’s were opsonised with rabbit anti-sheep IgG fraction (Sigma). These had better secondary staining characteristics than the IgM antibody and could be used as COS7 cells do not express Fc-gamma receptors.

For cytokine studies sRBCs were prepared in an identical way to that described above except the rabbit anti-sRBC IgM was dialysed in endotoxin-free PBS and filter sterilised and sterile endotoxin PBS substituted for the gelatin veronal buffer.

**Differential Staining of Internalised and External sRBC’s in Phagocytic Assay**

Following incubation of phagocytes with opsonised sRBC, phagocytosis was halted in ice and an external stain using Alexa 488 conjugated goat anti-rabbit IgG secondary antibody was added for 7 minutes (also capable of binding the light chain of the IgM rabbit anti-sheep sRBC primary antibody). Cells were then fixed in 4% paraformaldehyde for 15 minutes, permeabilised in 0.2% Triton X-100, and rewashed before adding the internal stain (Alexa 555 conjugated goat anti-rabbit IgG secondary antibody + DAPI for nuclear localisation) for 45 minutes. Cells were then rewashed and mounted on glass slides using Mowiol resin).

**Quantification of Adherent Monocytes in Adhesion Assay**

Monocytes adhering to ligand coated flat bottomed 96-well plates were fixed for 20 minutes with 1% gluteraldehyde. Fixed cells were the stained with 0.05% crystal violet (in 20% methanol) and then solubilised with 1% SDS, before measuring absorbance at 595nm (Thermo Multiskan ascent). Preliminary titration (not shown) using firmly adherent COS7 cells to uncoated plastic wells demonstrated a linear relationship between 595nm absorbance and cell number over the range for values reported in this study so data is presented as a percentage difference in adhesion.