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14. ABSTRACT

Two single-nucleotide polymorphisms (SNPs) in *ITGAM*, the gene encoding CD11b, associate with the risk of having lupus. One of these SNPs causes a ^{77}Arg→^{77}His amino acid change in the extracellular domain of CD11b and the other SNP causes a^{1146}Pro→^{1146}Ser change in its cytoplasmic domain. The goal of this project is to understand how these SNP-associated amino acid changes might affect the function of CD11b and explain their association with lupus. Using Epstein-Barr virus (EBV) transformed B cells and B cells from SLE patients, we will test the hypothesis that the ^{77}His and ^{1146}Ser variants alter CD11b mediated intracellular signaling in B cells, thereby change CD11b membrane mobility, clustering, and cytoskeletal association in ways that impact B cell biology and foster the development of lupus. To date we have made 2 major and new findings: First, we found that the SNP variant ^{1146}Ser residue is a target for phosphorylation by at least two kinases (ERK and GSK3β). Second, the presence of the SNP variant ^{1146}Ser residue is associated with increased surface expression of CD11b by EBV-B cells. We expect these changes in CD11b will have a major impact on CD11b signaling in EBV-transformed and primary B cells, helping understand why this SNP is associated with the risk of developing lupus.

15. SUBJECT TERMS
Mac-1, CR3, β2-integrin, B cell, lymphocyte, autoimmunity, SLE, lupus erythematosus, polymorphism

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1. INTRODUCTION:

In different racial and ethnic groups two single-nucleotide polymorphisms (SNPs) in *ITGAM*, the gene that encodes the α_M chain (CD11b) of the $\alpha_M\beta_2$ integrin receptor called Mac-1 (CD11b/CD18; **Fig. 1**), have consistently been shown to be associated with systemic lupus erythematosus (SLE). One of these (SNP rs1143679) leads to a ^{77}Arg→^{77}His amino acid exchange in the extracellular ligand-binding domain of CD11b while the other (SNP rs1143678) causes a^{1146}Pro→^{1146}Ser exchange in its cytoplasmic signaling domain (**Fig. 1**). The goal of this project is to understand how these SNP-associated changes in CD11b might affect the function of CD11b and the Mac-1 receptor on B cells, thereby altering B cell biology in a way that drives autoimmunity and SLE risk. To pursue this goal we are probing the impact of the ^{77}His and ^{1146}Ser variants on CD11b/Mac-1 function using Epstein-Barr virus (EBV) transformed B cells and B cells isolated from SLE patients. We hypothesize that ^{77}His and ^{1146}Ser will alter CD11b-mediated intracellular signaling and change Mac-1 membrane mobility, clustering, and cytoskeletal association, thereby altering B cell biology and fostering SLE development. To test these hypotheses we are pursuing three specific aims:

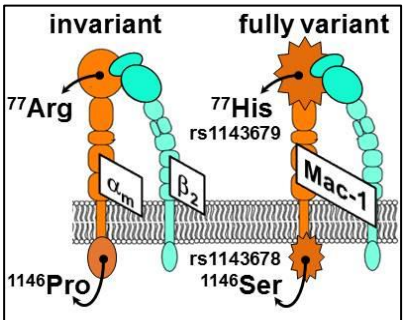

Figure 1. *ITGAM* encodes the α_M chain of Mac-1. We will study the impact on B cell functions of the SLE-associated extracellular ^{77}Arg→^{77}His exchange and the cytoplasmic ^{1146}Pro→^{1146}Ser exchange.

- Define the impact of the *ITGAM* ^{77}His and ^{1146}Ser variants on Mac-1 dependent signaling in B cells: Working hypothesis: ^{1146}Ser will change the extent and pattern of phosphorylation of the CD11b cytoplasmic tail and thus have a major impact on Mac-1 inside-out signaling (*i.e.* cell signaling needed to activate the Mac-1 receptor's ligand binding activity), whereas ^{77}His will affect outside-in signaling (*i.e.* cell signaling triggered by Mac-1 binding to ligand) and the downstream phosphorylation of signaling molecules. Approach: use EBV-transformed B cells from *ITGAM* genotyped healthy donors to assess the impact of ^{77}His and ^{1146}Ser on CD11b phosphorylation and downstream cell signaling after direct ligation of Mac-1 with ligands versus after indirect activation of Mac-1 via Toll-like receptor 4 (TLR4) and the B-cell receptor (BCR).
- Assess the impact of ^{77}His and ^{1146}Ser on Mac-1 cytoskeletal association, membrane mobility, and clustering on B cells: Working hypothesis: both SNP variants will perturb Mac-1 cytoskeletal associations, mobility, and clustering following Mac-1, TLR4, and BCR activation. Approach: by multispectral imaging flow cytometry (MIFC) we will compare the impact of *ITGAM* variants on Mac-1 cytoskeletal association, mobility, and clustering on EBV-B cells from *ITGAM* genotyped healthy donors.
- Confirm that *ITGAM* variation impacts Mac-1 biology in B cells from SLE patients: Working hypothesis: Mac-1 mediated functions of CD11b$^+$ B cells will be altered by the *ITGAM* variants in a manner consistent with their association to SLE. Approach: study peripheral blood B cells freshly isolated from healthy versus SLE affected donors with known *ITGAM* genotypes.

2. KEYWORDS:

Mac-1, CR3, β2-integrin, B cell, lymphocyte, autoimmunity, SLE, lupus erythematosus, polymorphism.

3. ACCOMPLISHMENTS:

The major goals of our project are presented in **Table 1**. During the most recent reporting period (15 September 2019 to 14 September 2019), despite some delays attributable to the ongoing COVID-19 pandemic (see part 5. CHANGES/PROBLEMS), we have made significant progress. Our accomplishments to date under each task and sub-task are described below. Accomplishments made between 15 September, 2019 to 14 September, 2020 are underlined.

- Subtask 1A: 100% complete. Our local IRB approval was renewed June 30, 2020.
- Subtask 1B: 25% complete. For our proposed studies EBV-B cells from donors with *ITGAM* invariant and 4 different *ITGAM* variant genotypes are needed (10 de-identified female healthy donors for each of the 5 genotypes). Although we have identified these EBV-B cells in our biobank we list this subtask as only 25% complete, since the viability of the frozen EBV-B cells can only be ascertained once they are retrieved, thawed, and expanded in cultures. So far we have tested specimens from 14 separate donors (8 invariant and 6 ^{1146}Ser variant) and all but 1 were expanded successfully. To expand B cells from frozen samples we thaw them quickly and propagate them for 7 days in RPMI 1640 + 10-15% FBS. By flow cytometry we

confirmed that the majority of cells (~90%) remain viable (live/dead staining shown in **Fig. 2A**). Our preliminary findings from two ^{1146}Pro and two ^{1146}Ser donors indicated that expression of CD11b (total surface CD11b probed using anti-CD11b antibody M1/70) is higher on EBV-B cells from patients with the ^{1146}Ser genotype (**Fig. 2, B and C**). Furthermore, heightened expression of CD11b was seen across all subpopulations of B cells with the ^{1146}Ser genotype (as assessed by staining for CD19 and CD27; **Fig. 2D**). These data validated that we can detect expression of CD11b by EBV-B cells and hinted at the possibility that the SLE-linked SNP rs1143678 alters expression of Mac-1 on B cells.

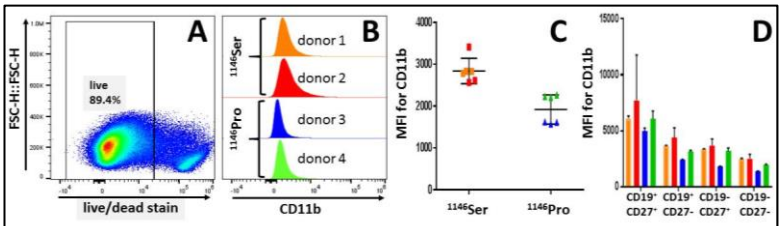

Figure 2. Viability of EBV-B cells is high (**A**) and they express different amounts of CD11b depending on their SNP rs1143678 genotype (**B-D** showing expression of CD11b for two 1146Pro (invariant) and two 1146Ser (variant) donors.

- During this reporting period we have studied additional specimens to validate that expression of CD11b is heightened on ^{1146}Ser variant CD19$^+$ EBV B-cells. Importantly, we discovered that the elevation of CD11b associated with ^{1146}Ser is accompanied by an ~15% increase in expression of CD24 (**Fig. 3A**). CD24 is known to be expressed at high levels by B-cell progenitors and mature resting B cells (CD24 is not expressed on plasma cells), so we sought to identify the B cell subpopulation(s) that might account for the overall increase in abundance of CD24 on CD19$^+$ EBV B-cells carrying SNP rs1143678. We found that the increased abundance of CD24 on ^{1146}Ser variant EBV B-cells is attributable to a significant increase in the number of CD19$^+$CD27$^+$CD24$^+$ memory B cells (Fig. 3B). **These new findings are the first to directly connect the SLE-linked SNP rs1143678 to a distortion in the proportions of B cell subsets;** an increased number of memory B cells might explain why SNP rs1143678 is associated with SLE.

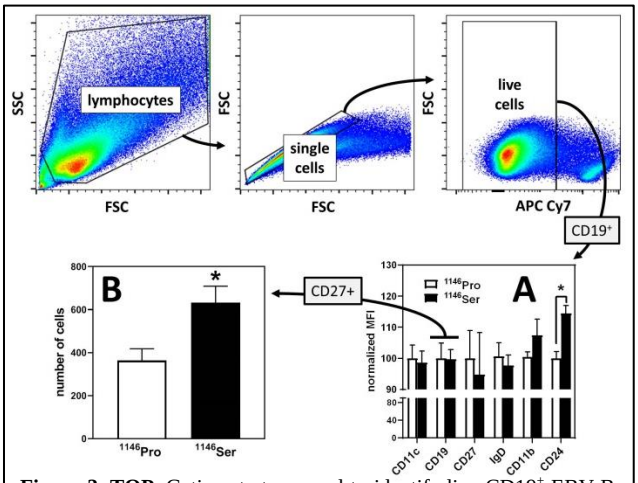

Figure 3. TOP. Gating strategy used to identify live CD19$^+$ EBV B-cells. **A.** Expression of B cell markers on CD19$^+$ EBV B cells from ^{1146}Pro (n=6) versus ^{1146}Ser donors (n=4). The mean + sem for mean fluorescence intensity (MFI) is normalized to ^{1146}Pro and the axis is broken. Asterisk indicates P=0.0109 for a 2-tailed 1-sample *t*-test. **B**. The number of CD19$^+$CD27$^+$CD24$^+$ memory B cells present in a sample of 10,000 CD19$^+$ B cells from the donors shown in panel **A**. The asterisk indicates P<0.05 for a 2-tailed 2-sample *t*-test.

- Subtask 1C: 100% complete.

- Subtask 1D: 20% complete. By testing combinations of various commercially available antibodies we found that the anti-CD11b antibody M1/70 that we use for flow cytometry (**Figs. 2 and 3**) could be used to capture and immunoprecipitate Mac-1 from B cell lysates, and that the anti-CD11b antibody 2LPM19C could subsequently be used to probe Western blots to detect the immunoprecipitated CD11b protein. For immunoprecipitation 1 x 10^7 EBV-B cells from an *ITGAM* homozygous (^{77}Pro/ ^{1146}Ser) donor were lysed in 500 µL of RIPA buffer containing protease and phosphatase inhibitors. Total protein in the resulting lysate was quantitated (BCA Protein Assay; Pierce) and 1 mg was pre-cleared by incubation overnight at room temperature with 20 µL of Protein A/G Magnetic Beads (Pierce). The beads were then removed by magnetic selection and the supernatant retained. The pre-cleared supernatant was then incubated sequentially with 10 µg of anti-CD11b clone M1/70 and 20 µL of fresh Protein A/G Magnetic Beads, each for 1 hr at room temperature. The beads were then removed by magnetic selection, gently washed, and the proteins retained by the beads eluted by exposure to low pH (Classic Magnetic IP/Co-Ip protocol; Pierce). Samples of the proteins eluted from the beads (30 µl) were subjected to non-reducing SDS-PAGE followed by electro-transfer to PVDF membranes, and the membranes were probed using 2LPM19C, anti-phospho-^{1142}Ser, and anti-phospho-^{1146}Ser. The results established that probing with 2LPM19C can detect both CD11b (band at ~175 *k*Da) and the intact Mac-1 heterodimer (CD11b/CD18 at ~220 *k*Da) (**Fig. 4;** leftmost strip). Utilizing our phospho-specific antibodies we confirmed previous reports that

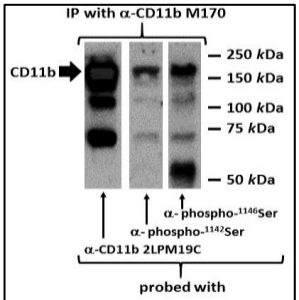

Figure 4. Western blots of EBV-B cell lysates from a donor expressing ^{1146}Ser. Immunoprecipitation (IP) was with antibody M1/70 and probing was with antibody 2LPM19C (detects CD11b), anti- phosphorylated ^{1142}Ser, or anti-phosphorylated ^{1146}Ser.

the invariant ^{1142}Ser is phosphorylated (**Fig. 4;** middle strip) and most importantly **we showed for the first time that the SLE-associated ^{1146}Ser residue is phosphorylated** (**Fig. 4;** rightmost membrane).

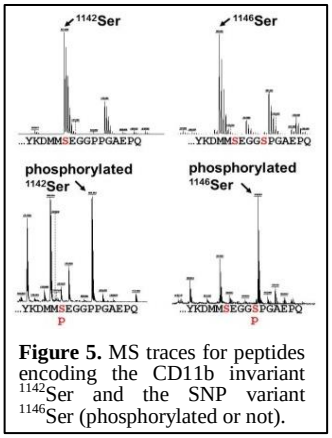

Figure 5. MS traces for peptides encoding the CD11b invariant ^{1142}Ser and the SNP variant ^{1146}Ser (phosphorylated or not).

- Subtask 1E: 25% complete. We subjected the synthetic CD11b peptides to tandem liquid chromatography–mass spectrometry (LC-MS) and confirmed we can detect the phosphorylated versus non-phosphorylated ^{1142}Ser and ^{1146}Ser residues (**Fig. 5**), and we can now immunoprecipitate Mac-1 from EBV-B cell lysates (**Fig. 4**). We excised the ~150-250 kDa range bands containing CD11b/Mac-1 from SDS-PAGE gels (see **Fig. 4**), subjected them to trypsin digestion, and ran the digest on LC-MS. By doing so we had hoped to confirm that the residues ^{1142}Ser and ^{1146}Ser in CD11b are phosphorylated in EBV B cells. To date we have been unable to detect phosphorylated ^{1142}Ser or ^{1146}Ser using LC-MS to analyze EBV-B cell lysates. Likely, this is because the amount of CD11b in the mixed protein lysate is insufficient for its identification by the MS associated peptide detection/identification software. We will hopefully be able to overcome this problem by further enriching the ~175 kDa proteins. **Despite this setback we have been able to detect many cytoskeletal/focal adhesion proteins whose abundance associates with *ITGAM* genotype (new findings described under subtasks 1G and 2J).**

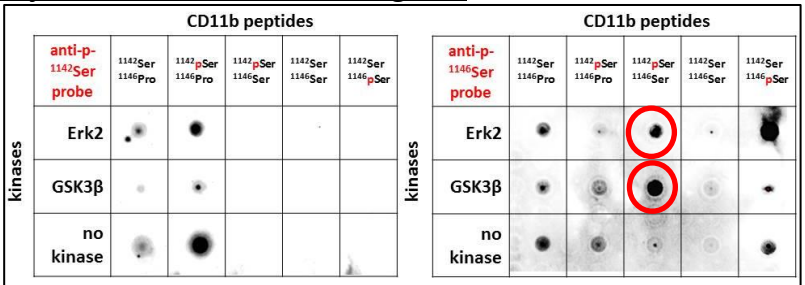

Figure 6. Synthetic peptides encoding the CD11b cytoplasmic tails invariant ^{1142}Ser and SNP variant ^{1146}Ser, phosphorylated or not, were incubated with Erk2 and GSK3β kinases and allowed to undergo *in vitro* phosphorylation reactions. The resultant peptides were blotted onto nitrocellulose membranes and probed with anti-phosphorylated ^{1142}Ser (left) or anti-phosphorylated ^{1146}Ser (right).

- Subtask 1F: 50% complete. We used our synthetic CD11b cytoplasmic tail peptides as substrates in both immunoblot and *in vitro* kinase assays. For immunoblots (**Fig. 6**) each peptide was co-incubated (24 hours at 37°C) with kinases (GSK-3β, Erk2, and CDK5/P25) in ATP containing buffer provided in the ADP-Glo kinase assay kit (Promega). After stopping the reaction and washing out excess substrates, the peptides were blotted onto nitrocellulose membrane and allowed to air dry. Each membrane was probed with either anti-phosphorylated ^{1142}Ser or anti-phosphorylated ^{1146}Ser for 2 hours, rinsed, and then incubated with secondary antibody for 1 hour. Immunoreactive peptides were illuminated using Pico West reagents and images captured and analyzed on a ChemiDoc Gel Imaging System (Bio-Rad). The results suggested that the **^{1146}Ser residue introduced by SNP rs1143678 can be phosphorylated (by ERK2 and GSK3β) but only when the invariant ^{1142}Ser is phosphorylated** (**Fig. 6**; red circle). For *in vitro* kinase assays (**Fig. 7**) the peptides were incubated with GSK-3β, Erk2, and CDK5/P25 for 2 hours at room temperature in ATP containing buffer as described for the immunoblots, and then luminescence was measured as a reporter of kinase activity. The results overall were not robust, but we reproducibly observed that GSK-3β phosphorylated the ^{1142}Ser/^{1146}p-Ser peptide (**Fig. 7**; blue bars) to a degree greater than ^{1142}Ser/^{1146}Ser peptide (**Fig. 7**; black bars). All kinases had greatly increased activity compared to negative control. **The combined results establish that GSK-3β can phosphorylate both the ^{1142}Ser and ^{1146}Ser residues.**

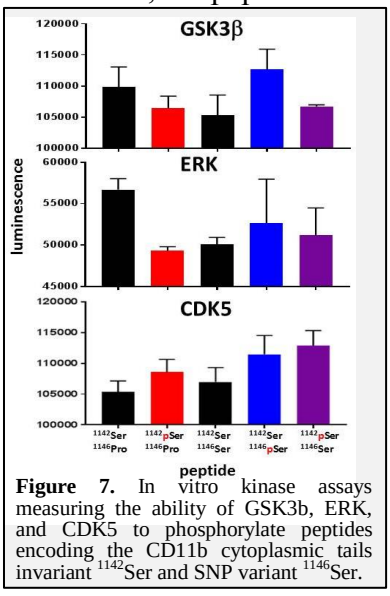

Figure 7. In vitro kinase assays measuring the ability of GSK3b, ERK, and CDK5 to phosphorylate peptides encoding the CD11b cytoplasmic tails invariant ^{1142}Ser and SNP variant ^{1146}Ser.

- Subtask 1G: 40% complete. The Comprehensive Flow Cytometry Facility (CFCC) houses the cytometry equipment we need to perform our proposed SCNP studies, but the CFCC stopped access to this equipment in order to comply with the 'social distancing' requirements put in place by our institute in response to the COVID-19 pandemic (see section 5. CHANGES/PROBLEMS). To sidestep this problem we used an alternative LC-MS-based proteomics approach and have made substantial progress on this subtask. Briefly, we immunoprecipitated Mac-1 from EBV-B cell lysates, isolated the CD11b/Mac-1 proteins by excising the bands that contained them from SDS-PAGE gels, subjected them to trypsin digestion, and performed LC-MS as described under subtasks 1D and 1E. The raw data obtained were subjected to analysis with Metacore

(Clarivate Analytics) to identify and quantify peptides/proteins and with GeneGo (Clarivate Analytics) to perform systems biology and pathway analyses. Thereby we were able to identify biological pathways/signaling networks that were over- or underrepresented in cells obtained from donors expressing the invariant ^{1146}Pro versus SNP variant ^{1146}Ser residues. **This unbiased approach provided new and compelling evidence that an interferon → JAK/STAT → antigen presentation pathway was over-represented in EBV B-cells from ^{1146}Ser donors (Fig. 8; top).** Since distortions in B-cell subsets and an 'interferon signature' have been observed in SLE patients, and since IFN-γ is a primary driver of the identified interferon → JAK/STAT → antigen presentation pathway, we tested whether ^{1146}Ser altered the expression of IFN-γ by EBV B-cells or changed their responsiveness to IFN-γ. We found that **EBV B-cells from donors expressing ^{1146}Ser have increased spontaneous secretion of IFNγ and increased expression of the IFNγR2 receptor Fig. 8; bottom). Since both B cells and IFNγ are known to play an important role in the pathogenesis of SLE, amplification of the interferon→JAK/STAT→antigen presentation pathway in individuals expressing ^{1146}Ser could explain their increased risk for progression to SLE.** In mouse models B cell-intrinsic IFNγ/STAT1 signaling has been shown to promote the formation of spontaneous autoimmune germinal centers, and antigen presentation by B cells has been shown to promote the development of SLE. Taken together our new findings indicate that the SLE-linked SNP rs1143678 (and the consequent ^{1146}Pro→^{1146}Ser exchange it causes in the cytoplasmic domain of CD11b) may lead to an increase in the antigen presenting capacity of B cells. This would drive both germinal center formation and autoantibody production and thereby increase the risk of lupus.

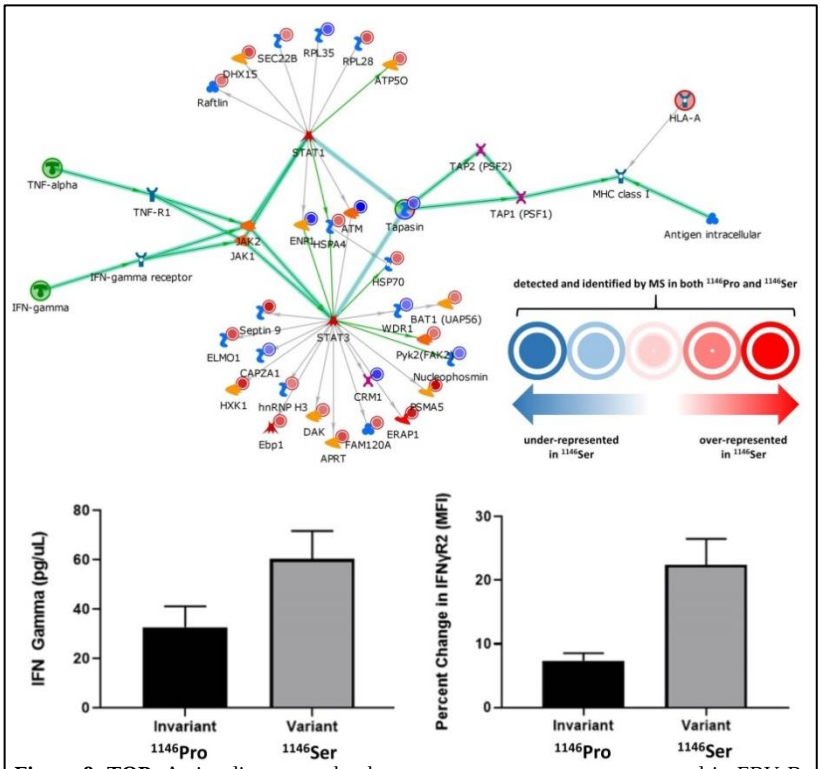

Figure 8. TOP. A signaling network whose components are over-represented in EBV B-cells from donors expressing the SNP variant ^{1146}Ser residue shows (from left to right) IFN-γ induced JAK/STAT activation culminating in antigen presentation. **BOTTOM.** EBV B-cells from donors expressing ^{1146}Ser have increased spontaneous secretion of IFNγ (**LEFT**) and increased expression of the IFNγR2 receptor after being placed into fresh medium and stimulated with 10 pg/ml exogenously administered IFNγ (**RIGHT**).

- Subtask 1H: 20% complete. In response to the COVID-19 pandemic the CFCC also limited our access to the imaging analyzer we need to perform multispectral imaging flow cytometry (MIFC) (see section 5. CHANGES/PROBLEMS for additional details). However as for subtask 1G, we used an alternative LC-MS-based proteomics approach and have made substantial progress on this subtask. Notably, our proteomics approach points to an over representation of cytoskeletal proteins participating in the actin remodeling complex in EBV B-cells expressing the ^{1146}Ser variant (data not shown). **This network is putatively involved in the formation of focal adhesions, which indirectly supports our hypothesis that ^{1146}Ser alters CD11b-mediated changes in Mac-1 membrane mobility, clustering, and cytoskeletal association. Interestingly, the ^{1146}Ser-associated changes in the cytoskeletal network predicted by our pathway analysis are quite similar to those seen in the autoimmune disease Wiskott-Aldrich syndrome (WAS), suggesting that a WAS-like pathway might be engaged in EBV-B cells with an ^{1146}Ser genotype. The implications of this finding are discussed further under Part 4.**
- Subtask 1I: 70% complete. We continue to work on a full-length manuscript describing the results shown in **Figures 1-8**. In the meantime an abstract on the same subject has been submitted and was accepted by the American College of Rheumatology for presentation at its annual meeting in November, 2020. See the appendix for a copy of the abstract.
- Subtask 2J and 2K: 20% complete. See Subtask 1H and section 5. CHANGES/PROBLEMS for additional details.

- Subtask 3L, 3M, 3N, and 3O: 0% complete. These subtasks will be pursued during the next reporting period.

- **This project continues to provide excellent opportunities for training and professional development.** Joseph Blake (PhD student) has been involved in all of the studies described above and he generated the bulk of the data, including the proteomics analyses. Joseph has recently taken a new undergraduate student under his wing, allowing him to hone his own mentorship skills.

- **How were the results disseminated to communities of interest?** An abstract describing the results shown in **Figures 1-8** has been accepted for presentation at the annual meeting of the American College of Rheumatology. See the appendix for a copy of the abstract.

- **What do you plan to do during the next reporting period to accomplish the goals?** To accomplish our goals and objectives during the next reporting period we will complete our ongoing investigations, submit at least one full-length manuscript and likely two, and pursue Task 3.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

- The major new discovery we have made is that a single amino acid change in a protein receptor that is known to be associated with the risk of lupus, causes changes in the biological function of the receptor. For the antibody producing B cells in your body, this amino acid change increases the amount of receptor expressed and alters how the receptor signals the antibody producing cell when it is activated. Our most recent findings suggest the overarching effect of the amino acid change is to shift B cells towards heightened IFNγ responsiveness and antigen presentation. This brings up the distinct possibility that the reason SNP rs1143678 is associated with lupus is because the resultant ^{1146}Ser change directly impacts CD11b and its operation in B cells. A novel observation is that it is the antigen-presenting function of B cells, and not their antibody producing ability, that seems to be affected most. This discovery is likely to make a major impact on our understanding of lupus and possibly point to new ways to treat this disease.

- **What was the impact on other disciplines?**

- CD11b is widely distributed on myeloid cells and B cell dysregulation is a characteristic of multiple autoimmune diseases, so our findings will likely have an impact also on infectious disease and other autoimmune diseases. So far the best example of this is Wiskott-Aldrich syndrome (WAS), a disease characterized by immune deficiency, eczema, and microthrombocytopenia leading to hypocoagulation. There is also an increased risk of inflammatory disorders in people with WAS and autoimmune diseases have been reported in 26 to 70 percent of WAS patients. These conditions have overlapping signs and symptoms with lupus and in fact WAS is used as an animal model for lupus in mice. The chance of developing lymphoma is also increased in people with WAS. Our MS analyses indicate a WAS-like pathway might be engaged in EBV-B cells with an ^{1146}Ser genotype.

- **What was the impact on technology transfer?**

- Nothing to Report.

- **What was the impact on society beyond science and technology?**

- Nothing to Report.

5. CHANGES/PROBLEMS:

- The major technical problem we have to overcome is our inability to detect phosphorylated ^{1142}Ser or ^{1146}Ser in EBV-B cell lysates using LC-MS. With refinement in technique we think this barrier will be overcome. An unforeseen problem was the COVID-19 pandemic; as a result of social distancing policies implemented by the University of Alabama at Birmingham (UAB), our research laboratory was shut for several months. Still now we are enduring 'limited operations'. Consequently (i) the Comprehensive Flow Cytometry Facility (CFCC) limits our access to the imaging analyzer we need to perform multispectral imaging flow cytometry, (ii) UAB continues to limit the amount of time Joseph Blake can spend on campus performing his thesis research, and (iii) UAB continues to limit the amount of time the PI can spend on campus. While this has had no effect on the direction of the project it has unfortunately slowed its tempo.

TABLE 1. Approved SOW

Task 1: Define the impact of the *ITGAM* ^{77}His and ^{1146}Ser variants on Mac-1 dependent signaling in B cells		
Subtask	**Details**	**Projected Timeline**
A: local IRB approval	Alexander J. Szalai, PhD and Jeffrey Edberg, PhD will ensure that all IRB requirements are met	achieved by month 3
B: identify EBV B cells from donors with different *ITGAM* genotypes	Jeffrey Edberg, PhD will identify EBV B cells representing the *ITGAM* invariant plus 4 *ITGAM* variant genotypes for study. Cells from 10 (de-identified) female healthy donors each.	identified by month 3
C: recruit a graduate student for this project	Alexander J. Szalai, PhD is part of the Immunology Admissions Committee and will thereby identify and mentor this student	identified by month 3
D: Western blots to detect phosphorylation of *ITGAM* residues ^{1142}Ser and ^{1146}Ser	Guided by Alexander J. Szalai, PhD the student will perform this task. Phosphorylated residues will be detected in EBV B cell lysates from *ITGAM* invariant and ^{1146}Ser (homozygous) donors. Cells from 3 donors of each genotype will be used	months 1-6
E: mass spectrometry to detect phosphorylation of *ITGAM* residues ^{1142}Ser and ^{1146}Ser	Guided by Alexander J. Szalai, PhD the student will generate B cell lysates as above and provide these to the Targeted Proteomic and Metabolomics Laboratory for trypsin digestion and mass spectrometry	months 1-6
F: *in vitro* phosphorylation studies	Guided by Chander Raman, PhD the student will use synthetic CD11b cytoplasmic tail peptides as substrates to test if purified GSK-3β (Abcam) phosphorylates ^{1142}Ser or ^{1146}Ser *in vitro* and if this is blocked by the selective GSK-3 inhibitor CHIR-98014 (Selleck)	months 3-9
G: signaling network analysis	Guided by Chander Raman, PhD the student will perform SCNP on EBV B cells as described in the Research Strategy	months 6-12
H: multispectral imaging flow cytometry of nuclear translocation and Mac-1 co-localization	Guided by Daniel Bullard, PhD the student will perform MIFC on EBV B cells as described in the Research Strategy	months 9-15
I: preparation of manuscript		months 9-12
Task 2: Assess the impact of ^{77}His and ^{1146}Ser on Mac-1 cytoskeletal association, membrane mobility, and clustering on B cells		
J: Mac-1 cytoskeletal association and clustering studies	Guided by Daniel Bullard, PhD the student will perform MIFC on EBV B cells as described in the Research Strategy	months 9-24
K: preparation of manuscript		months 18-24
Task 3: Confirm that *ITGAM* variation impacts Mac-1 biology in B cells from SLE patients		
L: identify donors with different *ITGAM* genotypes that are available for recall	Jeffrey Edberg, PhD will direct the UAB Participant & Clinical Interactions Resource of the UAB Center for Clinical and Translational Sciences to identify donors representing the *ITGAM* invariant plus 4 *ITGAM* variant genotypes for study.	months 18-35
M: recall donors with different *ITGAM* genotypes for phlebotomy	Jeffrey Edberg, PhD will direct the UAB Participant & Clinical Interactions Resource of the UAB Center for Clinical and Translational Sciences to recall donors for phlebotomy	months 18-35
N: isolation of primary B cells and performance of experiments	Guided by Alexander J. Szalai, PhD the student will isolate B cells and perform analysis as described in the Research Strategy	months 18-35
O: preparation of manuscript		months 30-36

6. PRODUCTS:

- **Publications, conference papers, and presentations.**

Journal publications.

Avery JT, TT Wright, RV Jimenez, Blake JL, BL Ruiz, TR Schoeb, AJ Szalai, and Daniel C. Bullard. Mice expressing the variant rs1143579 allele of ITGAM (CD11b) show impaired DC-mediated T cell proliferation. Mammalian Genome. 2019 Oct; 30(9-10):245-259. PMID: 31673770. This work made the cover of volume 30 and is relevant to the work being completed under this SOW, but was supported by other funding sources (National Institutes of Health grants 1R21AR069295 and 1R01DK099092). A copy of the published manuscript is included in the Appendix.

Other publications, conference papers, and presentations.

An SLE-linked ITGAM Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNγ Production and Antigen Presentation by B Cells. Joseph Blake, Jeffery Edberg, Alexander J Szalai, and James Mobley. American College of Rheumatology Annual Meeting, ACR Convergence 2020. November 5 – 9, 2020. A copy of the abstract is included in the Appendix.

- **Website(s) or other Internet site(s)**. Nothing to Report
- **Technologies or techniques**. Nothing to Report
- **Inventions, patent applications, and/or licenses**. Nothing to Report
- **Other Products.** Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	**Joseph L. Blake**
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	0000-0003-2298-2407
Nearest person month worked:	12 (includes remote work)
Contribution to Project:	Mr. Blake performed experiments that generated all of the data shown. His participation is beneficial to the overall aims of the study, and his participation will enhance/broaden his knowledge within the immunological research community. Joseph has already gained much experience working with the assays utilizing EBV B-cells.
Funding Support:	This award

Name:	**Alexander Szalai**
Project Role:	PI
Researcher Identifier (e.g.	0000-0001-6638-579X

ORCID ID):	
Nearest person month worked:	1.8
Contribution to Project:	All of the preliminary data described herein were generated under his direct project leadership. He provided scientific direction and oversight for all of the studies.
Funding Support:	NIH/NIAMS P50 AR060772 (PD/PI: Saag) *Investigations in Gout, Hyperuricemia, and ComorbidiTies (INSIGHT) Center of Research Translation* 09/20/2017 – 08/31/2022 (Co-Investigator: 0.6 CM)

Name:	**Jeff Edberg**
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-0248-7647
Nearest person month worked:	1
Contribution to Project:	Dr. Edberg identified the EBV-B cells from donors with specific *ITGAM* genotypes that we used.
Funding Support:	NIH/NCATS UL1 TR003096 (Kimberly) Center for Clinical and Translational Science (CCTS) 05/06/19 – 04/30/24 (Co-Investigator: 3.6 CM) Alabama Genomic Health Initiative (AGHI)No Number (Korf) 10/01/16 – 09/30/21 (Co-Leader of Data Management and Bio-banking Working Group: 2.4 CM)

Name:	**Dan Bullard**
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-3500-1057
Nearest person month worked:	1
Contribution to Project:	Dr. Bullard has extensive experience with the analyses of Mac-1 and its many ligands and as such, he participated in all aspects of the experiments performed by Joseph Blake.

Funding Support:	NIH/NIGMS 2 R25 GM086256 (Co-PIs: Bullard and Gavin) *UAB PREP Scholars Program* 04/1/18 - 03/31/23 (0.6 CM)

Name:	**Chander Raman**
Project Role:	Co-Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-7775-9988
Nearest person month worked:	1
Contribution to Project:	Dr. Raman's flow cytometry know-how and knowledge of GSK3β was used for the experiments described.
Funding Support:	DOD CDMRP W81XWH-16-1-0537 (PI: Bridges, SL Jr.): *The Role of the Interferon-Gamma-Jak/STAT Pathway in Rheumatoid Arthritis* 09/01/16 - 08/31/19 (Co-Investigator: 4.8 CM) NIH/NIAMS R01 AR071157 (PI: Yusuf): *Mechanisms Elicited by Type I Interferons in Cutaneous Photocarcinogenesis* 09/01/16 – 08/31/21 (Co-Investigator: 1.2 CM)

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - For **Dr. Szalai:** nothing to report.
  - For **Dr. Bullard:** nothing to report.
  - For **Dr. Edberg:** nothing to report.
  - For **Dr. Raman** the following grants is now in No Cost Extension:

    DOD CDMRP W81XWH-16-1-0537 (PI: Bridges, SL Jr.): *The Role of the Interferon-Gamma-Jak/STAT Pathway in Rheumatoid Arthritis*
- **What other organizations were involved as partners?** Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

- **QUAD CHARTS:** A copy of the Quad Chart is included in the APPENDICES.

9. APPENDICES:

- Copy of published manuscript: Avery JT, TT Wright, RV Jimenez, Blake JL, BL Ruiz, TR Schoeb, **AJ Szalai**, and Daniel C. Bullard. Mice expressing the variant rs1143579 allele of *ITGAM* (CD11b) show impaired DC-mediated T cell proliferation. *Mammalian Genome*. 2019.
- Copy of accepted abstract: An SLE-linked ITGAM Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNg Production and Antigen Presentation by B Cells. Joseph Blake, Jeffery Edberg, Alexander J Szalai, and James Mobley. ACR Convergence 2020. American College of Rheumatology Annual Meeting, November 5 – 9, 2020
- Copy of QUAD CHART:

Mice expressing the variant rs1143679 allele of ITGAM *(CD11b) show impaired DC-mediated T cell proliferation*

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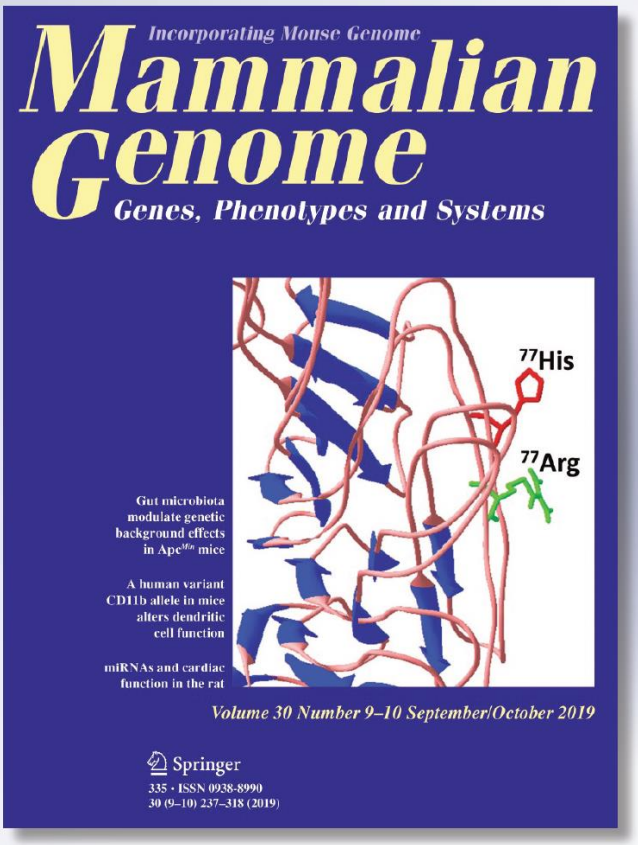

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Mice expressing the variant rs1143679 allele of *ITGAM* (CD11b) show impaired DC-mediated T cell proliferation

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Abstract

Genome-wide association studies (GWAS) and functional genomic analyses have implicated several *ITGAM* (CD11b) single-nucleotide polymorphisms (SNPs) in the development of SLE and other disorders. *ITGAM* encodes the α_M chain of the β_2 integrin Mac-1, a receptor that plays important roles in myeloid cell functions. The *ITGAM* SNP rs1143679, which results in an arginine to histidine change at amino acid position 77 of the CD11b protein, has been shown to reduce binding to several ligands and to alter Mac-1-mediated cellular response in vitro. Importantly, however, the potential contribution of this SNP variant to the initiation and/or progression of immune and inflammatory processes in vivo remains unexplored. Herein, we describe for the first time the generation and characterization of a mouse line expressing the 77His variant of CD11b. Surprisingly, we found that 77His did not significantly affect Mac-1-mediated leukocyte migration and activation as assessed using thioglycollate-induced peritonitis and LPS/TNF-α-induced dermal inflammation models. In contrast, expression of this variant did alter T cell immunity, as evidenced by significantly reduced proliferation of ovalbumin (OVA)-specific transgenic T cells in 77His mice immunized with OVA. Reduced antigen-specific T cell proliferation was also observed when either 77His splenic dendritic cells (DCs) or bone marrow-derived DCs were used as antigen-presenting cells (APCs). Although more work is necessary to determine how this alteration might influence the development of SLE or other diseases, these in vivo findings suggest that the 77His variant of CD11b can compromise the ability of DCs to induce antigen-driven T cell proliferation.

Introduction

Mac-1 (*ITGAM/ITGB2*, CD11b/CD18), a member of the β_2 integrin family of adhesion molecules, is expressed primarily by cells of the myeloid lineage (Tan 2012). ICAM-1, iC3b, RAGE, and CD40L are just a few of its more than 50 biologically important ligands (Rosetti and Mayadas 2016). This adhesion molecule plays an integral role in the recruitment and activation of neutrophils, monocytes, and macrophages during inflammation and participates in phagocytosis and cytokine production (Fagerholm et al. 2013; Herter and Zarbock 2013; Lim and Hotchin 2012). Additional evidence strongly suggests Mac-1 can modulate the immune response, either promoting or inhibiting it depending on the context (Ehirchiou et al. 2007; Han et al. 2010; Ren et al. 2004). For example, engagement of Mac-1 on certain subsets of dendritic cells (DCs) can, depending on the context, either amplify or restrict their ability to stimulate T cell proliferation (Behrens et al. 2007; Chen et al. 2008; Monrad and Kaplan 2007; Sandor et al. 2013; Schmidt et al. 2006; Skoberne et al. 2006; Varga et al. 2007). Thus, while splenic DCs isolated from *Itgam* null mice (mutants that lack expression of CD11b) showed an impaired ability to promote T cell proliferation (Ling et al. 2014), bone marrow-derived dendritic cells (BMDCs) from the same mutants had a decreased

Alexander J. Szalai and Daniel C. Bullard contributed equally to this work.

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capacity to suppress T cell proliferation (Bai et al. 2012). Likewise, loss or inhibition of Mac-1 has been reported to protect from or exacerbate inflammation and autoimmunity in different murine models, suggesting a complicated immunoregulatory role for this integrin in disease development (Bullard et al. 2005; Kevil et al. 2004; Leon et al. 2006; Soloviev et al. 2011; Stevanin et al. 2017).

Genome-wide association studies (GWAS) have now linked several *ITGAM* single-nucleotide polymorphisms (SNPs) with the risk and severity of disorders such as systemic lupus erythematosus (SLE), systemic sclerosis, and melanoma (Anaya et al. 2012; Hom et al. 2008; Lenci et al. 2012; Nath et al. 2008). One of these *ITGAM* variants, the non-synonymous SNP rs1143679 that results in a 77Arg → 77His change in the extracellular domain of human CD11b, has been shown to modulate certain Mac-1-mediated functions in vitro. For example, cell lines transfected to express human CD11b encoding the 77His variant showed impaired phagocytosis and adhesion and increased IL-6 generation compared to control cells expressing the invariant protein, which has an arginine at position 77 (MacPherson et al. 2011). In addition, primary cells (neutrophils and monocytes) from human donors expressing the 77His *ITGAM* variant exhibited reduced adhesion to iC3b, ICAM-1, and fibrinogen, decreased phagocytosis of opsonized particles, and altered cytokine expression (Fossati-Jimack et al. 2013; Reed et al. 2013; Rhodes et al. 2012; Rosetti et al. 2015; Zhou et al. 2013). Each of these findings has to be interpreted with some caution, however, because (i) in transfected cells expressing *ITGAM* variants, the function of CD11b ultimately depends on its ability to pair with CD18, whether expressed naturally or after co-transfection with *ITGB2*; and (ii) in primary cells, the potential impact of other *ITGAM* SNPs known to be in high linkage disequilibrium (LD) with rs1143679 is not always accounted for. Consequently, it might be that not all of the functional defects so far attributed to the rs1143679 variant are due to 77His per se. Indeed, our group showed that the SNP rs1143678, resulting in a proline to serine change at amino acid position 1146, compromises neutrophil adhesion and phagocytosis in the absence of the rs1143679 77His variant (Zhou et al. 2013). Lastly, in general, biologically meaningful effects might occur in vivo that might not be detectable in vitro and vice versa.

We took advantage of the high homology between human *ITGAM* and mouse *Itgam* to assess the impact of the rs1143679 77His variant on Mac-1-dependent processes in vivo. To accomplish this in complete isolation from other potentially confounding *ITGAM* variants in LD, we generated by gene targeting in embryonic stem cells mice expressing histidine at amino acid position 77 of the naturally expressed mouse CD11b protein. In contrast to prior reports of impaired ligand binding, adhesion, and migration of 77His transfected and primary cells in vitro, we found that compared to wild-type controls, mice expressing 77His showed no statistically significant changes in neutrophil and monocyte recruitment or the extent of tissue damage when subjected to acute inflammation models. However, expression of 77His in mice significantly diminished antigen-specific T cell proliferative responses. Importantly, this effect on T cells was also observed ex vivo in co-cultures using primary spleen DCs or bone marrow-derived DCs (BMDCs) from 77His mice as APCs. These are the first experiments to address the impact of 77His in vivo and they establish that expression of this variant form of CD11b can impair the ability of DCs to support a full T cell response in mice. Ongoing studies are aimed at identifying the specific Mac-1-dependent processes altered by 77His that might be responsible for this effect, and determining whether this effect is sufficient to alter the course of T cell-driven autoimmunity.

Materials and methods

Structural modeling of the human and murine CD11b proteins

To obtain evidence that the influence on CD11b of introducing the 77His variant should be comparable in the two species, and to thereby validate our genetic engineering approach, structural models of human and murine CD11b were rendered using the COACH meta-server for protein–ligand binding site prediction (Yang et al. 2013a, b). The respective invariant (77Arg in human, 77Pro in mouse) and variant (77His) CD11b structures were predicted using the multi-sources threader (MUSTER) algorithm (https://zhanglab.ccmb.med.umich.edu/MUSTER/) applied to the CD11c template 3k71G obtained from the protein data bank (PDB) library (https://www.wwpdb.org/). Each structure was then rendered and the invariant and variant versions aligned using the DeepView/Swiss PDBViewer v4.1.0 (https://spdbv.vital-it.ch/).

Generation of the 77His mouse line

The *Itgam* variant mouse line (C57BL/6 J) encoding histidine at amino acid position 77 (77His mice) was generated using an embryonic stem cell gene-targeting strategy as outlined in the *Results* section. Both conventional sequencing and pyrosequencing of genomic DNA isolated from tail biopsies were used to confirm the presence of the anticipated C to A nucleotide substitution in exon 3 of the targeted *Itgam* gene. Pyrosequencing was performed using the following primers: forward—GACAGGTGCCCTCTACCAGTG, reverse—TTGACAAGCCAGGGGTGTTCAC, and sequencing—TGAGACTCACCTTGCAG. To confirm the

C to A nucleotide exchange in *Itgam* transcripts, total RNA isolated from the spleens of heterozygous (CA) and homozygous (AA) mice was used as a template for the generation of cDNA (forward primer—TGGTCCAGCTTGGCGGAACC, reverse primer—CCTCTGGGAACTGCTGGGGC), cloned into the TOPOTA cloning vector (ThermoFisher Scientific), and then sequenced.

C57BL/6 J wild-type (WT) and flippase (FLP) mice (B6.129S4-Gt(ROSA)26Sortm1(FLP1)Dym/RainJ) were purchased from The Jackson Laboratory (Bar Harbor, ME). OT-II/CD45.1 mice were generated by crossing OT-II mice (B6.Cg-Tg[TcraTcrb]425Cbn/J) expressing a TCR-specific for ovalbumin (OVA) residues 323-339 [provided by Dr. Francis R. Carbone (Barnden et al. 1998)] with CD45.1 mice expressing the Ptprc^{a} allele on the C57BL/6 background (B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ; The Jackson Laboratory). Mice containing a CD11b null mutation were generated as previously described (Lu et al. 1997). This mutation was backcrossed for 11 generations onto the C57BL/6 J strain and homozygotes were generated by intercrossing. All animal procedures including euthanasia were approved by The University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Antibodies and flow cytometry

The following fluorochrome-conjugated anti-mouse antibodies (clones) were used: anti-CD11b (M1/70), -CD11c (N418), -CD45.1 (A20), -CD80 (16-10A1), -CD86 (GL-1), and -CD4 (RM4-5) all from BioLegend (San Diego, CA); anti-GR1 (RB6-8C5), -Ly6C (HK1.4), -CD3e (145-2C11), and -CD28 (37.51) all from eBioscience (San Diego, CA); and anti-MHC-II (M5/114.15.2) from BD Bioscience (San Jose, CA). LIVE/DEAD stain was used to determine the viability of cells and was purchased from Life Technologies (Eugene, OR). Flow cytometry was performed using an LSRII instrument and the acquired data were analyzed using FlowJo software.

Peritonitis model

Age- and sex-matched 77His and WT mice were injected i.p. with 1 mL of sterile 4% thioglycollate (T9032, Sigma Aldridge) or 1 mL of tissue culture grade PBS as a control. At 4, 24, or 48 h thereafter, mice were euthanized and 10 mL PBS was introduced into the peritoneal cavity. The resultant lavage fluid was collected and peritoneal exudate cells were harvested, counted, labeled using anti-CD11b, -Ly6c, and -GR1, and subjected to flow cytometry to enumerate neutrophils (CD11b^{+}/Ly6C^{+}/GR1^{high} cells) and monocytes/macrophages (CD11b^{+}/Ly6C$^{very\ high}$/GR1^{low} cells).

Dermal inflammation model

The dermal Shwartzman reaction was induced as described previously (Rothstein and Schreiber 1988). Briefly, age- and sex-matched 77His and WT mice received s.c. 100 µg of lipopolysaccharide (LPS from *Escherichia coli* serotype 055:B5; Sigma-Aldrich) in PBS (100 µl). Twenty-four hours later (day 1), recombinant TNF-α (0.3 µg/mouse; aa 80-235, R&D Systems) was injected s.c. at the same site. Control mice received two injections of PBS. Mice were euthanized 24 h later (day 2) and the skin at the injection site was excised, fixed in buffered 10% formalin/70% ethanol, and processed into paraffin. Thin sections (5 µm) were prepared and stained with hematoxylin and eosin (H&E) for microscopic evaluation, and neutrophilia, hemorrhage, and edema were then scored for their extent and severity in a blinded fashion. For each thin section inspected, the extent of each measure was scored 0 for absent, 1 for > 0–25%, 2 for 25–50%, 3 for 50–75%, and 4 for 75–100% affected. The severity of each measure was scored 1, 2, or 3 for mild, moderate, and severe, respectively, with increments of 0.5 for intermediate degrees (e.g., 2.5 for moderate to severe). The score was calculated as extent × severity. Scores for each section were averaged. Vasculitis was scored 1, 2, or 3 for mild, moderate, and severe, respectively, with increments of 0.5 for intermediate degrees for each affected vessel. The number of affected vessels having thrombi was counted. The *overall score* for each mouse was calculated as (neutrophil extent × severity) + (hemorrhage extent × severity) + (edema extent × severity) + sum of vasculitis scores + count of thrombi. Vasculitis severity also was assessed separately by averaging the individual vasculitis scores for each mouse.

In vivo T cell proliferation

OT-II/CD45.1 mice were euthanized and their spleens and lymph nodes collected. These were disrupted and after RBC lysis (Sigma-Aldrich, St. Louis, MO) CD4^{+} T cells were isolated by negative selection using the mouse CD4^{+} T cell isolation kit 19852A (Stemcell Technologies; Seattle, WA) and labeled with Celltrace CFSE (Invitrogen; Eugene, OR). 1×10^{7} CFSE-labeled T cells were injected into the tail vein of 77His or WT mice (day 0), and 24 h later (day 1), each mouse received i.v. 100 µg OVA (A-5503; Sigma Aldridge). On day 4, mice were euthanized and their spleens were harvested. Spleen cells were isolated and labeled with anti-CD4 and anti-CD45.1 antibodies, and CD4^{+}CD45.1^{+}CFSE^{+} OT-II T cells (events) identified by flow cytometry. To determine the percentage of T cells present in each proliferating generation, the following equation was used:

$$\frac{\text{Events in Generation} \times 2^{\text{Generation Number}}}{\sum \text{Transformed Events}}$$

In vitro T cell proliferation assays

Both splenic DCs ($CD11c^+$ cells) and bone marrow-derived dendritic cells (BMDCs) from 77His and WT mice were used as APCs for in vitro T cell proliferation assays. Splenic DCs were isolated from spleens by positive selection using the EasySep Mouse $CD11c^+$ Selection Kit II (18780A; Stemcell Technologies, Seattle, WA), and $CD4^+$ $CD45.1^+$ $CFSE^+$ OT-II T cells were isolated as described above. 2×10^6 CFSE-labeled T cells were co-cultured with 4×10^5 DCs (77His, WT, or CD11b−/−) in 96-well round-bottom plates in RPMI complete medium (RPMI 1640, 5% FBS, 2 mM L-GlutMAX, 50,000 U penicillin/streptomycin, 1% non-essential amino acids, 50 µM β-mercaptoethanol). Seventy-two or 120 h after supplementation with $OVA_{323–339}$ peptide (1.0 µM; New England Peptide, Gardner, MA), T cell proliferation (CFSE signal diminution) was assessed by flow cytometry and the percentage of T cells present in each proliferating generation was then determined using the formula described above for the in vivo T cell proliferation analyses.

BMDCs were generated as previously described (Jimenez et al. 2018). Briefly, bone marrow was flushed from tibias and femurs of 77His and WT mice and 1×10^6 cells were plated in 12-well flat-bottom plates and cultured in RPMI 1640 media supplemented with 5% FBS, 2 mM L-GlutaMAX, 50,000 U penicillin/streptomycin, 1% non-essential amino acids, 50 mM β-mercaptoethanol, and 20 ng/mL granulocyte–macrophage colony-stimulating factor (GM-CSF). The medium was changed on days 3 and 5. For antigen-specific T cell proliferation assays, BMDCs were stimulated/matured with 10 µg/mL LPS in the absence or presence of different concentrations of $OVA_{323–339}$ peptide (10, 20, 40, and 60 nM) on day 6. On day 7, BMDCs were harvested and used in co-culture assays with 2×10^5 CFSE-labeled $CD4^+CD45.1^+$ OT-II T cells. Cells were cultured at a 1:5 BMDC:T cell ratio in 96-well plates for 72 h, and T cell proliferation was measured by flow cytometry (CFSE diminution).

Analysis of costimulatory markers

WT and 77His mice were euthanized and their spleens were removed. After RBC lysis, spleen cells were incubated in medium containing RPMI 1640, 5% FBS, 2 mM L-GlutaMAX, 100 µg/mL pen/strep, 1X non-essential amino acids, and 55 µm β-mercaptoethanol (Fisher Scientific, Lenexa, KS). Cells were then treated with 1 µg/mL LPS or left untreated for 24 h, fixed in 4% paraformaldehyde, incubated with Fc block (BD Biosciences), and labeled with anti-CD11b, -CD40, -MHC-II, -CD80, -CD86, and -CD11c antibodies, and the expression of these markers were analyzed by flow cytometry.

Statistical analyses

Where appropriate, the data are presented as means ± their standard deviations. Differences between genotypes and treatments were assessed using Student's *t* tests or one-way ANOVA with post hoc Tukey's multiple comparisons tests. Non-parametric measures were assessed using the Mann–Whitney U test. Differences yielding a p value ≤ 0.05 were considered significant in all statistical analyses, which were performed using GraphPad Prism 7 software (Graphpad, La Jolla, CA).

Results

A predicted minor effect of the 77His substitution on the structure of human CD11b is reproduced in the structurally homologous mouse CD11b protein

Published studies using human leukocytes and transfected cell lines suggest that the 77His *ITGAM* variant significantly inhibits or alters Mac-1-dependent functions like adhesion, phagocytosis, and cytokine production (Fossati-Jimack et al. 2013; MacPherson et al. 2011; Maiti et al. 2014; Reed et al. 2013; Rhodes et al. 2012; Rosetti et al. 2012, 2015; Zhou et al. 2013). While these studies have been very informative, they cannot reveal how the 77His variant contributes to authentic immune and inflammatory processes since in vitro and ex vivo systems cannot recapitulate the different tissue microenvironments and biological stimuli encountered by leukocytes in vivo, nor can they effectively model all of the potential cellular interactions, trafficking patterns, and antigens present in the whole organism. To directly address this problem, we took advantage of the high homology between human *ITGAM* and mouse *Itgam* and developed a line of mice to investigate the in vivo impact of the rs1143679 variant allele on Mac-1-dependent responses. We first performed structural analyses to compare the predicted effects of the 77His substitutions on the conformation of the extracellular domain of the human and mouse CD11b proteins. Human *ITGAM* and mouse *Itgam* are highly homologous; they share 78.7% DNA sequence similarity and their encoded proteins have 74.8% direct amino acid identity. Importantly, this high homology is retained in the region that includes the 77His variant (Fig. 1; compare the sequences shown in panels A and A'). Protein modeling was used to render a predicted structure of the human and mouse CD11b β-propeller and

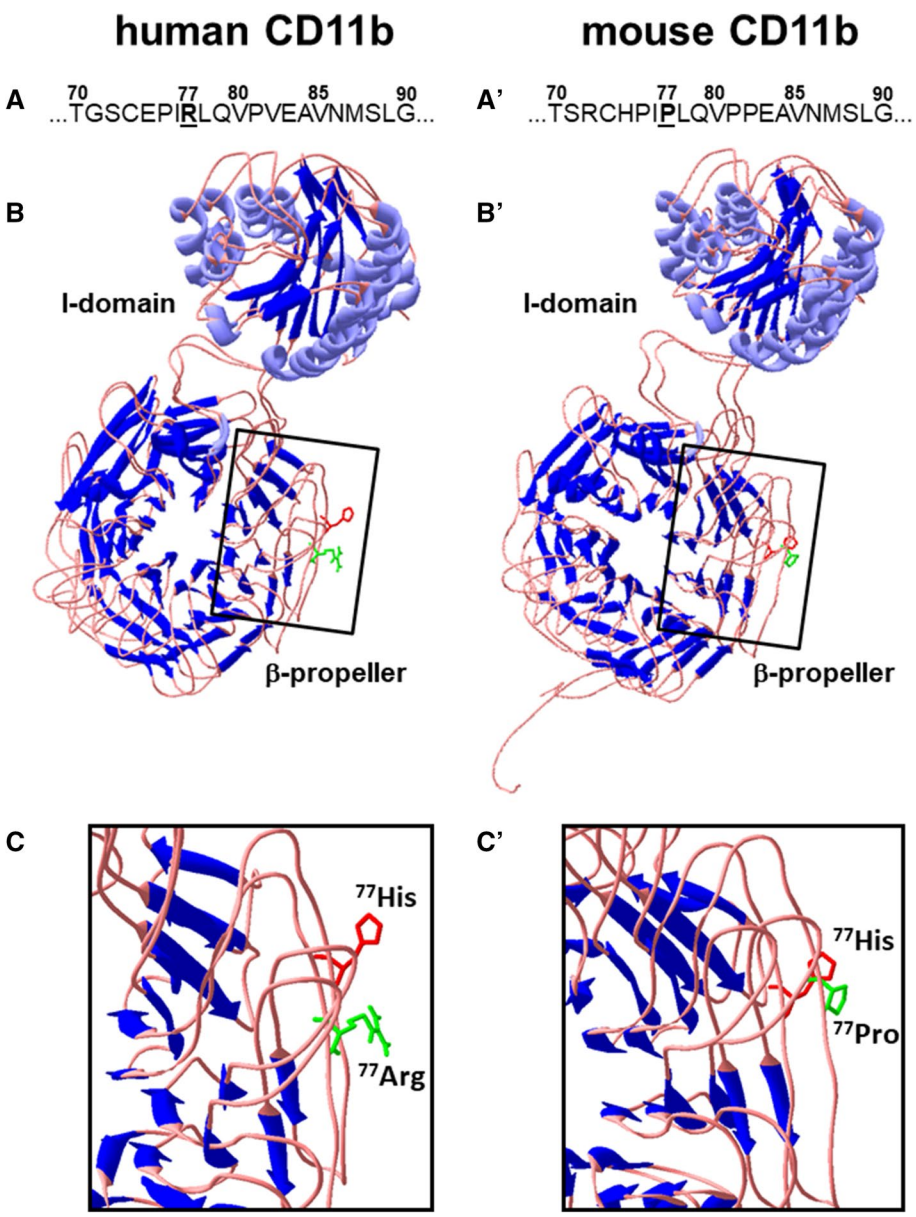

Fig. 1 Amino acid sequences and predicted structures of human and mouse CD11b and their respective 77His variants. **a** Amino acid sequence of the region of human CD11b adjacent to position 77 (the underlined bold residue). **b** The predicted structure of the human CD11b I-domain and β-propeller regions, with the predicted structures for the common 77Arg (green) and the rs1143679 SNP variant 77His (red) proteins overlaid. **c** The boxed area in B is expanded to show the relative positions of the human 77Arg versus 77His residues. **a′–c′** The wild-type (77Pro; green) versus the engineered (77His; red) versions of the analogous regions of mouse CD11b are shown. In all four situations, residue 77 is in an exposed position on the flank of the β-barrel, which might allow it to contribute to interactions with Mac-1 ligands. Tertiary structures were predicted with MUSTER using the 3k71G structure of CD11c as a template

I-domain regions (Fig. 1b and b′, respectively). Comparison of the predicted structures of the human invariant (77Arg) and variant (77His) proteins revealed only subtle changes (Fig. 1b and c). The predicted structure of the same region in mouse CD11b (Fig. 1b′) was very similar to that in human CD11b and, as for the human variant, introduction of a histidine at position 77 caused only subtle changes in mouse CD11b (Fig. 1c′). In all four situations, residue 77 is in an exposed position on the flank of the β-barrel, which might allow it to contribute to interactions with Mac-1 ligands.

Generation of the 77His mouse line

Our structural modeling suggested that the single 77Pro → 77His substitution in the mouse CD11b protein should not lead to any major tertiary structural changes that could potentially render Mac-1 incapable of interacting with its natural ligands. Accordingly, by gene targeting in embryonic stem cells, we generated mice expressing histidine at amino acid position 77 of the naturally expressed mouse CD11b. This strategy has the added advantage of allowing us to study the impact of 77His in complete isolation from other potentially confounding *ITGAM* variants known to be in high LD. We designed a replacement construct (Fig. 2a) to change the proline expressed at position 77

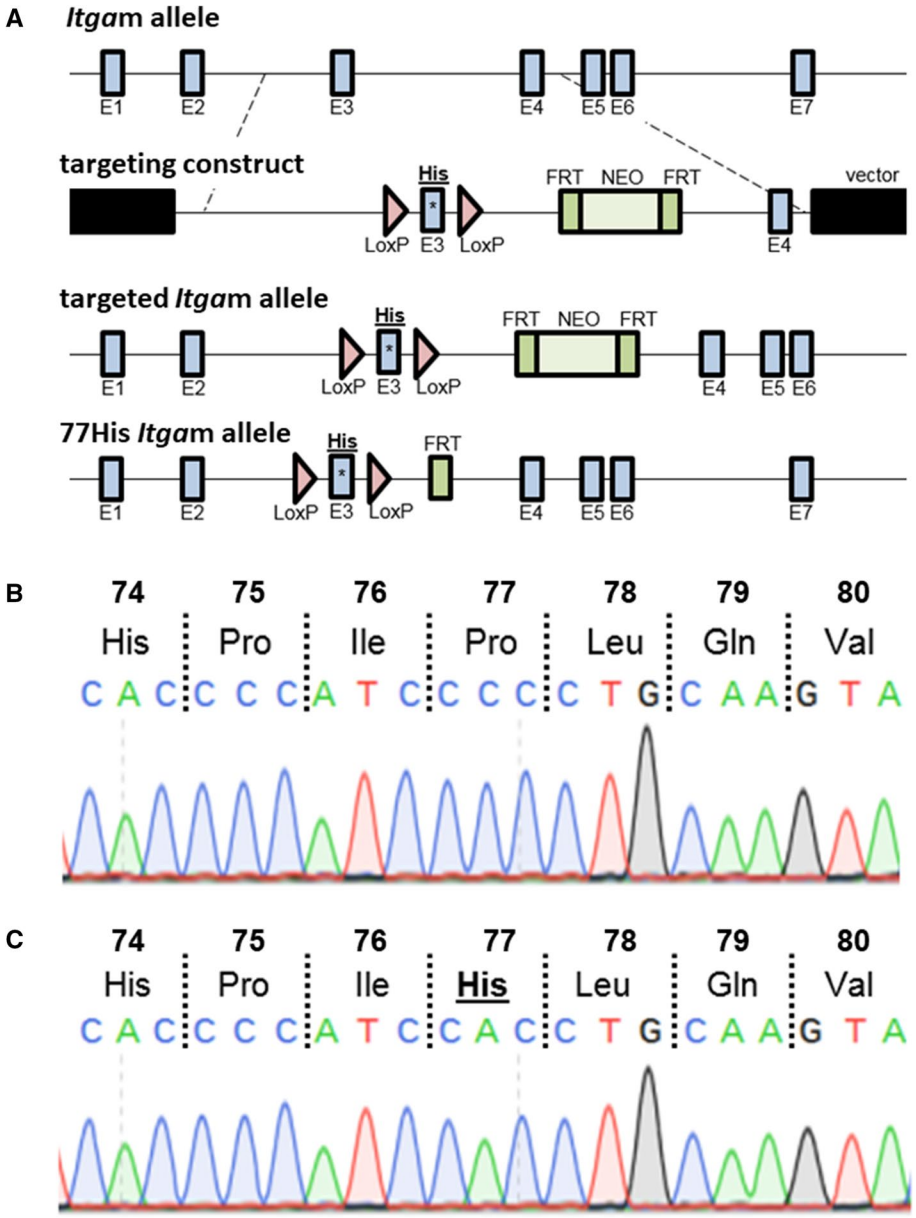

Fig. 2 Generation of the 77His variant C57BL/6 mouse. **a** The wild-type *Itgam* allele was targeted using a replacement construct that included *LoxP* sites to flank exon 3 (E3), wherein the 77His variant is encoded. The neomycin selection gene (NEO; located between E3 and E4 and flanked by FRT recognition sites) was removed by breeding founder mice to *FLP* recombinase-expressing mice. This yields the 77His *Itgam* allele. **b** DNA sequencing trace for a WT cDNA clone derived from the spleen of a 77His heterozygous mouse; note that Pro is encoded at position 77. **c** DNA sequencing trace for a variant cDNA clone derived from the spleen of a 77His homozygous mouse; note that His is encoded at position 77

in WT CD11b to a histidine, thereby mimicking the human rs1143679 SNP variant. In addition to the intended sequence change, the targeting construct included (i) flanking *FLP* recombinase recognition target (*FRT*) sites for subsequent removal of the *neomycin* selection gene and (ii) *LoxP* sites to generate a conditional *Itgam* allele for future studies. The targeting construct was introduced into C57BL/6 ES cells by electroporation, and correctly targeted clones (identified by PCR) were injected into blastocysts. After confirmation of germline transmission, mice with the correct nucleotide substitution (*i.e.,* C to A in codon 77 of the mature CD11b protein; see Fig. 2c) were identified by genomic sequencing of the exon 3 region. Heterozygotes were then crossed to mice expressing *FLP* recombinase. Their resulting 77His progeny, lacking the neomycin gene, were intercrossed to generate 77His homozygotes. We confirmed that the C to A nucleotide substitution was transcribed with high fidelity by sequencing cDNA clones generated from splenic mRNA of 77His heterozygous and homozygous mice (Fig. 2b and c, respectively), and we verified that Mac-1 expression on peripheral blood leukocytes and spleen cells from 77His mice was comparable to those from WT (Fig. 3a and b, respectively). To date, there has been no evidence of embryonic lethality or obvious visible phenotypes observed in the 77His mice.

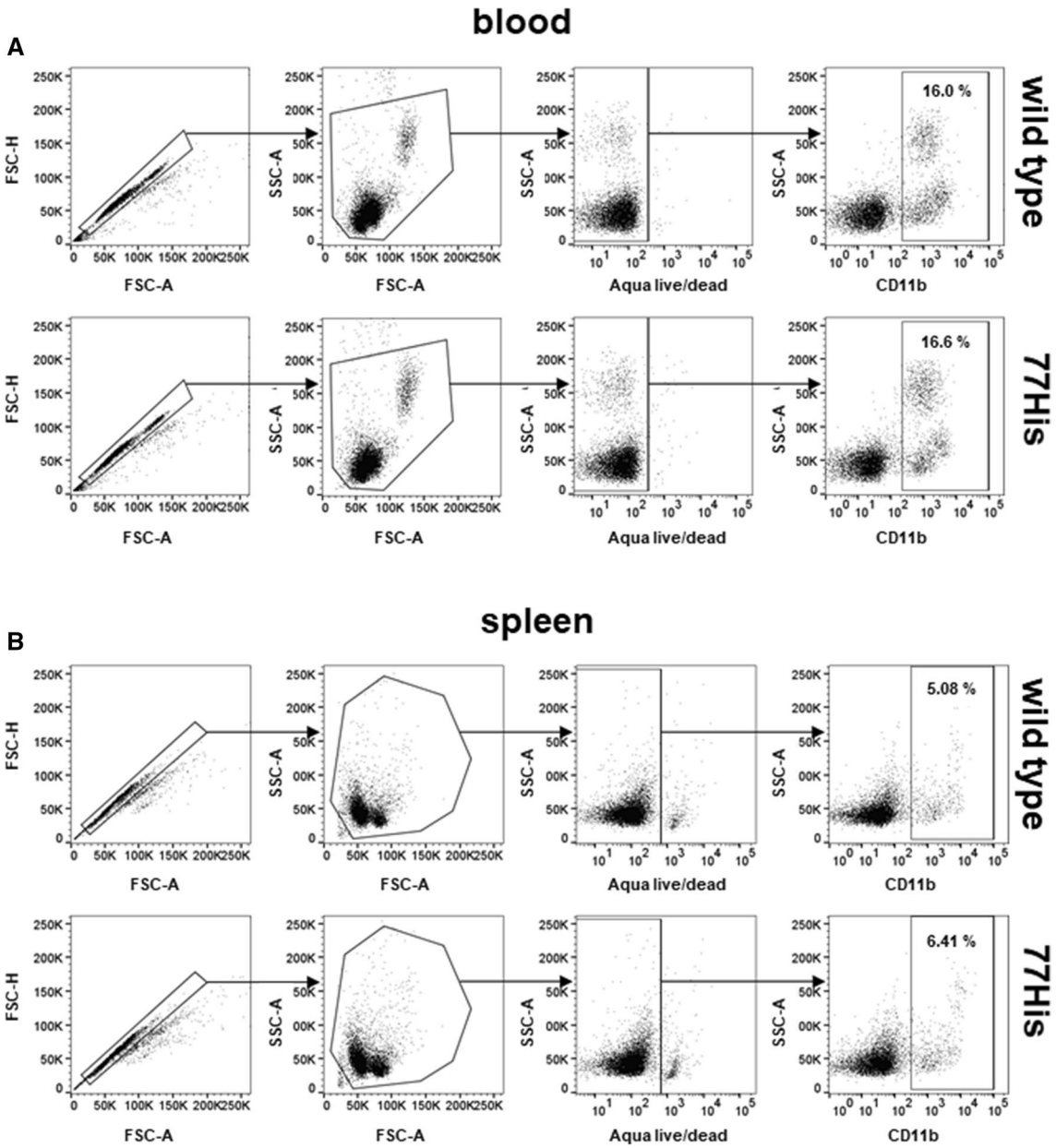

Fig. 3 Mac-1 expression by circulating and tissue resident myeloid cells in WT and 77His mice. Mac-1 expression was analyzed by flow cytometry of peripheral blood cells (**a**) and spleen cells (**b**) obtained from 77His and WT mice. In each instance, single cells were identified by FSC-A vs FSC-H, and cells of interest were gated by FSC-A vs SSC-A, and live cells (aqua Live/Dead negative) were analyzed. There was no difference in the proportion of $CD11b^+$ cells detected (percentages shown) or the level of CD11b expression (not shown) between genotypes

77His does not affect myeloid cell recruitment during localized inflammation

The importance of Mac-1 in the recruitment of neutrophils and monocytes to sites of inflammation has been well established (Coxon et al. 1996; Dirk Nolte et al. 1994; Jawhara et al. 2017; Mulligan et al. 1998; Tomasz Liberek et al. 2004), and ex vivo studies using human neutrophils and monocytes expressing 77His showed impairment of Mac-1 binding to ligands such as ICAM-1 (Rhodes et al. 2012; Rosetti et al. 2015; Zhou et al. 2013). To determine whether recruitment of neutrophils and monocytes in vivo was affected by 77His, we subjected mice to two different models of sterile localized inflammation. First, we induced peritonitis by injecting thioglycollate. Leukocytes were isolated from the peritoneum of 77His and WT mice by lavage at 4, 24, and 48 h, stained with antibodies for CD11b, Ly6c, and GR1, and neutrophils and monocytes

enumerated by flow cytometry as shown in Fig. 4a. 77His and WT mice were also injected with PBS as a control, which did not promote leukocyte recruitment into the peritoneal cavity at any of these time points (data not shown). We observed no significant differences in either the concentration or the frequency of either cell type recruited to the body cavity in 77His versus WT mice (Fig. 4b and c, respectively). We next compared the responses of 77His versus WT mice subjected to the Shwartzman reaction model of localized skin inflammation (Rothstein and Schreiber 1988). In this model, injection of LPS and TNF-α upregulates ICAM-1 and activates complement, thereby generating the Mac-1 ligand iC3b which, together with cytokine/chemokine production, promotes neutrophil recruitment to the site of injection. The recruited neutrophils subsequently degranulate, leading to fibrin deposition and hemorrhage (Hirahashi et al. 2006). Accordingly, 24 h after induction of the Shwartzman reaction, skin samples (Fig. 5a) were collected and neutrophil infiltration, fibrin deposition, hemorrhage/edema, and vasculitis were assessed. Although there was a distinct trend towards diminished overall scores for the Shwartzman reaction in 77His mice compared to WT (Fig. 5b), neither this difference nor the differences in each component of the overall score achieved statistical significance. The results of the two studies indicate that the 77His variant has little or

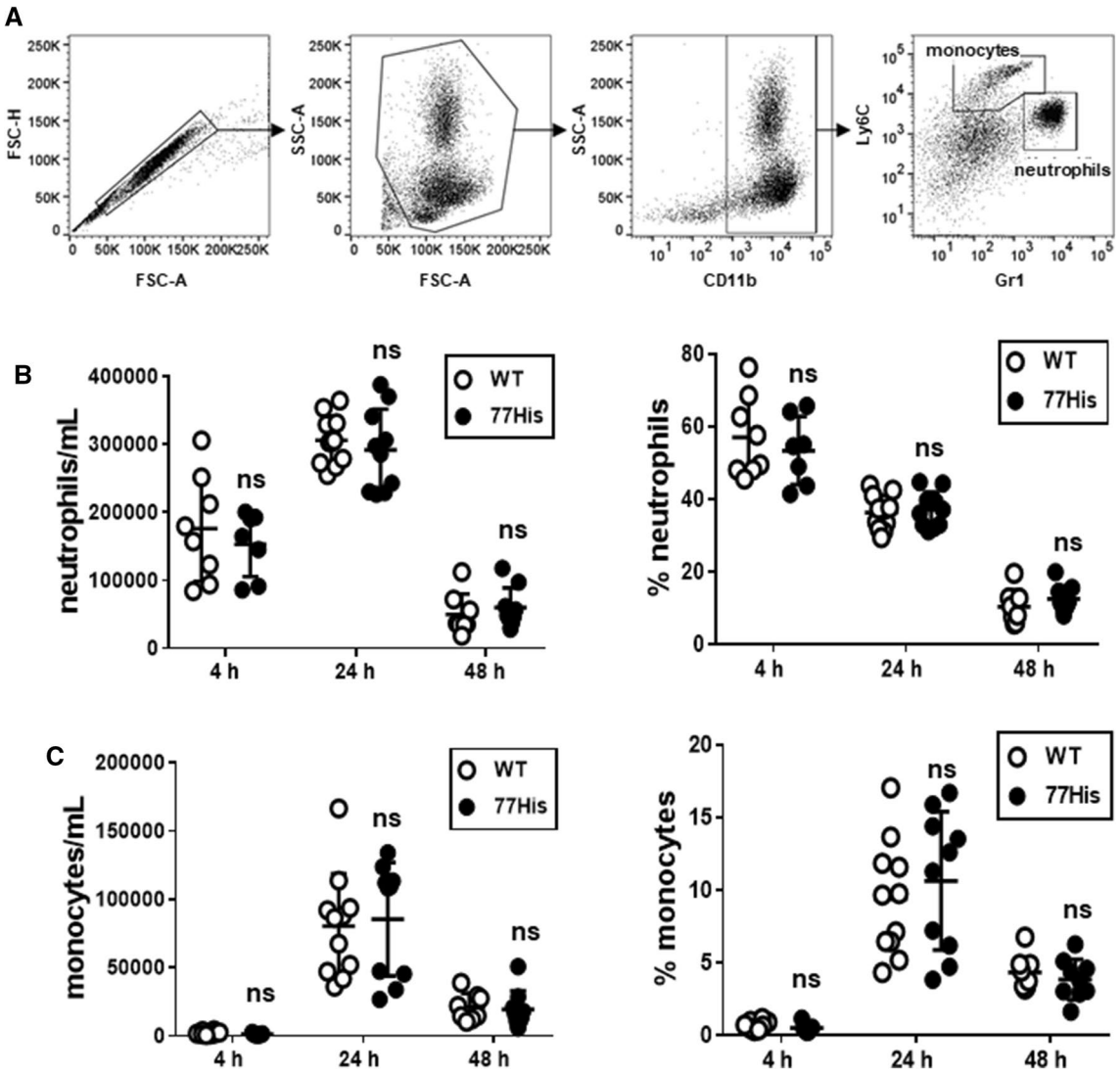

Fig. 4 No effect of the 77His variant on thioglycollate-induced peritonitis. **a** Four, 24, or 48 h after injection of thioglycollate i.p., the peritoneal cavity was lavaged and the fluid collected (see "Methods"). Monocytes ($CD11b^{+}$, $GR\text{-}1^{low}$, and $Ly\text{-}6c^{very\ high}$) and neutrophils ($CD11b^{+}$, $GR\text{-}1^{high}$, and $Ly\text{-}6c^{+}$) were enumerated by flow cytometry. The infiltration of the peritoneal cavity by neutrophils (**b**) and monocytes (**c**) was unaffected by 77His. The data shown are the mean ± standard deviation for $N = 7$–11 mice per time point per genotype. 'ns' indicates not significant ($p > 0.05$) for t tests comparing 77His to time-matched WT

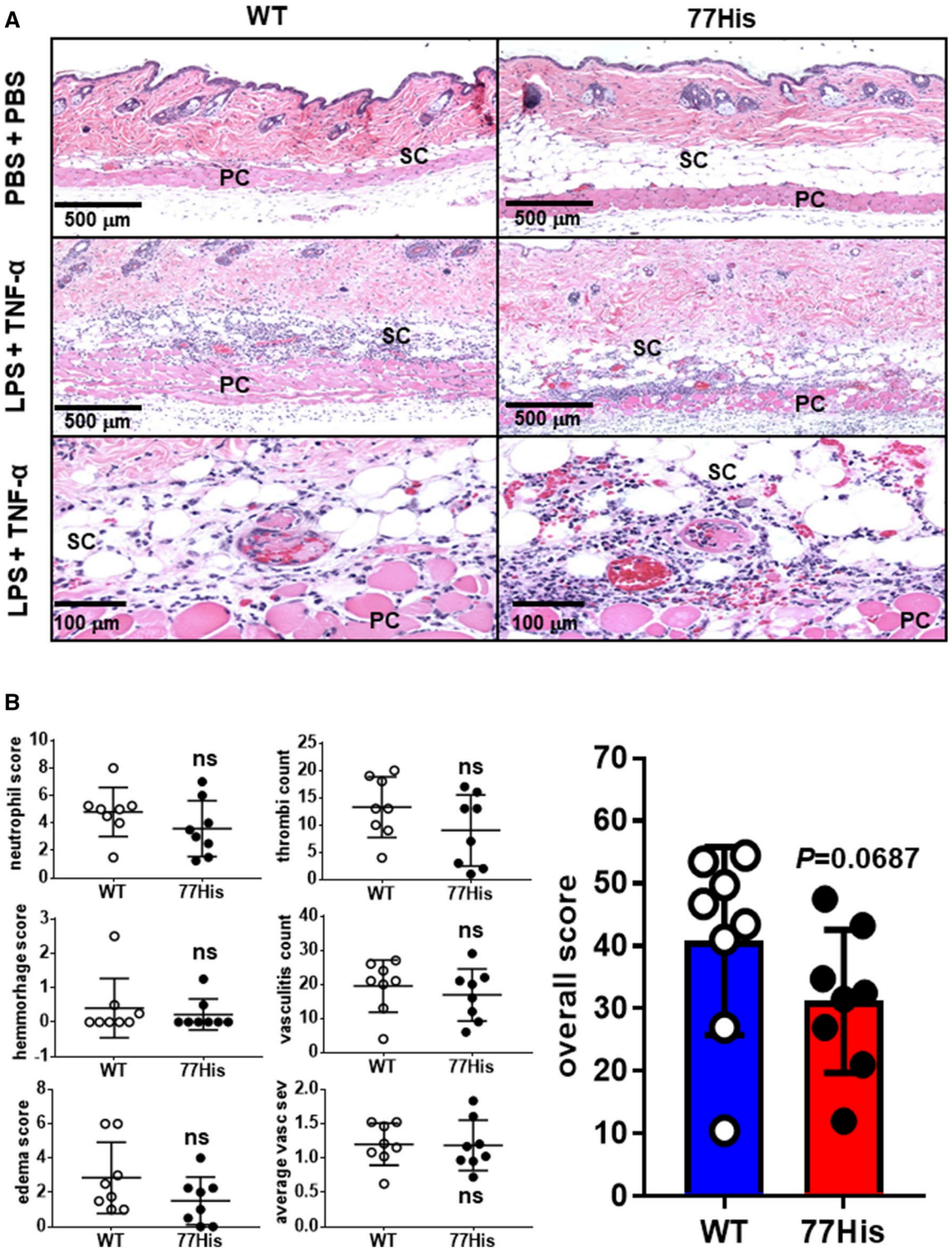

Fig. 5 Marginal effect of the 77His variant in the dermal Shwartzman reaction. **a** Mice were injected sequentially with LPS and (24 h later) TNF-α as described in the "Methods"; controls were twice injected with PBS. Twenty-four hours after the last injection, skin biopsies were collected, processed, and stained with H&E for assessment of tissue pathology. Representative thin sections (5 µm thick) from WT (left panels) and 77His mice (right panels) are shown. In LPS + TNF-α-treated mice of both genotypes, significant neutrophil recruitment in the subcutis (SC) and panniculus muscle (PC) was observed, as well as extensive vasculitis and thrombosis. **b** Scores for each individual component of dermal inflammation assessed (see "Methods") were not significantly different (ns) between 77His versus WT mice. The overall score for dermal inflammatory changes was reduced in 77His compared to wild type, but this difference also did not achieve statistical significance (the *p* value for a Mann–Whitney test is given). The mean ± standard deviation is shown for $N = 8$ mice per genotype

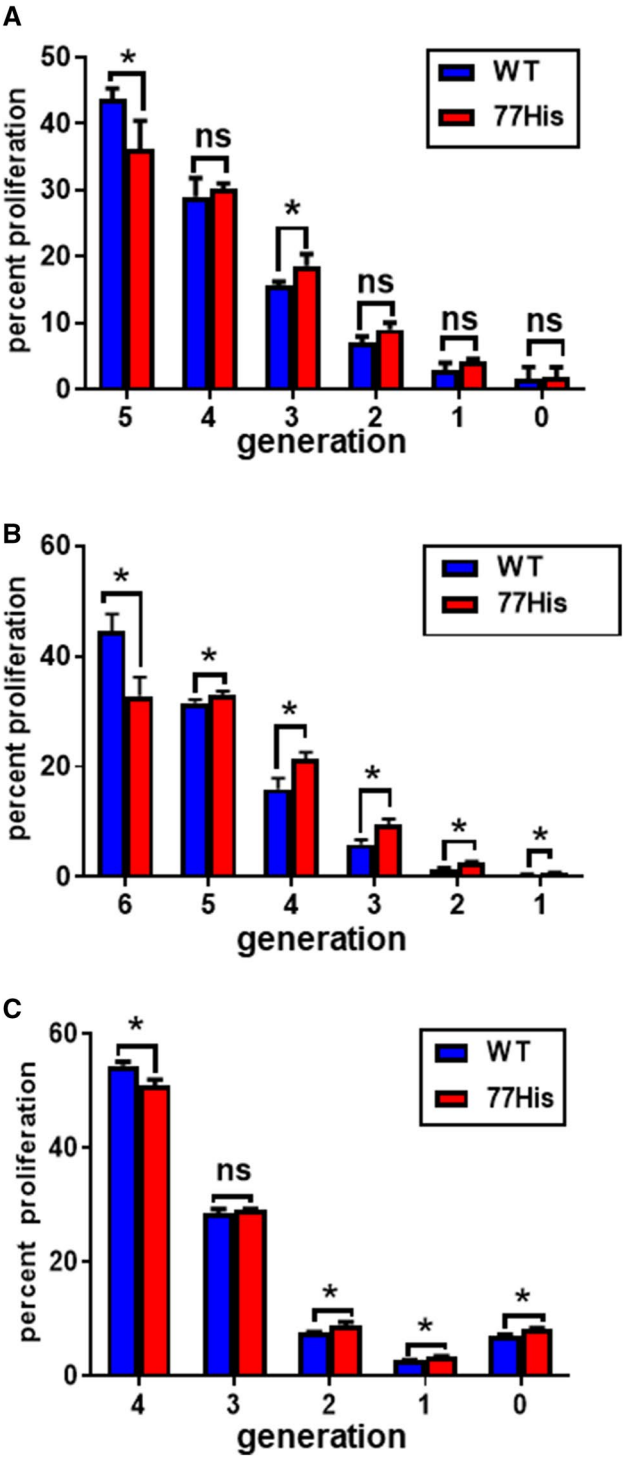

Fig. 6 77His compromises antigen-driven T cell proliferation in vivo ▶ in mice and in vitro in splenic DC/T cell cultures. **a** WT and 77His mice received i.v. $1x10^7$ CFSE-labeled $CD4^+CD45.1^+$ OT-II T cells, followed 24 h later by 100 µg OVA (i.v.). After 72 h, spleens were harvested and flow cytometry used to assess the proliferation of donor $CFSE^+$ T cells. T cell proliferation was reduced in 77His recipients, as indicated by a significant decrease in the percentage of T cells reaching generation 5. $N=3$ mice per genotype. Non-parametric Mann–Whitney U test; $p<0.05$ (*). **b** Dendritic cells were isolated from the spleens of 77His and WT mice (see "Methods") and used as APCs for CFSE-labeled $CD4^+CD45.1^+$ OT-II T cells. Co-cultures were provided with 1.0 µM $OVA_{323–339}$ peptide and T cell proliferation was assessed by flow cytometry after 120 h. T cell proliferation was reduced in co-cultures using 77His DCs compared to WT DCs at generation 6. The data shown are from a single in vitro trial, run in triplicate. Differences between genotypes were assessed using the non-parametric Mann–Whitney U test. Asterisks indicate $p<0.05$ (*). **c** Dendritic cells were isolated from the spleens of 77His and WT mice and used as APCs for CFSE-labeled $CD4^+CD45.1^+$ OT-II T cells as described for panel **b**, but T cell proliferation was assessed by flow cytometry after 72 h. T cell proliferation was reduced in co-cultures using 77His DCs, as indicated by a significant decrease in the proportion of T cells reaching generation 4. Similar results were observed across 3 separate experiments ($N=3$ mice per genotype for each experiment). Non-parametric Mann–Whitney U test; asterisks indicate $p<0.05$ (*). *Ns* not significant ($p>0.05$)

no impact on leukocyte recruitment after thioglycollate or LPS/TNF-α induced inflammation.

Dendritic cell function is impaired in 77His mice

Previous studies suggest an important role of Mac-1 in modulating the ability of DCs to promote/regulate T cell responses (Behrens et al. 2007; Chen et al. 2008; Monrad and Kaplan 2007; Sandor et al. 2013; Schmidt et al. 2006; Skoberne et al. 2006; Varga et al. 2007). To assess whether the 77His variant had any effect on antigen-specific T cell proliferation in vivo, 1×10^7 CFSE-labeled $CD4^+CD45.1^+CFSE^+$ OT-II T cells (donor cells) were administered i.v. to 77His and WT recipients. Twenty-four hours later, each mouse received i.v. 100 µg of OVA. After 72 h to allow for processing of OVA by recipient APCs and its presentation to donor OT-II T cells, spleens were harvested and the proliferation of the donor T cells was assessed. We reproducibly observed that OT-II T cells recovered from the spleens of 77His mice showed significantly reduced proliferation compared to OT-II T cells isolated from WT spleens (Fig. 6a). This effect was evidenced by a significant decrease in the percentage of OT-II T cells that had undergone five cell divisions in 77His compared to WT recipients. This result suggests that in vivo the single 77His change in CD11b is sufficient to compromise the ability of endogenous APCs to induce antigen-dependent proliferation of administered T cells.

To determine whether the observed reduction in donor T cell proliferation might be due to an effect of 77His on endogenous DCs per se, we isolated primary splenic DCs from untreated 77His and WT mice and used them as APCs in co-culture experiments (Fig. 6b). Co-cultures (splenic DCs plus CFSE-labeled $CD4^+CD45.1^+CFSE^+$ OT-II T cells) were provided with 1.0 µM $OVA_{323–339}$ peptide and T cell proliferation was assessed after 120 h. We found significant decreases in the percentage of OT-II T cells that had

undergone six cell divisions in 77His co-cultures compared to WT. A corresponding increase in the percentage of T cells was also observed at generations 1–5 in 77His co-cultures. We next investigated 77His and WT primary splenic DCs for their ability to promote antigen-specific T cell proliferation at a second time point (72 h of co-culture) (Fig. 6c). We observed a significant decrease in the proportion of OT-II T cells that had undergone 4 cell divisions in the presence of 77His DCs compared to WT. In addition, we saw a corresponding increase in the proportion of T cells that underwent 2 or fewer divisions in 77His co-cultures.

To further confirm these findings we next analyzed 77His BMDCs for their ability to promote antigen-specific T cell proliferation. LPS-stimulated 77His and WT BMDCs were incubated with varying concentrations of the OVA peptide for 24 h and then co-cultured with CFSE-labeled $CD4^+CD45.1^+$ OT-II T cells for 72 h (see "Materials and Methods"). In these assays too we observed an antigen concentration-dependent reduction in the number of proliferating T cells in co-cultures using 77His versus WT BMDCs (Fig. 7). The combined results show that the solitary 77His substitution in the CD11b protein is sufficient to compromise the ability of APCs in general, and DCs in particular, to promote antigen-dependent T cell proliferation in vivo and ex vivo.

One possible mechanism by which 77His leads to reduced T cell proliferation may involve altered expression of costimulatory molecules on DCs. Previously, ligation of Mac-1 has been shown to lead to elevated expression of MHC-II, CD86, and CD40 on DCs (Behrens et al. 2007). Thus, we next analyzed splenic DCs (defined as $CD11c^+$ cells) isolated from 77His and WT mice for the expression of MHC class II, CD40, CD80, and CD86. Expression of these markers was examined under both baseline conditions and after treatment with 1 µg/mL LPS for 24 h. We observed no significant differences in expression of MHC class II or any of these costimulatory molecules either at baseline or after LPS treatment (Fig. 8). Splenic $CD11c^+CD11b^+$ cells were also interrogated for expression of the same markers, but once again, no significant difference was identified between genotypes (data not shown).

Discussion

Genetic variants in many adhesion molecule genes have been shown to be highly associated with different inflammatory diseases (Anbarasan et al. 2015; de Lange et al. 2017; Raman et al. 2013). A significant challenge is deciphering whether any of these variants affect the expression, function, or activity of the encoded proteins and, if so, how that might contribute to increased disease susceptibility or severity of the associated diseases. GWAS have strongly

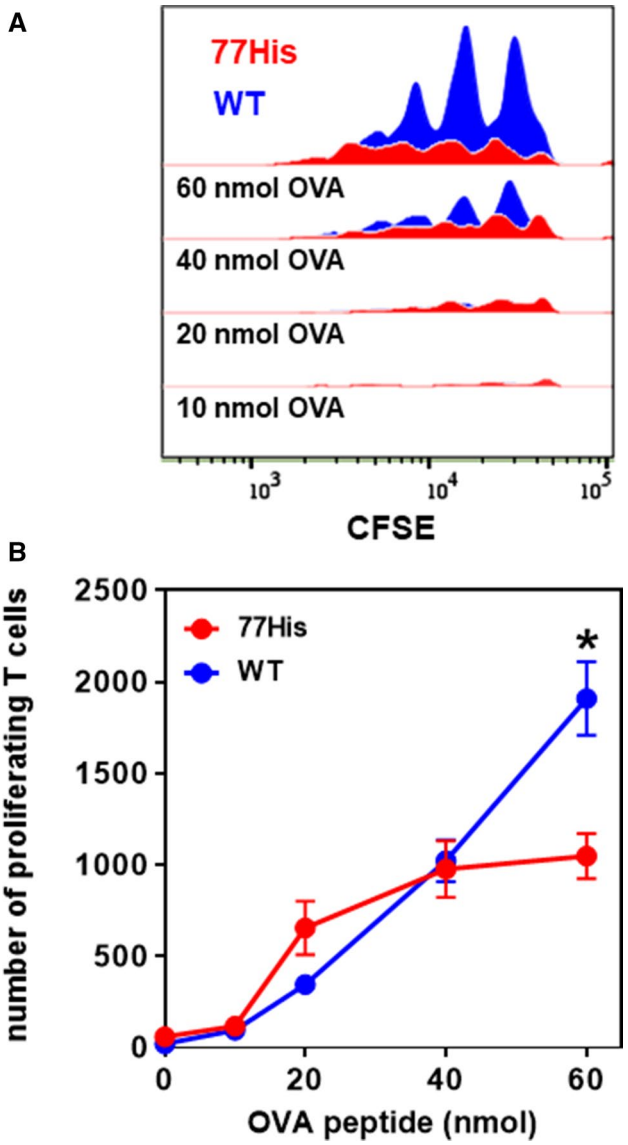

Fig. 7 Bone marrow-derived DCs (BMDCs) from 77His mice show impaired capacity to promote antigen-specific T cell proliferation. BMDCs generated from wild-type (WT) or 77His mice were used in co-culture assays to assess their capacity to support antigen-specific proliferation of OT-II cells. **a** Representative CFSE dilution histograms for proliferating $CD4^+$ T cells in co-cultures using WT 77His DCs in the presence of increasing amounts of OVA peptide. DCs were incubated with the indicated dose of OVA peptide for 24 h, rinsed, and co-cultured with $CFSE^+$ $CD4^+$ T cells. After 72 h of co-culture, T cell proliferation was assessed by flow cytometry (CFSE diminution). Note that the proliferation of T cells is reduced in the presence of 77His compared to WT BMDCs. **b** Pooled results (mean ± SEM for $N=2$ biological replicates with three technical replicates per dose of OVA) from experiments performed as in panel A. One-way ANOVA with post hoc Tukey's multiple comparisons tests; the asterisk indicates $p<0.0001$ for WT versus 77His at the indicated dose

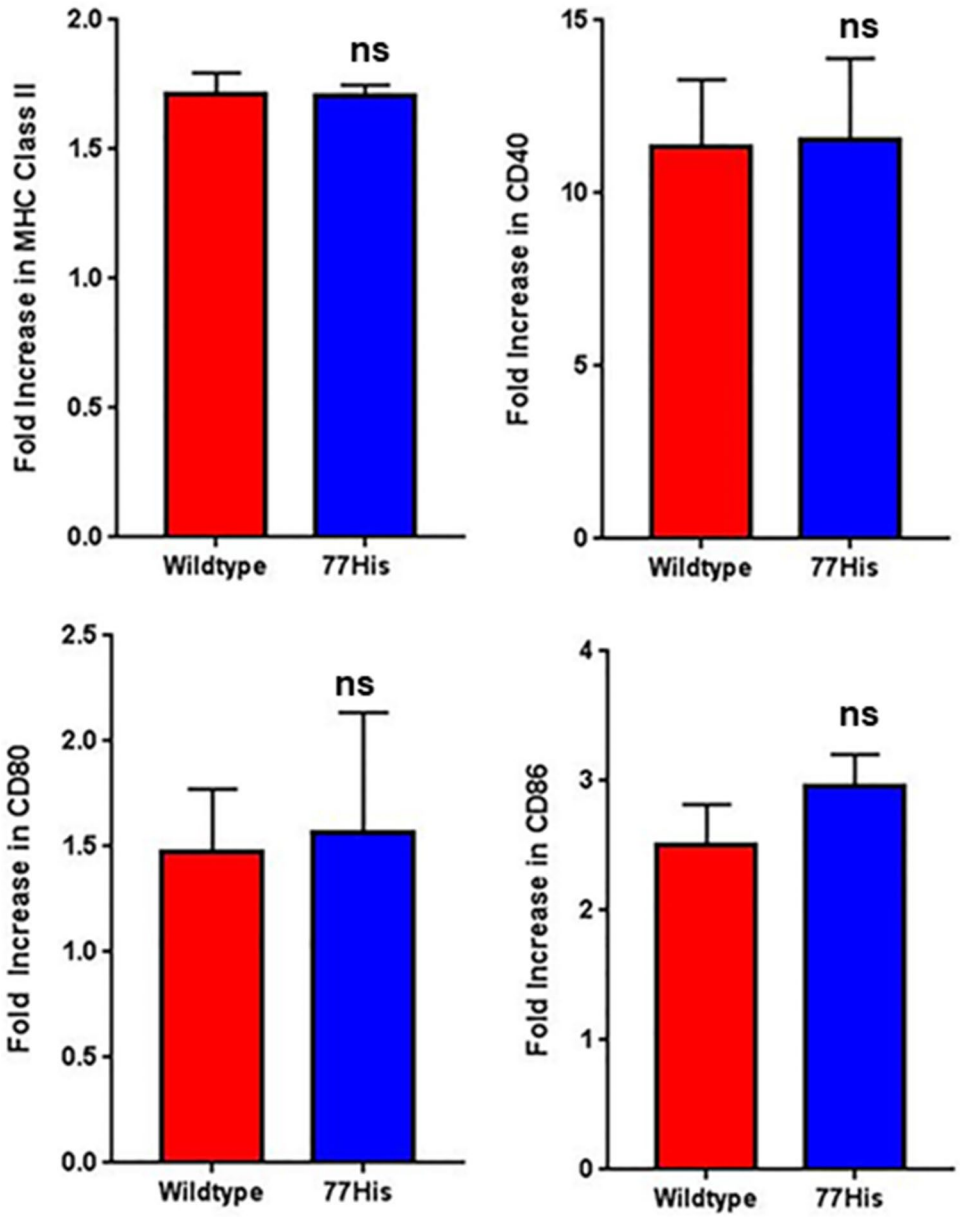

Fig. 8 No impact of 77His on splenic DC expression of MHC class II and costimulatory molecules. Splenic DCs ($CD11c^+$ cells) from 77His ($N=4$) and WT ($N=3$) mice were treated with 1 μg/mL LPS (serotype 055:B5) for 24 h or left untreated. The results are displayed as the fold increase in the expression of MHC class II, CD40, CD80, and CD86 (LPS/untreated)

implicated the SNP rs1143679 in the *ITGAM* gene, which results in an arginine to histidine change at position 77 in the extracellular domain of CD11b, with the risk of SLE, systemic sclerosis, and certain cancers (Anaya et al. 2012; Hom et al. 2008; Lenci et al. 2012; Nath et al. 2008). Functional genomic studies of transfected cell lines and primary human leukocytes have shown that the expression of 77His inhibits cell adhesion to substrates, their phagocytic capacity, and their cytokine regulation (Fossati-Jimack et al. 2013; Reed et al. 2013; Rhodes et al. 2012; Rosetti et al. 2015; Zhou et al. 2013). However, these model systems are all in vitro ones and thereby are unable to assess the effect 77His might have on the initiation and progression of immune and inflammatory processes. Further complicating the interpretation of the in vitro studies is the presence of additional *ITGAM* SNPs in high LD with rs1143679. For example, the variant allele for rs1143678, which encodes a serine at position 1146 in CD11b, has previously been shown to result in similar Mac-1-mediated defects in adhesion and phagocytosis to those reported for leukocytes expressing the 77His variant (Zhou et al. 2013). In many previous studies of 77His leukocytes, the presence/absence of 1146Ser was not specifically ascertained. Consequently, the alterations of cell functions observed in these in vitro investigations cannot be attributed to 77His per se with full confidence.

To sidestep these weaknesses, we report herein the generation of mice expressing a CD11b protein encoding histidine at amino acid position 77, thus mimicking the human

rs1143679 SNP variant. Granted mice are not humans, but despite this obvious drawback genetically engineered mice have been a powerful and useful tool for addressing phenotypic changes resulting from genetic alterations present in the human genome (Ernst 2016). In this study 77His mice were found to be viable and fertile with no obvious spontaneous phenotypes, and no difference in Mac-1 expression was observed on different leukocyte subsets from 77His mice compared to WT. We did not observe any significant effects of the 77His variant on outcomes in two different models of localized inflammation (peritonitis and the dermal Shwartzman reaction), even though previous studies of CD11b null mutant mice have revealed an important contribution of Mac-1 to leukocyte recruitment and the resultant tissue damage (Hirahashi et al. 2006; Jawhara et al. 2017). This suggests that the single 77His amino acid change is not sufficient to alter these underlying Mac-1-dependent processes in vivo, underscoring that the biology of the 77His variant is not necessarily the same as that of the null mutation.

It is possible that the 77His variant does affect Mac-1′s ability to promote leukocyte recruitment and activation in vivo, but this effect is masked due to redundancies in function with other adhesion molecules like LFA-1 (Ding et al. 1999). In addition, it is possible that the 77His variant per se may not specifically modify these inflammatory processes, but other *ITGAM* SNPS may play a role. As discussed above, rs1143678 is in high LD with rs1143679 and the amino acid change resulting from this SNP has been shown to decrease both neutrophil adhesion to purified ICAM-1 and firm adhesion to stimulated endothelial cells in vitro. The development of additional lines of *Itgam* SNP variant mice will be needed to determine the impact of other CD11b amino acid substitutions on Mac-1-dependent functions.

Mac-1 expression on certain DC subsets has been shown to regulate T cell proliferation (Bai et al. 2012; Behrens et al. 2007; Ling et al. 2014; Varga et al. 2007). For example, Ling et al. (2014) previously showed that this integrin is important in LPS-induced signaling by myeloid DCs and that loss of its expression by splenic DCs can reduce T cell proliferation in co-cultures. In the studies reported here, donor OT-II T cells administered to 77His recipients injected with OVA showed a significant reduction in proliferation when compared to OT-II T cells recovered from similarly treated WT mice. We also observed significantly diminished OT-II T cell proliferation in response to OVA peptide in co-culture experiments using 77His splenic DCs or BMDCs. This outcome was also observed for co-cultures using CD11b null mutant splenic DCs (data not shown). This suggests that in vivo the 77His variant is sufficient to compromise the ability of Mac-1 on DCs (and possibly other APCs) to promote antigen-specific T cell proliferation. Previous in vitro studies using cultured murine bone marrow-derived DCs (BMDCs) showed Mac-1-mediated inhibition of APC functions (Behrens et al. 2007; Ling et al. 2014; Varga et al. 2007), so we tested if the 77His variant also compromises the ability of LPS-stimulated BMDCs to restrict proliferation of T cells in the presence of anti-CD3 and anti-CD28 antibodies. BMDCs generated from 77His and WT mice expressed CD11b equally, and after maturation in response to LPS expressed equal amounts MHC-II (data not shown). In contrast to the results of our antigen-specific T cell proliferation experiments, the 77His variant did not significantly alter proliferation of CD3/CD28-stimulated $CD4^+$ T cells in co-culture assays with BMDCs (data not shown).

At this time, the mechanism by which this single amino acid substitution in murine CD11b leads to impaired antigen-specific T cell proliferation has not been identified. However this effect was not associated with diminished expression of MHC-II and costimulatory molecules by DCs, as was previously shown for cultured DCs from CD11b null mutant mice (Ling et al. 2014). It is possible that 77His compromises the ability of DCs to interact with one or more Mac-1 ligands, thereby altering DC intracellular signaling pathways and downstream expression of cytokines and chemokines involved in regulating T cell proliferation in vitro (Bai et al. 2012; Ling et al. 2014). Further investigations of the effects of the 77His variant on Mac-1-dependent inside-out and outside-in signaling pathways and cytokine expression in DCs are thus needed to determine whether they contribute mechanistically to the reduced T cell proliferation phenotype observed in these studies.

In summary, by substituting a proline at position 77 in the mouse CD11b protein with a histidine, we have developed and characterized a new line of mice expressing the human SNP rs1143679 variant allele. In two different acute inflammation models we detected no significant differences in leukocyte recruitment or tissue damage in 77His mice compared to WT, but we did observe (both in vivo and in vitro) that this variant inhibited the ability of APCs to promote antigen-specific T cell proliferation. Additional experiments using the 77His variant mice in other immune and inflammatory models are ongoing, and the results of those experiments will help determine the full impact of this solitary amino acid change in CD11b on Mac-1-dependent processes in vivo. Ultimately, it will be very informative to investigate whether 77His alters the development or progression of autoimmunity, which can be done by using various induced inflammatory disease models or by breeding 77His mice with SLE-prone strains of mice.

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Author contributions JTA is a primary author involved in the design, conception, and completion of all of the studies presented in the manuscript. RVJ was involved with DC cell culture and T cell proliferation experiments. JLB was involved with the BMDC:T cell proliferation experiments. TTW was involved with DC cell culture and analysis of costimulatory marker expression. BL was involved in T cell proliferation conception and design. TRS was involved in histological analysis. AJS was involved in study conception and design. DCB was the principal investigator, corresponding author, involved in study conception and design.

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Compliance with ethical standards

Conflict of interest The authors declare that the research presented was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal procedures including euthanasia were approved by The University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Research involving human and animal participants This article does not contain any studies with human participants.

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From: abstractsoutbox@rheumatology.org <abstractsoutbox@rheumatology.org>
Sent: Friday, September 11, 2020 3:00 PM
To: Blake, Joseph L <jblake16@uab.edu>
Cc: Szalai, Alexander J <aszalai@uabmc.edu>; Edberg, Jeffrey C <jeffreyedberg@uabmc.edu>; Mobley, James A (Campus) <mobleyja@uab.edu>
Subject: ACR Convergence 2020 Abstract Notification of Acceptance

Dear Joseph Blake,
We are pleased to inform you that the Abstract Selection Committee has accepted your abstract titled "An SLE-linked *ITGAM* Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNg Production and Antigen Presentation by B Cells" for presentation at the ACR Convergence 2020, to be held November 5 - 9.
ABSTRACT ACCEPTANCE INFORMATION
Presentation Type: **Poster**
Session: SLE â€" Etiology & Pathogenesis Poster
Date: Friday, November 6
Presentation Time: 9:00 AM - 11:00 AM
Abstract ID: 904181
Abstract Title: An SLE-linked *ITGAM* Gene Variant Changes Mac-1 Structure, Signaling, and Surface Expression and Enhances IFNg Production and Antigen Presentation by B Cells
PLEASE NOTE: *In rare instances, abstract presentation dates/times are subject to change. The ACR will also send out a final presentation confirmation email to all abstract presenters a few weeks before the meeting.*
This is a courtesy notification to all coauthors of the above abstract. All future communications regarding this abstract will be sent only to the presenting author. The primary contact for information about your abstract is the presenting author. Please visit our Abstract Presenter Information page for more details about abstract presentation.
Please note the following:

- The ACR is not able to provide additional information regarding the scoring of abstracts, or reviewer comments.
- This email was sent from a no-reply mailbox, and emails to this address will not be received; if you have any questions, please email abstracts@rheumatology.org.
- The ACR is not a party to author collaborations, and cannot facilitate communication between a presenting author and co-authors or any other stakeholders. It is the responsibility of the presenting author to communicate directly with the ACR and convey information to other stakeholders.

ACR Convergence Registration:

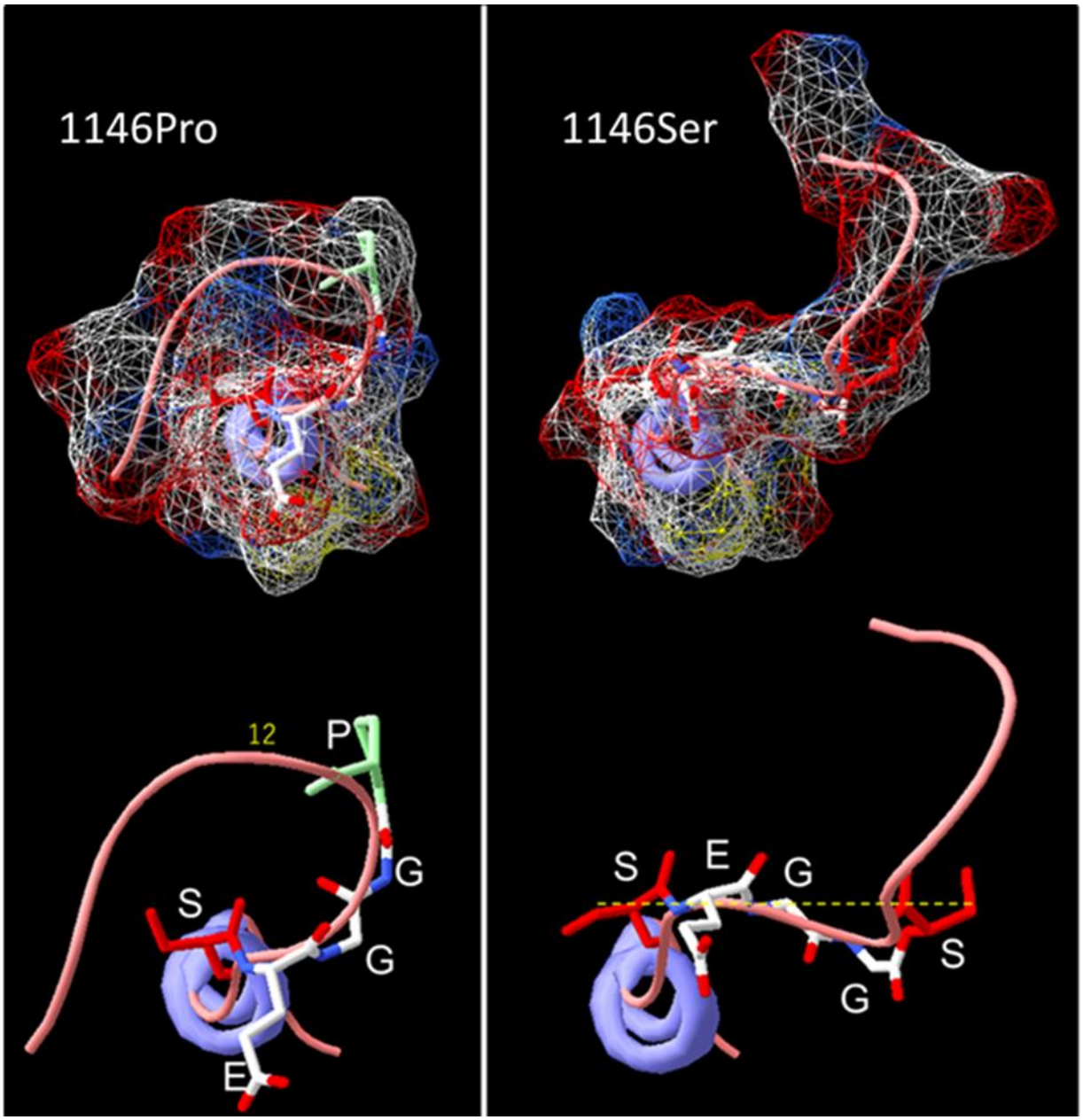
- Acceptance of an abstract does not automatically register the presenting author for ACR Convergence. All presenting authors are required to register for the meeting as a scientific attendee.
- Complete registration details are available on the Registration Page of the ACR Convergence website.
- If you have any registration questions, please contact the ACR Convergence 2020 Registration Support Center.

Congratulations on the acceptance of your abstract, and we look forward to seeing you at ACR Convergence!
Sincerely,
2020 Annual Meeting Planning Committee

Evidence that an SLE-linked *ITGAM* gene variant changes Mac-1 structure, signaling, and surface expression and enhances IFNγ production and antigen presentation by B cells.

SLE is a chronic and debilitating disease; in the USA with an estimated incidence of 3-10 per 100,000 people and currently affecting an estimated 300,000 people. The exact pathogenesis of SLE remains unknown, but there is a growing body of evidence that various genetic, hormonal, and environmental factors contribute to its occurrence and severity. Each is thought to modulate the activation and regulation of innate and adaptive immunity in ways that drive autoimmunity, the underlying cause of SLE. The hallmark of autoimmunity in SLE is B cell hyperactivity leading to the generation of autoantibodies, which together drives the deposition of deleterious immune complexes. SLE has strong familial clustering, high heritability, and high concordance rates between monozygotic versus dizygotic twins, suggesting it's a polygenic disease. Among the many different gene variants reportedly associated with SLE risk or severity are single nucleotide polymorphisms (SNPs) in the integrin alpha-M (*ITGAM*) gene, which are present in more than 1/3 of SLE patients. *ITGAM* encodes the protein CD11b, which pairs with CD18 and forms the Mac-1 receptor. Among the *ITGAM* SNPs associated to SLE is SNP rs1143678, encoding a P1146S amino acid substitution in the cytoplasmic tail of CD11b. Mac-1 is widely expressed by myeloid cells, and it is reported that SNP rs1143678 alters myeloid cell functions in ways that could propel SLE. Mac-1 is also expressed on lymphocytes, but comparatively little is known about its role in B cells or whether SNP rs1143678 has any impact on B cell biology. To fill this gap in knowledge we are investigating the impact of *ITGAM* SNP rs1143678 on Epstein-Barr Virus (EBV) transformed B cells from healthy human donors.

We report here three of our main findings. First, juxtaposition of the cytoplasmic tails of CD11b and CD18 - and their activation induced separation - are thought to be required for Mac-1-mediated cell signaling. Our *in silico* structural modelling (Fig. 1) predicts that the SLE-associated P1146S substitution significantly changes the conformation of CD11b's cytoplasmic tail. This shape-shift should allow CD11b to accommodate additional or new cytoskeletal/cytoplasmic binding partners, and thereby could influence Mac-1 signaling. Second, phosphorylation of 1142Ser (an invariant residue in CD11b) is thought to be required for Mac-1 signaling, but using *in vitro* kinase assays and Western blots we provide the first direct evidence that the SLE-associated 1146Ser residue is also phosphorylated (data not shown). Third, by mass-spectrometry combined with proteomics and network analyses, we obtained evidence that both IFNγ and antigen presentation pathways are enriched in B cells from donors expressing the *ITGAM* SNP variant (Fig 2). Indeed, compared to B cells from *ITGAM* invariant donors, B-cells from donors carrying the *ITGAM* SNP showed increased expression of CD11b, increased spontaneous secretion of IFNγ, and increased expression of the IFNγR2 receptor following stimulation (Fig 3). This trio of new findings together suggests that the *ITGAM* SNP rs1143678 significantly alters the biology of B cells, which might explain the strong association rs1143678 to SLE.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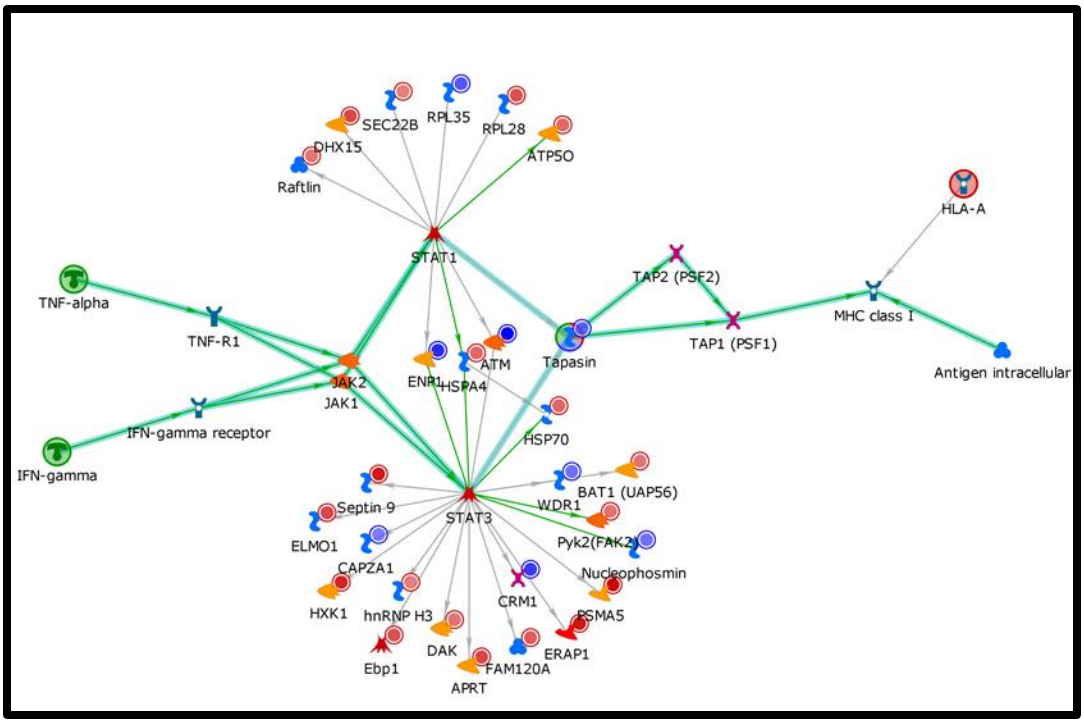
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TNF-R1
TAP1 (PSF1)
ATM
Tapasin
ENPL
HSPA4
JAK2
JAK1
Antigen intracellular
IFN-gamma receptor
HSP70
IFN-gamma
BAT1 (UAP56)
Septin 9
WDR1
STAT3
ELMO1
Pyk2(FAK2)
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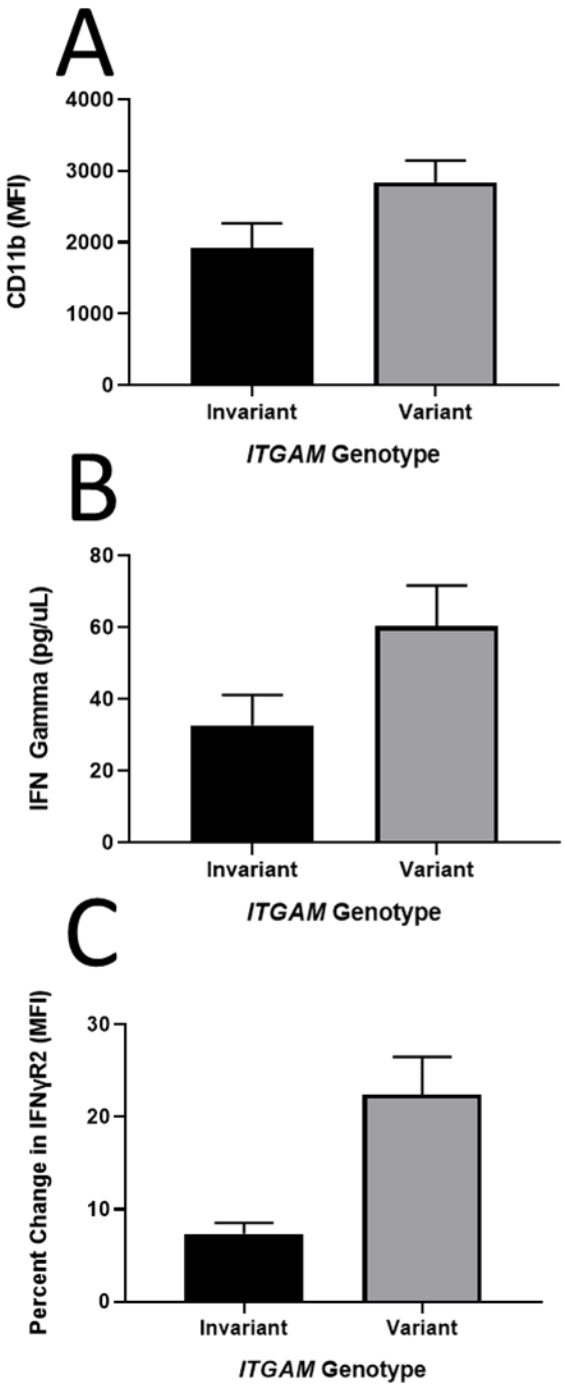
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3000
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CD11b (MFI)
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Variant
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IFN Gamma (pg/uL)
Invariant
Variant
ITGAM Genotype
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30
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10
0
Percent Change in IFNγR2 (MFI)
Invariant
Variant
ITGAM Genotype

Effects of *ITGAM* Genetic Variation on Mac-1-Mediated Functions of B Cells

Log # LR170037

Award Number W81XWH-18-1-0631

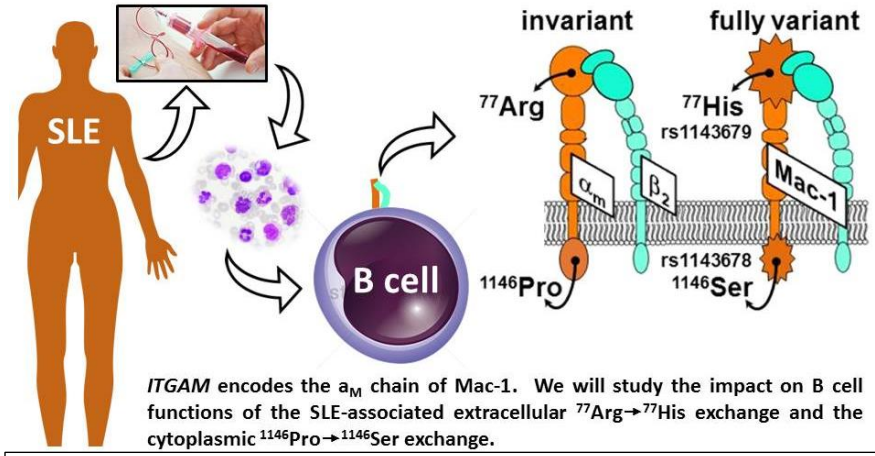
PI: Alexander J. Szalai **Org:** The University of Alabama at Birmingham **Award Amount:** $525,000

Study Aim(s)

- Define the impact of the *ITGAM* 77His and 1146Ser variants on CD11b dependent signaling in B cells.
- Assess the impact of 77His and 1146Ser on CD11b cytoskeletal associations, membrane mobility, and clustering on B cells.
- Show *ITGAM* variation impacts CD11b in B cells from SLE patients.

Approach

We have access to an already genotyped and already consented cohort of recallable healthy and SLE donors and a biobank of their Epstein - Barr virus (EBV) transformed B cells. EBV B cells from healthy donors will be used to assess the impact of *ITGAM* variation on CD11b phosphorylation and cell signaling and Mac-1 cytoskeletal association, mobility, and clustering. Clinical relevance will be validated using peripheral blood B cells freshly isolated from recalled healthy versus SLE affected donors. Approaches to be employed will include; tandem mass-spectrometry, single-cell network profiling, and multispectral imaging flow cytometry .



***ITGAM* encodes the a_M chain of Mac-1. We will study the impact on B cell functions of the SLE-associated extracellular ^{77}Arg→^{77}His exchange and the cytoplasmic ^{1146}Pro→^{1146}Ser exchange.**

We have provided the first evidence that the SLE-linked variant ^{1146}Ser residue in CD11b; (i) is a target for kinase-mediated phosphorylation of CD11b, (ii) associates with increased expression of CD11b by EBV-B cells, (iii) associates with an expansion of CD19$^+$CD27$^+$CD24$^+$ memory B cells, and (iv) intensifies an IFNγ→JAK/STAT→MHC pathway of antigen presentation resembling that seen in Wiskott-Aldrich Syndrome.

Timeline and Cost

Activities	Year 1	Year 2	Year 3
Define the impact of the *ITGAM* variants on CD11b dependent signaling in B cells.			
Assess the impact of 77His and 1146Ser on CD11b cytoskeletal associations, membrane mobility, and clustering on B cells.			
Show *ITGAM* variation impacts CD11b in B cells from SLE patients.			
Estimated Budget ($K)	**165**	**179**	**181**

Updated: October 9, 2020

Stated Goals/Milestones for Budget Year 2

■ accomplished ◘ ongoing ☐ not initiated

- ■ local IRB and HRPO approved
- ■ EBV B cells from donors with different *ITGAM* genotypes identified
- ■ graduate student Joseph Blake recruited to work on this project
- ■ Western blots detected phosphorylation of ^{1142}Ser and ^{1146}Ser
- ◘ mass spectrometry detected phosphorylation of ^{1142}Ser and ^{1146}Ser
- ■ *in vitro* kinase assays detected phosphorylation of ^{1142}Ser and ^{1146}Ser
- ◘ signaling network analysis
- ☐ multispectral imaging flow cytometry (MIFC)
- ◘ preparation of manuscript
- ◘ Mac-1 cytoskeletal association and clustering studies

Comments/Challenges/Issues/Concerns

Social distancing implemented due to COVID-19 has restricted our access to some equipment and our B cell. Nevertheless we have made substantial progress.

Budget Expenditure to Date

Projected: $343,854 Actual: $248,297