Macrophages of different tissue origin exhibit distinct inflammatory responses to mycobacterial infection

Running Title: Tissue origin may affect macrophage response

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Key Words: Mycobacteria, Tuberculosis, Macrophages, Cytokines, Flow Cytometry

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ABSTRACT

Macrophages display marked plasticity with functions in both inflammation and tissue repair. Evidence demonstrates that this spectrum of macrophage phenotypes is influenced by their local microenvironment and tissue origin. However, *in vitro* macrophage experiments often do not or cannot readily use macrophages from the most relevant tissue of origin. In this study we investigated if the origin of two C57BL/6 mouse macrophage cell lines of alveolar (AMJ2-C11) and peritoneal (IC-21) origin may influence their response to mycobacterial infection.

Both cell lines equally controlled growth of *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*, although expression of all proinflammatory cytokines and chemokines measured (TNF, IL-6, MCP-1, MIP-1α, MIP-1β, and RANTES) was significantly higher in AMJ2-C11 cells than IC-21 cells. During *M. tuberculosis* infection, IL-6, MCP-1, and RANTES expression increased 5-fold, and MIP-1β expression increased 30-fold. Additionally, AMJ2-C11 cells exhibited significantly higher iNOS activity than IC-21 cells, indicative of a more polarised M1 response. Expression of multiple surface markers were also assessed by flow cytometry. CD80 and CD86 were significantly upregulated in AMJ2-C11 cells and downregulated in IC-21 cells during *M. tuberculosis* infection.

Our results support the notion that the origin of tissue-resident macrophages influences their phenotype and antimicrobial response and demonstrate hereto unrecognised potential for these cell lines in *in vitro* studies.

INTRODUCTION

Macrophages are often the first leukocytes to interact with invading pathogens and form an integral part of the innate and adaptive immune systems. Macrophage activity is influenced by environmental stimuli and macrophage phenotype is often lineated into two categories. M1 macrophages are produced *in vitro* by stimulation with pathogen-associated molecules such as LPS, with or without GM-CSF or IFN-γ, and express proinflammatory cytokines including IL-1β, IL-6, IL-12, and TNF.[1, 2] By contrast, Th2-related or anti-inflammatory cytokines such as IL-4, IL-13, IL-10, and TGF-β stimulate M2 macrophages, expressing ARG1 and the anti-inflammatory cytokine IL-10.[3] Macrophage plasticity allows switching between the M1 and M2 phenotypes as stimuli change.[4] Recent studies have demonstrated that a binary M1-M2 phenotype classification does not universally apply to many tissue-resident macrophage subtypes, and that macrophage phenotype is strongly influenced by tissue of origin.[5]

Local tissue microenvironments and disease states generate gene expression profiles unique to tissue-resident macrophage subtypes, modulating the gene enhancer landscape of transplanted macrophages to produce a spectrum of M1-M2 phenotypes.[6] Retinoic acid produced in the mouse peritoneal cavity induces expression of the peritoneal macrophage-specific transcription factor GATA6, which in turn induces TGF-β and ARG1 production, both M2 markers.[7] GATA6 is downregulated when peritoneal macrophages are transplanted to the lung.[6] Peritoneal macrophages express typical M2 macrophage markers, including CD206 and TGF-β.[5, 8] In comparison, alveolar macrophages commonly express both M2 marker CD206 as well as M1 marker CD86 in steady state.[9] The percentage of alveolar macrophages expressing CD206 and M1-activation associated enzyme, inducible nitrogen oxide synthase (iNOS), is increased in smokers and chronic obstructive pulmonary disease patients.[10] Acute *Mycobacterium tuberculosis* infection drives mouse alveolar macrophages toward an $iNOS^+$ M1 phenotype, before switching to an $ARG1^+$ M2 phenotype as chronic infection persists.[11]

Many *in vitro* studies utilise immortalised phagocyte cell lines, such as human THP-1 or murine RAW 264.7 cells, or commonly differentiate macrophages from circulating human monocytes or mouse bone marrow progenitors. The polarity of mouse bone marrow-derived macrophages (BMDM) depends heavily on the cytokines used in their differentiation.[12] BMDMs differentiated with GM-CSF and M-CSF are phenotypically M1 and M2, respectively, the former expressing TNF and IL-6, whilst the latter secrete IL-10 and CCL2.[13] Studies in M-CSF deficient mice identified that M-CSF was essential for *in vivo* development of peritoneal macrophages, but not alveolar macrophages.[14] Secreted cytokine levels may vary markedly between macrophage cell lines and change during

extended culture.[15] Variability in steady state phenotype of model cells may influence the cell's response to *M. tuberculosis* infection.

Here we examine the response to mycobacterial infection by two immortalised C57BL/6 mouse macrophage cell lines of peritoneal and alveolar origin. We compare the ability of each cell line to control bacterial growth, demonstrating distinct proinflammatory cytokine expression patterns and differing iNOS activity in macrophage cell lines of different tissue origin. We demonstrate a more M1 phenotypic profile in the AMJ2-C11 cells compared to the IC-21 cells, and this also influences the surface receptor expression patterns associated with mycobacterial infection.

RESULTS

Macrophages from distinct origins retain control of mycobacterial growth

In order to assess the effect of tissue origin on the ability of macrophages to control mycobacteria, bacterial growth in AMJ2-C11 and IC-21 cultures were measured over time. Bacterial loads in *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG)-infected macrophages remained stable up to 24 hours post-infection (hpi), with both cell lines showing mycobacterial killing by 48 hours ($P < 0.05$, Figure 1a). Macrophages infected with *M. tuberculosis* exhibited stable bacterial load over the 48 hour infection, and there was no significant difference in capacity to control bacterial growth between the two cell lines (Figure 1b).

iNOS activity is influenced by macrophage tissue origin

The proinflammatory iNOS activity of the macrophage cell lines was compared through quantification of nitrite, a downstream product of NO. AMJ2-C11 macrophages exhibited more iNOS activity than IC-21 macrophages (Figure 1c). BCG infection of AMJ2-C11 cells induced significantly more nitrate than BCG infection of IC-21 cells. AMJ2-C11 cells produced similar nitrite levels during BCG and *M. tuberculosis* infection. No nitrite production by IC-21 cells was detected following *M. tuberculosis* infection.

Increased proinflammatory cytokines expression in alveolar macrophages

The inflammatory response of these macrophages during mycobacterial infection were investigated by measuring the cell lines' proinflammatory cytokine expression during BCG and *M. tuberculosis*

infection. Infection with the less virulent BCG induced more cytokine and chemokine expression in both cell lines compared to infection with the more virulent *M. tuberculosis*. In particular, production of the chemokines IL-6, TNF, MCP-1, and MIP-1β was considerably increased during BCG infection (Figure 2).

BCG infection induced a similar cytokine response from both cell lines (Figure 2a), although the production of the chemokines MIP-1α and MIP-1β was increased in IC-21 cells at 6 and 24 hpi, respectively, whilst RANTES production in AMJ2-C11 cells was almost 9-fold higher at 24 hpi (Figure 2a). Late IL-6 and MCP-1 expression was significantly higher at 48 hpi in AMJ2-C11 cells. A significant decrease in bacterial load at 24-48 hpi (Figure 1a) coincided with increases in cytokine expression, particularly MIP-1β, MCP-1, and IL-6.

However, *M. tuberculosis* infection induced differential responses between the cell lines. AMJ2-C11 cells expressed over 5-fold more IL-6, MCP-1, and RANTES, and more than 30-fold more MIP-1β at 48 hpi compared to IC-21 cells (Figure 2b).

Surface phenotype of alveolar macrophages indicates stronger proinflammatory response to mycobacterial infection

Cell surface marker expression is an indicator of macrophage subtype and inflammatory state. We quantitated the expression of 12 cell-surface proteins on AMJ2-C11 and IC-21 cells by flow cytometry, comparing median fluorescence intensity (MFI) between cells lines, uninfected and after infection with BCG and *M. tuberculosis*. A resazurin fluorescence assay confirmed no decrease in mitochondrial activity following mycobacterial infection, indicating cells remain viable at 24 hpi (Supplementary figure 1).

The expression of most markers was greater in uninfected IC-21 cells compared to uninfected AMJ2-C11 cells (Figure 3a). Only Ly6C expression was skewed towards AMJ2-C11 cells, and this difference was exaggerated upon mycobacterial infection. Expression of the M1 marker CD86 was higher in IC-21 peritoneal macrophages prior to infection, though AMJ2-C11 alveolar macrophages showed significantly higher expression after infection. Furthermore, uninfected IC-21 cells expressed more of the M2 marker CD206, as well as CD11b, CD24, and Siglec-F. Expression of these markers was not significantly different between cells lines during BCG infection, and was similar during *M. tuberculosis* infection. Expression of MHC-II was equivalent on the uninfected cell lines, however, this rose significantly on AMJ2-C11 cells following BCG infection. Expression of the M1 marker CD80, as well as CD11c, Ly6G, and immunoglobulin receptor, CD64, was greater on IC-21 cells under all conditions.

Infection with BCG and *M. tuberculosis* induced comparable responses in AMJ2-C11 macrophages, with increased expression of all tested markers (Figure 3). This activation was also reflected in the strong iNOS (Figure 1c) and cytokine response (Figure 2) from AMJ2-C11 cells to both bacterial species. In contrast, IC-21 cells downregulated Ly-6C upon BCG infection, and downregulated multiple inflammatory markers upon *M. tuberculosis* infection, including CD80 and CD86 (Figure 3). Moreover, downregulation of CD64 and CD11b by *M. tuberculosis* infected IC-21 cells could indicate a shift towards an anti-inflammatory phenotype. The comparable marker upregulation seen in AMJ2-C11 and IC-21 cells following BCG infection reflects the similar cytokine responses by both cell lines (Figure 2a). In contrast, the downregulation of inflammatory surface markers by IC-21 cells upon *M. tuberculosis* infection coincides with lower cytokine expression (Figure 2b) and undetectable iNOS activity (Figure 1c).

DISCUSSION

Mice are the most commonly used animal model of mycobacterial infection, with C57BL/6 the most common genetic background used. In this study we compared two C57BL/6 mouse macrophage cell lines of differing tissue origin, in response to *in vitro* mycobacterial infection. Whilst both the AMJ2-C11 alveolar macrophages and IC-21 peritoneal macrophages controlled the growth of BCG and *M. tuberculosis* to the same extent, AMJ2-C11 cells exhibited significantly greater proinflammatory cytokine expression and iNOS activity in response to the bacterial species. Additionally, AMJ2-C11 cells presented a more inflammatory surface phenotype during *M. tuberculosis* infection. The response to infection may be influenced by the tissue origin of the two cell lines.

When using primary macrophages or macrophage-derived cell lines for *in vitro* models of infection, it is important to consider both the site of *in vivo* infection and the origin of the macrophage cells. The IC-21 line was prepared from mouse peritoneal macrophages virally transduced *in vitro*.[16] In contrast, the AMJ2-C11 line was virally immortalised from characterised primary mouse alveolar macrophages[17] and readily expresses proinflammatory cytokines when activated.[18]

Development of a cell-based model of *M. tuberculosis* infection should take into account the basal alveolar macrophage phenotype and cellular changes associated with chronic disease.[10, 11] Here we illustrated the polarity and anti-microbial response of the alveolar and peritoneal macrophage cell lines, AMJ2-C11 and IC-21, during acute mycobacterial infection.

During acute *M. tuberculosis* infection *in vitro*, control of bacterial replication does not appear to be influenced by macrophage origin. THP-1 monocytic cells controlled *M. tuberculosis* load as efficiently as primary human alveolar macrophages over 4 days,[19] and MPI cells, a recently derived murine alveolar macrophage line, exhibit a similar infection pattern to THP-1, RAW 264.7, and BMDM cells.[20] Likewise, this study shows both AMJ2-C11 and IC-21 cells demonstrated comparable control of BCG and *M. tuberculosis* growth during a 48-hour infection. However, this may not replicate *in vivo* infection completely, as following intranasal infection in mice, alveolar macrophages were more permissive to intracellular *M. tuberculosis* replication than pulmonary interstitial macrophages.[21] *Ex vivo* lung and peritoneal macrophages comparably controlled *M. tuberculosis* H37Rv bacterial load up to 3 days, before bacterial growth accelerated in lung macrophages.[22] Extended *M. tuberculosis* infection of AMJ2-C11 cells may present similar results.

Cytokine secretion is central to macrophage antimicrobial and anti-inflammatory functions,[2] and pro-inflammatory M1 macrophages release cytokines, including TNF and IL-6, that are critical for anti-mycobacterial protection.[23] We observed significantly greater IL-6 expression from AMJ2-C11 cells, indicative of the M1-primed polarity seen in alveolar macrophages.[9] The alveolar cell line MPI also displays an M1 dominant phenotype, expressing high levels of TNF and IL-6 compared to BMDMs, when infected with *M. tuberculosis*.[20] Like AMJ2-C11 cells, human alveolar macrophages are known to express chemokines associated with an M1 phenotype, including MIP-1α, MIP-1β, and RANTES, following *M. tuberculosis* infection.[24] Although peritoneal macrophage-derived cell lines can also express these chemokines,[15] our results indicate infection-induced chemokine upregulation is delayed (Figure 2).

Alveolar macrophage-expressed MCP-1 recruits circulating monocytes during *M. tuberculosis* infection, aiding in bacterial dissemination.[25, 26] In the current study avirulent BCG induced higher MCP-1 expression on AMJ2-C11 cells than virulent *M. tuberculosis* H37Rv. Similarly, primary human alveolar macrophages expressed more MCP-1 when infected with avirulent *M. tuberculosis* H37Ra, compared to H37Rv.[24] We observed IC-21 cells expressed comparable MCP-1 to AMJ2-C11 cells during BCG infection, but significantly less during *M. tuberculosis* infection. In turn, IC-21 cells expressed more MCP-1 than BMDMs differentiated with M-CSF-containing L929 supernatant.[15] Tissue-resident macrophages are difficult to obtain in large numbers from small laboratory animals and mouse bone marrow is commonly used as a source of macrophages *in vitro* due to the quantity of cells generated. Although BMDMs may be a more practical for many laboratories than primary tissue-resident macrophages, basal phenotypic differences like chemokine expression should be considered when planning experimentation.

A number of surface protein markers are regularly used to characterise macrophage polarity, including CD206, CD80, and CD86.[2] The M2 marker CD206 is the mannose receptor, involved in post-infection remediation of inflammation, binding bacterial glycoproteins and heavily glycosylated anti-bacterial proteins such as myeloperoxidase.[27] CD206 also facilitates mycobacterial colonisation of macrophages.[28] CD80 and CD86 are inflammatory co-receptors required for antigen presentation and T-cell activation.[29] Pro-inflammatory M1 macrophages are known to express high levels of CD80 and CD86,[2] whereas only a subset of M2 macrophages express CD86.[30] We found that CD206, CD80, and CD86 levels were all higher for uninfected IC-21 compared to AMJ2-C11. However, whilst IC-21 upregulation of CD206 was notably higher during BCG infection, AMJ2-C11 cells showed significantly more upregulation of CD80 and CD86 during BCG and TB infection. Studies have shown that during acute *M. tuberculosis* infection, alveolar macrophages present an inflammatory phenotype, with high CD86 expression and strong iNOS activity,[11] similar to what was shown here with AMJ2-C11 alveolar macrophages. Yet, as the granuloma structures typical of chronic tuberculosis develop, macrophages decrease iNOS activity and upregulate the anti-inflammatory cytokine IL-10.[11] The subdued cytokine expression and weak iNOS activity of IC-21 cells during *M. tuberculosis* infection suggests these cells may not be an appropriate model of acute macrophage infection in the lung.

Our findings demonstrate that macrophage cell lines of different tissue-origin display marked differences in their response to infection, and this should be considered when utilising cell lines in *in vitro* assays. Mycobacterial infection induced significantly increased cytokine expression and iNOS activity in AMJ2-C11 cells, highlighting the M1/M2 phenotype of their alveolar macrophage origin. In contrast, IC-21 cells, whilst still controlling mycobacterial infection over the time frame of this experiment, displayed a more M2 phenotype, with lower cytokine expression and downregulation of proinflammatory markers. Our work supports the idea that acute *M. tuberculosis* infection models should consider using M1-like macrophages, particularly when using polarity-dependent readouts, such as cytokine expression and surface markers. The development of more alveolar macrophage-representative cell models, like the MPI cell line,[20] may also aid future tuberculosis research.

METHODS

Cell culture

Murine AMJ2-C11 cells of C57BL/6 origin (ATCC, Manassas, VA, USA) were maintained in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 25 mM HEPES, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 10% foetal bovine serum. Murine IC-21 cells of C57BL/6

origin (ATCC) were maintained in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 25 mM HEPES, 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 10% foetal bovine serum.

Bacterial cultures

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) Pasteur and *M. tuberculosis* H37Rv were grown in Middlebrook 7H9 broth (BD Biosciences, Franklin Lakes, NJ, USA) supplemented with 5 g L^{-1} bovine serum albumin (BSA), 2 g L^{-1} glucose, 4 mg L^{-1} catalase, 0.2% glycerol, and 0.05% Tween 20. Cell concentration was routinely determined by OD at 600 nm. CFU was determined by plating serial dilutions on Middlebrook 7H11 agar (BD Biosciences) supplemented with 5 g L^{-1} BSA, 2 g L^{-1} glucose, 4 mg L^{-1} catalase, 500 mg L^{-1} oleic acid, and 0.5% glycerol, and incubating for 3 weeks at 37 °C.

Macrophage infections with mycobacteria

AMJ2-C11 and IC-21 cells were stimulated with 100 U mL^{-1} IFN-γ (R&D Systems, Minneapolis, MN, USA) and infected with BCG or *M. tuberculosi*s at a multiplicity of infection of 5 or 1, respectively. Cells were washed after 4-6 hours to remove extracellular bacteria. At time points from 0-48 h post-infection, supernatant was removed for cytokine and nitrite quantification. Cells were lysed with 0.1% Triton X-100 solution for CFU determination. Lysates were plated on 7H11 agar and incubated at 37 °C for 21 days to determine CFU.

Cytometric bead array

The concentrations of IL-6, TNF, MCP-1, MIP-1α, MIP-1β, and RANTES in supernatants were determined by cytometric bead array (BD Biosciences) according to the manufacturer's protocol. Data were analysed using FCAP Array software (BD Biosciences).

Nitrite assay

Nitrite concentrations of supernatant samples were determined using Griess reagent, consisting of 3.85 mM *N*-(naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA), 58 mM sulphanilamide (Sigma-Aldrich), and 0.4 M phosphoric acid (Sigma-Aldrich) in water. Nitrite standards were prepared using Sodium nitrite (Sigma-Aldrich). Griess reagent was added to supernatant samples at a ratio of 1:1, and the immediate colour change was quantified by measuring absorbance at 550 nm using a FLUOstar plate reader (BMG Labtech, Ortenberg, Germany).

Flow cytometry

AMJ2-C11 and IC-21 cells were assessed by flow cytometry before infection and 24 hours after infection with BCG or *M. tuberculosis*. Cells were incubated with Fc Block (BD Biosciences) for 30

minutes and then stained with fluorochrome-labelled antibodies (see Supplementary table 1 for a list of antibodies, clones, fluorochromes, and manufacturers) for 30 minutes at room temperature in the dark. Stained samples were fixed with 10% neutral buffered formalin (Fronine, NSW, Australia). Data acquisition was performed on a BD Fortessa X20 using the BD FACS Diva software (BD Biosciences). Compensation and data analysis were performed in FlowJo v10 software (BD Biosciences). Cells were gated using forward scatter and side scatter to remove debris and dead cells (see Supplementary figure 2 for an example dot-plot). The expression of surface markers is presented as fold MFI difference, calculated as a ratio of AMJ2-C11 cells to IC-21 cells, or of infected cells to uninfected cells.

Cell viability

Macrophage viability was confirmed using a resazurin assay for mitochondrial activity. Macrophages were incubated with 5.5 mM resazurin (Sigma Aldrich) for 3 h at 37 °C. Conversion of resazurin to resorufin was evaluated by measuring fluorescence using a FLUOstar plate reader, excitation at 550 nm, emission at 590 nm.

Statistical analysis

GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for statistical analyses. CFU and cytokine concentration over time were compared by two-way analysis of variance with multiple comparisons post-test, corrected using the Šidák method. Nitrate concentration and flow cytometry MFI were compared using Student's *t*-test, corrected for multiple comparisons using the Holm-Šidák method.

ACKNOWLEDGMENTS

This work was supported by the National Health and Medical Research Council of Australia grants (APP1043225 and APP1153493). MTS was a recipient of a UTS Research Excellence Scholarship. We thank Dr Nilesh Bokil for his practical assistance.

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FIGURES

Figure 1. Bacterial growth in alveolar AMJ2-C11 and peritoneal IC-21 macrophage cultures infected with (**a**) BCG and (**b**) *M. tuberculosis*. Data are the mean ± SEM of 9 replicates, from 3 repeat experiments. (**c**) Supernatant nitrite concentration of AMJ2-C11 and IC-21 macrophage cultures during BCG and *M. tuberculosis* infection. Data are the mean ± SD of 9 replicate wells, from 3 repeat experiments. Dotted lines represent the assay's lower limit of quantitation. ** $P < 0.01$, by student's *t*-test, Holm-Šidák method corrected.

Figure 2. Increased cytokine expression by AMJ2-C11 alveolar macrophages compared to IC-21 peritoneal macrophages following infection with (**a**) BCG or (**b**) *M. tuberculosis*, relative to uninfected cells. Data are the mean ± SD of triplicate wells, representative of 3 repeat experiments. Repeat experiment data is shown in Supplementary figures 3 and 4. Asterisks represent significant difference between AMJ2-C11 and IC-21 expression by 2-way ANOVA with multiple comparisons post-test, Šidák method corrected. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. Expression of surface markers on AMJ2-C11 alveolar macrophages and IC-21 peritoneal macrophages during BCG and *M. tuberculosis* infection, as determined by flow cytometry. (**a**) Fold MFI difference for surface markers expressed on AMJ2-C11 and IC-21 cells, uninfected, and 24 hours after BCG and *M. tuberculosis* infection. Fold difference is a ratio of AMJ2-C11 MFI to IC-21 MFI (**b**) Change in surface marker expression of AMJ2-C11 cells following 24-hour mycobacterial infection. (**c**) Change in surface marker expression of IC-21 cells following 24-hour mycobacterial infection. Fold difference is a ratio of infected MFI to uninfected MFI. Data are the mean of triplicate wells. All coloured rectangles represent $P < 0.05$, student's *t*-test, Holm-Šidák method corrected.