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Immortalized MH-S cells lack defining features of primary alveolar macrophages and do not support mouse pneumovirus replication

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Abstract

The SV-40-transformed MH-S cell line maintains some, but not all, features of primary alveolar macrophages (AMs) from BALB/c mice. We show here that MH-S cells produce inflammatory cytokines IL-6 and CXCL10 in response to challenge with Gram-positive *Lactobacillus reuteri*, and to TLR2 and NOD2 ligands Pam3CSK4 and MDP, respectively. In contrast, although wild-type AMs are infected *in vivo* by pneumonia virus of mice (PVM), no virus replication was detected in MH-S cells. Interestingly, the surface immunophenotype of MH-S cells (CD11c⁺Siglec F⁻) differs from that of wild-type AMs (CD11c⁺Siglec F⁺) and is similar to that of immature AMs isolated from granulocyte macrophage-colony stimulating factor (GM-CSF) gene-deleted mice; AMs from GM-CSF^{-/-} mice also support PVM replication. However, MH-S cells do not express the GM-CSF receptor alpha chain (CD116) and do not respond to GM-CSF. Due to these unusual features, MH-S cells should be used with caution as experimental models of AMs.

Keywords

macrophage; inflammation; Siglec F; pneumovirus; pattern recognition receptor; granulocyte-macrophage colony stimulating factor

1.0 Introduction

Alveolar macrophages (AMs) are central to host defense against bacterial, viral, and fungal pathogens, provide routine environmental surveillance, and regulate, as well as initiate, inflammation [1 – 3]. AM activation, and the initiation of pro-inflammatory or regulatory programs, is highly dependent on the balance of signals from changing lung

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microenvironments [4 – 6]. This complex, multivalent regulatory environment can complicate the study of AMs in *in vivo* settings.

Mbawuike and Herscowitz [7] facilitated the study of AMs through development of the MH-S cell line. Adherent cells from bronchoalveolar lavage of BALB/cJ mice were infected with Simian Virus (SV)-40; the resulting immortalized cells expressed the surface receptors Mac-1 (CD11b/CD18) and produced IL-1 in response to lipopolysaccharide (LPS) stimulation. Recent reports on this cell line note constitutive expression of pattern recognition receptors (PRRs) toll-like receptor (TLR)2 and TLR4 [8], and the ability to phagocytose latex beads [9].

While MH-S cells, as well as primary AMs, typically respond to both intracellular and extracellular bacterial pathogens [10 – 13]. However, Reading and colleagues [14] reported that the BJx109 strain of the human viral pathogen, Influenza A (H3N2) readily infected both MH-S cells and primary macrophages from mouse bronchoalveolar lavage, while the PR8 strain of Influenza A H1N1 did not. Likewise, MH-S cells support robust replication and chemokine production from the orthopoxvirus, Modified Vaccinia virus Ankara (MVA), but no chemokine production from the vaccinia virus strain WR [15].

Our group maintains a strong interest in pneumonia virus of mice (PVM), a natural rodent pathogen of the same family (*Paramyxoviridae*) and genus (Pneumovirus) as human respiratory syncytial virus (RSV). PVM infection is lethal in numerous inbred strains of mice [16], with inflammatory pathology characterized by robust viral replication in the bronchiolar epithelium, granulocyte recruitment to the airways, and local production of proinflammatory mediators, including CCL2, CCL3 and CXCL1 [17, 18]. Using recombinant PVM (rK2-PVM) which incorporates the sequence encoding the far-red fluorescent protein, mKATE2, we have recently shown that AMs are a primary leukocyte target for this virus, and that AMs support both virus replication and productive infection *in vivo* [19]. Furthermore, application of immunobiotic *Lactobacillus* species to the respiratory tract, a regimen that results in robust and sustained survival in response to virus infection [20 – 23] limits PVM infection and virion release from AMs [19].

Given our interests in PVM infection, cross-protection associated with the administration of immunobiotic *Lactobacillus* species to the respiratory tract, ie...heterologous immunity, and interactions between these microbes at and within alveolar macrophages, a cell line that faithfully replicates these features (i.e., interactions with *Lactobacillus* species, limiting PVM infection) would be of significant utility to our ongoing studies. Here, we compare primary mouse AMs and MH-S cells with respect to their distinguishing surface markers, their ability to generate inflammatory cytokines in response to challenge with Gram-positive *Lactobacillus* species and related pattern recognition receptor ligands, as well as their ability to support replication of the PVM pathogen.

2.0 Methods

2.1 Cells and Mice

The mouse alveolar macrophage MH-S (CRL-2019) and peritoneal macrophage RAW 264.7 (TIB-71) cell lines were purchased from ATCC and maintained in culture at 37°C and 5% CO₂. Cells were grown in complete RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin; MH-S cells were also supplemented with 50 µM 2-mercaptoethanol. BALB/c and C57BL/6 mice (6 – 8 week old female) were from Charles River Laboratories, Frederick, Maryland Facility. Granulocyte macrophage – colony stimulating factor (GM-CSF) gene-deleted mice were as described [24] and obtained from Dr. June Kwon-Chung (NIAID). All mouse studies were approved by NIAID and carried out in accordance with ACUC guidelines.

2.2 Flow Cytometry

Bronchoalveolar lavage fluid (BALf) samples were collected from all mice in a total of 4 mL sterile phosphate buffered saline with 0.1% bovine serum albumin (PBS/BSA). Cells were centrifuged at 300 × g for 5 min and re-suspended in PBS/BSA. MH-S cells were detached with ice-cold 10 mM EDTA in PBS and washed×2 with PBS/BSA prior to being stained with fluorochrome-conjugated antibodies against CD16/CD32, Siglec F, CD45, CD11c, CD116 (GM-CSF receptor alpha chain), and MHC II-A/I-K (BD Bioscience, eBioscience, or R&D Systems). After incubation with antibodies for 30 minutes at 4°C, the cells were washed with 3 mL of PBS/BSA and then fixed in 4% paraformaldehyde. The samples were stored at 4°C in the dark until analysis. A minimum of 100,000 events was collected on an LSRII flow cytometer (BD Biosciences) and data were analyzed in FlowJo (Tree Star, Inc).

2.3 Viruses and Bacteria

PVM strain J3666 was prepared as previously described [25]. Recombinant rK2-PVM (strain J3666 with mKATE2 fluorescent tag) was generated as described [19]. *Lactobacillus reuteri* F275 (ATCC 23272) was grown overnight in Mann Rogosa Sharpe broth at 37°C; the ratio of OD₆₀₀ to colony forming units was determined experimentally [20]. Stationary phase cultures were heat-inactivated at 70°C for 30 min, washed with PBS/BSA, and stored at –80°C at 5 × 10⁹ cells / mL prior to use. Initial titration of (recombinant) rK2-PVM in tissue culture was performed by a modification of the TCID₅₀ assay in which virus infection is monitored by detection of the mKATE2 fluorescent marker (TCFD₅₀; see reference 19).

2.4 Proinflammatory Cytokines

Pattern recognition receptor ligands Pam3CSK4 and MDP were purchased from Invivogen. Release of IL-6 and CXCL10 from activated MH-S cells was evaluated by Duo-Set ELISA (R&D Systems).

2.5 Virus replication

Replication of native PVM strain J3666 in MH-S and RAW 264.7 cells was evaluated by quantitative RT-PCR using primers specific to the virus SH gene and mouse GAPDH with

standard curves as previously described [26]. Infection with rK2-PVM was evaluated by flow cytometry as described [19].

3.0 Results and Discussion

3.1. Surface immunophenotype of MH-S cells

MH-S cells exhibit a different surface marker phenotype than primary alveolar macrophages isolated from BALB/c mice [Figs. 1a and 1b]. Both primary alveolar macrophages and MH-S cells express the common leukocyte antigen CD45, but, in contrast to AMs, no sialic acid-binding immunoglobulin-type (Siglec) F was detected on the cells of the MH-S cells line. Murine Siglec F, the functional ortholog of human Siglec 8, recognizes the α 2–3-linked sialic acid, 6'-sulfated sialyl Lewis X, and is expressed primarily on mouse eosinophils and alveolar macrophages [27 – 29]. On eosinophils, Siglec F engagement modulates cell viability *ex vivo*, a feature that may limit allergic eosinophilia through induction of apoptosis [30, 31]. While the function of Siglec F on murine alveolar macrophages remains uncertain [32], the literature consensus is that it is one of the strong positive markers that define this resident population; in flow cytometric analyses, Siglec F is used in conjunction with CD11c or CD64 to distinguish AMs from Siglec-F⁺ eosinophils [33 – 35]. Notably, cultures of MH-S cells in media supplemented with (10%) cell-free bronchoalveolar lavage fluid or clarified lung homogenate from naïve BALB/c mice had no impact on Siglec F expression (data not shown).

Our observation that MH-S cells have features typical of macrophages but express no Siglec F suggests several possibilities. Among them, the specific target cell(s) that underwent SV-40 immortalization may not have been fully differentiated AMs; alternatively, SV-40 infection and/or long-term culture *ex vivo* may have resulted in selection for distinct sub-lineages with altered phenotypes. Indeed, Sankaran and Herscowitz [36] reported in 1995 that the MH-S cells were not (or were no longer) homogeneous with respect to expression of an original defining antigen, Mac-1 (CD11b/CD18). Guillems and colleagues [37] demonstrated that AMs in adult mice are replenished locally under homeostatic conditions; as such, one can surmise that there should be few, or no immature AMs in otherwise unperturbed adult mouse lung tissue. Nonetheless, the surface immunophenotype that we have characterized for MH-S cells (CD11c⁺Siglec F[−]) is similar to that reported for immature AMs in granulocyte macrophage-colony stimulating factor (GM-CSF) gene-deleted mice (Fig 2a; [37, 38]). This is an important finding, given that MH-S cells have been designated “wild-type” AMs in studies that include comparisons to AMs from GM-CSF gene-deleted mice [9].

As shown in Fig. 2b, GM-CSF receptor alpha chain (CD116) was detected on AMs from wild-type BALB/c and C57BL/6 mice, as well as on AMs from mice devoid of GM-CSF. Reed and colleagues [39] reported that phenotypically immature AMs from GM-CSF gene-deleted mice undergo maturation *in situ* in response to administration of GM-CSF, a finding indicating that the GM-CSF alpha receptor, as well as the shared GM-CSF / IL-5 / IL-3 beta signaling receptor are both expressed and functional in this strain. In contrast, GM-CSF receptor alpha was not detected on MH-S cells (Fig. 2b). Furthermore, no phenotypic

maturation (e.g., expression of Siglec F) was detected in response to administration of GM-CSF (data not shown).

3.2. MH-S cells produce inflammatory mediators in response to biochemical pattern recognition receptor ligands and Gram-positive *Lactobacillus*

As shown in Fig. 3, MH-S cells produce and release IL-6 and CXCL-10 in response to synthetic ligands for the pattern recognition receptors TLR2 and NOD2, as well as in response to challenge with heat-inactivated Gram-positive *Lactobacillus reuteri*. Although MH-S cells have been previously shown to express TLR2 [8], this is the first report that documents responses consistent with expression of the intracellular pattern recognition receptor NOD2. Like TLR2, NOD2 recognizes pathogen associated molecular patterns (PAMPs), which leads to transcription of NF- κ B-dependent gene targets [40]. Cytoplasmic NOD2 senses muramyl-dipeptide, a metabolite of extracellular bacterial peptidoglycan [41, 42], and can generate inflammatory responses that have been best characterized at the gastrointestinal mucosa [43]. We have also identified NOD2, along with TLR2, as the two principal receptors interacting with *Lactobacillus* at the respiratory mucosa [23; T. A. Rice, T. A. Brenner, *et al.*, ms in preparation] suggesting that this PRR is of broad interest to the study of host-microbe interactions, including those mediated by AMs.

We also report for the first time that MH-S cells generate an inflammatory response to *Lactobacillus* challenge. In addition to producing the proinflammatory mediators IL-6 and CXCL10, MH-S cells phagocytose recombinant green fluorescent protein expressing-*L. reuteri* [44; *unpublished findings*]. These findings contribute to the collective data indicating that MH-S cells interact with extracellular bacteria in a manner consistent with many other macrophage cell lines [10]. Equally important, MH-S cells are not only well-suited to the study of TLR2-dependent pathways, as shown here, they are also clearly capable of responding to NOD2-dependent ligands. As such, the MH-S cell line may be useful in modeling cross-talk between TLR2 and NOD2 and the role of these receptors, and their downstream signaling pathways, in mediating the protection elicited by *Lactobacillus* and its components.

3.3. The MH-S cell line does not support replication of pneumonia virus of mice (PVM)

PVM undergoes robust replication in the lung tissue of inbred strains of mice [16 – 18], as well as productive replication in alveolar macrophages *in vivo* [19]. PVM also replicates in the mouse peritoneal macrophage RAW 264.7 cell line [45, 19], although no replication was detected in MH-S cells challenged with PVM under similar conditions [Fig. 4a]. Similarly, no PVM replication was detected in MH-S cells incubated in full tissue culture medium supplemented with cell-free BAL fluid or tissue homogenate from the lungs of BALB/c mice [Fig. 4a], or in cells treated with GM-CSF (data not shown). Analogous results were obtained using rK2-PVM and detection via flow cytometry; as shown in Fig. 4b, one observes a substantial population of mKATE2⁺ primary AMs, while few to no mKATE2⁺ MH-S cells are detected.

As noted earlier, the results of virus replication studies in MH-S cell are variable and may be idiosyncratic [14, 15]. Of particular interest to this work, Miller and colleagues [46] detected

replication of the human pneumovirus pathogen, respiratory syncytial virus (RSV) strain A2 in the MH-S cell line, in association with production of proinflammatory chemokines. The explanation for these discordant results is not immediately clear. Although PVM is related to RSV, and both are viruses of the Family *Paramyxoviridae*, genus *Pneumovirus*, there is little direct amino acid sequence homology between their attachment (G) and fusion (F) virion surface proteins [47]. While the receptor(s) for PVM have not been characterized, the results presented here suggest the possibility that Siglec F might play a role in promoting infection, at least in targeted alveolar macrophages. As highlighted in a recent review [48], cell surface Siglecs as a group interact with numerous pathogens and pathogen-derived ligands. Interestingly, the macrophage cell surface protein Siglec-1 (Sialoadhesin; CD169), was initially characterized as an important receptor for porcine reproductive and respiratory syndrome virus (PRRSV) [49] although a recent study that monitored PRRSV infection in Siglec-1 gene deleted pigs suggested modification of this hypothesis [50]. Siglec-1 interacts with retroviruses HIV and murine leukemia virus and mediates virion transfer to T lymphocytes [51, 52]. To evaluate the manner in which Siglec F might facilitate PVM infection, we infected GM-CSF^{-/-} mice with recombinant rK2-PVM and isolated AMs (CD11c⁺CD116⁺SiglecF⁻; see Fig. 2a) for analysis. As shown in Fig. 4c, a significant fraction of AMs from GM-CSF^{-/-} mice are infected with recombinant rK2-PVM, indicating that Siglec F is not a requirement for PVM infection.

4.0 Conclusions

SV-40-immortalized MH-S cells derived from the airways of BALB/c mice maintain some, but not all, features of primary wild-type AMs. MH-S cells produce inflammatory cytokines in response to challenge with heat-inactivated Gram-positive *Lactobacillus reuteri*, and in response to the TLR2 and NOD2 ligands Pam3CSK4 and MDP, respectively. Interestingly, although MH-S cells have been reported to support replication of the human pneumovirus RSV, we detected no replication of the mouse pneumovirus pathogen, PVM, utilizing both qRT-PCR and flow cytometric-based assays. Of particular note, the surface immunophenotype of MH-S cells (CD11c⁺SiglecF⁻CD116⁻) is atypical, and differs significantly from wild-type AMs (CD11c⁺SiglecF⁺CD116⁺).

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Abbreviations

AM	alveolar macrophage
PVM	pneumonia virus of mice
RSV	respiratory syncytial virus

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Highlights

- MH-S cells are SV-40 immortalized alveolar macrophages (AMs) from BALB/cJ mice.
- Similar to wild-type AMs, MH-S cells respond to Gram-positive bacteria.
- MH-S cells also respond to the NOD2 ligand, MDP, and the TLR2 ligand Pam3CSK4.
- Unlike wild-type AMs, MH-S cells do not express Siglec F or GM-CSFRalpha (CD116).
- Unlike wild-type AMs, MH-S cells cannot be infected with pneumonia virus of mice.

A. **B.**

Figure 1. Cell surface immunophenotypes of alveolar macrophages from BALB/c mice and the immortalized alveolar macrophage MH-S cell line

A. BAL cells from BALB/c mice, **B.** MH-S cells, both evaluated with fluorochrome-conjugated antibodies against CD45, CD11c, Siglec F, and MHC II. Data presented as % maximum count vs. relative fluorescence, specific antibody, gray shaded; isotype control, open.

Figure 2. Cell surface immunophenotypes of alveolar macrophages from GM-CSF genedeleted (GM-CSF^{-/-}) mice and corresponding C57BL/6 controls; expression of GM-CSFRalpha
 A. BAL cells from GM-CSF^{-/-} and C57BL/6 mice evaluated with fluorochrome-conjugated antibodies against CD45, CD11c and Siglec F. B. BAL cells from GM-CSF^{-/-}, C57BL/6, and BALB/c mice, and MH-S cells evaluated with fluorochrome-conjugated antibodies against GM-CSFRalpha (CD116).

Figure 3. MH-S cells produce inflammatory mediators in response to biochemical pattern recognition receptor ligands and Gram-positive *Lactobacillus*

A. IL-6 (pg/mL) and **B.** CXCL10 (pg/mL) detected in supernatants of cultures of MH-S cells challenged for 4 hrs with (+) or without (-) TLR2 ligand Pam3CSK4 (100 ng/mL) and/or NOD2 ligand MDP (10 μ g/mL). **C.** IL-6 (pg/mL) or **D.** CXCL10 (pg/mL) detected in supernatants of cultures of MH-S cells challenged for 4 hrs with (-) diluent alone or (+) 1×10^8 cells heat-inactivated *Lactobacillus reuteri*; n = 5 per group, ** $p < 0.01$, *** $p < 0.001$.

B.

C.

Figure 4. MH-S cells do not support replication of pneumonia virus of mice (PVM)

A. Replication of PVM strain J3666 in cells of the RAW 264.7 line, 200 TCID₅₀ units to 10⁵ cells on day 0, shown as copies PVM per copies GAPDH [25]; replication of PVM strain J3666 in MH-S cells in response to challenge as above, ^aMH-S cells grown in medium containing 10% BAL fluid from BALB/c mice; ^bMH-S cells grown in medium containing 10% lung homogenate protein from BALB/c mice. **B.** Flow cytometric analysis of MH-S cells alone, MH-S cells inoculated at day 0 with 3 × 10⁴ TFCD₅₀ units (see Methods) recombinant PVM J3666 expressing mKATE2 (rk2-PVM) and evaluated on day 7; AMs

(CD11c⁺CD116⁺SiglecF⁺) isolated from airways of wild-type mice 5 days after intranasal challenge with rK2-PVM. C. Flow cytometric analysis of AMs (CD11c⁺CD116⁺SiglecF⁻) isolated from the airways of GMCSF^{-/-} mice 5 days after intranasal challenge with diluent alone (left panel) or rK2-PVM as in B.

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