Francisella tularensis Elicits IL-10 via a PGE$_2$-Inducible Factor, to Drive Macrophage MARCH1 Expression and Class II Down-Regulation

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Abstract

Francisella tularensis is a bacterial pathogen that uses host-derived PGE$_2$ to subvert the host’s adaptive immune responses in multiple ways. Francisella-induced PGE$_2$ acts directly on CD4 T cells to blunt production of IFN-$\gamma$. Francisella-induced PGE$_2$ can also elicit production of a $>$10 kDa soluble host factor termed FTMOsN (Frankisella tularensis macrophage supernatant), which acts on IFN-$\gamma$ pre-activated MØ to down-regulate MHC class II expression via a ubiquitin-dependent mechanism, blocking antigen presentation to CD4 T cells. Here, we report that FTMOsN-induced down-regulation of MØ class II is the result of the induction of MARCH1, and that MØ expressing MARCH1 “resistant” class II molecules are resistant to FTMOsN-induced class II down-regulation. Since PGE$_2$ can induce IL-10 production and IL-10 is the only reported cytokine able to induce MARCH1 expression in MØ, this factor then drives MØ IL-10 production to induce MARCH1 expression and drive resulting class II down-regulation. Since many human pathogens such as Salmonella typhi, Mycobacterium tuberculosis and Legionella pneumophila also induce production of host PGE$_2$, these results suggest that a yet-to-be-identified PGE$_2$-inducible host factor capable of inducing IL-10 is central to the immune evasion mechanisms of multiple important human pathogens.

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Introduction

F. tularensis is a bacterial pathogen that infects macrophages and uses host-derived PGE$_2$ to enhance bacterial growth and subvert the adaptive immune response [1]. Other clinically relevant human pathogens such as Salmonella typhi [2,3], Mycobacterium tuberculosis [4] and Legionella pneumophila [5] also elicit host PGE$_2$, and thus may employ a similar strategy to promote infection. Francisella-induced PGE$_2$ acts directly on CD4 T cells, reprogramming them to restrict production of pro-inflammatory cytokines such as interferon-$\gamma$ (IFN-$\gamma$) [1]. Moreover, blockade of Francisella-induced PGE$_2$ production in vivo allows for more robust IFN-$\gamma$ production and better control of pulmonary Francisella infection in mice [6].

Francisella-induced PGE$_2$, which is elicited by either the live vaccine strain (LVS) or human virulent SchuS4 strain of Francisella, also acts in an autocrine/paracrine fashion to drive production of a soluble MO factor that elicits the ubiquitin-dependent down-regulation of MHC class II and CD86 molecules expressed by IFN-$\gamma$ activated MO [7] (Figure 1). The factor within this F. tularensis MO supernatant (termed FTMOsN, pronounced foʊt-moʊ-sin) drives MO class II down-regulation by eliciting ubiquitination of the class II cytoplasmic tail, which results in class II trafficking to degradative intracellular compartments [7,8]. The resulting class II negative MO are thus greatly impaired in their ability to present antigens to CD4 T cells.

Until now, the identity of the active factor in FTMOsN and the mechanism of class II ubiquitination were unknown. Here, we establish that the mechanism of FTMOsN induced class II ubiquitination is via up-regulation of the ubiquitin ligase MARCH1. Studies with IL-10 knockout (IL-10$^{-/-}$) MØ revealed that Francisella (via elicitation of PGE$_2$) is inducing production of a yet-to-be-identified factor that is able to elicit MO IL-10 production to then induce MO class II down-regulation via a MARCH1-dependent mechanism.

Methods

Ethics Statement

Mice were house and used in strict accordance to the guidelines established by the Albany Medical College Institutional Animal Care and Use Committee. Animal protocols were reviewed and
FTMØSN contains a soluble factor of >10 kDa molecular mass that elicits the ubiquitin-dependent down-regulation of "reporter" MØ MHC class II molecules [7]. In this report, we establish that the active factor in FTMØSN is distinct from IL-10, but that "reporter" MØ class II down-regulation is driven by induction of reporter MØ IL-10 production, which drives MARCH1 expression and class II ubiquitination. This means that FTMØSN contains a factor distinct from IL-10, which is induced by F. tularensis-elicted PGE₂ and which is able to drive reporter MØ IL-10 production. doi:10.1371/journal.pone.0037330.g001

Generation of Bone Marrow-Derived Macrophages

Producer bone marrow-derived macrophages (BMMØ – Figure 1) were generated from B10.Br, C57Bl/6 or IL-10Δ (on the C57Bl/6 background [9]) mice as previously described [7]. Reporter BMMØ from B10.Br, MARCH1Δ [10], class II K→R [11] or IL-10Δ [9] mice were treated with 100 U/ml IFN-γ for 24 hours and then washed before exposure to FTMØSN or control supernatants.

Measurement of IL-10

IL-10 was measured by cytometric bead array (CBA, BD Biosciences, San Jose, CA catalog number 553090) IL-10 Flex Set according to the manufacturer’s directions.

Treatment of BMMØ with FTMØSN

FTMØSN was generated by exposure of producer BMMØ to F. tularensis LVS at a multiplicity of infection of 100:1 (PGE₂) as previously described [7]. FTMØSN was cleared of any bacteria and cellular debris by centrifugation and 0.2 μm filtration. Reporter MØ were exposed to 50% FTMØSN or control supernatants for 20–24 hours before washing and harvesting to generate whole cell lysates. The anti-IL-10 receptor blocking mAb 1B1.3A (BioXCell # BE005) was used at the indicated concentration.

Western Blot Analysis of MØ MHC Class II Levels

Whole cell lysates of treated or control BMMØ were analyzed for total MHC class II levels by SDS-PAGE and anti-class II β chain western blot as previously reported [7]. Blots were subsequently probed for GAPDH with a loading control.

RT-PCR Analysis of MARCH1 mRNA Levels

Total RNA was prepared using TRIzol (Invitrogen) and treated with DNase I to remove genomic DNA. RNA was converted to cDNA using standard procedures and reagents from Invitrogen. Real-time PCR primers used to amplify MHC class II and MARCH1 have been described previously [12] and primers for GAPDH were from Qiagen (QuantiTect primer #QT01658692). Real-time PCR was performed using an ABI Prism 7900HT Sequence detection system and QuantiTect SYBR green PCR kit (QIAGEN) according to the manufacturers instructions. In all experiments the normalized Ct values for the FTMØSN-treated macrophages were expressed relative to the normalized Ct values for the mock supernatant-treated macrophages.

Results

The Ubiquitin Ligase MARCH1 is Responsible for FTMØSN-induced Down-regulation of Macrophage Class II Expression

Previous work established that upon Francisella infection, MO make PGE₂, which works in an autocrine/paracrine fashion to elicit the production of a >10 kDa soluble factor termed FTMØSN. FTMØSN drives the ubiquitin-dependent down-regulation of MHC class II expressed by IFN-γ pre-activated reporter MO (Figure 1 and [7]). The goal of this report is to define the mechanism of class II down-regulation and characterize the active factor in FTMØSN.

One mechanism for the post-translational control of MHC class II expression in MO and dendritic cells (DC) is the regulated expression of the ubiquitin ligase MARCH1, which ubiquinates the cytoplasmic domain of class II molecules resulting in their intracellular sorting to degradative intracellular compartments [13,14]. To determine if MARCH1 might be involved in FTMØSN-elicted down-regulation of class II, the effect of FTMØSN treatment on reporter MØ MARCH1 mRNA levels was determined (Figure 2). Overnight treatment of IFN-γ-pre-activated reporter MO with FTMØSN results in the up-regulation of MARCH1 mRNA, but no significant change in the level of Aβ class II mRNA expression, consistent with a post-transcriptional mechanism for regulating MHC class II expression. The current lack of reliable anti-MARCH1 antibodies precludes direct analysis of the impact of FTMØSN treatment on MARCH1 protein levels. Nevertheless, the results presented in Figure 2 suggest that FTMØSN-induced up-regulation of MARCH1 may be responsible for the ubiquitin-dependent down-regulation of MHC class II molecules observed in FTMØSN treated MO [7].

To further investigate this possibility, the level of class II expression by FTMØSN-treated wild type and MARCH1Δ reporter MO was determined (Figure 3). The results show that MARCH1Δ MO are completely refractory to the class II down-regulatory effects of FTMØSN, confirming the notion that FTMØSN-induced MARCH1 drives class II down-regulation. To further confirm this scenario and determine if class II is a direct target for MARCH1-mediated ubiquitination, the impact of FTMØSN treatment on class II expression by reporter MO expressing class II molecules lacking the single cytoplasmic lysine ubiquitination site (i.e., Aβ K225R [11]) was examined (Figure 3). Consistent with the idea that class II is the direct target for MARCH1-mediated ubiquitination, MARCH1-positive MO expressing these non-ubiquitinatable class II molecules are refractory to FTMØSN-induced MHC class II down-regulation. Taken together, the results presented in Figures 2 and 3 establish that MARCH1-mediated ubiquitination of the cytoplasmic tail of class II β chains is directly responsible for the FTMØSN-induced down-regulation of reporter MO MHC class II expression [7].

FTMØSN Contains a Soluble Factor the Induces MØ IL-10 Production

IL-10 is the only cytokine that has been demonstrated to induce MARCH1 expression in both DC and human monocytes [13,14].

Figure 1. FTMØSN Production and Action. F. tularensis infected “producer” MØ make PGE₂, which acts in an autocrine/paracrine fashion to drive the production of FTMØSN (FTMØSN MØ supernatant). FTMØSN contains a soluble factor of >10 kDa molecular mass that elicits the ubiquitin-dependent down-regulation of “reporter” MØ MHC class II molecules [7]. In this report, we establish that the active factor in FTMØSN is distinct from IL-10, but that “reporter” MØ class II down-regulation is driven by induction of reporter MØ IL-10 production, which drives MARCH1 expression and class II ubiquitination. This means that FTMØSN contains a factor distinct from IL-10, which is induced by F. tularensis-elicted PGE₂ and which is able to drive reporter MØ IL-10 production.
Moreover, PGE₂ has been reported to induce MØ production of IL-10 [15] and FTMØSN contains some level of IL-10 (see below). Together, this suggests that IL-10 is likely the PGE₂-induced factor in FTMØSN responsible for inducing MØ MARCH1 expression. To test this possibility, the ability of FTMØSN from both wild type and IL-10 knock out (IL-10 D) producer MØ to drive MHC class II down-regulation by wild type reporter MØ was determined (Figure 4). Surprisingly, FTMØSN from both wild type and IL-10 D producer MØ drive the same degree of reporter MØ MHC class II down-regulation, demonstrating that the active factor in FTMØSN is not IL-10 (The absence of IL-10 in FTMØSN generated by IL10 D BMMØ was confirmed by CBA, see below).

The ability of FTMØSN from IL-10 D producer MO to drive MHC class II down-regulation either means that FTMØSN contains a factor distinct from IL-10 that is able to elicit MARCH1 expression and drive class II down-regulation or that the active factor in FTMØSN acts by inducing IL-10 production by reporter MO which then induces MARCH1 expression and class II down-regulation. To determine which of these scenarios is correct, BMMØ from IL-10 D mice were used as reporter MO (Figure 5). IL-10 D reporter MO fail to down-regulate MHC class II expression in response to either wild type or IL-10 D FTMØSN, whereas wild type reporter MO down-regulate class II in response to both forms of FTMØSN. These results indicate that IL-10 produced by reporter MO is central to FTMØSN’s ability to induce class II down-regulation.

Consistent with the possibility that FTMØSN is acting through induction of IL-10, analysis of both FTMØSN and secondary supernatants from FTMØSN-treated reporter MO revealed that these secondary supernatants contain approximately 10 fold more IL-10 than FTMØSN itself (Figure 6). Moreover, treatment of reporter MO with high dose recombinant IL-10 (100 ng/ml) results in down-regulation of MO class II (Figure 7), whereas lower doses of recombinant IL-10 had a limited effect on MO class II...
levels (not shown). Finally, treatment of wild type reporter MØ with a blocking anti-IL-10 receptor mAb blocks FTMOsN-induced class II down-regulation (Figure 8). Taken together, these results indicate that FTMOsN contains a PGE2-inducible factor distinct from IL-10 that acts to induce IL-10 release by reporter MØ. This reporter MØ-produced IL-10 then acts in an autocrine/paracrine fashion to induce MARCH1 expression to drive ubiquitin-dependent MHC class II down-regulation.

**Discussion**

Previous studies have established that *Francisella tularensis*-infected MO produce PGE2, which can both alter CD4 T cell cytokine production and drive the production of a soluble factor (i.e., FTMOsN) that elicits the ubiquitin-dependent down-regulation of MO MHC class II expression [7]. Since other clinically relevant human pathogens such as *Salmonella typhi* [2,3], *Mycobacterium tuberculosis* [4] and *Legionella pneumophila* [5] also elicit host PGE2, it was of interest to determine the mechanism of FTMOsN-induced MO class II down-regulation.

Post-translation control of MHC class II expression in DC and MO can be driven by MARCH1-mediated ubiquitination of the cytoplasmic tail of class II molecules [13,14], which results in altered intracellular class II trafficking away from recycling endosomes and toward degradative compartments [8]. To date, IL-10 is the only cytokine reported to induce antigen presenting cell MARCH1 expression and class II down-regulation [13,14]. Since PGE2 can induce MO IL-10 [13], we explored the possibility that a PGE 2 to IL-10 to MARCH1 axis is responsible for the FTMOsN-induced down-regulation of reporter MO class II expression (Figure 1).

Analysis of reporter MO exposed to FTMOsN revealed that they up-regulate MARCH1 mRNA expression. In addition, MARCH1Δ MO fail to down-regulate class II expression in response to FTMOsN. Finally, MO expressing class II molecules refractory to the action of MARCH1 (i.e., I-A β chain K225R [11]) fail to down-regulate class II in response to FTMOsN. Taken together, these results establish that the active factor in FTMOsN drives the ubiquitin-dependent down-regulation of MO class II by increasing MO MARCH1 expression, which results in the ubiquitination of the cytoplasmic domain of class II and its post-endocytic sorting into a degradative compartment.

To address the question of whether the MARCH1-inducing factor in FTMOsN is IL-10, IL-10Δ producer MO were exposed...
to *F. tularensis* and the resulting FTMOsN tested for activity. Surprisingly, FTMOsN from IL-10 knockout MO is able to drive reporter MO class II down-regulation, ruling out IL-10 as the active factor in FTMOsN. In contrast, IL-10α-deficient MO fail to down-regulate class II in response to FTMOsN (both wild type and IL-10α-deficient FTMOsN). These results indicate that PGE2 is inducing IL-10 via an indirect mechanism. More precisely, PGE2 is inducing the production of a >10 kDa soluble factor distinct from IL-10 (i.e., the active factor in FTMOsN), which then acts to induce reporter MO IL-10 production, thus leading to MARCH1 expression and class II down-regulation. While previous reports have demonstrated that PGE2 can induce IL-10 production by MO and DC [13, 16], the molecular mechanism of IL-10 induction remains undetermined. These current findings suggest a complex mechanism, minimally involving the production of at least one yet to be defined soluble factor. These results also suggest that Francisella might act on to override MO responsiveness to this undefined soluble factor, since Francisella-infected producer MO (which are bathed in FTMOsN) do not make as much IL-10 as non-infected reporter MO exposed to FTMOsN. This possibility will be addressed in future studies.

Presently, the identity of the IL-10-inducing active factor in FTMOsN remains unclear. Preliminary experiments using either knockout MO and/or neutralizing mAbs have ruled out TGF-β, IL-6, VEGF, MIP-1α, and leukemia inhibitory factor. Moreover, generation of supernatants from Francisella-infected gene knock-out producer MO has established that production of FTMOsN activity is independent of TLR-2 (which recognizes *F. tularensis*-derived lipoproteins [17]) and MO caspase-1 activity (which drives IL-1β production/release). Therefore, it is likely that a combined genomics/proteomics approach will be necessary to conclusively identify and characterize the PGE2-induced active factor in FTMOsN that elicits MO IL-10 expression to then drive MO class II down-regulation.

MHC class II restricted antigen presentation by MO is central to immune defense against many human pathogens and tumors. Moreover, many human pathogens and tumors elicit the production of significant levels of PGE2. The results presented in this report establish that PGE2 drives the production of a soluble MO factor distinct from IL-10 that induces MO IL-10 production to drive MARCH1 expression, culminating in down-regulation of MO class II molecules and limiting the ability of these cells to participate in an adaptive immune response. Future identification of the active factor in FTMOsN will provide greater insight into this mechanism of immune suppression common to both infectious agents and tumors, and will provide an additional target for new interventional therapies.

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**Author Contributions**

Conceived and designed the experiments: DH JEW JAH PAR JRD. Performed the experiments: DH JEW KAW. Analyzed the data: DH JEW KAW JAH PAR JRD. Contributed reagents/materials/analysis tools: DH JEW KAW JRD. Wrote the paper: DH JEW JAH PAR JRD.

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**Figure 7. Recombinant IL-10 Down-regulates Reporter MO Class II Expression.** Reporter MO were treated with FTMOsN or 100 ng/ml of recombinant IL-10 (rIL-10). After overnight culture, the levels of class II (II) and GAPDH (G) was monitored as in Figure 4. Shown are representative results from 1 of 3 independent experiments. doi:10.1371/journal.pone.0037330.g007

**Figure 8. Blocking the IL-10 Receptor Blocks FTMOsN-induced Reporter MO Class II Down-regulation.** Reporter MO were treated with 20 μg/ml anti-IL-10R blocking mAb for 20 min. at 37°C or left untreated before addition of an equal volume of undiluted FTMOsN. After overnight culture, the levels of class II (II) and GAPDH (G) was monitored as in Figure 4. Shown are representative results from 1 of 3 independent experiments. doi:10.1371/journal.pone.0037330.g008
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