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Fcγ receptors and toll-like receptor 9 synergize to drive immune complex-induced dendritic cell maturation

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ABSTRACT

Previous *in vivo* studies established that inactivated *Francisella tularensis* immune complexes (mAb-*iFt*) are a more protective vaccine against lethal tularemia than *iFt* alone. Subsequent *in vitro* studies revealed enhanced DC maturation marker expression with mAb-*iFt* stimulation. The goal of this study was to determine the mechanism of enhanced DC maturation. Multiparameter analysis of surface marker expression and cytokine secretion demonstrates a requirement for FcγR signaling in enhanced DC maturation. MyD88 was also found to be essential for heightened DC maturation, implicating MyD88-dependent TLRs in DC maturation. Upon further study, we discovered that TLRs 2 & 4 drive cytokine secretion, but surprisingly TLR9 is required for DC maturation marker upregulation. These studies reveal a separation of DC cytokine and maturation marker induction pathways and demonstrate that FcγR-TLR/MyD88 synergy underlies the enhanced dendritic cell maturation in response to the mAb-*iFt* vaccine.

1. Introduction

According to the World Health Organization, vaccines prevent 2–3 million deaths per year. However, there are many infectious diseases for which there is no vaccine, including tularemia [1]. Respiratory tularemia, which can be fatal in up to 60% of cases, is caused by the intracellular bacterium *Francisella tularensis* (*Ft*) [2]. We have previously demonstrated that opsonizing the inactivated *Ft* with an IgG monoclonal antibody enhances immunity and protection against mucosal challenge with *Ft* [3]. This vaccine approach is also known as an immune complex vaccine. Immune complexes have been shown to be potent activators of immunity both in the context of chronic inflammatory/autoimmune diseases (i.e. atherosclerosis, multiple sclerosis, lupus, rheumatoid arthritis, etc.), as well as vaccine responses [4–8]. It is therefore important to study how immune complexes elicit immune reactions in order to design effective vaccines with minimal side-effects.

Dendritic cells (DCs) are key players in eliciting adaptive immune cell activation; they link the innate and adaptive arms of immunity by presenting antigen and secreting factors such as cytokines. Because activation of T cells relies heavily on their interaction with DCs, the type of adaptive response generated is largely dependent on the DC maturation status [9]. At steady state, most DCs are considered immature; they are highly phagocytic and express very low levels of co-stimulatory molecules (e.g. CD40, CD80, CD83, & CD86). At this stage, MHC-restricted antigen presentation to T cells without co-stimulatory molecules results in tolerogenic responses. To elicit an immunogenic response, DCs must recognize an antigen as foreign through various pattern recognition receptors (PRRs). Signaling through these receptors will trigger induction of DC maturation which includes: increased proteolytic processing, decreased phagocytic capacity; increased surface expression of major histocompatibility complex class II (MHC II) and co-stimulatory molecules; and migration of DCs to lymph nodes where they can present antigen to T cells [10,11]. Studies using mAb-*iFt*-stimulated DCs/macrophages have shown increased maturation marker expression, secretion of IL-1β & TNF-α, and increased presentation to T cells [12–14]. We therefore sought to define the cellular/molecular mechanism of mAb-*iFt*-induced DC maturation.

Fc receptors are one type of receptor, which assist DC recognition of mAb-*iFt*; they bind the constant region (Fc) of antibodies thus allowing

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Abbreviations: *Ft*, *Francisella tularensis*; *iFt*, methanol-free formaldehyde-inactivated *Francisella tularensis*; mAb-*iFt*, immune complex containing inactivated *Francisella tularensis* and monoclonal antibody against *Francisella tularensis* lipopolysaccharide; BMDCs, bone marrow-derived dendritic cells; FcγR, Fc gamma receptor; FcR-γ, Fc receptor gamma chain

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DCs to bind and take up immune complexes. mAb-*iFt*, contains mouse IgG2a so our focus is on Fc receptors that bind IgG. There are three different classes of receptors which bind IgG: FcγRs, FcRn, and TRIM21 [15]. FcγRs are the only surface-expressed IgG receptor and therefore, likely the first of the three to bind IgG immune complexes. In mice there are four FcγRs: FcγRI, IIb, III, and IV; all of which, except FcγRIIb, are considered activating receptors based on the presence of an Immunoreceptor Tyrosine-based Activation Motif (ITAM) within the receptors' associated γ-chain. FcγRIIb, does not associate with Fc receptor γ-chain and instead contains an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) within its cytoplasmic tail. Because the activating receptors contain an ITAM, their signaling cascades are very similar to other receptors containing ITAMs (e.g. B cell receptor). Upon ligation and crosslinking of an activating FcγR, tyrosine residues in the ITAM are phosphorylated by an active Src family kinase. This phosphorylation creates a docking site for active spleen tyrosine kinase (Syk), that which subsequently phosphorylates downstream signaling mediators such as phosphatidylinositol-3kinase (PI3K) [15]. It is important to note however, that mAb-*iFt* could be recognized by receptors other than FcγRs.

Another receptor family involved in DC recognition of antigen is toll-like receptors (TLRs). TLRs are part of the pattern recognition receptor family which recognize repeating patterns within pathogens as foreign. The recognition of pathogens through TLRs triggers signaling cascades that include adaptor proteins such as myeloid differentiation primary response 88 (MyD88), kinases like interleukin-1 receptor-associated kinases (IRAKs), et cetera, which ultimately lead to activation of transcription factors such as nuclear factor kappa-light-chain-enhances of activated B cells (NF-κB). The signaling cascades triggered by TLR ligation can elicit DC maturation, migration, and therefore an enhanced ability to trigger immunogenic responses in adaptive immune cells. Studies have shown that macrophages stimulated with mAb-*iFt* have increased IL-6 and IL-1β secretion because *iFt* can be recognized/bound by TLR2 [14,16]. It is therefore likely that TLR ligation of mAb-*iFt* plays a role in DC maturation.

In this study, we demonstrate a requirement for both MyD88 and FcR γ-chain ITAM signaling in the maturation of mAb-*iFt*-stimulated bone marrow-derived DCs (BMDCs). Fc receptor γ-chain deficient and WT cells treated with a FcR γ-chain signaling inhibitor have decreased maturation in mAb-*iFt* stimulated cells. In addition, we see substantial reduction in the maturation of mAb-*iFt*-stimulated cells lacking MyD88. We explored this further and found, surprisingly, that mAb-*iFt*-stimulated BMDCs deficient in TLR2 or TLR4 have no changes in CD80, CD86, or MHC II expression when compared to the WT BMDCs. They do, however, have changes in IL-6, IL-2p70, and TNF-α secretion. Ultimately, we found that TLR9 is responsible for mAb-*iFt*-induced expression of DC maturation markers. These studies also reveal a separation of DC cytokine and maturation marker induction pathways, demonstrating that both aspects need to be examined when studying DC maturation. In addition, the requirement for both FcR γ-chain ITAM signaling and TLR2/4 & TLR9 in mAb-*iFt*-induced BMDC maturation reveals a FcγR-TLR/MyD88 synergy that underlies enhanced dendritic cell maturation in response to the mAb-*iFt* vaccine.

2. Materials and methods

2.1. Mice

Mice were bred under pathogen free conditions in Albany Medical College's Animal Resource Facility. To obtain human FcγRI (hFcγRI) heterozygote (−/+) mice, as well as hFcγRI (−/−) (WT) littermates for our experiments, hFcγRI (−/+) transgenic males were bred with age-matched WT C57BL/6 females obtained from Taconic Biosciences (Rensselaer, NY). This breeding strategy (Tg Males × WT Females) has continued for the last 14 years. All offspring are screened for the presence or absence of the hFcγRI transgene with PCR analysis of tail snips from the pups. The hFcγRI (−/+) mice were originally generated by backcrossing hFcγRI FVB/N mice onto a C57BL/6 background for 12 generations [1]. We obtained the hFcγRI (−/+) male mice from Medarex Inc. in 2005. In the few instances where our own WT mice were not available for use (Fig. S1), C57BL/6 mice were ordered from Taconic Biosciences (Rensselaer, NY). TLR2$^{-/-}$ (B6.129-*Tlr2*^{tm1Kir}/J), TLR4$^{-/-}$ (B6(Cg)-*Tlr4*$^{tm1.2Karp}$/J), and MyD88$^{-/-}$ (B6.129P2(SJL)-Myd88$^{tm1.1Defr}$/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). FcR-γ$^{-/-}$ (B6.129P2-*Fcer1g*^{tm1Rav} N12) mice were obtained from Taconic Biosciences. Mice were provided with water and food *ad lib*. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Reagents

Anti-*F. tularensis* LPS monoclonal antibody (mouse IgG2a) was purchased from Fitzgerald Industries (Acton, MA). Lipopolysaccharide from *E. coli* O111:B4 (LPS) was purchased from Millipore Sigma, (Burlington, MA). Poly(I:C), a TLR3 ligand; ODN 1826, a TLR9 agonist; ODN 2088, a TLR9 antagonist; and R406, a Syk inhibitor, were purchased from InvivoGen (San Diego, CA). Bio-Plex® kits were purchased from Bio-Rad (Hercules, CA). eFluor 450 conjugated anti-MHC Class II (I-A/I-E) Monoclonal Antibody (clone M5/114.15.2) was purchased from eBioscience (Waltham, MA). PE conjugated anti-CD80 Antibody (clone 16-10A1) and APC conjugated anti-CD86Antibody (clone Gl-1) were purchased from BioLegend (San Diego, CA). αTLR2 was purchased from InvivoGen. αFcγRI clone # 290322 was purchased from R&D Systems. αFcγRII/III (Mouse BD Fc Block) clone 2.4G2 was purchased from BD Biosciences (San Jose, CA). Mouse IgG2a Isotype Control clone UPC-10 for making mAb-beads was purchased from Millipore Sigma.

2.3. Buffers

ACK (ammonium-chloride-potassium) lysis buffer was composed of: 150 mM NH_4CL, 10 mM $KHCO_3$, 0.1 mM EDTA. BMDC culture media included: 10% heat inactivated FBS (HyClone, Marlborough, MA), 1% 100 mM sodium pyruvate (Millipore Sigma), 1% 200 mM L-glutamine (Gibco), 1% MEM non-essential amino acids (Millipore Sigma), 1%, penicillin–streptomycin 100x solution (HyClone), 0.05 mM 2-mercaptoethanol (Bio-Rad), RPMI 1640, and 50 ng/mL recombinant mouse Flt3 ligand (R&D Systems, Minneapolis, MN).

2.4. Bone Marrow-derived dendritic cells

Femurs were removed from FcR γ-chain$^{-/-}$ (FcR-γ$^{-/-}$), MyD88$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$ or wild-type C57BL/6 mice and crushed using a mortar and pestle containing RPMI1640 (Gibco, Gaithersburg, MD). Debris was removed via 70 μm nylon cell strainer, and the cells recovered by centrifugation (200*g*, 5 min.). The resulting cell pellet was re-suspended in ACK lysis buffer, centrifuged, and re-suspended in BMDC culture media; the media was replaced after 3 days. At 6 days, cells were mostly non-adherent; adherent and settled cells were lifted via scraping. All cells (adherent and non-adherent) were then re-suspended in fresh BMDC culture media, plated in 6 well plates (1×10^6 cells/well), and incubated overnight before stimulation.

2.5. Generation of stimuli

iFt: Inactivation of *Francisella tularensis* (*Ft*) and generation of mAb-*iFt* complexes were done essentially as described [13]. Briefly, *Ft* were grown on chocolate agar plates (BD Biosciences) and a single colony was picked and expanded in Mueller Hinton Broth (MHB) (BD Biosciences) for 24 h. The concentration of the bacteria was estimated by optical density (OD) at 610 nm and *Ft* was once again expanded in MHB overnight. The resulting culture was washed twice in PBS and fixed in 2% methanol-free formaldehyde (MFF) (Millipore Sigma). This inactivated *Ft* (*iFt*) was washed in PBS and re-suspended at a concentration between 4×10^9 and 4×10^{10} bacteria/mL. Fixation Inactivation of *Ft* was confirmed by lack of growth on chocolate agar plates for 7 days.

mAb-*iFt*: To generate mAb-*iFt*, 4×10^9 *iFt* were opsonized with 10 µg of anti-*F. tularensis* LPS monoclonal antibody in 1 mL PBS with rocking at 4 °C overnight. mAb-*iFt* immune complexes were then washed twice in PBS, aliquoted at a concentration of 4×10^9 bacteria/mL, and stored at −20 °C until use.

BSA- & mAb-beads: Glass beads (2 µm in diameter) were coated with either IgG-free bovine serum albumin (Sigma A0281) or mouse IgG2a isotype control via poly-L-lysine-dimethyl pimelimidate linkers as previously described [17].

2.6. BMDC stimulation

BMDCs (1×10^6) were stimulated with culture media, *iFt* (50/cell), mAb-*iFt* (50/cell), LPS (2.25 ng/mL), BSA- or mAb-beads (5–30 per cell), Poly(I:C) (5 µg/mL), or ODN 1826 (5 µM) for 24 h. For Syk inhibition studies, BMDCs were pretreated with 0.1, 0.3, 1, or 3 µM of R406 for 30 min before stimulation. For αTLR2 treatment (10 µg/mL) and TLR9 inhibition (10 µM ODN 2088), BMDCs were pretreated for 1 h before stimulation. For FcγR blocking, αFcγRI clone # 290322 and/or αFcγRII/III (Mouse BD Fc Block) clone 2.4G2 were added to the cells as a pre-treatment was for 20 min prior to stimulation.

2.7. Flow cytometry

Cells (5×10^5) were harvested, counted, and placed into wells of a u-bottom 96-well non-treated plate with 0.2 mg of IgG from human serum (Millipore Sigma) for 10 min on ice. Cells were then stained (MHC Class II, CD80, and CD86), fixed, re-suspended in 400 µl of PBS-BSA, and analyzed using FlowJo software. Cell debris was removed before gating on single cells (Fig. 1A). Unstained cells were then used to draw the positive/negative gates on the resultant histograms (i.e. Fig. 1B) Due to the differences in scatter generated by the beads, voltages had to be altered for the bead-containing samples; BSA-beads served as a negative control for gating. Therefore, when depicting the results from these experiments, fold change was used for comparisons (i.e. mAb-beads/BSA-beads & mAb-*iFt*/*iFt*). Cells incubated in media alone had a basal level of marker expression which was not present in unstained cells (and therefore not autofluorescence).

2.8. Cytokine analysis

BMDCs were stimulated as described above. At 24 h, the supernatants were collected and stored at −20 °C for cytokine analysis. Cytokine release was quantified using the Bio-Plex® multiplex assay per manufacturers' instructions with the exception that all assay volumes were reduced by half. Washes were completed with 100 µl of 1x wash buffer.

2.9. Data analysis

All data shown are in raw format to illustrate differences between groups/treatments. However, due to heteroskedasticity, all data was Log_{10} transformed prior to statistical analysis. In some cases where there were zeros as values, the number 1 was added to all data prior to Log_{10} transformation. Statistical analysis and post-tests are specifically indicated within each figure legend for clarity.

3. Results

3.1. Targeting iFt to FcγRs via mAb-iFt results in enhanced DC maturation

We previously reported that BMDCs stimulated with the immune complex, mAb-*iFt*, have higher expression of maturation markers/costimulatory receptors (i.e. CD80, CD83, CD86, and MHC II) compared to cells stimulated with inactivated *Francisella tularensis* (*iFt*) alone [12]. We wanted to not only confirm that mAb-*iFt*-induced maturation was enhanced compared to *iFt* stimulation, but also know whether increased maturation marker expression corresponded with increased cytokine secretion. Before stimulating the BMDCs, however, we wanted to analyze the phenotype and heterogeneity of our BMDCs. We followed the staining and gating strategy of Xu, et. al. to look for the presence of plasmacytoid DCs (pDCs), CD24^+ conventional DCs (cDCs), and/or CD11b^+ cDCs (Fig. S1) [18]. This analysis revealed that our BMDCs are a heterogeneous population composed of ~30% pDCs, and a variety of cDCs (25% CD24^+ and 61% CD11^+). Next, WT BMDCs were stimulated with *iFt*, mAb-*iFt*, or LPS for 24 h before supernatants were collected and cells stained for the maturation markers CD80, CD86, and MHC II. After removal of debris and selecting for single cells, the unstained cells were used to draw gates for CD80^+, CD86^+, and MHC II^+ populations individually. With this strategy, shown in Fig. 1 A & B, we found that BMDCs exposed to media alone (blue-shaded peaks) have a basal level of marker expression quite different than the unstained population (gray-shaded peaks). The other stimulants (*iFt*, mAb-*iFt*, and LPS) had further increases in each of these markers when compared to media alone. More specifically, *iFt* stimulation resulted in a trend toward higher expression of CD80 & MHC II, and significantly higher levels of CD86 compared to the cells incubated in media alone. mAb-*iFt*-stimulated cells had significantly higher expression of all markers when compared to media. The CD86 and MHC II levels seen in the mAb-*iFt*-stimulated cells also trended toward increased expression over the *iFt* alone, whereas the CD80 expression was significantly increased over the *iFt*-stimulated cells. Our positive control in this instance, LPS stimulation, was significantly increased over the media alone group in every instance. Soluble mAb, on the other hand, did not increase expression of CD80, CD86, or MHC II (Fig. S2). In addition, it is important to note that when we observed any increase in CD80, CD86, or MHC II, the entire peak on the histogram(s) of Fig. 1A shifted to a higher MFI rather than a specific DC population becoming enriched for CD80, CD86, or MHC II. This would imply that the BMDCs increase their maturation marker expression when stimulated regardless of DC subtype (plasmacytoid vs. conventional). Overall, these MFI bar graphs confirmed our previous studies; BMDCs stimulated with mAb-*iFt* have enhanced maturation.

We then questioned whether the MHC II^+ cells were also positive for CD80 and/or CD86. The answer to this question would make a large impact on the outcome of the overall immune response; if cells which upregulate MHC II expression do not have increased CD80/CD86 expression, a tolerant reaction would occur. Both MHC II and CD80/CD86 should be expressed on the cells if they are to trigger a protective immune response against *iFt*. In the previous study, we had shown a protective response in mice immunized with mAb-*iFt*. Thus, we predicted to see an increase in CD80 and/or CD86. However we wanted to confirm this in our *in vitro* BMDC system. We assessed MHC II^+ single cells for both CD80 & CD86 expression 24 h after the stimulation described above. As shown in the contour plots and the bar graphs of Fig. 1C, both the proportion and the frequency of cells expressing both CD80 and CD86 in MHC II^+ BMDCs was significantly increased compared to media, regardless of the stimulant used. There were, no significant differences found between the various stimulants when assessing the triple positive population (MHC $\text{II}^+\text{CD80}^+\text{CD86}^+$), however there is an increasing trend seen where *iFt* induces an increase over media, mAb-*iFt* stimulation further increases the triple positive population, and LPS stimulation induces largest triple positive population.

Surface expression of T cell ligands/coreceptors, however, is not the only requirement for activation of naïve T cells; cytokines are also needed to activate and inform the differentiation of T cells (e.g. $\text{T}_\text{H}1$ vs $\text{T}_\text{H}2$). Therefore, we analyzed the amount of secreted IL-6, IL-12p70, & TNF-α in the supernatants of cells in panels A–C using Bio-Plex®

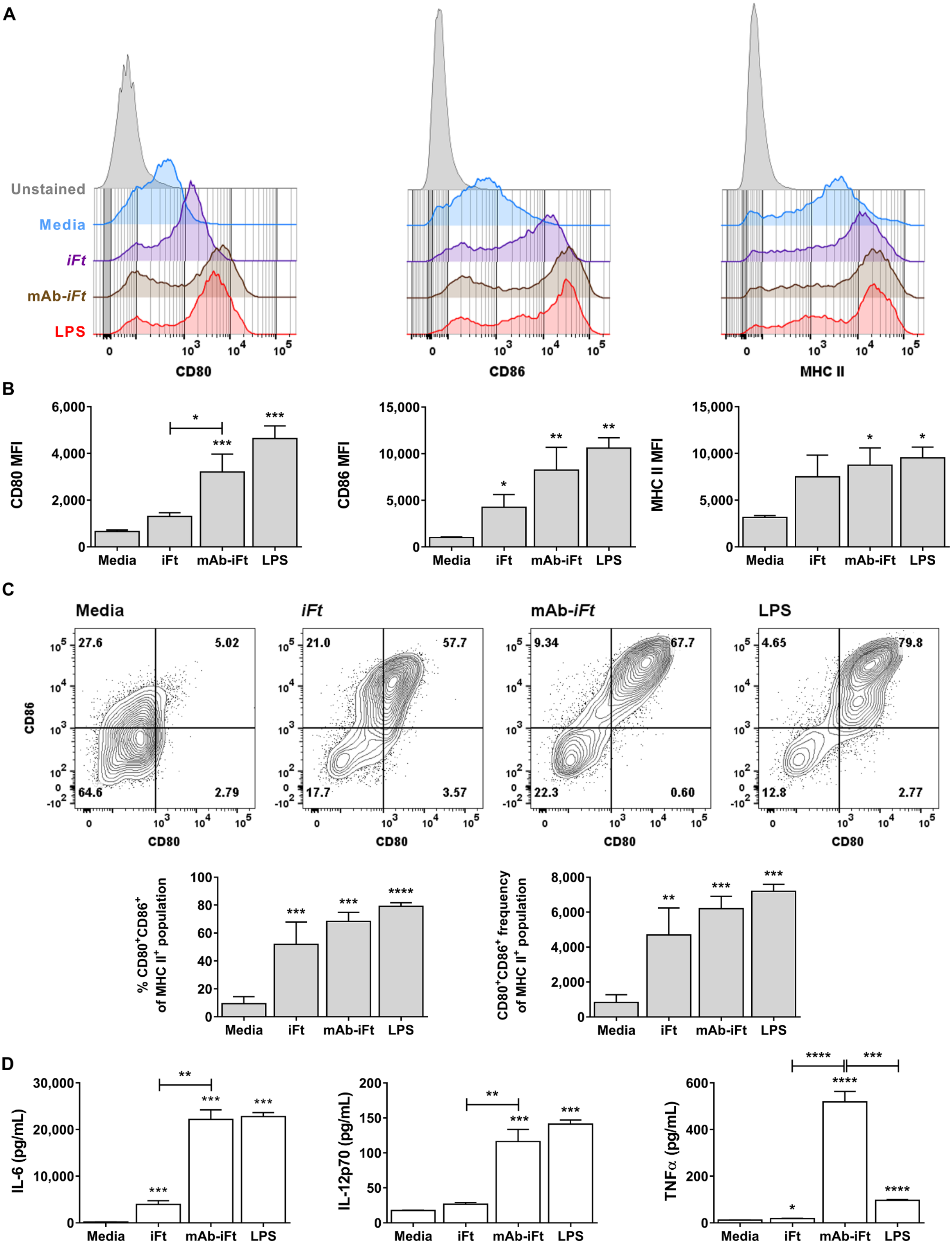

Fig. 1. Stimulation with mAb-*iFt* enhances BMDC maturation. WT BMDCs were stimulated with LPS (2.25 ng/mL), *iFt* (MOI 50), or mAb-*iFt* (MOI 50) for 24 h. Supernatants were collected for cytokine analysis (D) and cells were stained for CD80, CD86, and MHC II (A–C). (A) After gating out debris and doublets, unstained cells were used to draw gates on CD80$^+$, CD86$^+$, or MHC II$^+$ cell populations. Histograms representative of one experiment are shown. (B) Geometric mean fluorescent intensities (MFI) for CD80, CD86, or MHC II positive cells from the histograms in (A) were combined from three separate experiments and plotted as mean ± SEM. Further characterization of the MHC II$^+$ cells was then conducted by plotting CD80 vs. CD86 as shown in (C). Contour Plots are representative of one experiment whereas combined data from three separate experiments is shown in the bar graphs as mean ± SEM. (D) Cytokine analysis of BMDC supernatants was completed via Bio-Rad multiplex kit. Concentrations of IL-6, IL-12p70, and TNF-α from three different experiments are shown as mean ± SEM. All bar graphs were analyzed with an ordinary one-way ANOVA with a Tukey's post-test after Log$_{10}$ transforming the data to account for heteroskedasticity. $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$ compared to media unless noted with a bar.

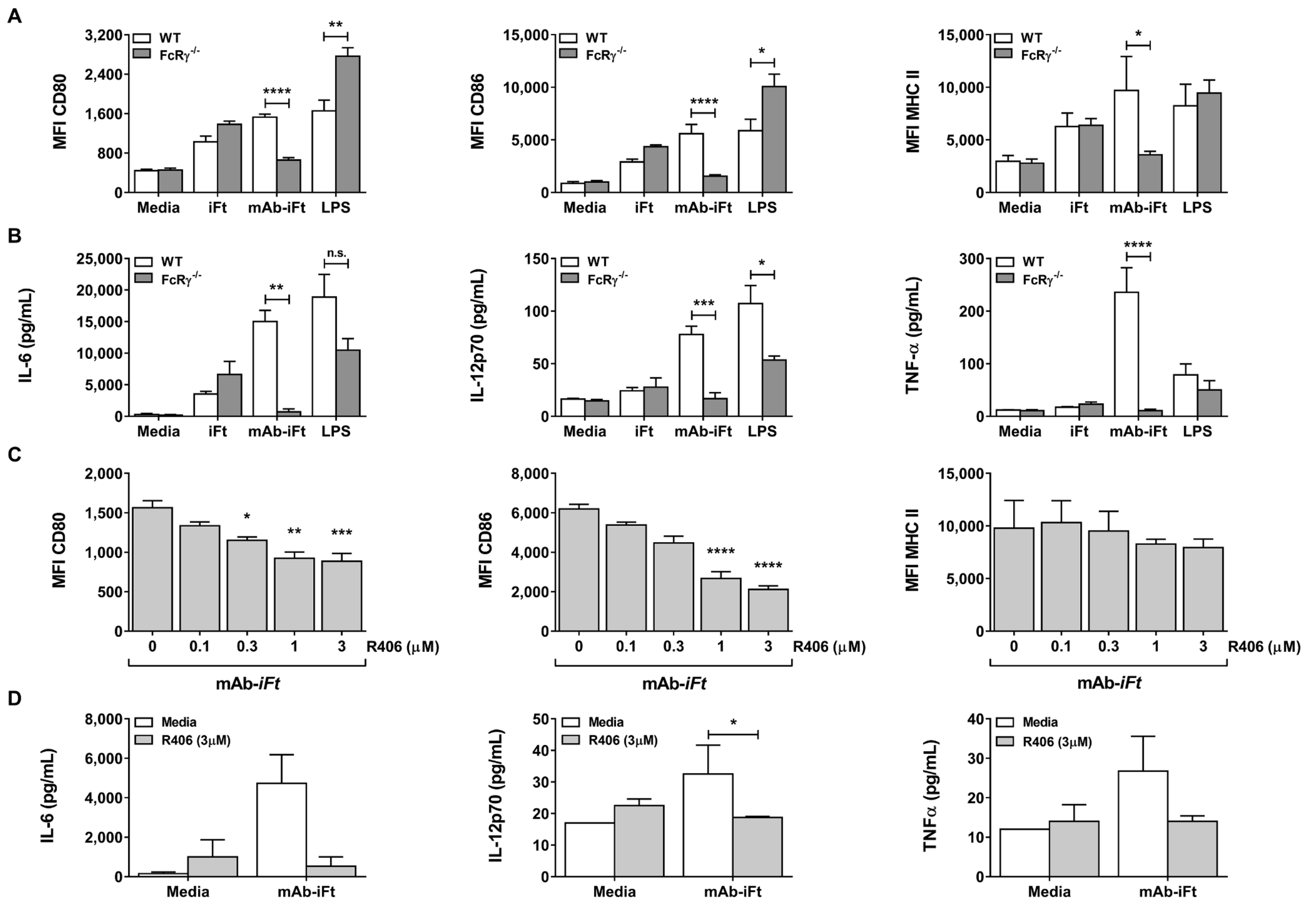

Fig. 2. BMDCs deficient in FcR-γ or treated with Syk inhibitor have decreased maturation compared to WT when stimulated with mAb-*iFt*. (A & B) WT (white bars) and FcR-γ$^{-/-}$ (gray bars) BMDCs and their corresponding supernatants were stimulated and studied as described in Fig. 1. (C & D) BMDCs were pretreated with R406 for 30 min and subsequently stimulated with media or mAb-*iFt* (50 *iFt*/cell) for 24 h. Supernatants were collected for cytokine analysis (D) and cells were stained for CD80, CD86, & MHC II (C). R406 was present for the entire duration of the experiment. Data are combined from three experiments where bars represent mean ± SEM. Transformed data from panels A & B were analyzed by two-way ANOVA with Sidak's post-test (WT vs. knockout). Transformed data from mAb-*iFt* stimulated cells of panel C were analyzed with an ordinary one-way ANOVA with a Dunnett's multiple comparison test (everything compared to 0 μM R406). Cytokine concentrations in panel D were transformed and then analyzed via two-way ANOVA with Sidak's post-test (WT vs. knockout).*$p \le 0.05$, **$p \le 0.01$, ***$p \le 0.001$, & ****$p \le 0.0001$.

multiplex immunoassay kits. Similar to the MFI analysis, *iFt*-stimulated cells had a slight increase in cytokine expression over media, whereas LPS- and mAb-*iFt*-stimulated cells secreted greater amounts of IL-6, IL-12p70, and TNF-α (Fig. 1D). Because cytokine secretion of IL-6, IL-12p70, and TNF-α, as well as maturation markers CD80, CD86, and MHC II expression were all greater in mAb-*iFt*-stimulated cells than *iFt*-stimulated cells, we concluded that targeting *iFt* to FcγRs in the form of an immune complex enhances multiple aspects of BMDC maturation.

3.2. Fc receptor gamma chain (FcR-γ) is required for mAb-iFt-induced BMDC maturation

Our previous *in vivo* studies with mAb-*iFt* demonstrated a requirement for Fc receptor gamma chain (FcR-γ) [3]. We therefore hypothesized that FcR-γ signaling is needed for the increase in DC maturation observed in Fig. 1. Thus, we stimulated both WT and FcR-γ knockout (FcR-γ$^{-/-}$) BMDCs with *iFt* or mAb-*iFt* for 24 h before collecting supernatants and staining for maturation markers. The resulting data (Fig. 2A & B) showed both heteroskedasticity and high variability in the effect of mAb-*iFt* on maturation when compared to *iFt* alone. Specifically, the difference between mAb-*iFt*-stimulated and *iFt*-stimulated MFIs shown in Fig. 2A is much smaller than previously noted in Fig. 1. It is important to note here that each bar in the figure shown is representative of three experiments (n = 3), each individual experiment including technical duplicates or triplicates which, when combined, exhibited very low variability. In addition, each experiment exhibited the same trend. Which leads us to believe that the variability seen is due to natural variation in different batches of BMDCs, *Ft*, and mAb-*iFt*. We consequently Log$_{10}$ transformed the data prior to statistical analysis to account for the heteroskedasticity. MFI and cytokine secretion data display notable decreases when comparing mAb-*iFt*-stimulated FcR-γ$^{-/-}$ cells (gray bars) to WT cells (white bars). This is not the case with *iFt*. Instead, FcR-γ$^{-/-}$ cells stimulated with *iFt* had small, insignificant increases in CD80 and CD86 expression and no changes in MHC II or cytokine secretion (Fig. 2). Interestingly, we observed a dramatic increase in CD80 and CD86 expression in FcR-γ$^{-/-}$ BMDCs stimulated with LPS (Fig. 2A) whereas cytokine secretion had a downward trend, especially in IL-12p70 where the decrease was significant (Fig. 2B). This was not entirely unexpected as Wei, et al. showed a decrease in IL-6 and TNF-α secretion in LPS-stimulated bone marrow-derived macrophages, a cell type similar to BMDCs [19]. In contrast, surface expression of the maturation markers CD80 and CD86 were increased in response to LPS, suggesting that maturation markers and cytokine secretion may be independently regulated in BMDCs. The observation of slight increases in CD80 and 86 MFIs in *iFt*-stimulated knockout cells was also unexpected and will require further study in the future, as we do not know the cause of this result. Nevertheless, the fact that mAb-*iFt*-stimulated FcR-γ$^{-/-}$ cells had reductions in BMDC maturation while the *iFt*-stimulated knockout cells did not, reveals that maturation of mAb-*iFt*-stimulated BMDCs requires FcR-γ while *iFt* alone does not.

It is important to note that FcR-$\gamma^{-/-}$ mice have been documented to have markedly reduced surface expression of Fc receptors [20]. Also, the inhibitory receptor, FcγRIIb, does not utilize γ-chain. It is therefore possible that the mAb-*iFt* cytokine results in Fig. 2B & D are due to an increase in mAb-*iFt* binding to the inhibitory receptor and subsequent skewing toward an inhibitory response in the DCs. In addition, the changes in LPS induced maturation in FcR-$\gamma^{-/-}$ BMDCs (Fig. 2A & B) were somewhat unexpected as stated above. Therefore, we decided to examine the role of activating FcγRs in BMDC maturation without altering the binding profile of the various stimuli. To accomplish this, we utilized a pharmacological inhibitor of FcR-γ signaling. As mentioned in the introduction, ITAM signaling of FcR-γ relies on phosphorylation by src family kinases. Syk is then recruited and activated by the phospho-ITAM in order to propagate the signaling cascade to other downstream effectors [15]. Inhibition of Syk would consequently result in abrogation of FcR-γ signaling. We therefore pretreated BMDCs with either media or varying doses of the Syk inhibitor R406 to assess the role of FcR-γ in another way. After pre-treatment, cells were incubated with media or mAb-*iFt* (in the continued presence of inhibitor) for 24 h prior to maturation marker staining and cytokine analysis. As Fig. 2C depicts, increasing amounts of R406 result in decreased CD80 and CD86 MFIs in mAb-*iFt*-stimulated cells. However, the MHC II MFI does not change with the amount of R406 suggesting that MHC II is not affected by FcR-γ signaling. Cytokine analyses also show decreases in the inhibitor pretreated BMDCs that are stimulated with mAb-*iFt*, in fact the decrease observed in IL-12p70 CD86 was significant. In the case of LPS stimulation, R406 pre-treatment significantly decreases CD80 and CD86 expression (Fig. S3) confirming the decreases observed in Fig. 2A & B. Cytokine data from LPS-stimulated cells, however, exhibited increases when cells were pretreated with R406. Together with the FcR-$\gamma^{-/-}$ results, these data suggest that enhanced maturation of mAb-*iFt*-stimulated BMDCs seen in Fig. 1 relies on FcR-γ ITAM signaling. However, it is important to note that FcR-γ is utilized by several receptors other than FcγRs such as some activating Fc receptors (e.g. FcεRI & FcαRI), TLR4, IL-3 receptor, TREM, Dectin-2, and others. [19,21,22] Thus, we used FcγR-selective blocking antibodies to determine their contribution to BMDC activation/maturation.

WT BMDCs were first incubated with 10 µg/mL of either an anti-FcγRI antibody, anti-FcγRIIb/III antibody, or both. Blocking antibodies remained present for the entirety of the experiment. αFcγRI or αFcγRIIb/III alone, or the two combined had no significant effect on the maturation status of BMDCs incubated with media alone. However, incubation with αFcγRIIb/III alone or in combination with αFcγRI decreased maturation marker expression and cytokine production in BMDCs stimulated with mAb-*iFt* (Fig. 3). This implicates FcγRIIb/III signaling in mAb-*iFt*-induced maturation. As FcγRIIb is inhibitory, the αFcγRII/III is likely blocking BMDC maturation via the activating FcγRIII. Thus, we conclude that FcγRIII mediates mAb-*iFt*-induced maturation in our BMDCs. This conclusion is in line with previous literature stating that FcγRIII in mouse cells is biased to binding immune complexes [23,24].

3.3. Monoclonal antibody-coated beads (mAb-Beads) do not elicit BMDC maturation

The fact that the Syk inhibitor R406 significantly reduces mAb-*iFt*-stimulated BMDC maturation (Fig. 2), and that blocking FcγRIIb/III decreases both maturation and cytokine secretion (Fig. 3), implicates FcγR signaling in BMDC maturation. To determine whether FcγR signaling alone is sufficient for mAb-*iFt*-enhanced DC maturation, we tested the ability of mAb-coated beads (lacking other ligands) to stimulate BMDCs. Glass beads (2 µm) were coated with either mouse IgG2a (mAb-beads) or bovine serum albumin (BSA-beads). mAb-beads are used routinely by the Lennartz laboratory to study FcR-mediated phagocytosis in bone marrow-derived macrophages; BSA-beads serve as a non-binding, non-internalized control [17]. By using mouse IgG2a, mAb-Beads should engage the same FcγR(s) as mAb-*iFt* but will lack the signaling contributed by the *iFt*. To account for any mechanical effects the glass beads may have on the BMDCs, we used BSA-coated beads which neither bind nor are internalized by bone marrow-derived macrophages [25]. After confirming mAb-bead binding/internalization via fluorescent microscopy (data not shown), BMDCs were stimulated for 24 h with *iFt*, mAb-*iFt*, BSA-beads, or mAb-Beads. Then supernatants were collected for cytokine analysis and cells were stained for maturation markers. As the presence of beads significantly shifted the scatter pattern of the BMDCs, it was necessary to use different voltages for the bead-containing samples, making direct comparison between BMDCs stimulated with mAb-*iFt* to those stimulated with beads impossible. To allow for comparisons between the two target types (mAb-*iFt* vs. mAb-beads), we report the normalized ratios (Fig. 4A). For all measures, the bead ratios were lower than their respective *iFt* measures, trending lower for CD80 and MHCII but significantly lower for CD86 (Fig. 4A). Release of all the measured cytokines by mAb beads was significantly lower than its' mAb-*iFt* counterpart (Fig. 4B). Together with the data from Fig. 1, we conclude that BMDC maturation requires input from both *iFt* and IgG, with the IgG input likely coming from FcγRIII signaling.

3.4. TLR2 and TLR4 plays a role in BMDC cytokine secretion, but does not influence surface maturation marker expression induced by mAb-iFt stimulation

We have shown thus far that mAb-*iFt* stimulation of BMDC results in an enhanced maturation status which requires FcR-γ signaling (Figs. 1–3). However, stimulating DCs with mAb-Beads results in little maturation (Fig. 4). One explanation for this could be the presence of toll-like receptor (TLR) ligands on mAb-*iFt*. Many studies have shown evidence for FcγR crosstalk/synergy with TLR, and in the context of mAb-*iFt* FcγRs have been shown to synergize with TLR2 in eliciting cytokine secretion from macrophages [14,16,26,27]. We therefore postulated that TLR2 could play a role in mAb-*iFt*-enhanced DC maturation. We stimulated WT and TLR2 knockout (TLR2$^{-/-}$) cells with LPS, *iFt*, or mAb-*iFt* as previously described. Surprisingly, we found that mAb-*iFt* stimulated cells had no significant changes to their maturation marker MFIs despite the absence of TLR2 (Fig. 5A). This came as a surprise to us, as TLR2 has been shown to play a role in mAb-*iFt* stimulation of macrophages [14,16]. *iFt*-stimulated TLR2$^{-/-}$ BMDCs, on the other hand, had significantly diminished MFIs across the board as expected and LPS-stimulated cells had unchanged MFIs.

Cytokine data from the TLR2$^{-/-}$ cells revealed that secretion of IL-6, IL-12p70, and TNF-α was diminished in the knockout cells compared to WT regardless of stimuli (Fig. 5B). This corroborated the MFI data of *iFt*-stimulated cells, and supported the results observed by other groups in mAb-*iFt*-stimulated macrophages. The IL-6 and IL-12p70 results for LPS, however, were a bit unexpected; in the presence of anti-TLR2 antibody (αTLR2), LPS-induced IL-6 secretion was reduced and IL-12p70 was significantly reduced. One possible explanation for our results could be LPS contamination with a TLR2 ligand, as has been previously reported [28]. We therefore pretreated our BMDCs with an αTLR2 that blocks ligand from binding to assess the role of TLR2 in BMDC maturation and cytokine secretion. We first verified the function of the antibody by stimulating BMDCs with Pam$_3$CSK$_4$ after pre-treatment with or without αTLR2 (data not shown). We then tested the αTLR2 in our system. Specifically, BMDCs were pretreated with αTLR2 and subsequently stimulated with LPS, *iFt*, or mAb-*iFt*. After 24 h of stimulation, maturation markers were quantified by flow cytometry. Surprisingly, only mAb-*iFt*-induced maturation was inhibited by αTLR2 pre-treatment (Fig. S4). This was contrary to our results using TLR2$^{-/-}$ cells, but Fc-FcγR interactions could be one explanation for this discrepancy. The αTLR2 is an IgG2a antibody and can therefore bind to

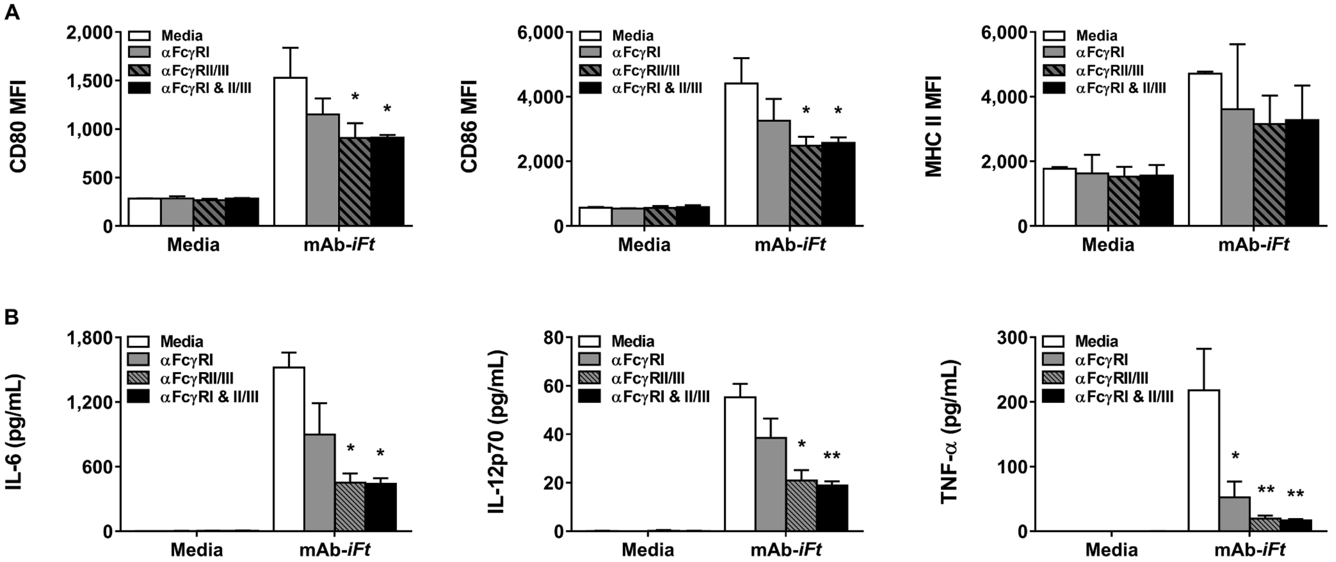

Fig. 3. Blocking mAb-*iFt* binding to FcγRs with specific antibodies reduces BMDC maturation induced by mAb-*iFt* stimulation. WT BMDCs were pretreated with 10 μg/mL of anti-FcγRI, anti-FcγRII/III, or both antibodies for 20 min and subsequently stimulated with media or mAb-*iFt* (50 *iFt*/cell) for 24 h. Supernatants were collected for cytokine analysis (B) and cells were stained for CD80, CD86, & MHC II (A). Blocking antibodies were present for the duration of the experiment. Data are combined from two experiments where bars represent mean ± SEM. MFI & cytokine data were Log_{10} transformed and subsequently analyzed via two-way ANOVA with Dunnett's multiple comparisons test (Media pre-treatment vs Antibody pre-treatment). *$p \leq 0.05$ & **$p \leq 0.01$.

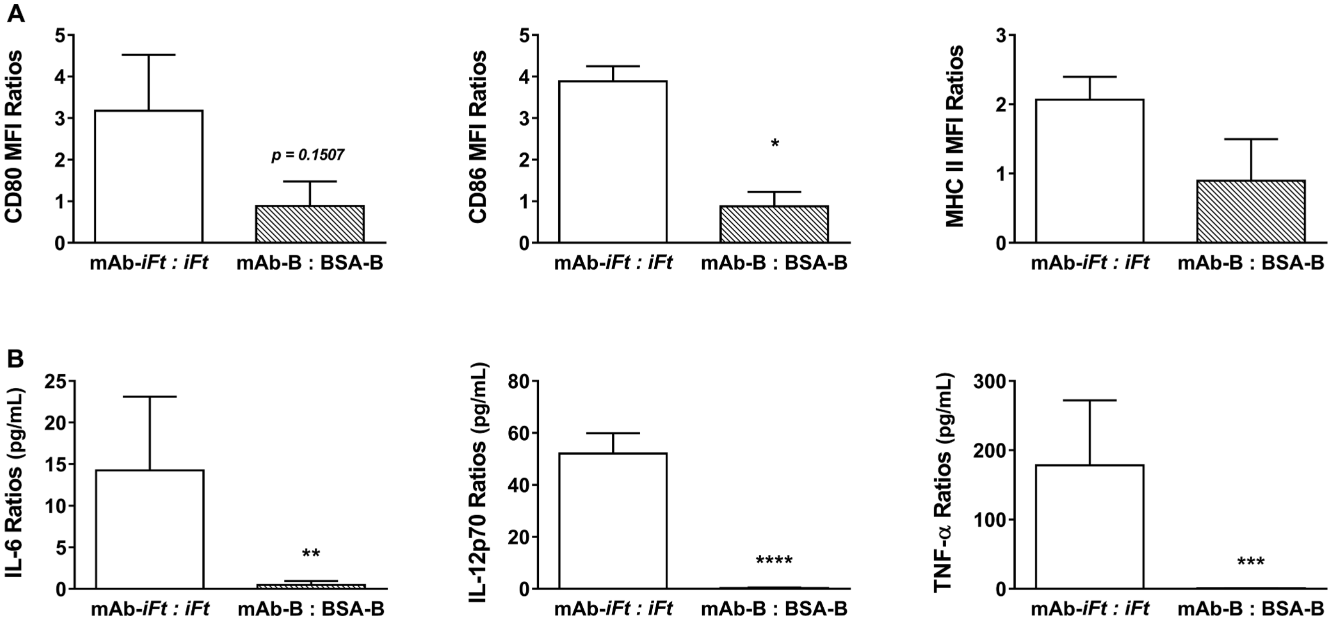

Fig. 4. Monoclonal antibody-coated beads (mAb-Beads) do not elicit BMDC maturation. WT BMDCs were stimulated with media, *iFt* (50/cell), mAb-*iFt* (50 *iFt*/cell), BSA-beads, or mAb-beads (5–30 beads/cell) for 24 h. Supernatants were collected for cytokine analysis (B) and cells were stained for CD80, CD86, and MHC class II (A). Data are combined from two experiments where bars represent mean ± SEM. Data were Log_{10} transformed to account for heteroskedasticity and then analyzed by unpaired two-tailed *t*-test (compared to media). *$p \leq 0.05$ & **$p \leq 0.01$.

FcγRs present on the BMDC surface. This could, in turn, block the ability of mAb-*iFt* to interact with FcγRs on the BMDC surface. Regardless, the MFI and cytokine secretion data together reveal a partial role for TLR2 in mAb-*iFt*-induced BMDC maturation, specifically the secretion of proinflammatory cytokines.

Because we found TLR2 to be only partially responsible for cytokine secretion and not maturation marker expression, we decided to test other surface-expressed TLRs. TLR2 can dimerize with TLRs 1 & 6 leaving only TLRs 4 & 5 to test. However, TLR5 is only known to recognize flagellin, which is absent in *iFt*. Therefore, TLR4 deficient BMDCs were stimulated in the same manner as the $TLR2^{-/-}$ cells. Conflicting results between $TLR2^{-/-}$ and αTLR2 studies, in addition to potential interactions between FcγRs and blocking antibodies, led me to believe the $TLR4^{-/-}$ cells to be the best model. As shown in Fig. S5, all cytokines concentrations were much lower in the knockout cells in comparison to the WT cells, much like that of $TLR2^{-/-}$ cells. As expected, only LPS stimulated BMDCs had decreased MFI values for CD80, CD86, and MHC II; no other stimuli had changes in co-stimulatory markers when TLR4 was absent (Fig. S5A).

3.5. Myeloid differentiation primary response 88 deficient ($MyD88^{-/-}$) BMDCs do not mature when stimulated with mAb-iFt

TLR2 & TLR4 deficient cells revealed the receptors' role in

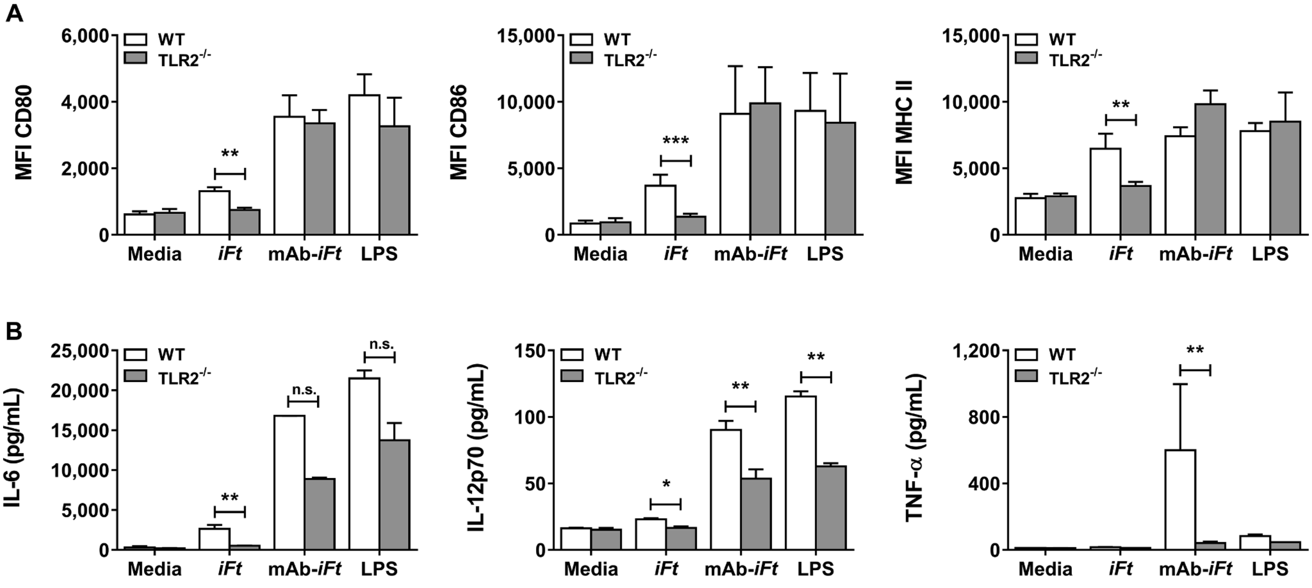

Fig. 5. TLR2 deficient BMDCs stimulated with mAb-*iFt* have decreases in cytokine secretion, but no changes in maturation marker expression. WT (white bars) or TLR2$^{-/-}$ (gray bars) BMDCs were stimulated as described in Fig. 1. Maturation marker expression was determined by fluorescent staining (A), while supernatants were analyzed via multiplex for cytokine concentrations (B). Data are combined from three experiments where bars represent mean ± SEM. Data were Log_{10} transformed to account for heteroskedasticity prior to two-way analysis of variance with Sidak's post-test (WT vs. knockout). $*p \le 0.05$, $**p \le 0.01$, & $***p \le 0.001$.

proinflammatory cytokine secretion (Fig. 5). These results, however, did not explain the MFI differences observed between *iFt* and mAb-*iFt* stimulation. We hypothesized that another TLR could be responsible for this difference. Myeloid differentiation primary response 88 (MyD88) is an adaptor protein involved in all TLR signaling with the exception of TLR3. Because of MyD88's heavy involvement in TLR signaling, comparing the response of WT to MyD88$^{-/-}$ cells would allow us to assess whether any TLR, other than TLR3, plays a role in mAb-*iFt*-induced BMDC maturation. BMDCs were stimulated with Poly(I:C) – a TLR3 agonist, LPS – a TLR4 agonist, *iFt*, or mAb-*iFt* for 24 h before the collection of supernatants and staining of cells for maturation markers. LPS, being a TLR4 ligand, has a significant decrease in CD80, CD86, and MHC II MFIs when MyD88 is absent (Fig. 6A, gray bars). Poly(I:C)-treated cells, on the other hand, were unaffected by an absence of MyD88, due to Poly(I:C)'s reliance on TLR3 for activation. *iFt* is known to be a TLR2 agonist, and so MyD88$^{-/-}$ cells had a dramatic drop in MFIs across the board. This is also the case for mAb-*iFt* stimulated knockout cells; MyD88$^{-/-}$ cells had significant decreases in CD80 and CD86 MFIs and a substantial decrease MHC II MFI in comparison to WT cells (Fig. 6A). Cytokine secretion from by WT and MyD88$^{-/-}$ cells

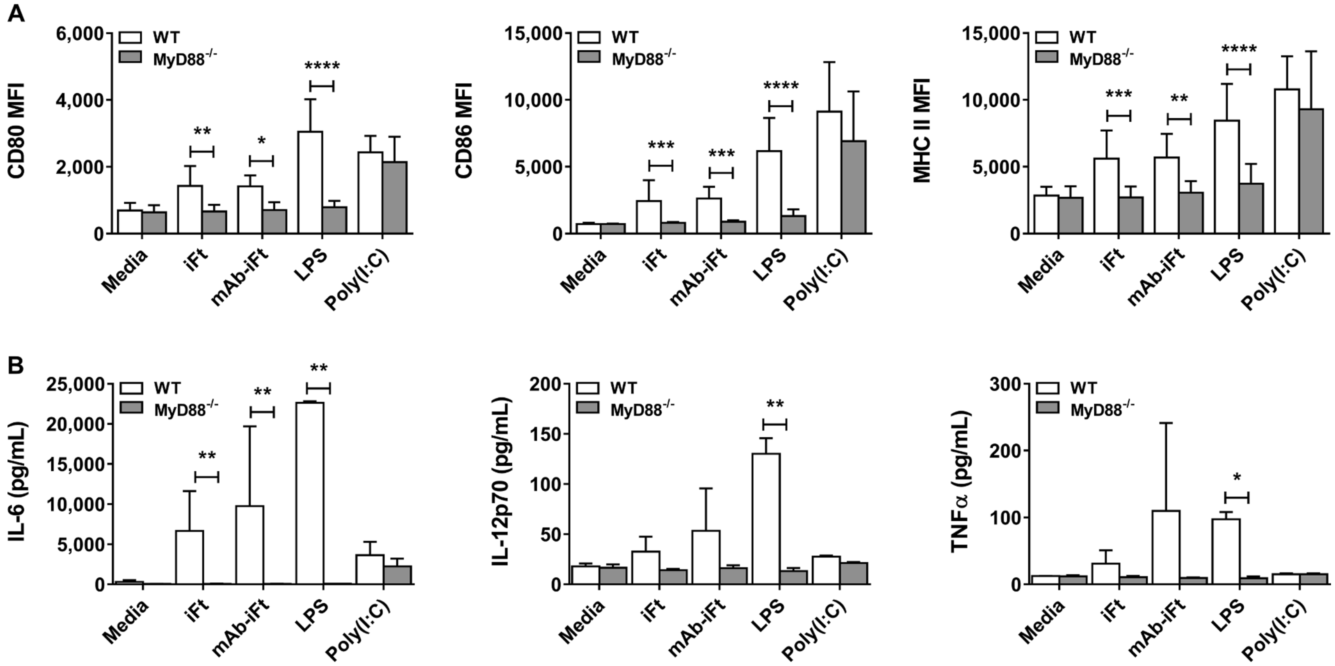

Fig. 6. MyD88 deficient BMDCs do not mature when stimulated with mAb-*iFt*. (A) WT (white bars) or MyD88$^{-/-}$ (gray bars) BMDCs were stimulated with *iFt* (50 *iFt*/cell), mAb-*iFt* (50 *iFt*/cell), LPS (2.25 ng/mL), or Poly(I:C) (10 µg/mL) for 24 h before being stained for maturation markers. (B) Cytokines analysis was completed on the BMDC supernatants via multiplex. Data are combined from three experiments where bars represent mean ± SEM and Log_{10} transformed to account for heteroskedasticity prior to analysis via two-way ANOVA with Sidak's post-test (WT vs. knockout). $*p \le 0.05$, $***p \le 0.001$, & $****p \le 0.0001$.

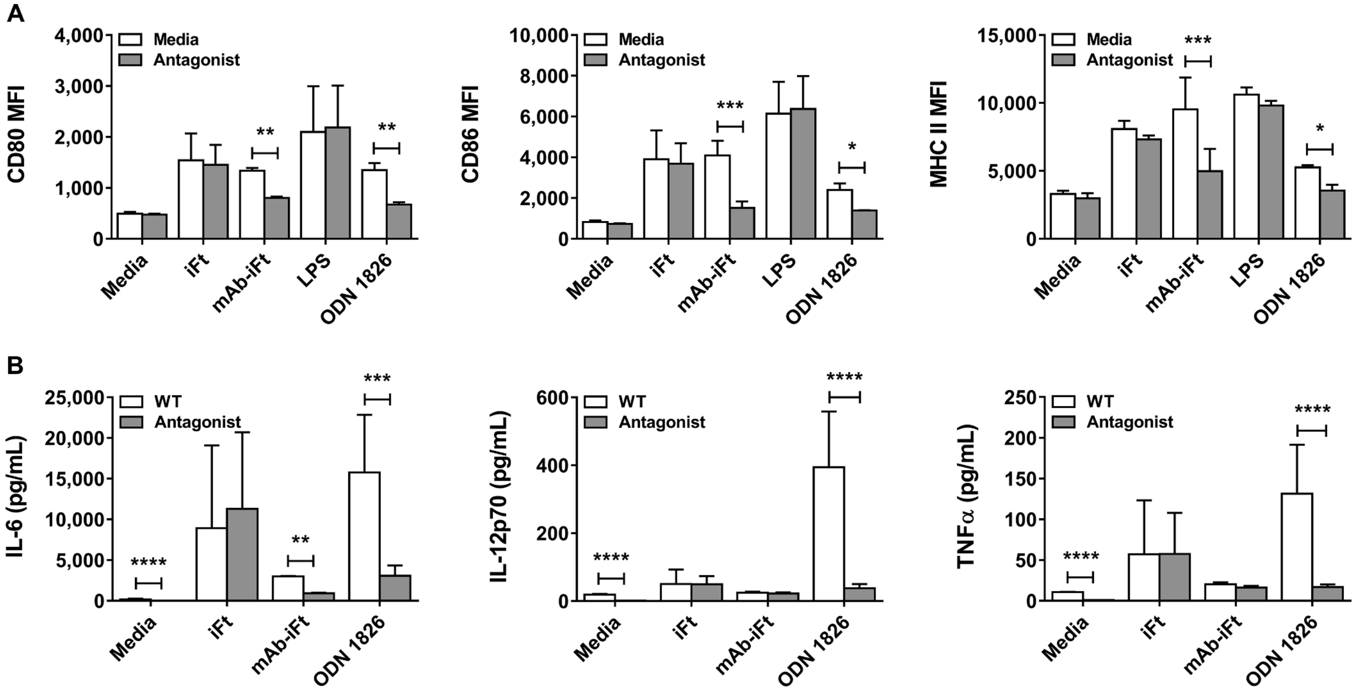

Fig. 7. mAb-*iFt*-stimulated BMDCs have reduced maturation marker expression when TLR9 signaling is blocked. (A) WT BMDCs were pretreated with either media (white bars) or the TLR9 antagonist ODN 2088 (10 μM, gray bars) for 1 h before being stimulated with *iFt* (50 *iFt*/cell), mAb-*iFt* (50 *iFt*/cell), LPS (2.25 ng/mL), or the TLR9 ligand ODN 1826 (5 μM) for 24 h. BMDCs were subsequently stained for maturation markers (A) and supernatants analyzed by multiplex for cytokines (B). Data are combined from three experiments where bars represent mean ± SEM. Data were Log_{10} transformed to account for heteroskedasticity and subsequently analyzed via two-way ANOVA with Sidak's post-test (media vs. antagonist). $*p \le 0.05$, $**p \le 0.01$, $***p \le 0.001$, & $****p \le 0.0001$.

showed similar trends. That is, secretion was significantly decreased across the board in MyD88$^{-/-}$ cells when compared to WT cells with the exception of Poly(I:C) stimulation, which was unaffected (Fig. 6B). Together these data show that MyD88 is critical for mAb-*iFt*-induced BMDC maturation.

3.6. The TLR9 antagonist, ODN 2088, inhibits enhanced maturation marker expression in mAb-iFt-stimulated BMDCs

As shown above, MyD88 is required for BMDC marker expression and cytokine secretion (Fig. 6), but TLR2 is not required for maturation marker expression (Fig. 5). We therefore postulated that an intracellular TLR other than TLR3 was required for the increase in maturation marker expression, as surface-expressed TLRs 2 and 4 showed no effect on mAb-*iFt*-induced maturation (Fig. 5 & S5). The only other surface-expressed TLR that could play a role (TLR5) binds flagellin, which *Ft* does not possess. We therefore turned to endosomal TLRs.

TLR9 is known to be activated by ligation of unmethylated CpG DNA motifs which are readily found in bacterial DNA, making TLR9 a logical candidate for our next studies. To examine TLR9′s role in BMDC maturation, we utilized a TLR9 antagonist to inhibit signaling. BMDCs were pretreated with culture media or the antagonist, ODN 2088, for 1 h prior to stimulation with LPS, *iFt*, mAb-*iFt*, or ODN 1826, a TLR9 agonist. As expected, MFIs of LPS-treated BMDCs remained unchanged regardless of pretreatment. TLR9 agonist-treated cells displayed decreased maturation marker MFIs in the antagonist pretreated cells (Fig. 7A, gray bars). The *iFt*-stimulated cells had some subtle differences between media and antagonist pretreated cells, none of which were large enough to imply a major role for TLR9 in *iFt*-induced BMDC maturation. Cells stimulated with mAb-*iFt*, on the other hand, had significant decreases in CD80, CD86, and MHC II MFIs when cells were pretreated with antagonist, suggesting a role for TLR9 in upregulation of maturation marker expression (Fig. 7A).

We then analyzed cytokine secretion in supernatants of the cells from panel A. As expected, cells stimulated with ODN 1826 (agonist) had a substantial decrease in cytokine secretion when pretreated with TLR9 antagonist whereas cells treated with LPS or *iFt* did not. Surprisingly, cells stimulated with mAb-*iFt* had practically no change in cytokine secretion when pretreated with the antagonist, with the exception of CD80 IL-6 (Fig. 7A). The LPS and ODN 1826 stimulation results in panel A confirm the effectiveness and specificity of the antagonist pretreatment; thus, we concluded that TLR9 plays a central role in mAb-*iFt*-induced BMDC maturation marker expression, but not in cytokine secretion (Fig. 6B).

4. Discussion

Our earlier studies with mAb-*iFt* demonstrated that intranasally administered mAb-*iFt* enhances protection against *Ft* challenge when compared to *iFt*. This enhanced protection required the constant region of IgG within mAb-*iFt*, FcR-γ, and FcRn [3]. Additional studies *in vitro* subsequently revealed increased binding, internalization, and presentation of mAb-*iFt* compared to *iFt* [12]. More recently, *in vivo* studies showed increased number and frequency of activated lung DCs, as well as frequency and number of gamma interferon-secreting effector memory CD4^{+} T cells in mAb-*iFt*-immunized mice [29]. Together these studies illustrate an importance for DC maturation in generating a protective immune response against *Ft*. Thus, the goal of this study was to determine the mechanism of maturation in mAb-*iFt*-stimulated DCs. As Fig. 1 shows, mAb-*iFt*-stimulated BMDCs exhibit higher maturation marker expression (though only CD80 was significantly increased when comparing *iFt* to mAb-*iFt* stimulation) and cytokine secretion compared to *iFt*-stimulated cells. Based on previous studies from our laboratory and others [3,12,14,16,29], we hypothesized that the enhanced maturation would rely on both FcγRs and TLR2. Subsequently, both our BMDC maturation marker expression and cytokine secretion results show that enhanced maturation of mAb-*iFt*-stimulated BMDCs relies not only on signaling through FcR-γ, FcγRs, TLR4 and TLR2, but also signaling through MyD88 and TLR9.

To determine the mechanism(s) of mAb-*iFt*-stimulated DC maturation, the degree of DC maturation needed to be assayed. It is important to note that DC maturation consists of several changes over time: phagocytic capacity decreases, proteolytic processing increases, expression of T cell costimulatory markers and MHC molecules increase, cytokine secretion increases, and migration to lymph nodes commences [10,30,31]. In this study, we chose to measure both maturation marker expression and cytokine secretion to assay DC maturation status. Many studies assay solely maturation marker expression levels to determine DC maturation. However, we decided to include cytokine secretion because numerous studies have shown that cytokines have an immense impact on T cell activation, for example, differential skewing of T helper phenotype with specific cytokine stimulation (e.g. IL-12 skews to T_H1 phenotype) [32–34]. Somewhat surprisingly, we show that when TLR4$^{-/-}$, TLR2$^{-/-}$ or TLR9 antagonist pretreated DCs are stimulated with mAb-*iFt* but changes in maturation marker expression do not equate to changes in proinflammatory cytokine secretion (Figs. 5 and 7). It should be noted however, that the variability and lack of mAb-*iFt*-induced cytokine signal in Fig. 7 makes interpretation difficult. In addition, studies have shown that not just cytokines, but the combination of costimulatory receptors with cytokines impact T cell skewing [35]. Together this demonstrates the importance of including cytokine secretion assays in addition to maturation marker studies in determining the maturation status of DCs. Furthermore, we find it intriguing that mAb-*iFt*-elicited cytokine secretion is dependent on the presence of TLR2 and TLR4 (Fig. 5 & Fig. S5), whereas maturation marker expression relies on TLR9 signaling (Fig. 7). However, both cytokine and maturation marker increases are dependent on the presence of MyD88 (Fig. 6). We surmise that perhaps the adaptor protein MyD88-adaptor-like (Mal a.k.a. TIRAP) could play a role in this discrepancy. Mal has been found not only to form a bridge between MyD88 and certain TLRs, but to also bind various phosphatidylinositol- or phosphatidylserine-rich regions of membrane, and conduct signaling functions independent of MyD88 [36–38]. In addition, this separation of cytokine secretion from co-stimulatory molecule expression could be due to differences in signaling from surface TLRs (i.e. TLR2 and 4) vs endosomal TLRs (i.e. TLR9). It would be quite interesting to conduct follow-up studies on how TLR2, 4 and 9 signal differently in the context of mAb-*iFt* stimulation by examining the role Mal/TIRAP plays (or doesn't play) for each receptor, as well as other potential differences in signaling between surface TLRs vs. endosomal TLRs.

The ability of DCs to present antigen to T cells is critical for effective immune activation and protection. Previous studies from our laboratory have shown, *in vitro*, that mAb-*iFt* stimulation of DCs results in more effective T cell activation. These same studies also discovered enhanced persistence of *Ft* antigen within DCs compared to stimulation with *iFt* alone [12]. In addition, Iglesias, et al. found that mAb-*iFt* binding to FcγRs leads to enhanced internalization, whereas *iFt* alone does not engage Fc receptors. Engagement of FcγRs by mAb-*iFt* (shown in Fig. 4) likely leads to altered intracellular trafficking compared to *iFt* alone, which could be an explanation for the differences previously observed in antigen persistence. Our studies herein could further imply differential trafficking as a possible mechanism for enhanced persistence. In addition to a need for FcγR engagement and signaling, which we show in Figs. 2–4, mAb-*iFt* maturation was shown to also require TLR9 (Fig. 7) to increase maturation marker expression. At steady state, TLR9 localizes in the endoplasmic reticulum in an inactive form. Upon cell stimulation, inactive TLR9 is processed/cleaved and ultimately migrates to endo-lysosomal compartments where it becomes activated [39,40]. The requirement for TLR9 in mAb-*iFt*-induced maturation therefore suggests that mAb-*iFt* traffics specifically to compartments that contain TLR9. *iFt* stimulation of TLR9 antagonist pretreated DCs, however, did not show any maturation deficiencies, suggesting that *iFt* either does not engage TLR9 or does not traffic to compartments containing TLR9. Unmethylated CpG motifs are a known TLR9 ligand which are readily found in bacterial DNA including that of *Ft*; it is thus more likely that *iFt* does not traffic to a TLR9-containing compartment. It has also been shown that B cell receptor signaling through phospholipase D can recruit TLR9-containing endosomes to the autophagosome where internalized BCR was located [41]. BCR signaling is nearly homologous to that of FcγRs due to the presence of ITAMs in the invariant CD79B and CD79A molecules that associate with the BCR, suggesting that engagement of FcγRs by mAb-*iFt* could lead to differential trafficking. Further support of this idea comes from studies from Means et al. This study shows that FcγRIIA in humans cooperates with TLR9, in fact FcγRIIA is required for the uptake of autoantibody immune complexes in patients with systemic lupus erythematosus and subsequent trafficking to TLR9-containing lysosomes [42]. It is thought that the mouse FcγRIII is the orthologue to human FcγRIIA [43]. It is therefore likely that the engagement of FcγRs by mAb-*iFt*, specifically FcγRIII as illustrated in Fig. 3, could lead to specific delivery of mAb-*iFt* to the compartment(s) containing active TLR9. This trafficking discrepancy and subsequent engagement of TLR9 could then lead to variances in the level and amount of antigen degradation, the amount and type of peptide loaded into MHC molecules, and antigen retention time. These variances can ultimately influence the amount and effectiveness of T cell activation. In fact, our previous studies have shown that mAb-*iFt* complex stimulation of APCs results in an increased rate of internalization, leading to increased antigen presentation and T cell activation, as compared to that of non-complexed *iFt* [12]. Alternatively, the requirement for TLR9 with mAb-*iFt*, but not *iFt*, could be due to simple probability. We know from previous studies that mAb-*iFt* is taken up both in greater amounts and more rapidly than *iFt* alone. Because there would be more mAb-*iFt* within a given cell than *iFt*, there is simply a higher probability that mAb-*iFt* would engage TLR9 than *iFt*. If true, then mAb-*iFt* might elicit more T cell activation because the DCs containing the complexes are more mature. This could result from more efficient (i.e. faster and more) internalization of mAb-*iFt* and/or differential intracellular trafficking when compared to *iFt* alone. However, the intracellular trafficking routes of each stimuli will need to be studied to determine which explanation holds true. In addition, future studies should investigate whether mAb-*iFt* engagement of FcγRs leads to the recruitment of TLR9.

All our mAb-*iFt* studies to date, including this one, suggest a need for FcγR ITAM signaling in mAb-*iFt* elicited protection, antigen presentation, or DC maturation [3,12]. It is important to note that *iFt* alone did not require FcR-γ to elicit an increase in maturation marker expression or cytokine secretion (Fig. 2), demonstrating that the receptor most likely binds the antibody portion of mAb-*iFt* and not *iFt* itself. It is therefore likely that the receptor that utilizes FcR-γ in our system is a FcγR. In fact, we did implicate the involvement of FcγRIII by using the 2.4G2 blocking antibody as a pretreatment (Fig. 3). Furthermore, there is ample evidence for synergy/crosstalk between ITAM-containing receptors, like FcγRs, and TLRs in the literature [16,26,27,44]. This data, along with our current study, suggests that FcγR/TLR synergy is also likely in the context of mAb-*iFt*. A review on FcR/TLR synergy by Lennartz & Drake discusses various ways in which synergy/crosstalk could occur such as signaling intermediates, transcriptional regulation, and post-transcriptional regulation [26]. Particularly relevant to this study are: tyrosine kinases (i.e. Syk & BTK); transcriptional regulation through NF-κB; and post-transcriptional regulation through micro-RNAs and/or long non-coding RNAs (lnc-RNAs). Our studies herein further emphasize the importance of this synergy by showing mAb-*iFt*-stimulated DC maturation relies on both MyD88 (Fig. 5) and ITAM signaling (Fig. 2). Future studies should therefore examine the mechanisms of synergy more in depth. Examination of the effects Syk/BTK, different NF-κB subunits and micro-RNA/lnc-RNA exert on mAb-*iFt*-elicited DC maturation would provide invaluable information on how this synergy occurs.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.103962.

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