Fc Receptor-Targeting of Immunogen as a Strategy for Enhanced Antigen Loading, Vaccination, and Protection Using Intranasally-Administered Antigen-Pulsed Dendritic Cells

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Abstract

Dendritic cells (DCs) play a critical role in the generation of adaptive immunity via the efficient capture, processing, and presentation of antigen (Ag) to naïve T cells. Administration of Ag-pulsed DCs is also an effective strategy for enhancing immunity to tumors and infectious disease organisms. Studies have also demonstrated that targeting Ags to Fcγ receptors (FcγR) on Ag presenting cells can enhance humoral and cellular immunity *in vitro* and *in vivo*. Specifically, our studies using an *F. tularensis* (*Ft*) infectious disease vaccine model have demonstrated that targeting immunogens to FcγR via intranasal (i.n.) administration of monoclonal antibody (mAb)-inactivated *Ft* (i*Ft*) immune complexes (ICs) enhances protection against *Ft* challenge. *Ft* is the causative agent of tularemia, a debilitating disease of humans and other mammals and a category A biothreat agent for which there is no approved vaccine. Therefore, using i*Ft* Ag as a model immunogen, we sought to determine if *ex vivo* targeting of i*Ft* to FcγR on DCs would enhance the potency of i.n. administered i*Ft*-pulsed DCs. In this study, bone marrow-derived DCs (BMDCs) were pulsed *ex vivo* with i*Ft* or mAb-i*Ft* ICs. Intranasal administration of mAb-i*Ft*-pulsed BMDCs enhanced humoral and cellular immune responses, as well as protection against *Ft* live vaccine strain (LVS) challenge. Increased protection correlated with increased i*Ft* loading on the BMDC surface as a consequence of FcγR targeting. However, the inhibitory FcγRIIB had no impact on this enhancement. In conclusion, targeting Ag *ex vivo* to FcγR on DCs provides a method for enhanced Ag loading of DCs *ex vivo*, thereby reducing the amount of Ag required, while also avoiding the inhibitory impact of FcγRIIB. Thus, this represents a simple and less invasive strategy for increasing the potency of *ex vivo*-pulsed DC vaccines against chronic infectious diseases and cancer.

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Keywords

Dendritic cell vaccine; Fcγ receptor-targeted vaccine; intranasal vaccine; vaccine platform; chronic infection; cancer

1. Introduction

Dendritic cells (DCs) play a central role in generating immunity to infection [1]. Specifically, DCs are highly efficient at taking up, processing, and presenting antigens (Ags) to naïve T cells [1]. This has lead to studies focused on DC-based vaccines against cancer and infectious diseases including HIV-1 and influenza [2–6]. Furthermore, numerous studies, including our own, have demonstrated that targeting Ag to Fcγ receptors (FcγRs) on Ag presenting cells (APCs), can enhance humoral and cellular immunity and protection against infectious diseases and cancer [6–12]. Consistent with these observations, engagement of FcγRs can induce DC maturation, a key event required for Ag processing and presentation to T cells [13, 14]. In this regard, studies from our laboratory have demonstrated that inactivated *F. tularensis* (i*Ft*) Ag can induce enhanced protection against *Ft* challenge, when targeted to FcγRs as mAb-i*Ft* complexes (ICs) administered intranasally (i.n.). The latter involves enhanced Ag binding to DCs, DC maturation, Ag processing/presentation by DCs, and i*Ft* trafficking to lymphoid tissues [7, 12, 14]. Thus, we hypothesized that the ability of *ex vivo* Ag-pulsed DCs to induce immunity/protection could be significantly enhanced by pulsing DCs with FcγR-targeted Ag administered i.n. Importantly, while parenteral immunization generally utilizes needle injection and is not optimal for stimulating mucosal immunity, i.n. immunization is less invasive and stimulates strong parenteral and mucosal immune responses [15]. Furthermore, recent studies have demonstrated Ag-containing DCs administered i.n., like Ag-containing peripheral DCs, migrate to lymphoid tissues [16, 17].

In this report, we demonstrate that immunization of mice i.n. with *ex vivo* FcγR-targeted Ag (mAb-i*Ft*)-pulsed bone marrow derived DCs (BMDCs) enhances humoral and cellular immune responses and protection against *Ft* challenge. Furthermore, despite the presence of the inhibitory FcγRIIB on DCs [18], protection is not impacted by FcγRIIB. Thus, these studies identify a vaccine strategy, which can be used to increase the potency of DC-based vaccines, as well as simultaneously induce mucosal and peripheral immunity, while bypassing the inhibitory impact of FcγRIIB.

2. Materials and Methods

2.1. Cells and reagents

RPMI 1640 medium (CellGro, Manassas, VA) supplemented with 10% FCS (HyClone, Logan, UT), 2mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessential amino acids (CellGro), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco Life Technologies, Carlsbad, CA), and 50 μM 2-ME (Bio-Rad, Hercules, CA) was used to generate BMDCs. The *Ft*-specific T cell hybridoma (FT256D10) is specific for an *Ft* ribosomal protein-derived peptide, and was provided by Dr. Jeffrey Frelinger (University of North Carolina at Chapel

Hill). The T cell hybridoma was maintained in RPMI 1640 medium containing 500 μg/ml of hygromycin B (CellGro). The mouse IgG2a anti-*Ft* LPS mAb used to make mAb-i*Ft* ICs was purchased from Fitzgerald (cat # 10-F02, clone M0232621, Acton, MA). Mouse recombinant Flt3 ligand (Flt3L) was obtained from R&D systems (Minneapolis, MN). Abs for flow cytometry: Anti-mouse CD11c, CD11b, CD8a, B220, CD3, CD4, MHC class II (I-A/I-E), CD40, CD83, CD80, CD86, DEC205, or IFN-γ, as well as isotype control Abs, were purchased from eBioscience (San Diego, CA). Luminex Bio-Plex assay kits were purchased from Bio-Rad (Hercules, CA).

2.2. Mice

C57BL/6 and FcγRIIB knockout (KO) mice on a C57BL/6 background were purchased from Taconic Laboratories (Hudson, NY). Mice (6 to 8 weeks of age) were housed in the Animal Resources Facility at Albany Medical College and provided with ad lib water and food during the course of each experiment. Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee utilizing NIH standards.

2.3. iFt (Ag) and mAb-iFt ICs

Inactivated *Ft* LVS (i*Ft*) (immunogen) was prepared as previously described using endotoxin-free PBS [7, 14, 19]. The mAb-i*Ft* ICs were generated by adding i*Ft* to DCs in tissue culture medium, followed by mAb to a final concentration of 1 μg/ml. Unless otherwise indicated in the figure legend, i*Ft* was used at a ratio of 10 i*Ft*/cell. In all experiments comparing i*Ft* versus mAb-i*Ft*, i*Ft* amounts were equalized between i*Ft* and mAb-i*Ft* preparations.

2.4. BMDCs

BMDCs were generated from mouse BM precursor as previously described [14, 20]. BMDC phenotype was verified by flow cytometry (> 93% CD11c+, high CD11b and B220, very low CD8a).

2.5. Immunization and challenge

BMDCs (3×10^6 cells/ml) in RPMI 1640 medium were pulsed with medium, i*Ft, or* i*Ft plus* 1 μg/ml anti-*Ft* LPS mAb at 37°C for 3 hours. BMDCs were then washed 3 times in PBS. Each mouse was then anesthetized and administered i.n. either 30 μl of PBS (vehicle) or 3×10^6 pulsed BMDCs. Before and after immunizations, serum was collected and analyzed for the presence of *Ft*-specific Ab. To measure *Ft*-specific Ab in BAL, immunized mice were sacrificed 2 and 4 weeks post-boost. In challenge experiments, immunized mice were infected i.n. 2 and 4 weeks post-boost with $4 \times LD_{50}$ of live *Ft* LVS. Survival was monitored for 28 days.

2.6. BMDC maturation and cytokine secretion

BMDCs were cultured overnight in RPMI medium alone, or with i*Ft*, or mAb-i*Ft* ICs. Cells were then harvested, stained, and analyzed for maturation markers (CD40, CD80, CD86, CD205, and MHCII) via flow cytometry as previously described [14]. To monitor cytokine secretion, BMDCs were cultured with medium, i*Ft,* or mAb-i*Ft* for 3 days at 37°C. Culture

supernatants were then collected and analyzed for cytokines/chemokines using the Luminex Bio-Plex assay system (Bio Rad).

2.7. Ag presentation

BMDCs were pulsed overnight at 37°C with medium, i*Ft, or* mAb-i*Ft* ICs. Cells were then washed twice with medium to remove unbound i*Ft* and adjusted to 2×10^6 cells/ml. In a 96 well plate, 100 µl of 2×10^5 pulsed BMDCs were co-cultured with 100 µl of 1×10^5 *Ft*-specific T cells (FT256D10). The plate was then incubated at 37°C for 24 h and supernatants were collected. Supernatants were then assayed for IL-5 (secreted by these cells in response to Ag) using Luminex.

2.8. ELISA

The production of anti-*Ft* Ab in response to immunization was measured by ELISA as previously described [19].

2.9. Recall responses

Single cell suspensions from spleen and mediastinal lymph nodes (MLNs) were prepared from immunized mice at 2 and 4 weeks post-boost. In the case of MLN cells, due to limited numbers of cells, MLNs cells were pooled from mice in each immunization group. Cells were cultured in 96-well plates at 3×10^5 cells (200 µl)/well for 3 days in the presence of medium or i*Ft*. Supernatants were subsequently harvested and analyzed for cytokines by Luminex assay.

2.10. CD4 and CD8 T cell frequencies

Splenocytes and lung cells (3×10^6) were harvested from immunized mice and cultured overnight with i*Ft*, ionomycin (50 ng/ml) (Sigma-Aldrich), and phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) (750 ng/ml). Monensin solution (eBioscience) was then added to the cultures for 4 hours. Cells were then washed with FACS buffer (PBS containing 0.1% BSA and 0.02% Sodium azide) and stained for CD3, CD8, and CD4. Subsequently, cells were washed and fixed on ice with 2% paraformaldehyde in PBS for 30 minutes. Cells were then resuspended in permeablization buffer [PBS containing 0.1% saponin (Sigma) and 0.09% sodium azide (Sigma)] for another 30 minutes on ice and stained with anti-IFN-γ Ab. The number of CD4+ and CD8+ T cells positive for IFN-γ was then quantified using an LSRII flow cytometer.

2.11. Statistics

The log-rank (Mantel–Cox) test was used for survival curves. One-way analysis of variances or the unpaired, one-tailed Student's t-test was used for the remaining figures. Data analyses were performed using GraphPad Prism 4 (San Diego, CA).

3. Results

3.1. Mice immunized with mAb-iFt-pulsed BMDCs are better protected against Ft LVS challenge

To evaluate the impact of administering BMDCs pulsed *ex-vivo* with FcγR-targeted mAb-i*Ft* ICs versus non-FcγR-targeted i*Ft* i.n. on protection, mice were immunized with PBS or BMDCs pulsed *ex-vivo* with medium, i*Ft*, or mAb-i*Ft*. Mice were subsequently challenged with a lethal dose of *Ft* LVS ($4 \times LD_{50}$) i.n. 2 and 4 weeks post-boost and monitored for 28 days for survival. As shown in Fig. 1A, mice immunized with mAb-i*Ft*-pulsed BMDCs and challenged 2 weeks post-boost were 100% protected compared with mice immunized with i*Ft*-pulsed BMDCs, which were 37% protected. No protection was observed when administering PBS or BMDCs pulsed with culture medium. Similarly, 4 weeks post-boost (Fig. 1B), mice immunized with mAb-i*Ft*-pulsed BMDCs exhibited 87% survival compared to 50% survival for the i*Ft*-pulsed BMDC group.

3.2. Pulsing BMDCs ex vivo with mAb-iFt ICs enhances DC maturation, cytokine secretion, and Ag presentation

In order to study the effect of FcγR-targeted mAb-i*Ft* ICs versus non-FcγR-targeted i*Ft* on BMDC function, we examined DC maturation following exposure to medium, i*Ft*, or mAb-i*Ft* for 24 hours *in vitro*. As demonstrated in Figs. 2A and 2B, the expression of the maturation markers CD40, CD80, CD86, CD205, and MHCII were substantially increased for i*Ft*-pulsed BMDCs. However, a significant increase in CD40, CD80, CD205, and MHCII expression above that of i*Ft* was also observed when pulsing BMDCs with mAb-i*Ft*. Similar results were observed when measuring IL-12p70, IL-6, IL-10, IFN-γ, TNF-α and MCP-1 in response to i*Ft* versus mAb-i*Ft*-pulsed DCs (Fig. 2C). In contrast, as shown in Figure 2D, T cell activation in response to i*Ft* alone was relatively low (approximately twice that of the medium control), while there was approximately an 80-fold increase in T cell activation compared to that of i*Ft*, when BMDCs were pulsed with mAb-i*Ft*.

3.3. Intranasal administration of BMDCs pulsed ex vivo with mAb-iFt ICs enhances Ag-specific B and T cell responses

Humoral immunity can play a key role in protection against extracellular and intracellular bacterial infections, including *Ft* infection [7, 21–26]. In addition, IgA is the predominant Ab found at mucosal sites. Thus enhancement of pathogen-specific Abs provides one possible means of enhancing protection against mucosal infection [7]. Sera were thus collected from mice immunized i.n. with BMDCs pulsed with medium, i*Ft*, or mAb-i*Ft* 2 and 4 weeks post-boost, and *Ft*-specific Abs were measured by ELISA. In the case where mAb-i*Ft*-pulsed BMDCs were administered, total *Ft*-specific Ab was enhanced above that of i*Ft*-pulsed BMDCs (Figs. 3A). The case was similar when examining *Ft*-specific IgG (Fig. 3B), IgG2c (Fig. 3C), IgG1 (Fig. 3D), and IgA (Figs. 3E and 3F).

IFN-γ also plays a key role in controlling *Ft* infection [7, 19, 27, 28]. In addition, pro-inflammatory cytokines such as IL-17 and TNF-α can confer protection against a variety of extracellular and intracellular bacterial pathogens including *Ft* [29–32]. As shown in Figure 4, cells from MLN of mice immunized with mAb-i*Ft*-pulsed BMDCs exhibited increased

production of IFN-γ (Fig. 4A), IL-5 (Fig. 4C), IL-17A (Fig. 4E), and TNF-α (Fig. 4G), as compared to that of i*Ft*-pulsed BMDCs. Similar enhancement of cytokine levels from splenocytes of mice immunized with mAb-i*Ft*-pulsed BMDCs were also observed, with the exception of IL-5 (Figs. 4B, 4D, 4F, and 4H). To further define the IFN-γ-producing T cell subsets being enhanced, we analyzed IFN-γ+ CD4+ and IFN-γ+ CD8+ T cell populations from lung and spleen of immunized mice. In all cases, immunization with mAb-i*Ft*-pulsed BMDCs increased the frequencies of IFN-γ+ CD4+ and IFN-γ+ CD8+ T cells over that of i*Ft*-pulsed BMDCs (Figs. 5A and 5B).

3.4. FcγRIIB has no impact on the enhanced protection induced by ex vivo mAb-iFt-pulsed BMDCs

FcγRIIB is the only inhibitory FcγR present in humans and mice [33, 34]. FcγRIIB mediates its inhibitory effect through an immunoreceptor tyrosine-based inhibitory motif (ITIM) in the cytoplasmic domain [35]. A number of studies have demonstrated that DCs derived from mice lacking FcγRIIB generate stronger immune responses *in vitro* and *in vivo* than that of WT mice [36, 37]. Since mAb-i*Ft* ICs are capable of engaging FcγRIIB as well as activating FcγRs (I and III) on BMDCs, we wished to determine whether FcγRIIB limits the level protection we are able to achieve when administering mAb-i*Ft*-pulsed BMDCs. As indicated in Figures 6A through 6D, the level of protection against *Ft* challenge is unchanged, regardless of whether FcγRIIB is present or absent on DCs pulsed with i*Ft* (Figs 4A and 4B) or mAb-i*Ft* ICs (Figs. 4C and 4D). Given the latter, we considered the possibility that FcγR signaling events initiated via the mAb-i*Ft* IC pulse are not involved in the enhanced protection we observed, but rather that enhanced Ag (i*Ft*) loading onto the DC surface is primarily responsible for the increased potency of the mAb-i*Ft*-pulsed BMDC vaccine. Thus, we loaded BMDCs with equal amounts of i*Ft* using either i*Ft* or mAb-i*Ft* ICs. As indicated in Figure 6E, by increasing the concentration of i*Ft* 30 fold above that of mAb-i*Ft*, BMDCs from both the i*Ft* and mAb-i*Ft*-pulsed DC groups bound equal amounts of i*Ft*. Furthermore, in this case, DCs pulsed with either i*Ft* or mAb-iFt were equally effective at inducing protection against *Ft* challenge (Fig. 6F).

4. Discussion

DCs play a critical role in generating immunity to infection [1]. Furthermore, the use of DCs pulsed *ex vivo* with Ag as a treatment strategy for cancer and chronic infections is currently a focus of intense investigation. Thus, the identification of approaches, which improve the effectiveness of DC-based vaccines could have a significant impact on DC-based vaccine development. Based on previous studies using soluble mAb-i*Ft* ICs as i.n. immunogens [7], we postulated that targeting Ags to FcγR on DCs would enhance the efficacy of DC-based vaccines. Here, we describe for the first time the use of FcγR-targeted Ag-pulsed DCs administered i.n. to enhance the potency of *ex-vivo* Ag-pulsed DC vaccines. Specifically, targeting i*Ft* to FcγR on BMDCs *ex-vivo* and administering them i.n., significantly enhances protection versus that of BMDCs pulsed with non-targeted i*Ft*. However, somewhat surprisingly, FcγR signaling appears to play a minimal role in the enhanced protection observed. Specifically, when the amount of i*Ft* loaded onto the surface of BMDCs is equalized to that of mAb-i*Ft*-pulsed BMDCs, both i*Ft* and mAb-i*Ft*-pulsed BMDCs are

equally effective at stimulating protection. The apparent lack of a requirement for FcγR signaling may be partially explained by the observation that non-targeted i*Ft* stimulates substantial BMDC maturation and cytokine production. This is likely due to the presence of endotoxins, including *Ft* LPS and other bacterial products, which are i*Ft* components. However, despite the stimulatory capacity of non-targeted i*Ft*, DC maturation and cytokine secretion are further enhanced when i*Ft* is targeted to FcγR on BMDCs. The latter is significant, since purified protein Ags are preferred for vaccine purposes but are poorly immunogenic and require adjuvant [38]. Importantly, this requirement for adjuvant can be overcome by targeting protein Ags to FcγR [7, 12], which, as observed, also stimulates DC maturation and cytokine secretion [13, 14, 39].

In regard to immune mechanisms involved, *Ft*-specific IgG and IgA Abs in sera and BALF of mice immunized with mAb-i*Ft*-pulsed BMDCs, versus that of mice immunized with i*Ft*-pulsed BMDCs, are significantly enhanced. Furthermore, production of IFN-γ, IL-17A, and TNF-α are also enhanced. While the role of Abs in protection against *Ft* infection is controversial, Abs do mediate protection against *Ft* infection under some circumstances [25, 26, 40]. In contrast, it is widely accepted that cellular immunity, in particular the production of IFN-γ and development of a Th1-type T cell response, is critical for generating protection against *Ft* [7, 19, 41–43]. A number of studies have also demonstrated a protective role for IL-17 following *Ft* infection [29–31, 44]. Thus, the fact Ab, IFN-γ, and IL-17 responses are enhanced via immunization with mAb-i*Ft*-pulsed BMDCs, not only explains why enhanced protection against *Ft* is observed when utilizing mAb-i*Ft*-pulsed BMDCs, but also suggests a broader potential for application of this strategy [2]. It is also important to note that another key advantage of utilizing DC-based vaccines in combination with FcγR targeting of Ag is the improved ability of DCs to process and present exogenous Ag through both the MHC Class II and MHC Class I pathways (cross-presentation), thereby stimulating CD4 and CD8 T cell responses, respectively [39]. CD8 T cell responses are not only important for controlling *Ft* infection [27], but also numerous intracellular infections and cancer. Thus, of substantial significance is the balanced induction of both CD4 and CD8 T cell responses induced by mAb-i*Ft*-pulsed DCs.

Finally, the inhibitory receptor FcγRIIB present on DCs can negatively regulate Ag-specific immune responses [8, 45]. In our previous protection studies utilizing soluble mAb-i*Ft* ICs administered i.n., *Ft*-specific IgA production was enhanced while production of *Ft*-specific IgG was inhibited [7]. This observation is consistent with mAb-i*Ft* ICs engaging FcγRIIB and thereby negatively regulating IgG production. However, when utilizing mAb-i*Ft*-pulsed DCs administered i.n., FcγRIIB had no significant impact on the level of protection, which is also reflected by the enhanced production of *Ft*-specific IgG, in direct contrast to what we observed using soluble mAb-i*Ft* ICs. The latter is a critical distinction between these two approaches. Specifically, a significant additional advantage of using FcγR-targeted Ags in combination with this DC-based vaccine strategy is the ability to bypass FcγRIIB-mediated inhibition and enhance both Ag-specific IgG and IgA production. The ability to do the latter may be explained in part by observations that FcγRIIB expression is reduced as a consequence of DC maturation, which is enhanced by mAb-i*Ft*. In addition, under some circumstances, FcγRIIB can act to enhance Ag presentation by DCs [18, 46, 47].

Alternatively, studies have suggested that in the case of DC-based vaccines, transfer of Ag to endogenous APCs is required *in vivo* [48], thus eliminating a direct role for FcγRIIB signaling in APC-mediated T cell activation. In addition, the latter may be further enhanced by DC lysis via DC-CD8 T cell interaction, thereby also contributing to reduced CD8 T cell responses over time [49]

5. Conclusions

These studies demonstrate the efficacy of a strategy for increasing the potency of DC-based vaccines by targeting immunogens to FcγR *ex-vivo*, prior to i.n. administration. Advantages of this approach include: Enhanced protection against infection; Enhanced mucosal, peripheral, humoral, and cellular immune responses; The ability to overcome FcγRIIB-mediated inhibition; The ability of DCs to act as "natural adjuvants" [50]; Less invasive administration [(i.n.) versus needle-based injection. In conclusion, this approach provides a unique ability to utilize FcγR-targeting to enhance Ag loading, immune responses, and protection generated by DC-based vaccines, while avoiding the inhibitory impact of FcγRIIB.

Acknowledgments

Abbreviations

DCs	Dendritic cells
BMDCs	Bone Marrow-Derived Dendritic cells
mAb	Monoclonal Antibody
Ft	*F. tularensis*
i*Ft*	Inactivated *Ft*
IC	immune complex
Ag	Antigen
Ab	Antibody
FcγR	Fcγ receptors
APCs	Ag presenting cells
i.n.	intranasal
BALF	bronchial alveolar lavage fluid
MLN	mediastinal lymph node

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Highlights

- <u>Efficacy:</u> Effective i.n. vaccination via DCs pulsed *ex-vivo* with FcγR-targeted Ag is demonstrated.

- <u>Immune Responses:</u> Mucosal, peripheral, humoral, and cellular immune responses are enhanced.

- <u>Immune Advantages:</u> Traditional adjuvant is not required and inhibition via FcγRIIB is absent.

- <u>Clinical Advantages:</u> Ability to use a less invasive i.n. route and stimulate CD4 and CD8 responses.

- <u>Applications:</u> Vaccines and therapeutics against chronic infectious diseases and cancer.
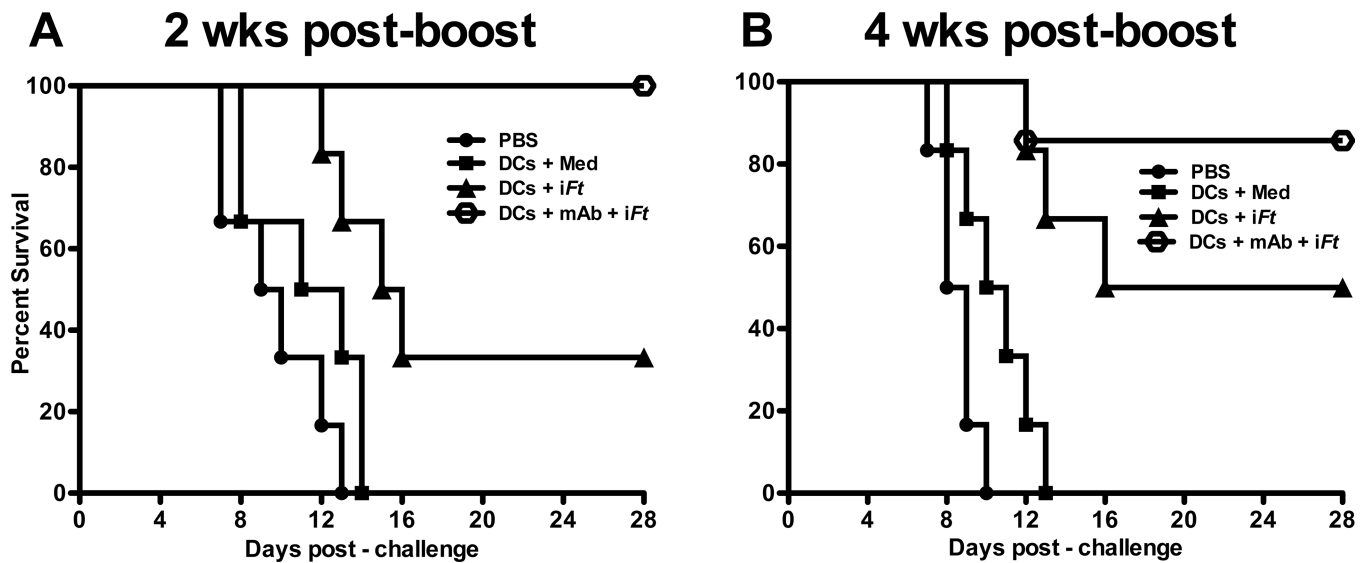
Figure 1. Mice immunized with mAb-i*Ft*-pulsed BMDCs are better protected against a lethal *Ft* LVS challenge

BMDCs pulsed with medium, i*Ft*, or mAb-i*Ft* (10 i*Ft*/cell) were washed with PBS. Pulsed cells (3×10^6 cells/dose) were then suspended in 30 μl of PBS and administered to C57BL/6 mice (n=6) i.n. as droplets in alternating nares. Mice were primed and boosted with the Ag-pulsed BMDCs on days 0 and 14. Two weeks (A) and 4 weeks (B) post-boost, mice were challenged i.n. with $4 \times LD_{50}$ (2×10^4 CFU) of *Ft* LVS in a volume of 20 μl of PBS. Survival was monitored for 28 days post-infection. The vehicle control groups received PBS only. Data presented are from a single experiment containing 6 mice per group. Each figure depicts results from a single experiment. The figure in panel A is representative of four independent experiments. The figure in panel B is representative of two independent experiments. In the case of panel A, the p value for the difference between i*Ft* versus PBS is p=0.0045 and for i*Ft* versus mAb-i*Ft* 0.018. In the case of panel B, the p value for the difference between i*Ft* versus PBS is p=0.0006 and for i*Ft* versus mAb-i*Ft* 0.2108.

A DC maturation
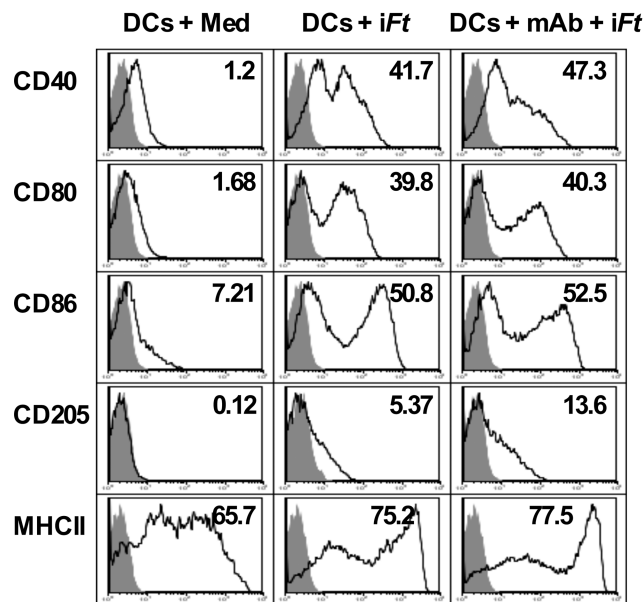
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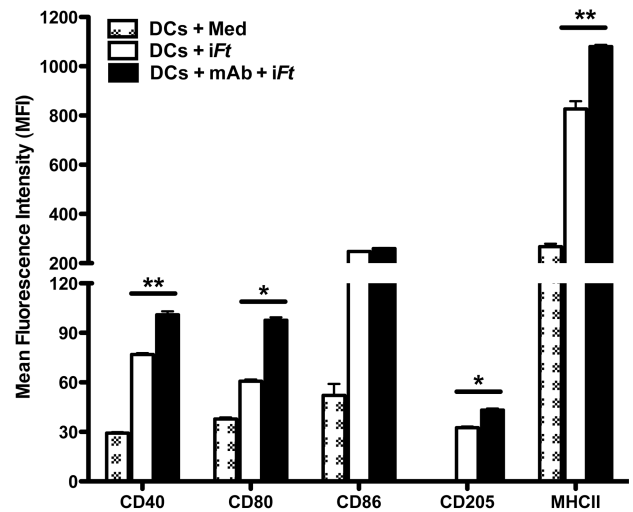
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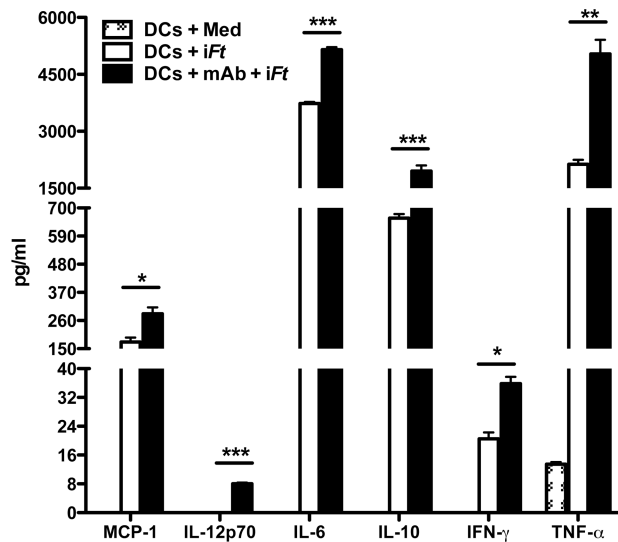
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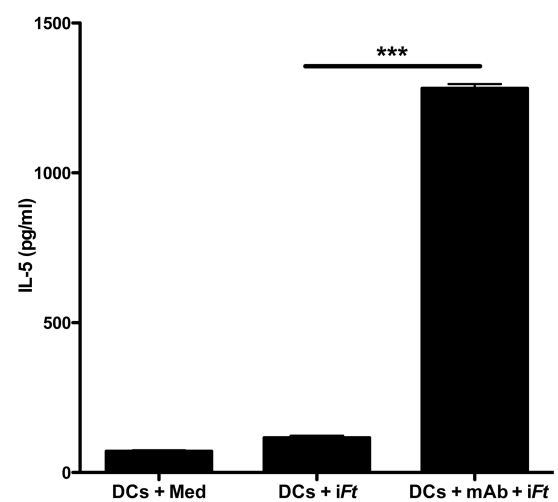
Figure 2. Pulsing BMDCs *ex-vivo* with mAb-i*Ft* enhances *in vitro* i*Ft*-induced BMDC maturation and Ag presentation

BMDCs (5×10^5) were pulsed overnight with medium or i*Ft* (10 i*Ft*/cell) in the presence or absence of mAb (1 μg/ml). Cells were then washed and stained for DC maturation markers. Acquisition was performed using an LSRII flow cytometer and data was analyzed using FlowJo software (Tree Star). (A) The grey peak represents isotype controls. Numbers in the right hand corner of each histogram indicate percent positive cells for the indicated DC maturation marker. (B) The maturation data are analyzed and expressed as Mean

Fluorescence Intensity (MFI). Data in A and B are representative of four independent experiments. (C) BMDCs (2×10^5) were stimulated with medium or i*Ft* (100 i*Ft*/cell), in the presence or absence of mAb (1μg/ml) for 3 days. Culture supernatants were then collected on day 3 and screened for cytokines using the Luminex assay. Data presented are from a single experiment, but are representative of results from three independent experiments. (D) BMDCs were stimulated overnight with medium or i*Ft* (2.5 i*Ft*/cell) in the presence or absence of mAb (1 μg/ml). Pulsed BMDCs were then washed 3 times with medium and co-cultured with an *Ft*-specific T cell hybridoma (FT256D10) for 24 hours. Culture supernatants were subsequently harvested and assayed for the presence of IL-5, a cytokine secreted by this T cell hybridoma in response to Ag recognition. Figures presented are from single experiments and are representative of results from three independent experiments. Data are presented as the average of three replicate samples ± SD (*: P< 0.01, ** P< 0.005, *** P<0.0005).
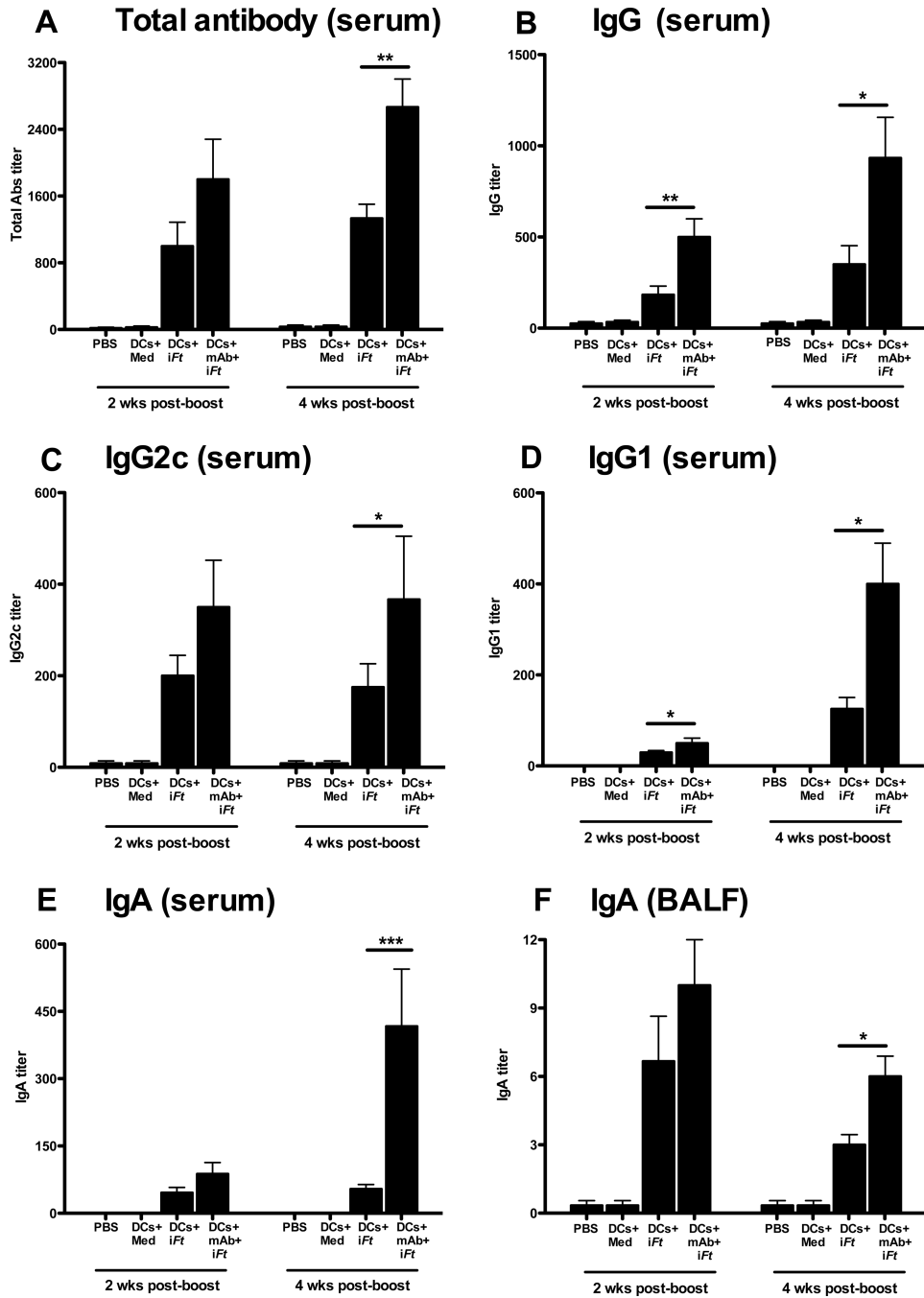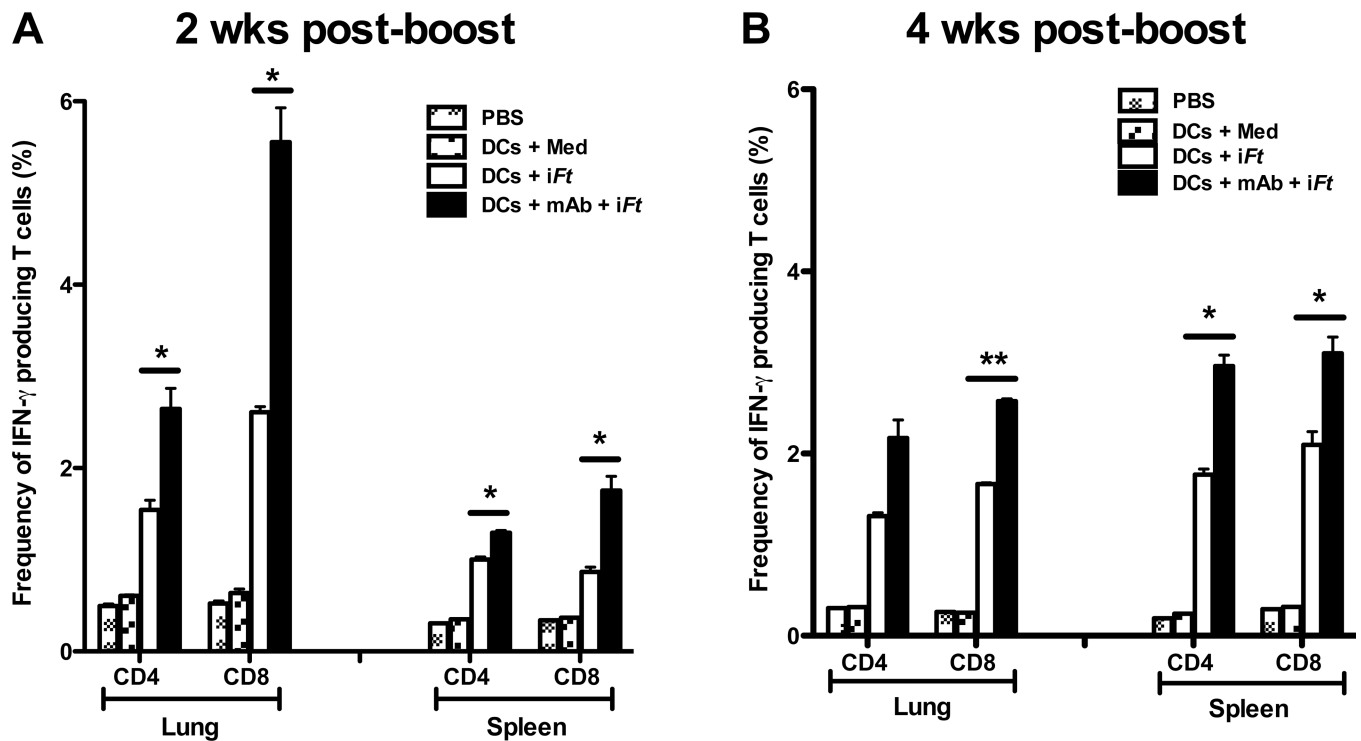
Figure 3. Mice immunized with mAb-i*Ft*-pulsed BMDCs exhibit higher *Ft*-specific Ab titers
Sera and BALF from immunized mice (n=4) were analyzed for total *Ft*-specific Ab (IgG, IgG1, IgG2c, and IgA) by ELISA. Ab titers were calculated using the reciprocal of the endpoint dilution of the test serum. Figures presented are from a single experiment, but are representative of results from two independent experiments. Data are presented as the average of four individual mice ± SD (*: P< 0.01, ** P< 0.005, *** P<0.0005).
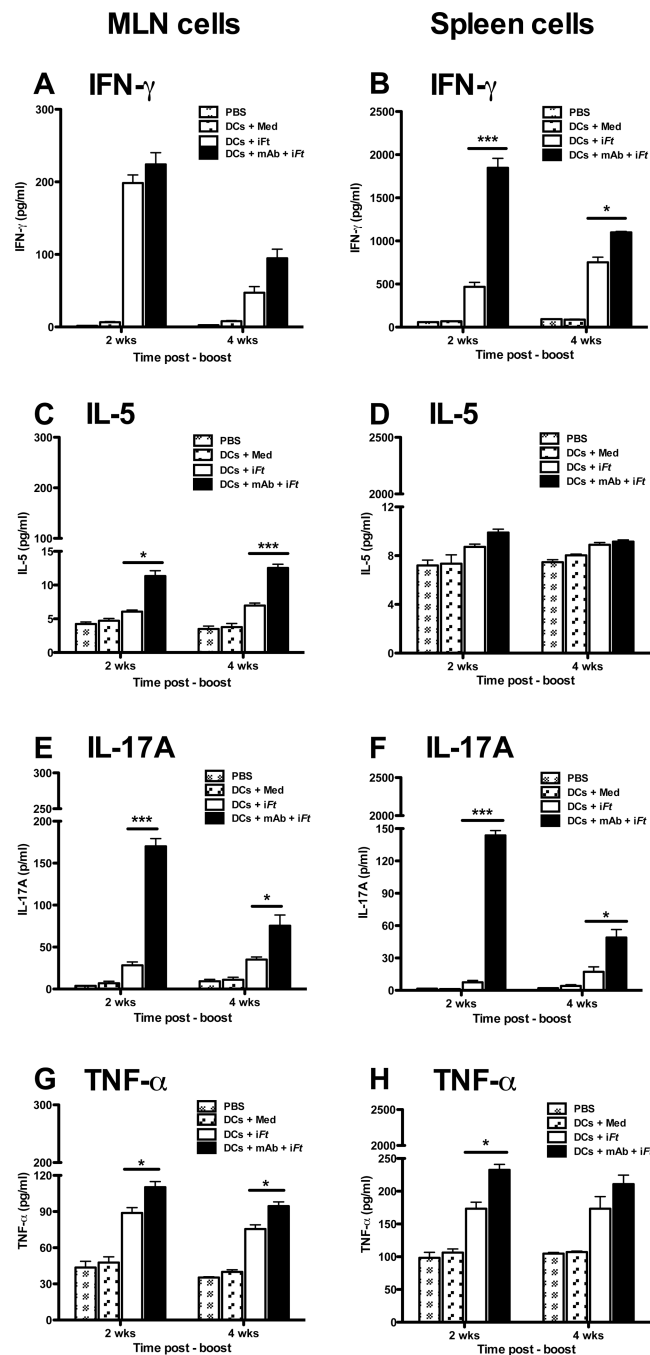
Figure 4. MLN and spleen cells from mice immunized with mAb-i*Ft*-pulsed BMDCs exhibit increased cytokine levels upon restimulation with i*Ft in vitro*

Single cell suspensions (3×10^5) of MLN or spleen cells from immunized mice (n=4) were added to wells of a 96 well plate and re-stimulated *in vitro* with medium or i*Ft* (5 i*Ft*/cell) for 3 days. Culture supernatants were harvested on day 3 and analyzed for cytokines using the Luminex assay. Figures presented are from a single experiment, but are representative of results from two independent experiments. Data are presented as the average of two (MLN cells) or four (Spleen cells) individual mice ± SD (*: P< 0.01, ** P< 0.005, *** P<0.0005).

Figure 5. The frequency of IFN-γ producing CD4 and CD8 T cells is enhanced in mice immunized i.n. with mAb-i*Ft*-pulsed BMDCs

Splenocytes and lung cells from immunized mice were stimulated *in vitro* with i*Ft* (5 i*Ft*/cell) combined with PMA and Ionomycine overnight, and then stained for CD4 and CD8 surface markers and intracellular IFN-γ. Samples were acquired using an LSRII flow cytometer and analyzed with FlowJo software. Percentages of IFN-γ+ CD4+ and IFN-γ+ CD8+ T lymphocytes at 2 weeks post-boost (A) and 4 weeks post-boost (B) are shown. Figures presented are from a single experiment, but are representative of results from two independent experiments. Data are presented as the average of four individual mice ± SD (*: P< 0.01, ** P< 0.005, *** P<0.0005).
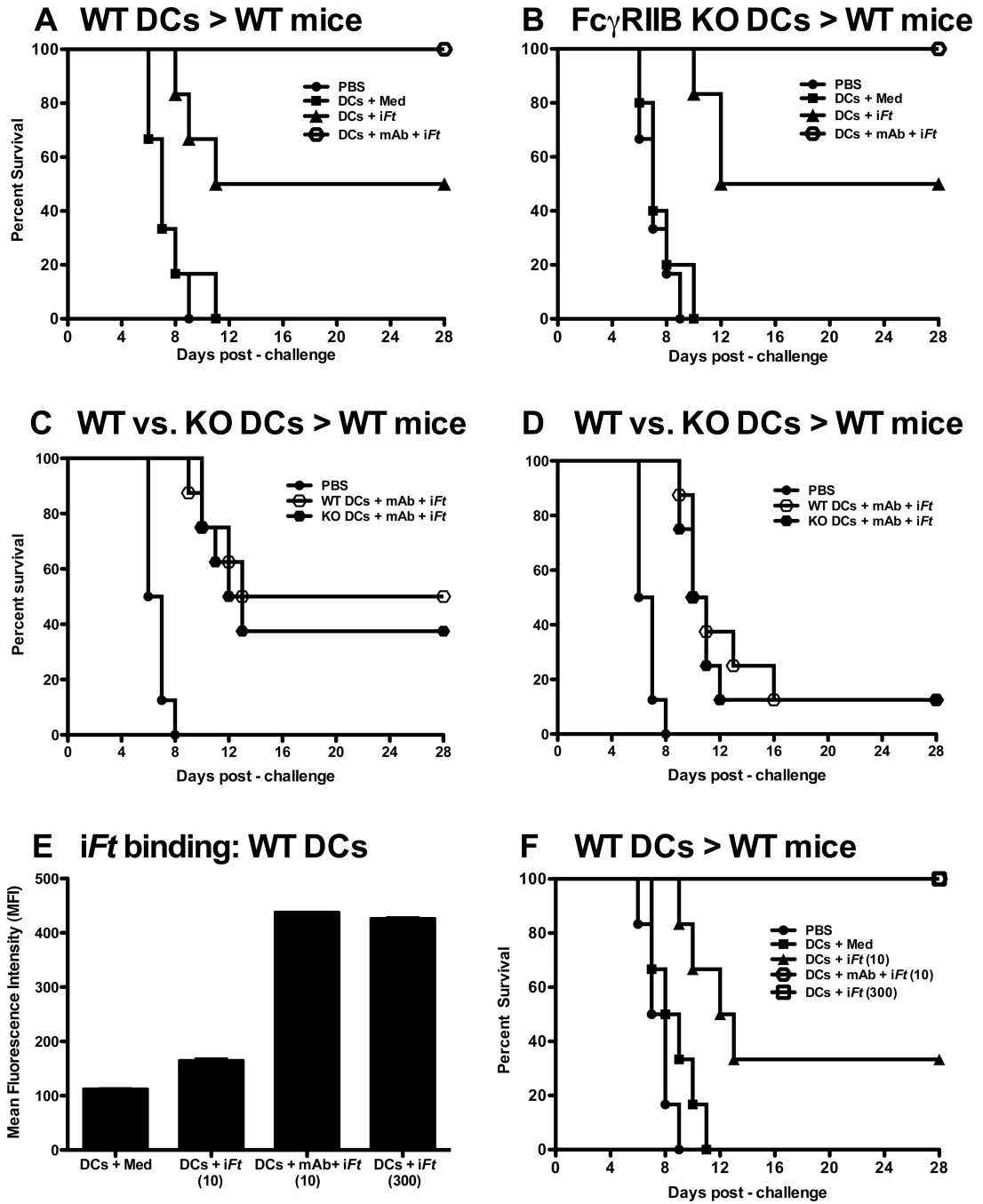
Figure 6. The inhibitory receptor (FcγRIIB) has no impact on the enhanced protection induced by *ex vivo* mAb-i*Ft*-pulsed BMDCs while enhanced i*Ft* (Ag) loading plays the primary role in the enhanced protection observed with mAb-i*Ft*-pulsed BMDCs

C57BL/6 mice (n = 6) were i.n primed and boosted with Ag pulsed BMDCs differentiated from WT mice (A) or FcγRIIB KO mice (B). Two weeks post-boost, mice were challenged i.n. with $4 \times LD_{50}$ of *Ft* LVS prepared in 20 µl of PBS. Survival was monitored for 28 days post-infection. Data are representative of two independent experiments. (C and D) C57BL/6 mice (n = 8) were immunized i.n. and boosted two weeks later with PBS or mAb-i*Ft* pulsed BMDCs (10 i*Ft*/cell) from WT or FcγRIIB KO mice. Two weeks post-boost, mice were

challenged i.n. with $8 \times LD_{50}$ of *Ft* LVS (C) or $16 \times LD_{50}$ of *Ft* LVS (D) in 20 μl of PBS. DCs pulsed with PBS (the vehicle control group) were challenged with $4 \times LD_{50}$ of *Ft* LVS. Survival was monitored for 28 days post-infection. (E) BMDCs were pulsed with medium, i*Ft* (10 i*Ft*/cell), mAb-i*Ft* (10 i*Ft*/cell) or an amount of i*Ft* (300 iFt/cell), which produced i*Ft* binding equivalent to that of mAb-i*Ft*. In this case, GFP-expressing *Ft* was used to produce i*Ft* in order to more easily monitor i*Ft* binding by flow cytometry. *Ft* inactivation/fixation does not alter GFP fluorescence. Pulsed BMDCs were then washed 3 times with PBS and binding to BMDCs analyzed with an LSRII flow cytometer and FlowJo software. Data are presented from a single experiment as the average of three replicate samples ± SD. (F) C57BL/6 mice (n=6) were primed and boosted i.n. with BMDCs pulsed with i*Ft* (10 i*Ft*/cell or 300 i*Ft*/cell) or mAb-iFt (10 i*Ft*/cell). At days 0 and 14, 3×10^6 pulsed BMDCs in 30 μl of PBS were administered i.n. to mice. Two weeks post-boost, mice were challenged i.n. with $4 \times LD_{50}$ of *Ft* LVS in 20 μl of PBS. Survival was monitored for 28 days post-infection. A vehicle control group received PBS alone. Figures presented in panels A, B, E, and F are from a single experiment, but are representative of results from two independent experiments.