Multiple mechanisms mediate enhanced immunity generated by mAb-inactivated *F. tularensis* immunogen

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We have previously demonstrated that immunization with the inactivated *Francisella tularensis*, a Category A intracellular mucosal pathogen, combined with IgG2a anti-*F. tularensis* monoclonal antibody (Ab), enhances protection against subsequent *F. tularensis* challenge. To understand the mechanism(s) involved, we examined the binding, internalization, presentation, and *in vivo* trafficking of inactivated *F. tularensis* in the presence and absence of opsonizing monoclonal Ab. We found that when inactivated *F. tularensis* is combined with anti-*F. tularensis* monoclonal Ab, presentation to *F. tularensis*-specific T cells is enhanced. This enhancement is Fc receptor (FcR)-dependent, and requires a physical linkage between the monoclonal Ab and the inactivated *F. tularensis* immunogen. This enhanced presentation is due, in part, to enhanced binding and internalization of inactivated *F. tularensis* by antigen(Ag)-presenting cells, and involves interactions with multiple FcR types. Furthermore, targeting inactivated *F. tularensis* to FcRs enhances dendritic cell maturation and extends the time period over which Ag-presenting cells stimulate T cells. *In vivo* trafficking studies reveal enhanced transport of inactivated *F. tularensis* immunogen to the nasal-associated lymphoid tissue in the presence of monoclonal Ab, which is FcRn-dependent. In summary, these are the first comprehensive studies using a single-vaccine protection model/immunogen to establish the array of mechanisms involved in enhanced immunity/protection mediated by an FcR-targeted mucosal immunogen. These results demonstrate that multiple cellular/immune mechanisms contribute to FcR-enhanced immunity. *Immunology and Cell Biology* (2013) 91, 139–148; doi:10.1038/icb.2012.66; published online 18 December 2012

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*F. tularensis* (Ft) is a Category A mucosal pathogen that represents a significant bioterror threat.¹ We have previously demonstrated that intranasal (i.n.) administration of immune complexes (ICs), composed of inactivated Ft (iFt) and IgG2a monoclonal Ab (mAb) specific for iFt lipopolysacharide (LPS), in which free/non-iFt bound mAb has been removed (mAb-iFt ICs), enhances the immune response to, and protection against, Ft challenge.² We further demonstrated that the enhanced protection observed was Fc receptor (FcR)-dependent. In addition, this was the first study to demonstrate that targeting an immunogen to FcR i.n. enhances protection against a mucosal infectious disease challenge. Subsequent studies have verified the potential for using FcR-targeted immunogens as effective mucosal vaccines.³⁻⁶

Although numerous mechanisms, including enhanced antigen (Ag) presentation, have been predicted to be involved in FcR-enhanced immune responses, comprehensive studies using a proven FcR-targeted mucosal vaccine strategy to actually define these specific mechanisms have not been conducted.⁷⁻¹⁰ Some of the proposed mechanisms involve include the following: it has been proposed that complement-mediated binding of Ab (Ab)–Ag ICs to APCs, not FcR binding, may be responsible for Ab–Ag IC-mediated immune enhancement. However, this has been contradicted in studies using FcR knockout mice, in which FcRs were required for immune enhancement.⁹ Alternatively, upregulation of major histocompatibility complex (MHC) class II and second signal molecules, as a result of FcR crosslinking on APCs, could also lead to enhanced Ag presentation. However, in previous *in vitro* studies using human IgG-Ag ICs and monocytes as APCs, we did not observe increased expression of these molecules as a consequence of IC-FcR interaction.¹¹ Studies using dendritic cells (DCs), however, have indicated that Ab–Ag ICs can induce DC maturation,¹² although this was later contradicted by a study, suggesting that IC interaction with the inhibitory FcR (FcγRIIB) blocks DC maturation induced by ICs.¹³ In addition, as previously indicated above, more efficient Ag-binding

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and internalization is believed to have an important role in IC-enhanced immune responses. However, this is dependent on not only the concentration of ligand, but the valency of the ligand, the number of FcR expressed on the APC surface, and whether or not the particular FcγR is occupied with serum IgG.9,14 In the latter case, the amount of FcR crosslinking required to induce internalization appears to actually be reduced when FcγRIIa is occupied.9,14 Engaging FcR on APCs can also induce cytokine production.15-17 The cytokine milieu can then determine the type and degree of the immune response. For example, studies, in which the activating FcγRIIA on monocyte-derived DCs was ligated, resulted in secretion of interleukin (IL)-10 and IL-6 (stimulates B cells and plasma cells), and tumor necrosis factor α and IL-8 (chemoattractants). However, the cytokines produced can vary with the source of DCs and the ratio of activating and inhibitory (FcγRIIB) receptors engaged.12,18 Furthermore, the impact of Ag targeting to FcR on Ag persistence, which is observed when Ag is targeted to the B cell receptor,19 or Ag trafficking to lymphoid tissues in vivo,8,9 remains unclear. More recently, and contrary to prior belief, studies have clearly demonstrated the neonatal FcR (FcRn), which binds IgG Fc, is present in the epithelium of the nasal mucosa in adult mice,4 and that targeting Ags to FcRn via this route also enhances the immune responses to, and protection against, mucosal pathogens.5,13 This is also consistent with our previous studies, indicating the presence of FcRn is critical for the enhanced protection that we observe when utilizing mAb-iFt ICs as protective mucosal immunogen.2

Thus, we sought to clarify the mechanisms involved in FcR-enhanced immunity, including the specific role of FcRn, by conducting a comprehensive analysis of the numerous potential mechanisms involved in FcR-targeted vaccination using a single FcR-targeted model immunogen, which has been proven to be effective in not only enhancing the immune response in vivo, but also producing protection against subsequent infectious disease challenge.

RESULTS
Enhanced protection against Ft challenge, following i.n. administration of mAb + iFt ICs
We have previously demonstrated that mAb-iFt ICs from which free/unbound mAb has been removed via centrifugation, when administered i.n., enhance protection against subsequent i.n. challenge with Ft.2 Furthermore, enhanced protection was not observed when administering either mAb alone or F(ab′)2 mAb-iFt ICs.2 To facilitate the conduct of these specific studies, as well as the future use of this approach as a vaccine strategy, we first determined whether anti-iFt mAb + iFt ICs, in which free/unbound anti-iFt mAb is not removed (mAb + iFt ICs), could be used in place of mAb-iFt ICs (free/unbound mAb removed).2 In Figure 1a western blot was utilized to verify the formation of ICs, following incubation of anti-iFt mAb with iFt. To accomplish this using western blot, it was necessary to remove free/unbound mAb, as was done in our previously published studies. Importantly, the incubation steps before centrifugation were the same in both our published studies in which free mAb was removed (mAb-iFt ICs) and the present study in which free mAb is not removed (mAb + iFt ICs). Thus, the ICs detected in Figure 1a reflect ICs formed regardless of whether free/unbound mAb is or is not removed. Most importantly, the sole purpose of this experiment was to verify IC formation. In fact, ICs were formed and there was a significant increase in IC formation in the presence of 5 μg versus 1 μg of mAb (Figure 1a). We then immunized and boosted mice i.n. with these mixtures and challenged 14 days later i.n. with either Ft LVS (Figure 1b) or Ft SchuS4 (Figure 1c). Similar to studies using mAb-iFt ICs (free/unbound mAb removed),2 a significant enhancement of protection against both strains of Ft was observed with mAb + iFt ICs (free/unbound mAb present). Also, consistent with previous studies using mAb-iFt ICs, while mAb + iFt ICs made with 1 μg mAb were sufficient to protect against Ft LVS challenge, mAb + iFt ICs made with 5 μg mAb were required to protect against Ft SchuS4 challenge.

**Figure 1** Enhanced protection is observed using anti-iFt mAb + iFt ICs. Before immunization, 1 × 10^9 iFt organisms were incubated at 4 °C overnight with 0, 1 or 5 μg ml⁻¹ of anti-iFt mAb in PBS. Following the incubation, iFt and mAb + iFt ICs were washed (to remove free mAb) and analyzed by SDS PAGE and western blot to verify mAb-iFt IC formation (a). 2 × 10^7 iFt organisms alone or complexed with anti-iFt mAb were administered i.n. to C57BL/6 mice. The mice were boosted on day 21 and challenged on day 35 with either 20 000 c.f.u. of Ft LVS (b) or boosted on days 14 and 28 and challenged on day 42 with 21 c.f.u. of Ft SchuS4 (e). Survival was monitored for 21 days. The P-value for iFt versus mAb + iFt ICs in B (Ft LVS challenge) was <0.05. The P-value for iFt versus mAb + iFt ICs in C (Ft SchuS4 challenge, 5 μg) was <0.02.
enhancement was eliminated if the iFt ICs alone does not stimulate the enhanced T cell activation (Figure 2b) concentration dependent. Alternatively put, mAb in the Ft supernatants of T cells and APCs incubated with iFt ICs may enhance bacteria in the presence of anti-iFt mAb and iFt ICs versus iFt alone (Figures 2a–d). This increase was dependent on both mAb (Figure 2e) and iFt (Figure 3f) concentration. Furthermore, ~50% of the mAb-mediated iFt binding could be blocked with monomeric IgG2a (Figure 3g). Monomeric IgG2a primarily blocks the high-affinity FcγRI. The mAb 2.4G2, which blocks FcγRII and FcγRIII, blocked ~70% of the mAb-iFt binding (Figure 3h). When combining monomeric IgG2a with mAb 2.4G2, nearly 100% of mAb-mediated iFt binding was eliminated (Figure 3i). Thus, enhanced binding of iFt to APCs in the form of mAb + iFt ICs represents another contributing mechanism by which mAb + iFt ICs impact the immune response via FcR-mediated delivery of Ag to APCs.

A physical linkage between mAb and Ag is required for FcR-mediated enhancement of Ag presentation by APCs

We also considered the possibility that the impact of mAb + iFt ICs on the presentation of iFt was not dependent on iFt being directly targeted to FcγR, but rather due to signaling events initiated by crosslinking FcγR. Thus, we determined whether mAb + iFt ICs added to APCs could enhance the presentation of an Ag other than iFt, specifically hen–egg lysozyme (HEL). In this case, an HEL-specific T cell hybridoma (LY50.5) was cultured in the presence of APCs and increasing concentrations of HEL containing either media, iFt, mAb + iFt ICs or mAb alone. After 24 h, supernatant was collected and IL-5 levels were measured. As shown in Figure 4, the presence of mAb + iFt ICs did not impact the level of IL-5 produced by the T cell hybridoma in response to HEL, regardless of whether media, iFt, mAb + iFt ICs or mAb alone was present (Figure 4a). The latter was not due to a failure of the mAb + iFt ICs to mediate enhanced presentation of iFt (Figure 4b). Furthermore, when HEL-specific mAb was bound to HEL, HEL presentation was enhanced (Figure 4c), as was the presentation of HEL, when iFt was chemically labeled with HEL and anti-iFt mAb bound (Figure 4d). Finally, consistent with the inability of mAb + iFt ICs to stimulate enhanced presentation of HEL, neither the MHC class II expression nor the expression of second signal molecules on these APCs, were enhanced in the presence of mAb + iFt ICs (data not shown). Thus, crosslinking FcR with mAb + iFt ICs alone is insufficient to enhance Ag presentation by APCs. Rather, a direct linkage between the Ag being presented and the mAb is required.

The rate of iFt internalization is increased in the presence of anti-iFt mAb

Another mechanism by which iFt processing and presentation could be enhanced, is via an increased rate of iFt internalization by APCs. To address this possibility, the amount of iFt internalized by APCs was...
Cells were pulsed for 1 hour with 200 iFt organisms/APC at 4°C, and then washed and incubated at 37°C for varying periods of time. Once the incubation was complete, the cells were cooled immediately on ice, washed and incubated with pronase in order to remove the non-internalized iFt. Once the non-internalized iFt was removed from the surface of the cells, they were washed again and fixed. The mean fluorescence intensity was then determined for each time point. As shown in Figure 5, it took ~3–4 h for internalization to be detected in the form of increased fluorescence of pronase-stripped cells, when pulsing with iFt alone (Figure 5a). However, the increase was much stronger and more rapid when anti-iFt mAb was present, occurring in 5 minutes or less (Figure 5b). Importantly, the rate of internalization for iFt alone remained similar even when the number of organisms/APC was increased by 10-fold (Figure 4c). Thus, not only is iFt binding to APCs enhanced in the presence of iFt-specific mAb, but also enhanced is the rate of iFt internalization by APCs.

**Figure 3** Anti-iFt mAb enhances FcR-dependent binding of iFt to APCs. Z projections of Balb/c MØs incubated for 2 h at 4°C with either iFt alone or anti-iFt mAb + iFt ICs. The iFt (green) (GFP-expressing inactivated Ft organisms) can be seen on the cells’ surface (red) labeled with CTB Alexa 647 and the nuclei (blue) have been stained with DAPI. The images were acquired on an Olympus IX 81 confocal microscope (a–d). Balb/c MØs were incubated at 4°C for 2 h in the presence of either increasing concentrations of anti-iFt mAb and 5 x 10⁵ iFt organisms per well (e) or increasing concentrations of iFt organisms plus or minus 1 µg ml⁻¹ of anti-iFt mAb (f). Before the addition of the iFt organisms in the presence or absence of anti-iFt mAb, FcR were blocked for 1 h at 4°C with monomeric IgG2a (FcγRI) (g), F(ab')₂ 2.4G2 mAb (FcγRII and FcγRIII) (h) or both IgG2a and F(ab')₂ 2.4G2 mAb (i). Binding of GFP-expressing iFt organisms was detected by flow cytometry using a BD LSRII flow cytometer (e–i). Results are representative of three independent experiments. (*** ) P-value <0.001.
Enhanced presentation of iFt persists in the presence of anti-iFt mAb

As we observed an increased T cell response, increased binding and more rapid internalization of the iFt in the presence of anti-iFt mAb, we wanted to know how these changes impact the kinetics of Ag processing and presentation. To examine this, APCs were allowed to take up and process iFt in the presence and absence of mAb for 0, 1, 2, 4, 8, 22 and 26 h. Following each incubation time point, APCs were fixed and incubated with T cells as a read out of iFt processing. After 24 h of APC plus T cell culture, the supernatant was collected and IL-5 production was measured by ELISA.

**Mechanisms involved in Fc receptor-enhanced immunity**

**Figure 4** A physical linkage between mAb and Ag is required for FcR-mediated enhancement of Ag presentation by APCs. HEL-specific T cell hybridoma cells (1×10^5 cells per well) were cultured with C3H/HeN MØs (2×10^5 cells per well) in the presence of media, 5×10^3 iFt organisms per well alone or plus 1 µg ml^-1 of anti-iFt mAb and increasing amounts of HEL (a). Ft-specific T cell hybridoma cells (1×10^5 cells per well) were cultured with Balb/c MØs (2×10^5 cells per well) in the presence or absence of 1 mg ml^-1 of HEL in media, 5×10^3 iFt organisms per well alone or plus 1 mg ml^-1 of anti-iFt mAb (b). HEL-specific T cell hybridoma cells (1×10^5 cells per well) were cultured with C3H/HeN MØs (2×10^5 cells per well) in the presence of media, 0.1 mg ml^-1 of HEL, 0.1 mg ml^-1 of HEL plus anti-HEL mAb (2D1), or anti-HEL mAb alone (2D1) (c). HEL-specific T cell hybridoma cells (1×10^5 cells per well) were cultured with C3H/HeN MØs (2×10^5 cells per well) in the presence of media, 2×10^7 iFt organisms plus or minus anti-iFt mAb or 2×10^7 HEL-conjugated iFt organisms plus or minus anti-iFt mAb (d). Production of IL-5 by Ft-specific T cells was measured in all figures (a-d) as an indicator of T cell response to iFt by CBA. Data was normalized between experiments by expressing the results as percent maximal response. Results in 2A and 2B represent the combined data from two independent experiments. All other results are representative of three independent experiments. (*** ) P-value <0.001.

**Figure 5** Internalization of iFt is enhanced in the presence of anti-iFt mAb. Balb/c MØs (2×10^5 cells) were pulsed with 200 iFt organisms/MØ in the absence (a) or presence (b) of anti-iFt mAb for 1 h at 4°C. After the pulse, the unbound iFt was removed and the MØs were incubated at 37°C to allow internalization of the iFt. At the end of each 37°C incubation time point, the non-internalized iFt was stripped from the cell surface using pronase, and the amount of internalized iFt was measured by flow cytometry (a, b). In addition, MØs were also pulsed with 2000 iFt organisms/MØ in the absence of anti-iFt mAb (c). The experiment was then carried out as in (a) and (b). Internalization is reflected as increased MFI, following incubation and pronase treatment, as compared with pronase-treated cells at time zero. Results are representative of three independent experiments. (**) P-value <0.01, (****) P-value <0.001.
was measured. As early as 4 hours after iFt pulse, the T cell response in the presence of mAb was increased above that of iFt alone (Figure 6a). Furthermore, the length of time processed iFt was available on the surface of the APCs for presentation was also increased (Figure 6a). To further investigate the latter observation, APCs were allowed to internalize and process the Ag for 8 h, then the non-bound/non-internalized Ag was washed away and the cells were either fixed immediately or fixed after 15, 18, 21 or 36 h of additional incubation.

As demonstrated in Figure 6b, when iFt is internalized in the presence of anti-iFt mAb, up to 36 h after free Ag is removed, an enhanced response by T cells to processed iFt is observed, as compared with APCs pulsed with iFt alone. Thus, the enhanced iFt presentation induced by mAb + iFt ICs begins as soon as 4 h post iFt addition and also persists up to 36 h after the iFt pulse.

Enhanced iFt binding, presentation, and iFt persistence is also observed with DCs in the presence of mAb + iFt ICs

As all the studies up to this point had focused on peritoneal exudate cells/MØs as APCs, we wanted to know whether mAb + iFt ICs would have a similar impact on DCs. Interestingly, the level of enhanced binding (Figure 7a) and enhanced presentation (Figure 7b) was even more dramatic when using DCs as APCs. In addition, in contrast to...
MOs, the expression of MHC class II and second signal molecules (DC maturation markers) was increased in the presence of mAb + iFt ICs versus iFt (Figure 7c). However, iFt alone also induced a significant increase in the expression of these markers (Figure 7c).

Furthermore, the higher levels of iFt presentation by DCs, induced in the presence of mAb + iFt ICs, also demonstrated similar persistence to that of MOs (Figures 6 and 7d). Thus, with the exception of MHC class II, CD80, and CD86 expression, which is enhanced by mAb + iFt ICs on DCs, but not on MOs (data not shown), the impact of mAb + iFt ICs on iFt binding, presentation, and Ag persistence, is similar to that of MOs.

Enhanced localization of iFt to NALT in vivo

It had been previously hypothesized that one potential impact of targeting Ag to FcRs in vivo may be to enhance Ag trafficking to lymphoid organs. Given that our immunizations are i.n., we wanted to test this hypothesis with regards to nasal-associated lymphoid tissue (NALT). Utilizing quantitative PCR (qPCR) to detect iFt localization after i.n. administration of iFt and mAb + iFt ICs, we observed nearly a 10-fold increase in the amount of iFt trafficking to the NALT by 30 min post-administration of mAb + iFt ICs, as compared with iFt alone (Figure 8a). Furthermore, prior indications that FcRn can have a significant role in Ag transport across the epithelium in adults,24–28 was confirmed in the case of mAb + iFt ICs (Figure 8b). Specifically, when using FcRn knockout mice in similar trafficking studies, the enhanced trafficking of mAb + iFt ICs observed with wild-type mice (Figure 8a) was eliminated (Figure 8b).

Thus, another mechanism by which mAb + iFt ICs exert their effect on the immune response is enhanced via transport of iFt to the NALT via FcRn.

DISCUSSION

Numerous studies have demonstrated that targeting Ag to FcR can enhance the immune response to Ag.12,24–28 In 2008, we demonstrated for the first time, that targeting Ag to FcR i.n. could also enhance protection against a subsequent challenge with the highly virulent mucosal pathogen Ft.2 Utilizing this model, we now provide the first comprehensive study examining the potential mechanism(s) that contribute to this immune enhancement.

Numerous studies have suggested one mechanism of immune enhancement involves enhanced presentation of the FcR-targeted immunogen,17 which is generally believed to be the primary contributor. However, a comprehensive study using established FcR-targeted protective immunogens, such as mAb-iFt or mAb + iFt ICs, has not been conducted to determine if this is in fact the case. In this study, we verify that FcR-dependent enhanced presentation is a significant component of the enhanced response to FcR-targeted iFt (Figure 2), and that targeting of iFt to FcR on APCs enhances both iFt binding and internalization (Figures 3 and 5). We also demonstrate that the observed FcR-mediated enhancement in iFt presentation requires a physical linkage between iFt and the mAb that engages the FcR (Figures 4a–d). In other words, simply crosslinking FcR is not sufficient to enhance Ag presentation. This is consistent with the idea that enhanced Ag binding and Ag internalization is critical to the enhanced presentation observed. The fact that crosslinking MO FcR with mAb + iFt ICs did not enhance expression of MHC class II or second signal molecules (data not shown) is also consistent with this observation and previous observations by this laboratory in which similar results were obtained using human IgG-Ag ICs and human monocytess.13 However, the impact of ICs on DC maturation is more controversial. Although some studies have indicated that DC maturation can be enhanced in the presence of ICs,12 more recent studies have suggested ICs inhibit DC maturation via engagement of FcγRIIB, the inhibitory FcR.13 In fact, despite the failure of mAb + iFt ICs to enhance MHC class II and second signal molecule expression on MOs, DC maturation (indicated by upregulation of both MHC class II and second signal molecules) was enhanced in the presence of mAb + iFt ICs (Figure 7c). The latter is likely explained by differences between these and other published studies, and the numerous variables involved. Specifically, the study, which demonstrates inhibition, utilized ICs composed of OVA and rabbit anti-OVA IgG, combined with human monocyte-derived DCs, as opposed to our studies, which utilized ICs composed of mouse IgG2a mAb + iFt ICs and mouse DCs. In this regard, the size of the ICs, the valency and differences in inter- and cross-species binding of IgG isotypes to FcR all significantly impact which FcRs (activating versus inhibitory) are engaged.10,13,17 It is likely in our case, engagement and activation of the stimulatory FcRs dominates that of the inhibitory FcR, resulting in the enhanced DC maturation we observe.
This paper also demonstrates for the first time that targeting Ag to FcR results in Ag presentation persisting at higher levels for an extended period of time (Figures 6 and 7). As with enhanced Ag binding and presentation, this was observed for both MØs and DCs (Figures 6 and 7). In fact, a similar observation has been made when targeting Ag to the B cell Ag receptor. Thus, our data demonstrate that a number of mechanisms targeting Ag to the B cell Ag receptor. Thus, this may represent a significant difference in cellular infiltration (nearly absent) between PBS, iFt or mAb + iFt IC-immunized mice (data not shown).

Nevertheless, it is likely that the inflammatory response is enhanced within the NALT following administration of ICs, as reflected in the resultant enhanced humoral and cellular immune responses. It has also been proposed that an additional mechanism by which FcR-targeted immunogens enhance the immune response is by facilitating the trafficking of Ag to lymphoid tissues. In the case of mAb + iFt ICs, this does hold true, in that iFt transport to the NALT was enhanced in the presence of anti-iFt mAb and was FcRn-dependent. (Figure 8b). In fact, since our original publication in Ft mice, suggesting that FcRn was playing a key role. Thus, our data support the hypothesis that mAb + iFt ICs first engage FcRn for trans-epithelial transport to the NALT, followed by engagement of FcγR on APCs within the NALT. However, further trafficking studies will be needed to specifically verify this hypothesis. It should also be noted Ag's can be transported by M cells, which thus may also have a role. In fact, in regard to the latter, we have recently published studies in which engagement of FcRn cannot occur, but never the less enhanced mucosal immunity is still observed when targeting the immunogen to FcγRI on mucosal APCs.

In summary, by utilizing a proven FcR-targeted protective mucosal immunogen, we demonstrate that a number of mechanisms contribute to the enhanced immunity and protection observed when targeting an immunogen to FcR. Specifically, FcR targeting mediates enhanced Ag binding, as well as more rapid Ag internalization, leading to enhanced activation of Ag-specific T cells. Consistent with this, Ag must be physically attached to the FcR-targeting component (in this case mAb) to observe enhanced presentation. In addition, not only is the T cell response enhanced, but also that enhancement is maintained over an extended period of time. Furthermore, DC maturation is also enhanced, as well as the FcRn-mediated trafficking of iFt to NALT. Thus, the impact of targeting Ag to FcR is multi-pronged with each mechanism involved, having the potential to significantly contribute to enhanced immunity and protection. By understanding and further optimizing each of these events, one has the potential to further improve on FcR targeting as an effective mucosal vaccine platform. Furthermore, the fact that mAb + iFt ICs (free mAb present) are as effective as mAb-iFt ICs (free mAb removed) (Figure 1) will further facilitate the application of this specific strategy in a clinical setting.

METHODS

Reagents

The Ag HEL and Pronase were purchased from Sigma (St Louis, MO, USA). Endotoxins were removed from HEL using Detoxi-Gel from Pierce (Rockford, IL, USA). Cell-Tak was purchased from BD Biosciences (San Diego, CA, USA) and used as directed by the vendor. Cholera Toxin Subunit B (recombinant)-Alexa Fluor 647 conjugate was purchased from Invitrogen (Carlsbad, CA, USA). The mouse IgG2a anti-Ft LPS mAb used to make mAb + iFt ICs was purchased from Fitzgerald (cat # I0-F02, clone# M0232621, Acton, MA). F(ab')2 mAb against Ft LPS was prepared using an F(ab')2 preparation kit from Pierce, according to the vendor instructions. Anti-mouse F4/80 Ab was purchased from Invitrogen. Anti-mouse CD16/32 (2.4G2) F(ab')2 Ab was purchased from BD Biosciences. Anti-mouse MHC class II (I-A/I-E), CD83 and DEC205 Ab were purchased from eBioscience (San Diego, CA, USA). Anti-mouse CD80 and CD86 Ab were purchased from BioLegend (San Diego, CA, USA). IgG2a isotype control Ab was purchased from MP Biomedicals, LLC (Solon, OH, USA). All other isotype control Abs were purchased from BioLegend.

Cells

Either peritoneal exudate cell/MØs or DCs were used as APCs in these studies. Peritoneal exudate cell/MØs were obtained from Balb/c or C3H/HeN mice. DCs were derived from Balb/c bone marrow cells and cultured for 1 week in the media, as described below, supplemented with 50 ng ml⁻¹ of FRTL (R&D Systems, Minneapolis, MN, USA). 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 hybridoma was cultured in RPMI 1640 (CellGro, Manassas, VA, USA) containing 10% FBS (HyClone, Logan, UT, USA), 2 mM L-glutamine (CellGro), MEM non essential aminos (CellGro), 1 mM sodium pyruvate, 50 μM 2-ME, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ Streptomycin (Gibco Life Technologies, Carlsbad, CA, USA), and 500 μg ml⁻¹ of hygromycin B (CellGro). The HEL-specific T cell hybridoma (Ly50.5) and Balb/c bone marrow-derived DCs were cultured in the same media without the addition of Hygromycin.

Mice

Balb/c, C57BL/6, and C3H/HeN mice were purchased from Taconic Laboratories (Hudson, NY, USA). All mice were housed at the Animal Resources Facility at Albany Medical College. The mice were used at 6–10 weeks of age. All protocols were reviewed by the Albany Medical College Ethics Committee utilizing NIH standards.

Inactivation and labeling of Ft

Inactivated Ft (iFt) was generated by growing GFP-expressing Ft LVS in MHB medium (BD Biosciences) in 5% CO₂ up to a density of 1 × 10⁹ c.f.u. ml⁻¹. The culture was then spun down at 22 000 g for 20 min in 4°C, and washed three times with PBS, resuspended in 2% paraformaldehyde (Sigma) and incubated 2 h at room temperature on a rocker. Bacteria were then washed three more times with PBS and 1 × 10⁹ organisms were plated on a chocolate agar plate (BD Biosciences) to confirm inactivation. The plate was then incubated for 7 days at 37°C. HEL labeling of iFt was done by using a Rapid Conjugation Kit (AbD Serotec, Raleigh, NC, USA). Briefly, 1 × 10¹⁰ iFt organisms were resuspended in 200 μl of PBS and 20 μl of modifier reagent (provided with the kit) along with 500–1000 μg of HEL dissolved in PBS. This mixture was then incubated at room temperature overnight. The following day, 20 μl of quencher (provided with the kit) was added to the iFt organisms and this was incubated for 30 min at room temperature. Thereafter, the labeled iFt was washed with PBS two times. The final concentration of iFt organisms was determined by optical density (OD) at 610 nm, HEL labeling was verified by ELISA and anti-iFt mAb binding to the HEL-labeled iFt was verified by flow cytometry and ELISA.

Generation of mAb + iFt ICs

To generate mAb + iFt ICs, 1 × 10⁹ iFt LVS organisms were incubated at 4°C overnight on a rocker with 0, 1 or 5 μg ml⁻¹ of IgG2a anti-iFt LPS mAb in
PBS. Following the incubation, iFt or mAb + iFt ICs were administered to mice i.n.

SDS-polyacrylamide gel electrophoresis and western blot analysis
The IgG2a anti-Ft LPS mAb and iFt were incubated, as previously indicated\(^2\) and as briefly described above. To avoid the detection of free mAb by the goat anti-mouse mAb used to detect ICs, following the incubation of mAb + iFt ICs, free/unbound mAb was removed via centrifugation. Samples of iFt (10 µg (\(\sim 1 \times 10^6\) organisms of iFt or mAb + iFt ICs)) were mixed with Laemmli sample buffer and boiled for 10 min before resolution through 4–12% gradient SDS-polyacrylamide gel electrophoresis pre-cast gels (Invitrogen). The running buffer was NuPAGE MES SDS buffer from Invitrogen; gels were run at 120 V. Resolved samples were transferred to nitrocellulose membranes. Membranes were blocked for 1 h with PBS, 0.05% Tween 20, 2.5% horse serum and 1% casein. Biotinylated goat anti-mouse Ig heavy (\(\gamma\)) chain from Southern Biotech (Birmingham, AL, USA) was used as the primary Ab for overnight incubations at a dilution of 1:1000 in blocking buffer; streptavidin-conjugated HRP at a dilution of 1:5000 for 1 h was used for detection. Development of the chemiluminescent substrate (SuperSignal West Pico, Pierce) was visualized using an Alpha Innotech imaging system in movie mode. Densitometric analysis of developed blots was performed on the same system. Following development of the Ig heavy chain signals, we re-probed the membranes for analysis of developed blots was performed on the same system. Following the re-probing, the membranes for total FopA (a constitutively-expressed protein) were quantified using a constitutively-expressed Francisella protein (and quantified the data as surface mAb/total FopA. These ratios were normalized to the corresponding ratios from mAb-iFt (1 µg of mAb).

Immunization and challenge studies
C57BL/6 mice were immunized on days 0 and 21 (Ft LVS challenge) or days 0, 14 and 28 (Ft SchuS4 challenge) with 2 \(\times 10^7\) iFt organisms alone or in the form of mAb + iFt ICs utilizing IgG2a anti-Ft LPS mAb. On day 35 (Ft LVS) or day 42 (Ft SchuS4), the mice were challenged with 20,000 c.f.u. of live Ft LVS or 21 c.f.u. of live Ft SchuS4. Following challenge survival was monitored for 21 days.

Ag presentation assays
APCs (2 \(\times 10^5\)) from Balb/c mice and the Ft-specific T cell hybridoma cells (FT25 6D10) (1 \(\times 10^5\)) were added in T cell medium to the wells of a 96-well plate containing iFt alone or iFt + mAb, while whole anti-iFt mAb (1 µg ml\(^{-1}\)), an F(ab\(^\prime\))\(_2\)-anti-iFt mAb or an IgG2a isotype control Ab. The plate was then incubated at 37°C in 5% CO\(_2\) in a humidity chamber for 24 h and supernatants were collected. The supernatants were then assayed for IL-5 using a FACScanto flow cytometer (BD Biosciences). Alternatively, to demonstrate FcR specificity of the observed binding, the APCs were pre-incubated with non-specific monomeric mouse IgG2a (blocks Fc\(\gamma\)RI), F(ab\(^\prime\))\(_2\)-anti-mouse CD16/32 (2.4G2) (blocks Fc\(\gamma\)RII and Fc\(\gamma\)RIII), or both IgG2a and F(ab\(^\prime\))\(_2\)-2.4G2 mAb, for 90 min on ice before adding iFt or minus anti-iFt mAb. To visualize iFt binding using fluorescence microscopy, after the 2-h incubation and subsequent washes, the cells were resuspended in PBS-azide containing cholera toxin subunit B-Alexa Fluor 647 conjugate (5 µg ml\(^{-1}\)), which labels cell membranes, incubated for 15 min at 4°C and then fixed. After fixation, cells were attached to a coverslip previously coated with CellTak (BD Biosciences) and mounted on a slide. Observation and image acquisition was done using an Olympus IX 81 confocal microscope (Olympus America Corporation, Center Valley, PA, USA).

Internalization studies
Internalization of iFt in the presence and absence of anti-iFt mAb was visualized by flow cytometry. In the first instance, APCs (2 \(\times 10^5\)) were pulsed with 200–2000 iFt organisms/APC plus and minus 1 µg ml\(^{-1}\) anti-iFt mAb for 1 h at 4°C in HEPES-RPMI-10% FBS. After the pulse, the cells were washed three times with 250 µl of cold HEPES-RPMI, resuspended in culture media, and incubated at 37°C for the desired time. Once the incubation was complete, the cell suspension was cooled immediately on ice and washed two times with PBS BSA 0.1% azide. The cells were then resuspended in 100 µl of 400 µg ml\(^{-1}\) pronase (in PBS plus 0.1% azide (prevents further iFt internalization)) and subsequently incubated at 37°C for 15 min to strip from the cell surface the non-internalized iFt. After Pronase treatment, the cells were washed and fixed with 2% parafomaldehyde. The samples were analyzed on a FACSCanto flow cytometer (BD Biosciences).

Surface marker expression by APCs
APCs (2 \(\times 10^5\)) were added to the wells of a non-binding surface 96-well plate and incubated at 37°C in 5% CO\(_2\) in the presence of iFt alone or anti-iFt mAb + iFt ICs. After 24 h, the cells were washed three times with PBS-BSA-azide, resuspended in blocking buffer (PBS-BSA-azide plus 30 µg ml\(^{-1}\) of normal mouse IgG (Sigma)) and incubated on ice for 30 min. Cells were then washed three times with PBS-BSA-azide and fluorescently labeled Ab to MHC class II, F4/80, CD80, CD83, DEC205 or CD86 or their corresponding isotype controls were added. The cells were then incubated on ice for 30 min, washed and then fixed with 2% parafomaldehyde. Cells were then analyzed by flow cytometry on an LSRII flow cytometer (BD Biosciences).
In vivo trafficking of iFp and iFr in mAb-iFp ICs
Wild-type C57BL/6 mice or Fcrn-deficient mice on the same genetic background, were immunized i.n. with either iFp (2 × 10^7 organisms per mouse) or anti-iFp mAb-iFp ICs at a mAb concentration of 1 µg mL^{−1}. At 30 min intervals for a period of 3 h, a single mouse from each group was euthanized, and the NALT was harvested. To detect the trafficking of iFp, total Ft genomic DNA was isolated from the NALT samples using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA). A Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (Hercules, CA, USA) was used to amplify and detect the Francisella gene, fopk, as an indication of iFp trafficking to the NALT. The amount of target Francisella DNA was determined based upon calculations using the standard curves established with plasmids containing the cloned fragment of the Ft fopk gene. More specifically, total Ft genomic DNA was obtained by using the DNeasy Blood and Tissue kit (Qiagen), and the 600 bp fopk gene was amplified by PCR. Both the forward (5′-ACTTATAGCCGCTTGACTAAAAGGAC-3′) and reverse (5′-CTGGCTGTATTTAAAGCAATTGAAGGAGG C-3′) primers were designed and purchased by Integrated DNA Technologies (Corvalle, IA, USA). The desired PCR product (5′-TACTTATTAGCGTTT GACCTAACAGGACACATTGGGTCCTCAAGATGAAACTGGGCAAGT GATTATTGATCAACTGGGTCCTAGACGTAAGCTGACGGTCTGCA TGGAATCTCTACCTCTCTCACAGTGGCTGTGGCTCAATTTCATATGGTTTAAACATCTAATACTTCTAGG CTTGACAGTACACAAACTAAGGCTGACAGTAATGTTCAGAAGCT GGGTGTGTTGTTGGCTTAAGTTGTTAAACTACTATTGTCTCTAT CTTGGCGAGAATCTAACATATAAGTGTAAACTTCTAGTAAATGTC TTAGATGTATTACATGTATTAGATGTATTAGATGTATTAGATGT ATTATGGCTATACGCTATACGCTATACGCTATACGCTATACGCTA ATGGCCGGAGCGCTACATAGGGTTGCGATTTAATGTTTCACTCTT GTGTGCGAGAATCTAACATATAAGTGTAAACTTCTAGTAAATGTC TTAGATGTATTACATGTATTAGATGTATTAGATGTATTAGATGT ATTATGGCTATACGCTATACGCTATACGCTATACGCTATACGCTA ATGGCCGGAGCGCTACATAGGGTTGCGATTTAATGTTTCACTCTT

Statistical analysis
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. GraphPad Prism 4 provided the software for the statistical analysis (San Diego, CA, USA).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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