REVIEW

Arch. Immunol. Ther. Exp., 2009, **57**, 311–323
PL ISSN 0004-069X
DOI 10.1007/s00005-009-0040-y

Fc receptor-targeted mucosal vaccination as a novel strategy for the generation of enhanced immunity against mucosal and non-mucosal pathogens

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Received: 2009.01.15, **Accepted:** 2009.03.30, **Published online:** 2009.08.18

Abstract

Numerous studies have demonstrated that targeting immunogens to Fcγ receptors (FcγR) on antigen (Ag)-presenting cells (APC) can enhance humoral and cellular immunity *in vitro* and *in vivo*. FcγR are classified based on their molecular weight, IgG-Fc binding affinities, IgG subclass binding specificity, and cellular distribution and they consist of activating and inhibitory receptors. However, despite the potential advantages of targeting Ag to FcR at mucosal sites, very little is known regarding the role of FcR in mucosal immunity or the efficacy of FcR-targeted mucosal vaccines. In addition, recent work has suggested that FcRn is present in the lungs of adult mice and humans and can transport FcRn-targeted Ag to FcγR-bearing APC within mucosal lymphoid tissue. In this review we will discuss the need for new vaccine strategies, the potential for FcR-targeted vaccines to fill this need, the impact of activating versus inhibitory FcγR on FcR-targeted vaccination, the significance of focusing on mucosal immunity, as well as caveats that could impact the use of FcR targeting as a mucosal vaccine strategy.

Key words: vaccine, mucosal immunity, Fc receptors.

Abbreviations: Ab – antibody/antibodies, Ag – antigen(s), APC – Ag-presenting cell(s), DC – dendritic cell(s), MØ – macrophage(s), Th – T helper cell(s), CTB – cholera toxin B, FcR – Fc receptor(s), mAb – monoclonal Ab, ITAM – immunoreceptor tyrosine-based activation motif, ITIM – immunoreceptor tyrosine-based inhibition motif, TT – tetanus toxoid, KO – knockout.

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INTRODUCTION

An effective humoral or cellular immune response to most pathogens requires antigen (Ag)-presenting cells (APC), such as dendritic cells (DC), macrophages (MØ) and, in the case of humoral immunity, B cells, to process Ag from the pathogen and present it to T cells, thereby inducing T-cell activation (Tew et al. 1997; Vogel 2000; Yewdell and Bennink 1990). Two major pathways for Ag processing and presentation have been described (Neefjes and Momburg 1993). When foreign proteins are synthesized by APC (for example during viral infection), Ag peptides are formed in the cytoplasm and delivered to the endoplasmic reticulum, where they bind to a pocket in the MHC class I molecule. The MHC class I-bound Ag is subsequently brought to the cell surface, where the combination of peptide and MHC class I is recognized by CD8 cytotoxic T cells, a component of the cellular immune response. Ag, internalized by endocytosis [an antibody (Ab)-Ag complex, for example], is fragmented in either the endosome or lysosome. The resulting fragments bind to an MHC class II molecule and are returned to the cell surface, where the combination of peptide and MHC class II is recognized by CD4 T helper cells (Th). Depending on a number of factors, including Ag density and the cytokine milieu, Th1 and/or Th2 subsets are generated (London et al. 1998). Th1 cells have been defined based primarily on their ability to secrete interleukin (IL)-2 and interferon (IFN)-γ. Because IFN-γ activates MØ, Th1 cells are also generally associated with the generation of a cellular immune response. Th2 cells have been defined primarily based on their ability to secrete IL-4 and IL-5. Since the latter cytokines play a role in B-cell activation and differentiation, Th2 cells have been associated with the generation of the humoral immune re-

sponse. However, cytokines secreted by both Th1 and Th2 cells can influence humoral immunity by directing the generation of distinct Ab isotypes (London et al. 1998; Romagnani 2000). In addition, MØ and DC can present endocytosed/exogenous Ag via both MHC class I and MHC class II molecules using as yet poorly defined pathways (Kovacsovics-Bankowski et al. 1993; Rock 1996). Thus, in addition to the generation of humoral immunity, use of exogenous Ag can, in some cases, lead to the stimulation of both CD4 Th1 and CD8 T cells, both of which are important in providing protection against intracellular pathogens (Anthony et al. 1989; Leiby et al. 1992; Sjostedt et al. 1996; Yee et al. 1996). Thus, depending on whether a vaccine targets the MHC class I or MHC class II pathway and/or stimulates Th1 versus Th2 T-cell subsets, one can influence the generation of cellular versus humoral immunity, respectively. Therefore, when generating a vaccine against a given pathogen, one must consider not only whether cellular or humoral immunity will be required, but also which specific Ag processing pathway(s) must be targeted to generate the desired response. Ideally, in many cases vaccines which generate both humoral and cellular immunity are the most desirable, such as is the case with HIV and many other intracellular pathogens (2007; Moore and Hutchings 2007; O'Hagan et al. 2001; Robinson 2007).

In addition, the route of immunization can significantly influence the generation of mucosal versus peripheral immunity and must therefore also be an important consideration in the generation of the majority of vaccines. Specifically, while most pathogens enter through mucosal sites, most vaccines are administered through parenteral routes. However, parenteral immunization is often ineffective in stimulating mucosal immunity. In contrast, mucosal immunization frequently generates both mucosal and peripheral immunity (Arulanandam et al. 1999; McGhee and Kiyono 1994).

HUMORAL VERSUS CELLULAR IMMUNITY IN VACCINE DEVELOPMENT

As indicated above, the type of immunity required for protection against infection can vary, based on the pathogen involved. Ab is usually effective against extracellular bacterial pathogens such as *Staphylococcus*, *Streptococcus*, and *Neisseria.* Cellular immunity is most often required to protect against intracellular pathogens, including *Listeria monocytogenes*, *Legionella*, *Mycobacterium*, and *Rickettsia* as well as viral and some parasitic infections (Rocha et al. 2004). With regard to vaccines against extracellular pathogens, their success is most often associated with their ability to induce high-affinity neutralizing Ab that rapidly binds to the pathogen and prevents it from adhering to or entering the host cell (Law and Hangartner 2008; Weltzin and Monath 1999). Polysaccharide vaccines, as well as whole killed pathogens, are known to elicit specific Ab responses following vaccination (Lesinski and Westerink 2001). However, the protective ability of polysaccharide vaccines is questionable as they are frequently poorly immunogenic and elicit T cell-independent Ab of low affinity, such as IgM (Lesinski and Westerink 2001). This problem has been addressed with the development of conjugate vaccines in which capsular polysaccharide Ag are chemically coupled to proteins such as diphtheria or tetanus toxoid (TT) (Jennings 1992). Linking polysaccharides to protein transforms such immunogens into T-dependent Ag which stimulate T cell-dependent Ab responses. This drives isotype switching and the production of high-affinity polysaccharide-specific IgG Ab, which can block pathogen interaction with host cells, enhance opsinophagocytosis, and mediate complement-dependent lysis of pathogens. Whole killed pathogens can also generate T-dependant Ab responses, including Ag-specific IgG. However, problems associated with whole cell killed vaccines have been their reactogenicity/toxicity and lower immunogenicity compared with live vaccines, therefore requiring multiple immunizations (Singh and O'Hagan 2002; Vogel 2000).

With regard to vaccines against intracellular pathogens, such as viral infections, it is generally believed that protection requires the generation of CD8 T-cell responses, while CD4 Th1 T cells are generally believed to be required in the case of intracellular bacterial infections (Appay et al. 2008; London et al. 1998; Sant et al. 2007). However, studies suggest Ab may also play a role in protection against intracellular bacterial and parasitic infections (Casadevall 1998; Edelson and Unanue 2001; Feng et al. 2004; Li et al. 2001; McSorley and Jenkins 2000; Yager et al. 2005). With regard to viral infections, Ab can neutralize virus before it interacts with host cells. As with viral infections, most intracellular bacterial pathogens also have an extracellular phase during which Ab can bind antigenic determinants on these pathogens and prevent binding to host cell receptors. In such cases, Ab-mediated opsinophagocytosis and complement-mediated lysis can also occur (Vogel 2000; Yager et al. 2005). In this regard our own studies have provided evidence that Ab can play a crucial role in protection against infection with the intracellular pathogen *Francisella tularensis* (Rawool et al. 2008). Specifically, we observed up to 100% protection against *F. tularensis* challenge following intranasal (i.n.) immunization with inactivated *F. tularensis* organisms. This protection was dependent in part on Ab in that the protection was eliminated when immunizing mice that lacked IgA. In a similar study by others, serum Ab were capable of conferring protection against lethal respiratory tularemia when passively administered via the intraperitoneal route 24–48 h post-challenge (Kirimanjeswara et al. 2007). Numerous other studies have also demonstrated that Ab can play a significant role in resolving infections by intracellular pathogens (Casadevall 1998; Edelson and Unanue 2001; Feng et al. 2004; Li et al. 2001; McSorley and Jenkins 2000; Yager et al. 2005). However, despite the ability of Ab to provide protection against both extracellular and intracellular pathogens, Ab-me-

diated protection is often insufficient in many intracellular infections. HIV infection is an example of this in that evidence suggests that the generation of both humoral and cellular immunity will likely to be required of any successful vaccine against this organism (2007). Furthermore, while evidence suggests Ab can play a significant role in protection against *F. tularensis* infection, recent studies in our laboratory also suggest this requirement can be overcome through the use of adjuvant, and thereby the generation of a sufficiently high cellular (CD4 Th1) immune response (submitted for publication). Thus a vaccine strategy which generates both humoral and cellular immunity at mucosal and peripheral sites provides the greatest potential for a universally useful vaccine.

THE NEED FOR VACCINE ADJUVANTS

An ideal vaccine would involve the administration of a purified nontoxic protein derived from the pathogen known to generate protective immunity. While such molecules do exist in the case of some pathogens, purified proteins administered alone do not generally stimulate an effective immune response. In such cases, vaccine adjuvants are required to boost or enhance specific immune responses and to ultimately generate a protective immune response (Kensil et al. 2004; McCluskie and Weeratna 2001; Vogel 2000).

Vaccine adjuvants can be divided into two broad categories, immune modulators and delivery systems (Kensil et al. 2004). Immune modulators include a large collection of molecules such as bacterial products [lipopolysaccharides, peptidolglycans, lipoproteins, DNA (CpGs), and enterotoxins], plant products (saponins and glycosylceramides), and cell products (heat-shock proteins and cytokines). Mechanisms of action can include immune-cell activation, up-regulation of costimulatory molecules (required for efficient T-cell activation), and induction of cytokines involved in immune regulation (Vogel 2000). However, most immune modulators have broad specificity for many cell types, increasing the potential for toxic side effects and thus causing significant safety concerns. Vaccine delivery systems are more limited in number (currently there are three) (Kensil et al. 2004). Delivery systems function primarily by slowing Ag release at the site of injection and/or enhancing Ag uptake by APC. Such adjuvants currently approved for clinical use are limited. For example, the only delivery system currently approved for human use in the United States is mineral salts, specifically alum. However, while alum induces a potent Ab (humoral, Th2-type) immune response, it is generally ineffective in boosting cellular (Th1-type or CD8 T-cell) responses (Petrovsky 2006; Singh and O'Hagan 2002; Vogel 2000). The latter responses are critical for the control of intracellular (viral and bacterial) infections. Another delivery system consists of emulsions (Ag mixed with oil and water). MF59 is such an adjuvant and has been approved for use in Europe. However, similar to mineral salt (alum), MF59 stimulates a potent humoral (Th2-type) immune response, but fails to stimulate cellular immune responses. A third delivery system consists of particulate Ag. In particulate form, Ag can stimulate both humoral and cellular immune responses. The most researched approach within this group involves the incorporation of Ag into lipid--containing vesicles. However, this strategy also has its limitations. The lipid particles can be difficult and thus costly to manufacture, resulting in a vaccine product which poorer countries cannot afford. Furthermore, as with many of the immune modulators, there is broad specificity for many (if not most) cell types. The latter not only increases the potential for toxic side effects, but also decreases the efficiency of Ag delivery to APC. In turn, the lack of delivery efficiency increases the amount of Ag required per dose, and thus the cost of the vaccine. Thus the search for safer and more effective vaccine adjuvants and/or vaccine strategies that can simultaneously generate humoral and cellular immunity continues.

MUCOSAL ADJUVANTS

The adjuvants discussed above are generally used with parenteral immunogens. However, parenteral immunization normally does not produce effective mucosal immunity (McGhee and Kiyono 1994). However, the majority of pathogens, in particular respiratory and gut--associated organisms, enter through mucosal sites resulting from disruption of epithelial barriers, disease, drug therapy, or injury (Svanborg et al. 1999). IgA is the most prevalent immunoglobulin in mucosal secretions and is believed to contribute significantly to mucosal defense by binding to and neutralizing bacteria and toxins at mucosal sites (Keren 1987; Russell and Mestecky 1988). Although innate mechanisms often suffice to protect against mucosal pathogens, pathogens have also established immune evasion strategies, including the ability to neutralize complement and inhibit phagocytosis (Coombes et al. 2004; Finlay and McFadden 2006). For this reason, an effective adaptive immune response, including the generation of both humoral and cellular immune responses, is often critical to resolving mucosal infections. (Cooper et al. 2002; Klinman et al. 1999; Marriott 1997). Consequently there is a need for safe and effective mucosal immunization strategies and/or safer and more effective mucosal vaccine adjuvants.

Two well-known and extensively studied mucosal vaccine adjuvants which are currently being tested and utilized in some countries are cholera toxin B (CTB) and IL-12. CTB is particularly effective in the induction of protective Ab (Isaka et al. 2004; Isaka et al. 1999; Isaka et al. 2003; Jackson et al. 1993; Wu and Russell 1998). In addition, CTB also enhances cellular immunity, although the precise impact of CTB on Th1 versus Th2 responses can vary significantly. For example, i.n. and oral administration of CTB tends to drive Th2-like responses (Braun et al. 1999; Marinaro et al. 1995; Toida et al. 1997), while transcutaneous and intravaginal

routes tend to elicit Th1 responses (Anjuere et al. 2003; Luci et al. 2006). However, not only does the route of immunization influence the ability of CTB to stimulate cellular immunity, but also the type of Ag used (Holmgren et al. 2003). On the other hand, IL-12 is a cytokine which favors the development of Th1 T-cell responses (Arulanandam et al. 1999). It has been shown that IL-12, when administered i.n. in combination with protective immunogen, can reduce bacterial burden and increase survival of mice following *S. pneumoniae* challenge (Sun et al. 2007). The protection observed is IFN-γ (Th1) dependent. In a similar study, use of IL-12 as a mucosal adjuvant co-administered with a pneumococcal conjugate vaccine provided significant Th1-dependent protection against subsequent lethal challenge with type 3 and type 14 *S. pneumoniae* serotypes (Lynch et al. 2003). However, while both CTB and IL-12 can be effective mucosal adjuvants (Areas et al. 2004; Boyaka et al. 1999; McCluskie and Weeratna 2001), there remains significant concern regarding their safety in humans.

Fcγ RECEPTORS AS POTENTIAL VACCINE TARGETS TO ENHANCE HUMORAL AND CELLULAR IMMUNE RESPONSES

Fcγ receptors (FcγR) are classified based on their molecular weight, IgG-Fc binding affinity, IgG subclass binding specificity, and cellular distribution. Three major subtypes of FcγR have been described in mice and humans: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (Tables 1 and 2). In addition, a fourth subtype, FcγRIV, has been more recently identified in mice (Table 1), but due to its exclusive expression in mice it will not be discussed further.

FcγRI is a high-affinity FcγR which binds monomeric and multimeric IgG. Thus FcγRI is normally occupied by serum IgG *in vivo*. FcγRI is constitutively expressed on MØ and DC (professional APC) (Fanger et al. 1997; Graziano et al. 1995; Guyre et al. 2001; Guyre et al. 1989; van de Winkel and Anderson 1991), while FcγRII and FcγRIII (low-affinity FcγR which bind multimeric IgG only) are constitutively expressed on a more diverse population of cell types. When cross-linked by Ab-Ag complexes, for example, FcγR transduce signals to the interior of the cell which can be stimulatory or inhibitory in nature. The type of signal is determined by signal transduction motifs in the cytoplasmic domains of the FcγR. The signaling motif responsible for generating stimulatory signals is called the immunoreceptor tyrosine-based activation motif (ITAM), while the motif responsible for generating inhibitory signals is called the immunoreceptor tyrosine-based inhibition motif (ITIM) (Daeron 1997; Gessner et al. 1998).

Activating receptors

FcγRI is an activating receptor and contains an ITAM in its cytoplasmic domain, as is also the case for FcγRIIA, an FcγRII isoform, and FcγRIII. Numerous studies have demonstrated that targeting Ag to FcγR *in vitro* and *in vivo* can enhance humoral and cellular immune responses (Adamova et al. 2005; Gosselin et al. 1992; Guyre et al. 1997; Heijnen et al. 1996; Heyman 2000; Keler et al. 2000; Liu et al. 1996; Rawool et al. 2008; Regnault et al. 1999; Snider et al. 1990; Wallace et al. 2001; Walsh et al. 2003). This has been demonstrated utilizing Ab-Ag complexes (Heyman 2000; Rawool et al. 2008; Wernersson et al. 2000), FcγR-specific-Ag chemical conjugates (Gosselin et al. 1992), and anti-FcγR-Ag fusion proteins (Adamova et al. 2005; Liu et al. 1996; Wallace et al. 2001). However, the mechanisms involved in Ab-Ag complex-enhanced (FcγR-mediated) Ag presentation *in vitro* and Ab production *in vivo* are not well understood (Amigorena and Bonnerot 1999; Heyman 2000). It has been proposed that complement-mediated binding of Ab-Ag complexes to APC, not Ab-Ag complex binding to FcγR, may be responsible for Ab-Ag complex-mediated enhancement of the immune response (Heyman 2000). However, this has been disproved using FcγR knockout (KO) mice in which activating FcγR were absent and complement function was normal (Wernersson et al. 1999). Specifically, using FcγR KO mice in place of normal mice eliminated enhanced Ab production due to immunization with Ab-Ag complexes. In addition, studies in which complement activity was eliminated failed to eliminate Ab-Ag complex-enhanced immune responses (Heyman 2000). Alternatively, upregulation of MHC and second signal molecules as a result of FcγR cross-linking on APC could play a role in FcγR-mediated immune enhancement. However, in previous *in vitro* studies using human Ab-Ag complex and monocytes as APC, a role for increased expression of these molecules due to Ab-Ag complex-FcγR interaction was not evident (Jelley-Gibbs et al. 1999). Studies using DC, however, have indicated that Ab-Ag complex can induce DC maturation. The latter is characterized by increased expression of the DC maturation marker CD83, MHC class II, and costimulatory molecules (Boruchov et al. 2005). Consequently, DC maturation is accompanied by an enhanced ability to process and present Ag (Bayry et al. 2005). In addition to inducing DC maturation, more efficient Ag binding and internalization is believed to play an important role in Ab-Ag complex-enhanced immune responses and is not only dependent on the concentration of the ligand, but also on the valency of the ligand, the number of FcγR expressed on the APC surface, and whether or not FcγR is occupied with serum IgG (Guyre et al. 2001; Heyman 2000). In the latter case the amount of FcγR cross-linking required to induce internalization appears to actually be reduced (Guyre et al. 2001; Heyman 2000).

Engaging FcγR on DC can also induce cytokine production. The cytokine milieu can determine the type and degree of response. Studies in which the activating FcγRIIA on monocyte-derived DC was ligated resulted in secretion of IL-10 and IL-6 (stimulates B cells and plasma cells) and tumor necrosis factor (TNF)-α and IL-8

(chemoattractants). However, the cytokines produced can vary with the source of DC and the ratio of activating and inhibitory (FcγRIIB) receptors engaged (Bayry et al. 2005; Boruchov et al. 2005). More importantly, while *in vitro* studies are valuable for defining mechanisms, *in vivo* studies are more informative when trying to understand clinical potential. Specifically, when this laboratory targeted Ag to hFcγRI *in vivo* in the form of an anti-hFcγRI-Ag fusion protein, increased production of both Th1 (IL-2, IFN-γ, TNF-α) and Th2 (IL-4, IL-5) cytokines was observed (Adamova et al. 2005). As previously discussed, Th1 cytokines can be important in generating and maintaining the cellular immune response against intracellular pathogens, while Th2 cytokines can play a role in the humoral response against extracellular pathogens (London et al. 1998; Romagnani 2000).

It must also be emphasized that additional factors may also be involved in FcγR-enhanced immunity. For example it has been proposed that increased localization of Ab-Ag complex to follicular DC in lymphoid follicles may explain Ab-Ag complex-enhanced immunity *in vivo* (Heyman 2000). In fact, preliminary studies in our laboratory suggest there is enhanced localization of Ab-Ag complexes to nasal-associated lymphoid tissue following i.n. immunization of mice with Ab-Ag complexes (unpublished data). It is also possible that targeting Ag to FcγR not only enhances Ag binding and uptake, but Ag processing and presentation as well, including alterations in Ag trafficking, although the latter has not yet been thoroughly investigated. It should also be noted that characteristics of the monoclonal Ab (mAb)-Ag complexes themselves, such as valency, Ab isotype, and subclass, also impact the ability of particular mAb-Ag to bind to particular FcγR and thus enhance immunity (van de Winkel and Anderson 1991). Furthermore, when targeting Ag to FcγR, adjuvant is not required and both cellular and humoral immunity can be enhanced, which are key advantages of this particular vaccine strategy (Adamova et al. 2005; Guyre et al. 1997; Heijnen et al. 1996).

As previously indicated, adjuvants can limit vaccine stability, consistency, versatility, and the ability to generate all the desired responses. Thus, targeting Ag to FcγR may make it possible to generate protection against both extracellular and intracellular pathogens in the absence of adjuvants, and their associated limitations. Whether this strategy will be superior to the many other strategies being used or under development will require direct comparisons. However, published studies thus far which compare FcγR targeting of Ag with the use of more traditional adjuvants appear favorable. For example, in the absence of alum, tetanus toxin C (TTC) fragment-Fc fusion protein was superior to the commercial vaccine (TT plus alum) in inducing TT-specific Ab *in vivo* (Chargelegue et al. 2005). This is despite the many limitations of utilizing Fc to target Ag to FcγR, including the potential for interaction with the inhibitory FcγRIIB. In addition, when mice were immunized with the TTC-Fc fusion protein and then challenged with tetanus toxin, they were protected. Studies targeting "weak Ag" to human FcγRI (hFcγRI) in hFcγRI transgenic mice demonstrated that Ag-specific Ab responses could only be obtained when Ribi (a commercial adjuvant composed of bacterial products) and hFcγRI-targeted Ag were used in combination. Surprisingly, Ribi plus Ag in the absence of hFcγRI targeting failed to generate an Ag-specific Ab response (Keler et al. 2000). The latter study, while not directly comparing hFcγRI targeting of Ag with the use of adjuvant, suggests another potential application of the FcγR-targeting strategy. Specifically, utilizing FcγR Ag-targeting in combination with other adjuvants may augment the immunopotentiating activity of FcγR--targeted immunogen. In addition, studies have also shown protection can be generated against viral challenge when immunizing mice with fusion proteins containing viral Ag linked to Fc (Takashima et al. 2005). Thus evidence strongly suggests that targeting vaccine Ag to activating FcγR offers a number of unique advantages over current vaccine strategies.

Inhibitory receptors

A significant concern regarding the use of Ab-Ag complexes as vaccines is their ability to interact with Fc -γRIIB, the inhibitory FcγR. FcγRIIB, an FcγRII isoform, expresses an ITIM in its cytoplasmic domain. As a result, FcγRIIB, the only FcγR expressed on B cells, is unique among FcγR in that it is the only FcγR with the ability to inhibit B-lymphocyte activation and thus Ab production (Daeron 1997; Gessner et al. 1998).

FcγRIIB is expressed as two common isoforms, designated as FcγRIIB-1 and FcγRIIB-2. The two differ in their cytoplasmic tails. FcγRIIB-1, in humans, contains a 19-amino-acid insert spliced out of the FcγRIIB-2 isoform (Brooks et al. 1989). Importantly, however, the ITIM sequence is retained in both FcγRIIB-1 and FcγRIIB-2. In the mouse, the length of the FcγRIIB-1 insert is slightly longer, but otherwise FcγRIIB-1 is similar in mouse and human. (Hibbs et al. 1986; Ravetch et al. 1986). As indicated in Tables 1 and 2, FcγRIIB-1 and FcγRIIB-2 are expressed on many cell types, but FcγRIIB is the only FcγR expressed on B cells. In addition, FcγRIIB-1 predominates on B cells and mast cells and, due to the insert discussed above, has a limited capacity to endocytose bound Ab-Ag complexes (Cassel et al. 1993; Ravetch and Kinet 1991). FcγRIIB-2 predominates in cells of mononuclear/phagocyte lineage and both human and murine FcγRIIB-2 are capable of internalizing Ab-Ag complexes upon receptor cross-linking (Miettinen et al. 1992; Miettinen et al. 1989). Regardless of the ability of FcγRIIB to internalize Ab-Ag complexes, Ab-Ag complex interaction with FcγRIIB will induce signaling through the ITIM motif.

Importantly, due to its inhibitory capacity, FcγRIIB has the potential to counteract any advantage of targeting Ag to activating FcγR, in particular when using Ab--Ag complex as immunogen. Not only can co-ligation of ITAM-containing FcγR and FcγRIIB by Ab-Ag comple-

xes result in a dominant inhibitory signal on cells that express both activating and inhibitory FcγR (Ono et al. 1996; Tridandapani et al. 1997), but B cells express only FcγRIIB which, when cross-linked, can inhibit B-lymphocyte activation and thus Ab production (Fridman 1993; Heyman 2000; Nimmerjahn and Ravetch 2008). In addition, FcγRIIB is expressed on DC, where it also plays an inhibitory role (Boruchov et al. 2005) and thus can have a negative impact on T-cell activation. Thus FcγR--targeting strategies which utilize the IgG-Fc domain to target Ag to FcγR can bind FcγRIIB on B cells and DC and potentially dampen Ag-specific Ab responses as well as DC-mediated T-cell activation. Despite this, numerous studies, including our own, demonstrate the efficacy of targeting Ag to FcγR (Adamova et al. 2005; Amigorena and Bonnerot 1999; Celis et al. 1984; Gosselin et al. 1992; Guyre et al. 1997; Heijnen et al. 1996; Heyman 2000; Jelley-Gibbs et al. 1999; Moore et al. 2003; Rafiq et al. 2002). In fact, recent studies by others have demonstrated that enhanced immune responses can be generated with mAb-Ag complexes despite mAb-Ag interaction with FcγRIIB. Thus it appears that while Ab-Ag interactions with FcγRIIB can limit the degree of immune enhancement, they do not prevent it (Getahun et al. 2004). Furthermore, the Ab isotype utilized may influence the degree of FcγRIIB involvement. Specifically, surface plasmon resonance (SPR) analysis was conducted to measure the interaction of the IgG subclasses with activating and inhibitory FcγR pairs. These results suggest that the activity of activating versus inhibitory FcγR is strongly influenced by the IgG subclass involved (Nimmerjahn et al. 2005; Nimmerjahn and Ravetch 2005). Thus selecting the appropriate IgG subclass to generate Ab-Ag complexes could minimize Ab-Ag complex engagement of FcγRIIB while enhancing the engagement of activating FcγR.

TARGETING FcR AT MUCOSAL SITES

Very little is known about the role FcγR play in mucosal immunity, in particular as it applies to protection against lethal infection with a mucosal pathogen. Studies from two laboratories have suggested a role for FcγR. In one case, i.n. immunization with hepatitis B surface Ag-Ab complex enhanced Ag-specific IgG1 production (McCluskie et al. 1998). In another case, i.n. immunization of mice with *Streptococcus mutans*-Ab complex influenced the immunoglobulin isotype and specificity of the host humoral immune response against the Ag (Brady et al. 2000; Rhodin et al. 2004). In both cases the involvement of FcγR was implied, but not proven.

In contrast, studies focused on the IgG-binding neonatal Fc receptor (FcRn) (Tables 1 and 2) have provided strong evidence for a role of this receptor in mucosal immunity and also cause to believe that FcγR on mucosal APC may also play a role. Specifically, it has been established that FcRn is present in the lungs of adult mice and humans and can transport IgG from lumen to underlying mucosa-associated lymphoid tissues. As a re-

Table 1. Characteristics of mouse FcγR and FcRn

	FcγRI (CD64)	FcγRIIB (CD32)	FcγRIII (CD16)	FcγRIV	FcRn
Molecular weight	70 kDa	40–60 kDa	40–60 kDa	28 kDa	40 kDa
Constitutive expression	monocytes macrophages DC	monocytes macrophages DC basophils mast cells neutrophils B cells	monocytes macrophages DC basophils mast cells neutrophils NK cells eosinophils	monocytes macrophages DC neutrophils	monocytes macrophages DC epithelial cells endothelial cells muscle cells skin cells
IgG binding affinity	10^8–10^9 M^{-1}	10^6–10^7 M^{-1}	10^7 M^{-1}	10^7 M^{-1}	pH-dependent
IgG binding hierarchy	IgG2a>>>1,2b,3	IgG1=2a=2b>>>3	IgG1=2a=2b>>>3	IgG2a>2b	IgG
Signal motif	activating/ITAM	inhibitory/ITIM	activating/ITAM	activating/ITAM	non-activating non-inhibitory
Ab-Ag mediated functions	Ab-Ag uptake Ag presentation cell activation phagocytosis ADCC	Ab and immune cell inhibition (B cells, DC, mast cells, macrophages, granulocytes)	Ab-Ag uptake Ag presentation cell activation degranulation	Ab-Ag uptake Ag presentation cell activation	Ab-Ag uptake Ag presentation Ag processing epithelial IgG transport IgG recycling prolonging IgG half-life

Compiled from information contained in the following references: (Gessner et al. 1998; Nimmerjahn et al. 2005; Nimmerjahn and Ravetch 2007; Nimmerjahn and Ravetch 2008; Qiao et al. 2008; Roopenian and Akilesh 2007).
Abbreviation: ADCC – antibody-dependent cell-mediated cytotoxicity.

Table 2. Characteristics of human FcγR and FcRn

	FcγRI (CD64)	FcγRII (CD32)	FcγRIII (CD16)	FcRn
Molecular weight	72 kDa	40–43 kDa	50–80 kDa	40–45 kDa
Isoforms	FcγRIa FcγRIb FcγRIc	FcγRIIa FcγRIIb FcγRIIc	FcγRIIIa FcγRIIIb	
Constitutive expression	monocytes macrophages DC	monocytes macrophages DC Langerhans cells B cells neutrophils eosinophils basophils platelets	**FcγRIIIa** monocytes macrophages NK cells γδ T cells neutrophils mast cells, eosinophils **FcγRIIIb** neutrophils eosinophils	monocytes macrophages DC epithelial cells endothelial cells
IgG binding affinity	10^8–10^9 M^{-1}	10^7 M^{-1}	10^7 M^{-1}	Ph-dependent
IgG binding hierarchy	IgG1>3>4>>2	**FcγRIIa** IgG3>1=2>>4 **FcγRIIb1** IgG3>1>>>2 **FcγRIIb2** IgG3>1>>2>4	IgG1=3>>>2=4	IgG
Signal motif	activating/ITAM	**FcγRIIa** activating/ITAM **FcγRIIb** inhibitory/ITIM	**FcγRIIIa** activating/ITAM **FcγRIIIb** none: GPI anchor	non-activating non-inhibitory
Ab-Ag mediated functions	Ab-Ag uptake Ag presentation cell activation phagocytosis oxidative burst ADCC	**FcγRIIa** Ab-Ag uptake Ag presentation cell activation phagocytosis oxidative burst degranulation ADCC **FcγRIIb** B cell inhibition mast cell inhibition	**FcγRIIIa** Ab-Ag uptake Ag presentation phagocytosis ADCC apoptosis degranulation **FcγRIIIb** oxidative burst ADCC	Ab-Ag uptake Ag presentation Ag processing epithelial IgG transport IgG recycling prolonging IgG half-life

Compiled from information contained in the following references: (Dijstelbloem et al. 2001; Gessner et al. 1998; Ivan and Colovai 2006; Roopenian and Akilesh 2007; Salmon and Pricop 2001).

sult, it has been proposed that FcRn may function in the adult human to shuttle IgG or Ab-Ag complex across epithelial barriers for immune surveillance, host defense, or both (Bitonti et al. 2004; Spiekermann et al. 2002). More importantly, follow-up studies by the same research group have demonstrated that FcRn-mediated transport of IgG-Ag complex from the intestinal lumen to the intestinal lamina propria leads to IgG-Ag capture by FcγR-bearing APC (Yoshida et al. 2004). Our own studies have also demonstrated a requirement for FcRn in Ab-Ag complex-enhanced mucosal immunity against *F. tularensis* in that protective immunity generated by Ab-Ag complexes administered i.n. to wild-type mice was eliminated when immunizing and challenging FcRn KO mice (Rawool et al. 2008). However, the overall lack of knowledge regarding the role of FcγR in mucosal immunity leaves a significant gap in our knowledge of mucosal immunity in general and our ability to use a knowledge-based approach in immunogen design, including the design of FcR-specific-Ag fusion proteins to target specific FcγR types at mucosal sites. Importantly, the ability to target specific FcγR types would provide another means of avoiding immunogen interaction with inhibitory FcγRIIB.

THE POTENTIAL FOR CLINICAL APPLICATION OF FcR TARGETING AS A MUCOSAL VACCINE STRATEGY

As suggested above, there are two potential mechanisms/approaches by which Ag could be targeted to FcR

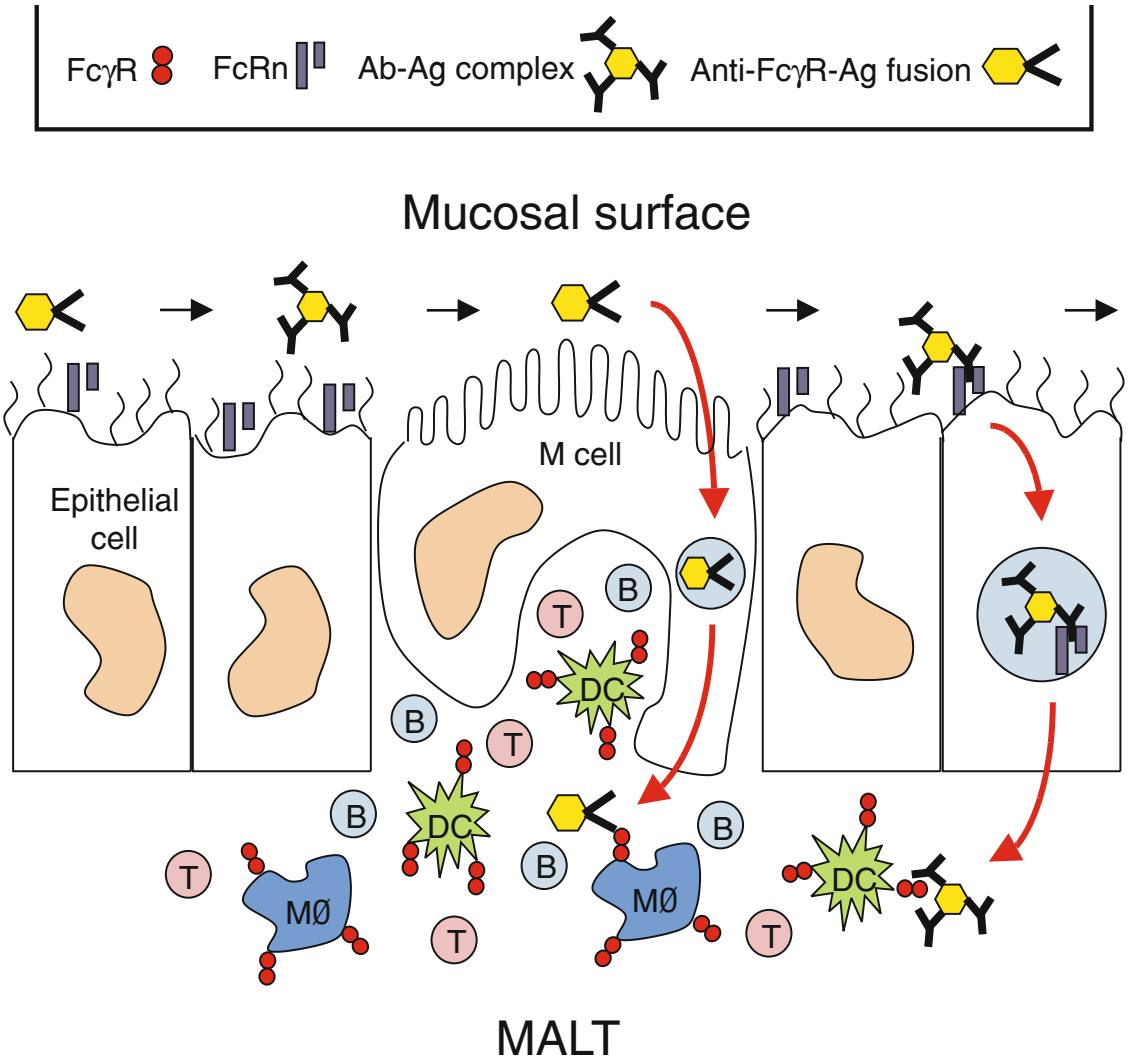

Fig. 1. Overview of the possible mechanisms/strategies by which FcγR-targeted immunogens could trafficed to mucosa-associated lymphoid tissue (MALT), specifically FcγR-expressing mucosal APC, following mucosal administration. Anti-FcγR-Ag fusion proteins would likely be transported via M cells, while Ab-Ag complexes could traffic either through M cells or through epithelial cells utilizing FcRn.

at mucosal sites (Fig. 1). One could utilize Ag-specific mAb as vaccine adjuvants, in a manner similar to that of alum. Specifically, Ag-specific mAb could be combined with Ag prior to vaccine administration. IgG subclasses could be selected which maximize interaction with activating FcγR and FcRn while minimizing interaction with inhibitory FcγRIIB. Alternatively, one could generate FcR-specific-Ag fusion proteins targeted to those FcR known to stimulate the desired immune response most effectively. In regard to the use of mAb as adjuvant, a number of mAb are currently being used as immune adjuvants in the clinic to treat cancer (Armstrong and Eck 2003). The choice of mAb isotype would likely reflect the need to target specific or multiple FcR, with an emphasis on excluding FcγRIIB involvement. Mouse IgG2a has proven efficacious in the mouse model (Rawool et al. 2008). Like mouse IgG2a, human IgG1 has a broad binding specificity for human FcR, including human FcRn (Ober et al. 2001), and thus would likely be the isotype and subclass of choice. It must also be emphasized, however, that human mAb would be required in human vaccines. Importantly, the technology is currently available to generate human Ag-specific mAb (Presta et al. 2002; Vaughan et al. 1998), further supporting the feasibility of this approach in humans. Specifically, three primary strategies are available to accomplish this. First, one can humanize mouse mAb. This involves generating a mouse mAb to the desired Ag and then replacing all but the variable region sequences with human sequences (Gonzales et al. 2005). One can also generate human mAb using phage display (Vaughan et al. 1998). Finally, one can utilize mice genetically modified to produce human Ab in response to immunization with the desired Ag (Gonzales et al. 2005; Vaughan et al. 1998).

The decision to use mAb-Ag complexes or anti-FcR--Ag fusion proteins as immunogens would necessarily be limited by the availability of identified protective Ag for a given pathogen. Specifically, the use of mAb would be the method of choice for FcR-targeted vaccines when

a protective protein Ag has not been identified for a given pathogen, such as is the case with *F. tularensis*. In such instances, use of inactivated organisms plus pathogen-specific human mAb as adjuvant (mAb-Ag complexes) would be a necessary choice. Importantly, inactivated organisms are currently in use as vaccines in numerous cases and have proven safe and effective when appropriate precautions are in place to assure complete inactivation (Zimmerman et al. 2003). However, should a protective protein Ag be identified for a given pathogen, FcR-specific-Ag fusion proteins could be generated, although it would be necessary to fully humanize the FcR-targeting component. Ultimately, this strategy could also be combined with other adjuvant strategies, such as CTB (mucosal immunization), alum (parenteral immunization), or other adjuvants, thereby further enhancing vaccine efficacy dependent on the specific requirements of the pathogen involved.

CONCLUSION

There is a need for novel strategies in vaccine development, in particular for mucosal immunization. In this regard, numerous studies have clearly demonstrated FcR-targeted vaccines can enhance humoral and cellular immune responses *in vitro* and *in vivo* (Adamova et al. 2005; Gosselin et al. 1992; Guyre et al. 1997; Heijnen et al. 1996; Heyman 2000; Keler et al. 2000; Liu et al. 1996; Rawool et al. 2008; Regnault et al. 1999; Snider et al. 1990; Wallace et al. 2001; Walsh et al. 2003). While significant additional study is needed to define the role of FcR in mucosal immunity, recent evidence clearly indicates the use of FcR-targeted vaccines at mucosal sites can enhance protection against mucosal infection. Specifically, Ab-inactivated *F. tularensis* complexes administered i.n. optimally protected mice against mucosal challenge with *F. tularensis*, whereas immunization of mice with inactivated *F. tularensis* alone did not (Rawool et al. 2008). Furthermore, ongoing studies in our laboratory indicate FcR-specific-Ag fusion proteins administered i.n. can enhance protection against mucosal infection with *S. pneumoniae*. In addition to the general lack of knowledge regarding the role of FcR in mucosal immunity, other limitations of this approach are the ability of Ab-Ag complexes to interact with inhibitory FcγRIIB and the potential for mAb and anti-FcR-Ag fusion proteins to generate immune responses to the FcR targeting components themselves. However, in the latter case the ability to generate Ag-specific human mAb, and to humanize FcR-specific fusion proteins, will significantly minimize such complications. Targeting specific FcR utilizing select IgG subclasses or FcR type-specific-Ag fusion proteins should also further reduce the impact of FcγRIIB engagement on immune enhancement.

In conclusion, although caveats do exist in regard to targeting immunogens to FcR at mucosal sites, they can be overcome. Thus the use of FcR-targeted immunogens has significant potential in many respects. It offers the potential to eliminate the need for traditional adjuvants (and their associated problems), substantially reduce the amount of Ag required to vaccinate an individual (reducing cost and potential toxicity), and provide a mechanism for simultaneously enhancing humoral and cellular (Ab and Th cell) immune responses against a variety of mucosal and peripheral (extracellular and intracellular) pathogens.

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