

Immunoregulatory functions of immune complexes in vaccine and therapy

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Abstract

Clinical and experimental preparations of IgG/soluble antigen complexes, as well as those formed following antibody therapy *in vivo*, are multifaceted immune regulators. These immune complexes (ICs) have been tested in humans and animal models, mostly in forms of experimental or clinical vaccination, for at least a century. With intensified research on Fc γ receptor-mediated immune modulation, as well as with immune complex-directed antigen processing, presentation, and inflammatory responses, there are renewed interests of using ICs in vaccines and immunotherapies. Currently, IC-based immune therapy has been broadly experimented in HBV and HIV viral infection control and antitumor treatments. However, mechanistic insights of IC-based treatments are relatively recent subjects of study; strong efforts are needed to establish links to connect laboratory findings with clinical practices. This review covers the history, mechanisms, and *in vivo* outcomes of this safe and effective therapeutic tool, with a clear aim to bridge laboratory findings with evolving clinical applications.

Keywords adjuvant; Fc γ R; immune complex; immune therapy; vaccine

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See the Glossary for abbreviations used in this article.

Introduction

Immune complex, as a form of autoimmune disorder and a tool in clinical therapy, has been an important topic of modern immunology. Binding of antigen by antibody, an immunologically simple event, changes properties of the original ligand, resulting in modulated antigen processing, presentation, receptor signaling, and inflammatory responses. On a larger scheme, antibody immune therapy has become one of the successfully implemented biomedical technologies in modern health care. Immunoglobulin-based new drugs such as rituximab, ipilimumab, and alemtuzumab are among

the highly effective in cancer treatments (Sondak *et al*, 2011; Scott *et al*, 2012). Intravenous immunoglobulin, IVIG, is also a choice treatment of autoimmunity, pediatric infections, and antibody deficiencies (Schwab & Nimmerjahn, 2013). In contrast, use of classically defined IC represents an old subcategory among the list of options. ICs in vaccine development and clinical interventions are becoming increasingly sophisticated, largely attributable to the newly gained understanding of Fc γ receptors (Fc γ R; Nimmerjahn & Ravetch, 2008; Ravetch, 2010). However, IC-mediated functions are not solely controlled via those receptor engagements. For instance, its ability to transport antigen is also a consideration of anatomic location; its roles at different stages of immune induction and memory formation are modulated by additional spatiotemporal factors.

Over 100 years ago, IC vaccines were started as a practice of using antisera complexed to toxoids of *Corynebacteria diphtheria* or *Streptococcus pyogenes* to decrease the side effects in human vaccination (Copeman *et al*, 1922; Olitzki, 1935). One concern of this approach was that antigens coated with antibodies might have reduced exposure of surface sites, leading to limited antibody production. This concern was proven unnecessary by subsequent experiments (Olitzki, 1935). In 1950s, Terres *et al* reported that mice immunized with human or bovine serum albumin were sensitized faster if a specific rabbit antibody was also introduced to the recipients, an effect not seen with antigen alone or in combination with a control antiserum (Terres & Wolins, 1959a, 1961). The enhancement was optimal in a slight antigen excess (Terres & Wolins, 1959b, 1961; Terres *et al*, 1960). After another three decades, using peptides from HIV gp120 to rejuvenate CD4 T cells in HIV carriers, Berzofsky *et al* reported that addition of antibodies recognizing these peptides substantially increased the proliferative index of CD4⁺ T cells in the patients (Berzofsky *et al*, 1988).

In the meantime, immune complexes were casted in a negative shadow, mainly because of their frequent presence in sites of autoimmunity and inflammation, such as lupus and membranous nephropathy. Collectively called type III hypersensitivity, IC deposition at rates faster than their clearance leads to robust activation of the complement system and Fc receptor signaling. As consequences, mononuclear cell degranulation, antibody-dependent cellular cytotoxicity (ADCC), and release of proinflammatory cytokines may

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Glossary

Self-tolerance breaking

While immune tolerance refers to the lack of immune response to an antigen as a result of central (thymic selections) or peripheral (lack of co-stimulation) tolerance education, an extrinsic trigger, such as the same antigen presented to the host in an immune complex, can initiate specific immune recognition against the tolerized antigen. The end results can be detrimental in cases of autoimmunity, or beneficial, as in the immune attack on tumor antigens.

Cross-presentation

The ability of antigen contained in endo/phagocytic vesicles to be presented by MHC class I molecules on the APC surface. This is in contrast with the conventional understanding that class I antigens originate from endogenous protein synthesis. This is an intensely studied and contested topic, as several leading hypotheses, including models of “ER–phagosome fusion”, “endocytic/plasma membrane recycling”, and “cytosolic translocation”, are still being verified.

Toxoids

Richard Friedrich Johannes Pfeiffer classified bacterial toxins into endotoxins that were believed to be sequestered by the cell wall and exotoxins that were thought to be released into the surroundings. Those toxins, particularly the latter, can be thermally or chemically deactivated, that is, with formalin, to produce toxoids to be used as a form of deactivated vaccine. This is an old term with decreasing popularity in modern literature.

Fc and Fc γ receptor (Fc γ R)

Fc, fragment crystallizable region, simply refers to the constant region of antibodies of any type. Fc γ Rs are the cell surface receptors expressed mostly by cells of the immune system that have binding specificities to the Fc region of γ -immunoglobulins.

Biological retention

Refers to any substance retained in a biological system. Used in the context of antibody induction or antibody-based treatments, the term usually refers to the deposition of IC in tissues that can either serve as a source for persistent antigenic stimulation, usually with desirable outcomes, or induce uncontrolled immune attacks, as often seen in autoimmunity.

Plasmablast

Less mature plasma cell precursors, which could still undergo cell division.

Chemotaxis

Generically refers to the movement of a biological entity in response to chemical stimulus in their environment. Immunologically, this term refers to the immune cell movement driven by their surface expression of chemotactic receptors to or away from a gradient of cognate chemokines.

Erythroleukemia

A form of acute myeloid leukemia that involves the cells that give rise to the erythrocytes.

Antigenemia

Persistence of antigen in circulating blood. Antigenemia is used as an indication for severity of infection or as a measurement for efficacy of immune intervention.

result (Nydegger, 2007). Some efforts were made to characterize the pathogenic ICs. It was found that the IC-mediated attacks were mainly driven by inflammatory cytokines such as TNF- α (Warren *et al*, 1989). It was also found that ICs formed with low affinity antibodies tended to be present in high levels in the circulation and deposited more readily into subendothelia of renal capillary loops (Koyama *et al*, 1978). This was confirmed by mice genetically bred to produce only low and high-affinity antibodies, as ICs chronically formed in the former were deposited in glomeruli (Devey & Steward, 1980).

The early work was not illuminated from mechanistic insights. A landmark turning point for IC's immune regulatory function was the discovery of Fc γ Rs. In an attempt to understand the molecular nature of antibodies adsorbed to splenocytes reported by Boyden & Sorkin (1960), Belkin and Benacerraf suggested in 1966 that this affinity was mainly afforded by the heavy chain of antibodies, implicating a structural presence on the surface of macrophages interacting with the Fc portion of IgG (Berken & Benacerraf, 1966). In the 1980s, all common forms of Fc γ R were identified (Ravetch & Kinet, 1991), which subsequently developed into the current paradigm of Fc receptor biology (Nimmerjahn & Ravetch, 2007). Mouse activating Fc γ R family consists of Fc γ RI, RIII, and RIV, while human counterparts include Fc γ RI, IIa, IIc, IIIa, and IIIb. Except for human Fc γ RIIIb, these receptors transmit activating signals via an ITAM motif on the associated common γ -chain or intrinsically present in their cytoplasmic domains. Both species have an additional inhibitory receptor, Fc γ RIIb, that signals via a cytoplasmic ITIM domain. Meticulous work by Ravetch *et al* gradually delineated the subtype specificities and binding strengths to IgG subtypes. In addition, the presence of lectin-based type II Fc γ Rs (DC-SIGN and CD23) that are sensitive to the glycosylation state of Fc has been demonstrated in

recent years (Ravetch, 2010; Bohm *et al*, 2014). Relevant to ICs, Fc γ R signaling has implications in at least three aspects: (i) the activating and inhibiting receptors, their affinities, and the emerging lectin-based type II Fc γ Rs; (ii) the subclasses of antibodies binding to these receptors; and (iii) the highly diverse expression of these receptors on host cells and their levels of expression at different stages of immune activation. These considerations are becoming indispensable to modern applications of ICs (Nimmerjahn & Ravetch, 2005, 2008; Guillems *et al*, 2014). Under this backdrop, this review aims to provide a utilitarian look on ICs from a perspective of clinical application, to complement the current picture of antibody immune therapy.

Mechanisms of immune regulatory functions of IC

Cross-presentation and cellular immunity

For extracellular antigens, the default antigen presentation pathway takes in soluble cargos via endocytosis, processed in progressively acidic endolysosomal vesicles by pH-dependent chain activation of cathepsins (Amigorena & Savina, 2010), for loading onto the class II molecules. Solid particles engulfed via phagocytosis or soluble cargos endocytosed via receptor-mediated uptake can be routed into MHC class I cross-presentation via several mechanisms (Lizee *et al*, 2003; Burgdorf *et al*, 2007; Joffre *et al*, 2012). The latter activates CD8⁺ T cells, known as the cross-presentation, that becomes the mechanistic basis of cellular immunity against antigens originated outside antigen-presenting cells (APCs).

The involvement of ICs in cross-presentation was initially noted in an assay to study the *in vivo* tumor killing by antibodies. EG7 tumor was inhibited or eradicated with an antibody against CD4

expressed by these cells. The antibody did not kill the tumor directly; the effect was entirely dependent on CD8⁺ T cells and Fc γ R γ -chain (Vasovic *et al*, 1997). A logical explanation today is that binding of CD4 by the antibody facilitated tumor antigen processing by DCs and drove antigen-specific CD8⁺ T-cell activation. As a vivid confirmation, in the RIP-mOVA mice whereby OVA is transcriptionally controlled by rat insulin promoter, infusion of OVA-specific CD8⁺ T cells (OT-I) was not efficient to trigger destruction of β -cells. Addition of an OVA-specific IgG antibody, however, led to severe autoimmune diabetes, suggesting that the presence of ICs can overcome the tolerance to “self” antigens. As expected, the breakdown of self-tolerance required the presence of Fc γ R common γ -chain (Harbers *et al*, 2007).

Cross-presentation of external antigens is enhanced by antigen association with surface receptors on DCs (Shakushiro *et al*, 2004; Burgdorf *et al*, 2007; Chatterjee *et al*, 2012). Independent studies arrived at a similar conclusion that the presence of ICs facilitated the antigen uptake by two to three logs (Amigorena & Bonnerot, 1999; Schuurhuis *et al*, 2002). However, the real enhancement in antigen presentation measured by the degree of T-cell activation was in the order of 4–5 logs (Regnault *et al*, 1999). γ -chain ITAM domain-mutant NOTAM mice have normal surface expression of Fc γ Rs. This to some extent rescued the IC uptake; however, cross-presentation of OVA IC was lost (Boross *et al*, 2014). That report seems to indicate that the ITAM is not required for uptake and yet behaves in part to direct intracellular antigen trafficking. Interestingly, in DCs, Syk deficiency downstream of ITAM phosphorylation disrupted not only cross-presentation of IC antigen but antigen uptake as well (Sedlik *et al*, 2003). The difference is likely due to the fact that Syk is associated with a broader spectrum of inhibition on phagocyte signaling (Mocsai *et al*, 2010). Deep mechanistic understanding of how ICs facilitate cross-presentation is currently limited. In one report, IC-mediated enhanced cross-presentation required TAP proteins and was sensitive to proteasome inhibition, suggesting the involvement of cytosolic targeting of internalized antigens (Regnault *et al*, 1999). In some systems, IC-mediated antigen uptake retained the complex at early endosome, rather than permitting further maturation to more degradative lysosome (Chatterjee *et al*, 2012; Cohn *et al*, 2013). Although the molecular nature of this “shallow” targeting is unknown, the findings are in agreement with the contemporary understanding that reduced cargo digestion is the main advantage of DCs in antigen presentation over other APCs (Joffre *et al*, 2012; Kotsias *et al*, 2013; Hari *et al*, 2015).

CD8⁺ DC subsets in the mouse periphery are usually considered to be superior in cross-presentation, and a developmentally overlapping population of CD103⁺ DCs is known for similar functions. These cells are phenotypically mirrored by human CD141⁺ DCs (Platzer *et al*, 2014). However, how these cells are involved in IC-mediated cross-presentation is not well understood (Joffre *et al*, 2012). Not in strict sense an standard IC, OVA linked to an antibody specific for a C-type lectin receptor DNGR-1 found on CD8⁺ conventional DCs elicited strong CD8⁺ T-cell activation and eliminated OVA-positive B16 tumor *in vivo* (Sancho *et al*, 2008). Yet, when delivered into the host, OVA conjugated to anti-DCIR-1 and anti-DEC205, expressed by CD8⁻ and CD8⁺ DCs respectively, suppressed OVA-expressing B16 tumor to a similar extent (Neubert *et al*, 2014). As IgG1 antibodies used in this study carried Fc mutation (N297A) that disrupts Fc γ R binding, both preparations might

function merely as a delivery vehicle. This paper therefore did not address the effect of Fc γ R signaling in cross-presentation. In another report, Bevan's group suggested that Fc γ R-mediated IC cross-presentation was only essential in CD8⁻ DCs, while IC uptake and processing by CD8⁺ DCs were not affected by the absence of Fc γ Rs (den Haan & Bevan, 2002).

DCs can rapidly engulf ICs and sustain the stimulation capacity for a long period after a short pulse (Bonifaz *et al*, 2004). In influenza virus-challenged mice, the absence of extracellular availability of ICs did not strongly affect the primary CTL response to flu NP antigen. However, the recall response was limited. Transfer of immune serum from control-infected mice restored the long-term CD8⁺ T activation to NP antigen (Leon *et al*, 2014). Crossing μ MT (B-cell-deficient) mice to γ -chain knockout mice, NP-specific CD8 response upon viral challenge was reduced. Transfer of immune serum restored both primary and secondary responses in μ MT mice, yet failed to do so in the double-deficient recipients, suggesting the essential role of Fc γ R signaling in cross-presentation. This observation was confirmed by a recent study that the ICs associated antigens were present in the endocytic vesicles from early endosome to lysosome one day after the uptake, with dissociation of antigen taking place along the route (Liu *et al*, 2016).

Helper T cells and antibody responses

Fc γ R-mediated IC signaling likely supports a simultaneous activation of both CD8⁺ and CD4⁺ T cells. For instance, prostate-specific antigen-containing ICs, when targeted via Fc γ R, led to a combined activation of both CD4⁺ and CD8⁺ T cells, while mannose receptor targeting was more associated with CD4⁺ T-cell response alone (Berlyn *et al*, 2001). Regarding intracellular antigen trafficking, Syk signaling is an essential event for efficient MHC class II epitope peptide loading, as in the case of cross-presentation. A mutation in Fc γ R that disrupts Syk association failed to deliver internalized antigen in ICs to lysosome (Bonnerot *et al*, 1998). Syk deficiency resulted in reduced MHC class II/DM molecule association with internalized antigens (Le Roux *et al*, 2007). As evidence of IC-facilitated class II antigen presentation, a viral glycoprotein rabies G as a free antigen entered Rab5⁺ early endosome with a kinetics similar to that of antibody (ARG1)-bound protein. Thereafter, the IC became localized to Rab9⁺ late endo/lysosome but at no time was co-localized with Rab11 (slow recycling endosome) and Rab4 (rapid recycling endosome) markers, while the free antigen was more associated with Rab11⁺ vesicles (St Pierre *et al*, 2011). Therefore, ICs may facilitate antigen delivery to vesicles in late endocytic maturation steps for MHC class II presentation. This notion inevitably disagrees with the “shallow” targeting reported in cross-presentation as discussed previously. This discrepancy possibly resulted from different antibodies used in experiments, which could have preferences in Fc γ R subtype association, leading to distinct routes of trafficking. However, considering ICs in general enhance both class I and class II antigen presentation, one can postulate that binding of antibodies in an uncharacterized manner intercepts the robust proteolytic “grinding” in the endolysosomal system, saving antigens at multiple stages for eventual surface presentation.

One main mechanism for enhanced antigen presentation of ICs to B cells is longer biological retention. This is most evident in the trapping of ICs by follicular dendritic cells (FDCs) (Szakal *et al*, 1988a,b; Kosco *et al*, 1989). It was reported that great quantities of

ICs attached to the long membrane processes of FDCs (Szakal *et al*, 1988b). FDCs have high Fc γ RIIb expression at protein level (Qin *et al*, 2000) and reports suggested that ICs were internalized by Fc γ RIIb (Bergtold *et al*, 2005), consistent with the observation that FDCs and B cells only express this Fc receptor. The ICs internalized by Fc γ RIIb were retained in the cytoplasm and antigens within the complex were kept in their native form and were made available to B cells via surface recycling (Bergtold *et al*, 2005). The exact order of baton exchange has been reported. ICs were initially trapped by lymph node subcapsular macrophages; B cells then acted as an intermediary fetching and sending the complexes to FDCs (Phan *et al*, 2007). FDCs received complement-coated ICs from non-specific B cells, and following endocytosis sorted, the complexes to a non-degradative compartment for extended resurfacing (Heesters *et al*, 2013). Furthermore, FDC-associated ICs played a role for BCR hypermutagenesis to generate high-affinity antibodies (Wu *et al*, 2008).

Type II Fc γ Rs are also implicated in B-cell activation. IgG is glycosylated at asparagine 297 with a biantennary core glycan that serves as the base for further addition of sugar moieties, including fucose, galactose, bisecting *N*-acetylglucosamine, or sialic acid, etc. This complex glycosylation event controls IgG conformational state and determines the preferential binding to either type I or type II Fc γ Rs. For instance, sialylation reduces the binding of Fc to all type I while increasing the binding to type II receptors (Pincetic *et al*, 2014). Treatment to remove this moiety suppressed ankle swelling in a model of serum transfer arthritis and prolonged survival of a mouse strain from an autoantibody-induced lupus-like disease (Albert *et al*, 2008). Targeting OVA to DCs via DEC-205 without concomitant activation via CD40 induced IgG1 antibody response and but a suppressed DTH (delayed type hypersensitivity) response. When the antiserum from those mice was transferred to new hosts that were challenged with the antigen in the full complement of anti-CD40, an extensive reduction of antigen-specific IgG1, IgG2b, and IgG2c was detected, accompanied by a reduced DTH response as well. These results suggested that antibodies produced in the original hosts without DC activation signal were immunosuppressive. Indeed, the antibody produced without anti-CD40 had a N297 sialylation rate higher than those with the treatment, due to higher levels of sialyltransferase in the plasma cells (Oefner *et al*, 2012). As this reduction in antibody production and DTH response was independent of Fc γ RIIb, the involvement of type II receptors was implicated (Oefner *et al*, 2012). Type II Fc γ R engagement is an important consideration in vaccination. In a trivalent inactivated influenza vaccine (TIV)-immunized individuals, mass-spec analysis did not show a significant glycosylation pattern change in the recipients. Interestingly, the subtle differences in glycosylation in the existing antibody pool were found to be a predictor of responders and non-responders to the vaccine (Wang *et al*, 2015a). In addition, it was recently reported that in human volunteers, TIV led to a peak production of HA-specific sialylated IgG from plasmablast one week after the immunization, followed by a drop in abundance by week three. This elevation was resulted from glycosyltransferase expression in these early responding B cells. Importantly, this sialylated IgG, via CD23, triggered Fc γ RIIb expression, which is a known threshold setter for BCR signaling (Wang *et al*, 2015b) resulting in altered antibody-binding affinity. The pattern of Fc glycosylation is associated with protection efficacy of a given antibody.

Palivizumab, a humanized mAb against respiratory syncytial virus (RSV) derived from animal cell culture, has a heterogeneous glycosylation pattern. The same antibody derived from plant cells (*Nicotiana benthamiana*) that had their xylosyl- and fucosyltransferase activities deleted produced G0-dominated glycans, palivizumab-N. This form of glycosylation showed enhanced Fc γ RIII binding and better protection against RSV challenge (Hiatt *et al*, 2014).

Regulation of antigen-presenting cells

ICs can directly activate APCs via activating Fc γ Rs. Upon IC binding to these receptors, the ITAM/Syk interaction is the primary signaling axis in phagocyte activation, leading to the full spectrum of signaling events involving PI3Ks, PLCs, MAPKs, and NF- κ B (Mocsai *et al*, 2010). Accordingly, ICs can trigger DC expression of CD40, CD86, IL-6, IL-13, and unexpectedly IL-2 (Matsubara *et al*, 2006; Perreau *et al*, 2008; Boross *et al*, 2014). In addition, ICs themselves were sufficient to trigger CD11c⁺ DC aggregation and entrance into lymphatic vessels for migration to LNs in a CCR7/MMP-9-dependent manner (Clatworthy *et al*, 2014). While in general the activation of APCs is linked to the Fc γ R signaling, it has been recently suggested that once the ICs are inside the cell, cytosolic antibody receptor TRIM21 can bind to the Fc portion and activate a K63-specific Ubc13-mediated ubiquitination event. This event can independently activate NF κ B, AP-1, and IRF pathways, adding another level of complexity to IC signaling (McEwan *et al*, 2013).

Immune-suppressive effects of ICs are equally noted. The small immune complexes formed in IVIG can inhibit macrophage responses to IFN- γ , an effect mainly mediated by IgG Fc binding to Fc γ RIII, and surprisingly independent of Fc γ RIIb (Park-Min *et al*, 2007). In complement C5a-induced inflammatory recruitment, IgG1 ICs suppressed the neutrophil and macrophage chemotaxis. This inhibition was a result of an unexpected collaboration between ITIM tyrosine phosphorylation in Fc γ RIIb itself and a ITAM phosphorylation of Syk downstream of dectin-1 (Karsten *et al*, 2012). In contrast to the conventional understanding that ICs formed in chronic infections cause inflammatory response by Fc γ R cross-linking, it was recently found that in mice with clone 13 LCMV (long term) infection, the presence of ICs resisted CD4⁺ T- and B-cell depletion by anti-CD4 and anti-CD20 (rituximab) antibodies and blocked the activation of DCs by agonistic anti-CD40. The effect can be explained by competitive inhibition in the long-term presence of excessive ICs (Wieland *et al*, 2015). A similar report in the same issue of immunity confirmed the finding and demonstrated additional defects in cross-presentation. It was shown in the latter that the suppression was dependent on CD4⁺ T-supported antibody production (Yamada *et al*, 2015). This conclusion somewhat echoes the observation that in influenza infection, peak of antibody production formed ICs that blocked antibody-dependent phagocytosis (Astry & Jakab, 1984), both pointing to IC's negative impact on immune activation.

To summarize the discussion on the immune regulatory functions of IC engagement of Fc γ Rs, Fig 1 categorizes the immunologic effector functions of IC uptake by DCs and macrophages, and Fig 2 illustrates the known APC functional regulations and antigen processing pathways.

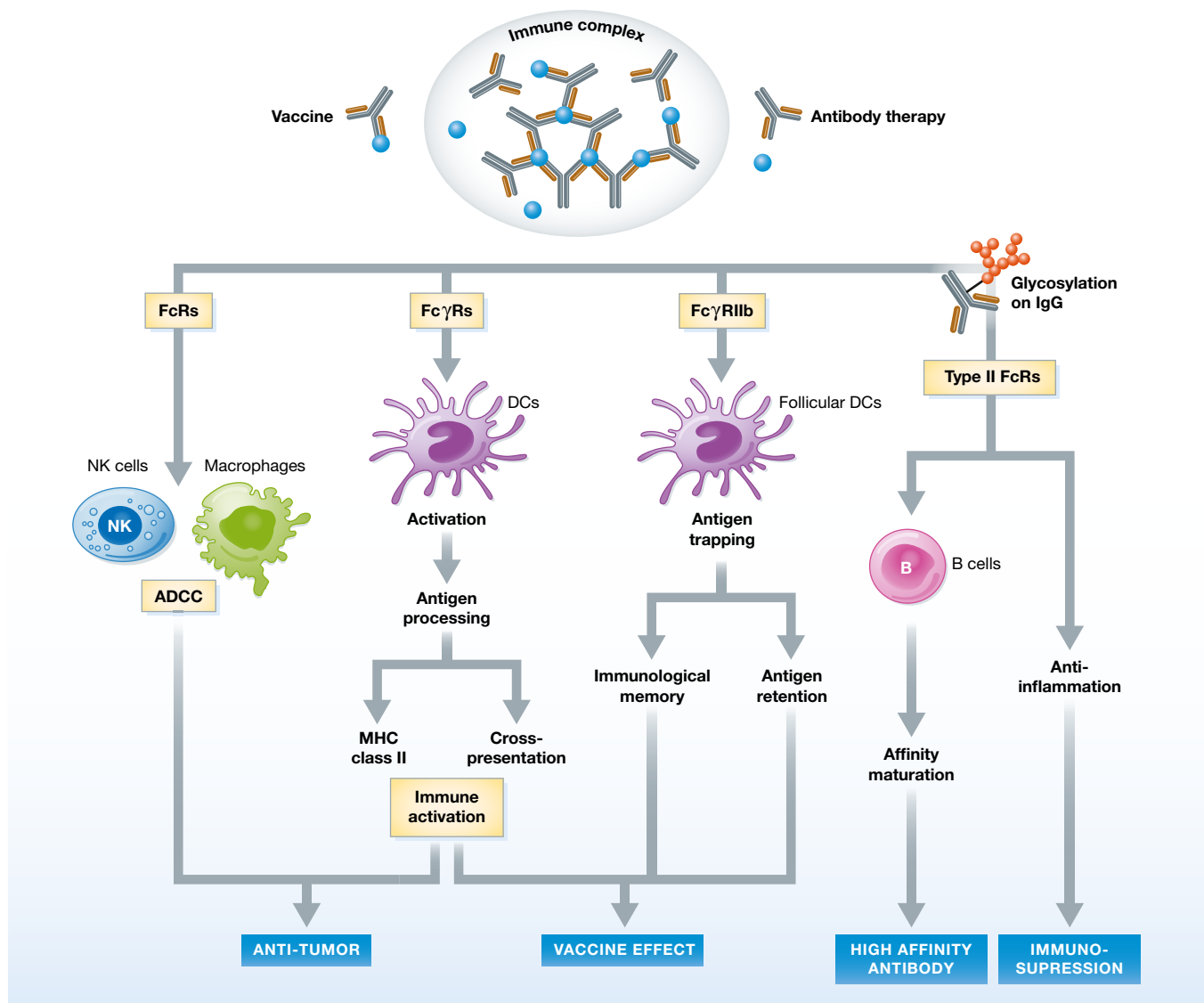


Figure 1. Immunologic regulation of ICs in vaccine and tumor therapy.

ICs preformed as vaccine preparations or as a consequence of antibody binding to endogenous antigens can signal via activating receptors to induce ADCC in macrophages and NK cells, leading to lysis of tumor or infected cells. The same signal via $Fc\gamma R$ s on dendritic cells results in enhanced antigen uptake and upregulated antigen presentation, both to MHC class II-restricted $CD4^+$ T cells and to $CD8^+$ T cells via cross-presentation. ICs retained in lymphoid tissues via deposition or $Fc\gamma RIIb$ -mediated endocytosis extend the antigen availability for B-cell activation, resulting in increased antibody response and immune memory induction. Different glycosylation patterns on Fc regulate the preferential binding to type I or type II $Fc\gamma R$ s, controlling the state of inflammation. Sialylation of Fc with preferential binding to type II $Fc\gamma R$ in particular helps to set the threshold for the production of high-affinity antibodies.

Experimental and clinical applications of ICs

IC vaccines in persistent viral infections

In HIV infection, experimental work on ICs started by using antibodies targeting the viral epitopes, as an approach to form ICs *in vivo*. ICs formed by antibodies recognizing HIV CD4 binding sites (CD4bs) and V3 region of gp120 have been promising, eliciting strong neutralizing antibodies against the virus in mice (Visciano *et al*, 2008; Hioe *et al*, 2009). One of V3 region-specific antibodies mAb 654-D showed high affinity to large variants of gp120 subtypes. The binding of this antibody significantly increased the accessibility of V3 region by other mAbs, suggesting

that the attachment of 645-D opened up the V3 region that is masked by other structures, a common detection-averting strategy of HIV (Kwong *et al*, 2002; Pan *et al*, 2015). This IC elicited anti-V3 neutralizing antibody in BALB/c mice (Hioe *et al*, 2009). IC formed with mAb A32, binding to gp120 outside the CD4 binding site, induced neutralizing antibodies against a panel of HIV strains, although the enhancement over gp120 alone was not pronounced. In the presence of cholera toxin and LPS as adjuvants, the breadth of the resulting antisera in neutralizing diverse HIV isolates was widened (Liao *et al*, 2004).

Recently, broadly neutralizing antibodies (bNAb) against HIV-1 have seen great success. bNAb-producing B cells identified in HIV

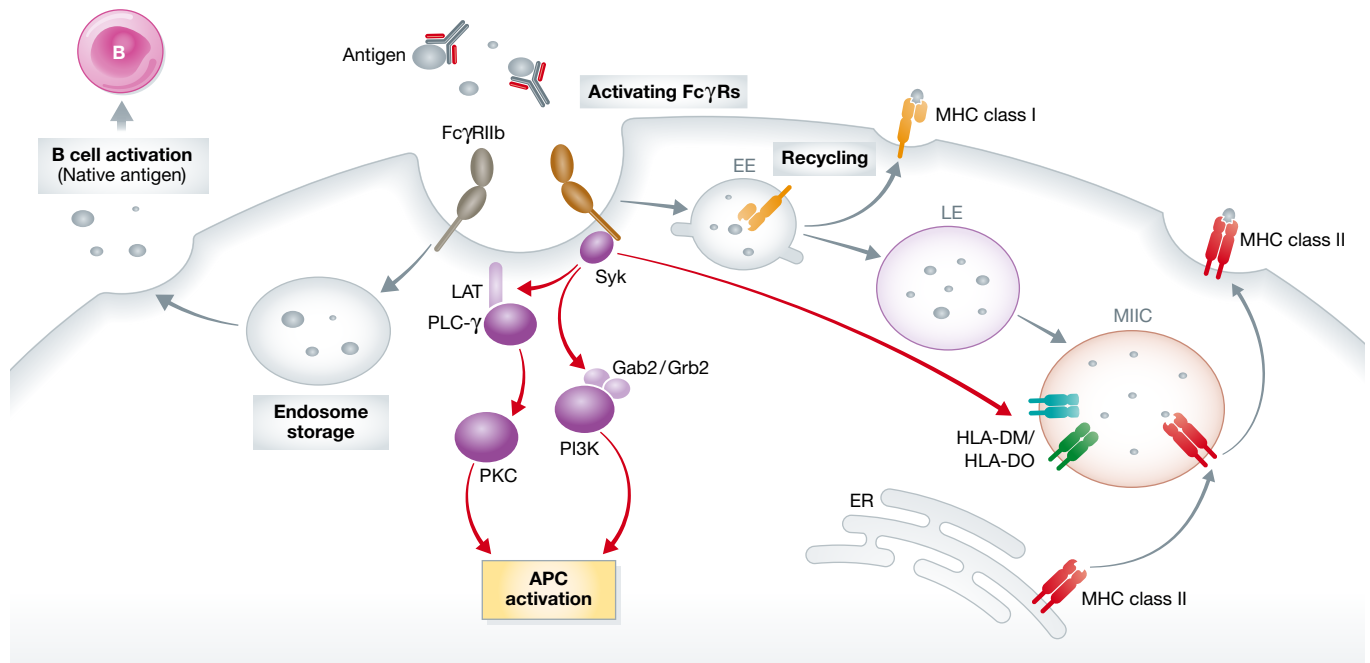


Figure 2. Fc γ R ligation and the fate of antigens.

Antigens in ICs entering APCs via different Fc γ Rs show distinct trafficking patterns. In general, Fc γ RIIb-mediated uptake traps the cargo in non-degrading vesicles for prolonged extracellular release of antigens in their native forms. This feature is important for B-cell activation. Activating Fc γ R-mediated entry may direct the cargo into two routes. One is through the shallow early endosomes whereby the antigens are recycled to the cell surface in complex with MHC class I molecule for cross-presentation. The other route leads the antigens to the MHC class II compartment (a branch of late endo/lysosomal compartment) and epitope peptides generated in this harsher environment are loaded onto MHC class II molecules for conventional CD4 T-cell activation. The ITAM motif present in Fc γ Rs (intrinsic or in the common γ -chain) recruits Syk and activates signal transducers of PLC, PKC, and PI3K leading to APC activation. Syk signaling may assist the class II peptide exchange regulated by class II-like DM/DO molecules in the MHC class II compartment.

carriers by high-throughput screening are capable of neutralizing a large number of HIV isolates (Walker *et al*, 2009; Wu *et al*, 2010). To eradicate the latent pool of HIV in the carriers, one strategy was to reactivate the latent virus in the presence of antiretroviral treatment followed by immune therapies. bNAbs were shown to suppress HIV viremia in humanized mice and reduced the HIV DNA in the infected lymphocytes (Horwitz *et al*, 2013). However, the generic treatment was followed by viremia rebound after termination, suggesting the presence of a persistent pool of cells with intact virus. In humanized mice, delivery of bNAb cocktail (TriMix antibodies with 3BNC117, anti-CD4bs; PG16, anti-V1/V2 loop region; and 10-1074, anti-V3) 4 days after HIV inoculation significantly delayed the viremia rebound, with a statistically significant increase in aviremic period. Furthermore, TriMix bNAbs with a combination of three viral inducers (shock and kill) significantly suppressed viremia rebound over a long period of up to 105 days in comparison with antibody alone or with in combination with a single inducer (Halper-Stromberg *et al*, 2014). Implicating the role of ICs, TriMix antibodies carrying Fc mutation that disrupts human and mouse Fc γ R binding showed shortened delay in viremia rebound (Halper-Stromberg *et al*, 2014). The definitive proof of Fc γ R engagement by bNAbs was elegantly demonstrated in a recent study. Human antibodies with Fc domains swapped with mouse Fc subtypes were used in a model of HIV entry inhibition assay. While the Fc swapping minimally affected the Fab binding, mouse IgG2a Fc was found to be most potent in blocking HIV entry in an *in vivo* analysis.

Accordingly, in mice lacking all Fc γ α -chain or common γ -chain/Fc γ RIIb, the advantage of IgG2a over a non-FcR-binding mutant Fc was lost (Bournazos *et al*, 2014). To mimic the human infection, a mouse model with all Fc γ Rs replaced with human Fc γ Rs was used. Human HIV-specific IgG1 antibodies with Fc domain engineered to carry high-affinity mutations to activating Fc γ Rs (Fc γ RIIIa and Fc γ RIIa) were highly protective against HIV entry in comparison with Fc mutants that diminish the Fc γ R binding (Bournazos *et al*, 2014). Beyond direct eradication of HIV, in a mouse model to approximate human mother to child transmission, FrCasE virus-infected neonatal mice develop erythroleukemia once reaching adulthood. Neonatal delivery of a specific mAb 667 boosted antibody protection and T-cell response that resulted in survival of treated mice in comparison with the total mortality in untreated controls. This protection was mediated by ADCC and formation of virus/antibody ICs (Michaud *et al*, 2010).

Viral hepatitis B is another persistent infection treated with ICs as therapeutic vaccine. In a HBV vaccination model, ducklings were infected with duck hepatitis B virus (DHBV). They were treated with IC (DHBsAg complexed to rabbit anti-DHBs) linked to *Staphylococcus aureus* bacteria as a solid matrix complex. About 70% and 50% of treated ducks were cleared of viremia and antigenemia after three injections (Wen *et al*, 1994). The efficacy of IC-based vaccines in HBV infections was studied in mice in 1980s. ICs formed with non-specific IgG or IgM antibodies showed minimal enhancement, as did the Fab fraction of those specific antibodies (Celis & Chang, 1984).

HBsAg ICs stimulated T cell's avidity, which in turn induced antibody production *in vivo* (Celis et al, 1987). IC-mediated effects were followed by a seroconversion to HBsAb positivity in HBsAg-transgenic mice, indicating self-antigen tolerance breaking (Zheng et al, 2001). Subsequently, DCs induced from chronic hepatitis B patient PBMCs with GM-CSF/IL4 were stimulated by either HBsAg, antibody, or IC *in vitro*. Expression of functional markers including MHC class II, CD80/CD86, and CD40 was found increased only with the IC stimulation. Furthermore, when T cells from the patients were incubated with the stimulated DCs, higher levels of IFN- γ and IL-2 were observed in the IC-stimulated group (Yao et al, 2007). Using a lentivirus vector coding HBsAg either alone or fused to Fc domain of mouse IgG2a, the fusion version induced much higher CD8⁺ T-cell activation in recipient mice, as well as elevated CD4⁺ T-cell response and antibody production (Hong et al, 2011). To simplify the vaccination protocol, Meng et al produced HBsAg-Fc (from IgG1) fusion protein and used it to immunize HBsAg-transgenic mice. This resulted in a predominant production of Th1 cytokines and reduced serum HBsAg level (Meng et al, 2012). Encouraged by these experimental findings, human HBsAg IC was adsorbed to alum and used to immunize 14 HBV chronic hepatitis B patients in a pilot study. The recipients showed substantial reduction in serum HBV DNA and HBeAg was converted to negative (Wen et al, 1995). In a phase 1 clinical trial, healthy subjects receiving various amounts of HBsAg IC preparations developed IgG1 and IgG3 antibodies against the immunogen with a simultaneous increase in serum IFN- γ and IL-2 (Xu et al, 2005). From this outcome, one of the doses was used to immunize chronic hepatitis B patients. Five of ten patients responded and showed ≥ 2 logs decrease in serum HBV DNA, with a loss or marked reduction of HBeAg and an appearance of anti-HBeAg; and two of these patients developed anti-HBsAg antibodies (Xu et al, 2005). In a phase 2 clinical trial, HBeAg-positive chronic hepatitis B patients were immunized with six injections of the IC. At the end of post-trial follow-up, HBeAg seroconversion rate was 21.8% (17/78) and 9% (7/78) in the IC and placebo groups, respectively ($P < 0.05$; Xu et al, 2008).

IC in other microbial infections

In addition to the vaccine trials against HIV and HBV, smaller scale efforts to control other infections have been reported. CTL response against influenza virus infection is reduced in aging populations. While young BALB/c mounted strong cytolytic response with no discernible differences in intensity to flu NP antigen following inoculation of live, killed H1N1 virus, or a preparation of the virus plus a NP-specific antibody, only the IC significantly restored the same response in the aged mice. This enhanced killing was associated with increased IFN- γ production in both CD4⁺ and CD8⁺ T cells (Zheng et al, 2007). Due to its rapid spread and high rate of mutagenesis, the emphasis of influenza infection control has been to generate bNAbs against conserved regions of hemagglutinin (HA) (Okuno et al, 1993, 1994; Ekiert et al, 2011). One version of the antibody, CT149, targeting the stem region of HAs of both type I and type II isolates blocked the membrane fusion. In addition, the binding by this antibody induced Fc γ R-dependent ADCC, suggesting the involvement of ICs (Wu et al, 2015). For Ebola infection, several B-cell clones of highly potent GP-specific neutralizing antibodies from a disease survivor have been tested. These antibodies also mediated ADCC via Fc γ R as mutants in the Fc that interfered with

the receptor binding lost the cytotoxicity (Corti et al, 2016). *Francisella tularensis* (FT) is the causative pathogen of tularemia. DCs coated with monoclonal antibody-treated FT delivered intranasally protected hosts from subsequent challenges. This effect was not affected by the absence of Fc γ RIIb, suggesting a preferential engagement of activating Fc γ Rs (Pham et al, 2014). In malaria infection, it was found that the Fc γ R binding was critical for the uptake of IC (McClintock et al, 2005). In one study, the mortality of *Plasmodium berghei* infection that is often lethal was prevented if the antiserum from immune mice was transferred into infected mice. This protection was eliminated in the absence of Fc γ R γ -chain (Pleass, 2009). In addition, Phoolcharoen et al transiently expressed Ebola virus GP1 glycoprotein fused to an antibody heavy chain from a humanized GP1-specific antibody in *Nicotiana benthamiana* (tobacco plant). The purified fusion protein induced high titers of Ebola-specific IgG antibodies, similar to those induced by GP1 virus-like particles, suggesting a scheme of vastly increased production capacity for IC preparations (Phoolcharoen et al, 2011). Urgent productions of ICs to protect populations under threat from rapidly emerging viral infections such as Middle East respiratory syndrome (MERS) are also being tested (Lu et al, 2015).

Although ICs are in general considered to be conducive to antiviral immunity, it should be noted that in some cases the presence of virus-specific antibodies can increase infectivity, a phenomenon called antibody-dependent enhancement (ADE) of viral infection (Tirado & Yoon, 2003). In the STEP trials, HIV *gag*, *pol*, and *nef* gene cassettes were inserted into the adeno vector, however individuals with preexisting immunity against the virus showed greater risk of HIV infection following the immunization (Buchbinder et al, 2008). The same adverse effect was seen for additional viruses, such as dengue (Goncalvez et al, 2007), measles (Iankov et al, 2006), and over a dozen others (Suhrbier & La Linn, 2003). Fc γ R-facilitated virus entry and a skewed cytokine profile may be the main culprits. These are important considerations in assessing ICs' immune regulatory functions.

IC in tumor immunity

Cancer immunotherapy has seen tremendous strides with technological developments in the immune checkpoint blockage. The basic concept is to reduce negative regulators of adaptive immunity, mainly targeting CTLA-4 and PD-1/PD-L1. Another modality of antibody-based cancer therapy is to target surface factors critical to tumor cell growth, such as EGFR and VEGF, of which optimized Fc γ R ligation is an important consideration (Nimmerjahn & Ravetch, 2012). Concurrent to these progresses, IC-based intervention has remained an alternative due to its low side effects. Antibody binding to tumor surface antigens triggers ADCC and Fc γ R engagement is an important activation signal for tumor lysis (Moalli et al, 2010). A large number of experiments have been carried out using B16 or its variants as the tumor model. Here, ICs have been shown to mediate tumor suppression via prophylactic, therapeutic and memory-inducing effects (Rafiq et al, 2002). In mice challenged with B16 tumor-expressing OVA antigen (MO4), injection of DCs pulsed with OVA-containing IC, but not with OVA or antibody alone, inhibited the tumor growth. Furthermore, treatment with DCs loaded with ICs cured 40% of mice bearing established tumors. Protection mediated by DC/IC immunization was long-standing and induced memory responses. In recipients that had been vaccinated with OVA

Table 1. A select list of published laboratory and clinical assessments of IC-based immune therapy and vaccination.

Complex	Species	Disease targeted/model	Observations	References (authors, year)
Antitumor				
Specific mIgG2A/hCD20	Mouse (humanized)	EL4 expression hCD20	Tumor clearance; via ADCC; FcγRIV is required; engineered selective engagement of hFcγRIII is effective	DiLillo and Ravetch (2015)
Specific mIgG1/NYU-seo-1	Mouse	Colon epithelial tumor	Tumor inhibition; CD8 required; enhanced by chemotherapy; epitope spreading	Noguchi et al (2012)
Fusion of OVA to anti-DEC205 antibody, DEC205 targeting etc.	Mouse	B16, HER2-neu bearing tumor	Enhanced tumor resistance, involvement of both CD4 and CD8 responses, enhanced by anti-CD40	Wang et al (2012); Charalambous et al (2006); Bonifaz et al (2004)
OVA epitope conjugated to anti-DNGR-1 (mouse or rat)	Mouse	B16	Tumor inhibition and prevention; metastasis inhibition; enhanced antigen uptake by CD8α ⁺ DCs	Sancho et al (2008)
Specific mIgG2a mAb/HER2-neu	Mouse	Her2 transgenic mouse	IC-mediated uptake of antigen by DCs; specific CD8 expansion; Fc required	Kim et al (2008); Wolpoe et al (2003)
Polyclonal rIgG/cell surface OVA	Mouse	Self-antigen tolerance breaking	Breaking CD8 tolerance of OVA transgene; requires FcγR γ-chain and complement C3	Harbers et al (2007)
IgG2A (TA99)/Gp75	Mouse	B16	Tumor clearance; FcγRIV is required	Nimmerjahn and Ravetch (2005)
Specific mAb hIgG1 (B4-B)/syndecan-1	Human	Myeloma	CTL induction to unrelated testis antigen epitopes; reduced with anti-FcγR antibodies	Dhodapkar et al (2002)
Specific rIgG/OVA	Mouse	B16 (OVA expressing)	Mouse survival; FcR-γ chain is required, absence of FcγIIb reduces tumor burden	Kalergis and Ravetch (2002)
Specific rIgG/OVA	Mouse	B16 (OVA expressing)	Reduced tumor establishment; FcγR-γ required; TAP and β2m required; MHC class II required	Rafiq et al (2002)
Vaccine				
bNAb/Ebola GP	Macaque	Ebola	Protection against Ebola challenge, ADCC	Corti et al (2016)
bNAb/flu HA	Mouse	Influenza model	Protection against influenza challenge, ADCC	Wu et al (2015)
IgG/TIV	Mouse/human	Influenza	Early production of sialylated IgG set the threshold for subsequent high-affinity and protective antibody production.	Wang et al (2015b)
Chicken polyclonal serum/NDV	Chicken	Newcastle disease	Protection against viral challenge; some preparations reduced protection	Yosipovich et al (2015); Rautenschlein et al (2007); Pokric et al (1993)
bNAb/HIV gp120	Mouse (humanized)	HIV model	Longer period of aviremia after treatment; requires Fc portion for the effect	Halper-Stromberg et al (2014)
Engineered bNAb/gp120	Mouse (humanized)	HIV model	Better protection against HIV entry; bNAb with Fc engineered to bind activating FcγR (humanized in mouse) are more effective	Bournazos et al (2014)
Specific mIgG2a/FT	Mouse	<i>Francisella tularensis</i>	Protection from subsequent challenges	Pham et al (2014)
Humanized mAb/RSV	Cotton rat	RSV	GO glycosylation is linked to better protection against RSV challenge	Hiatt et al (2014)
Pre-made HBsAg IC	Human	HBV	Induction of specific IgG1 and IgG3; reduced serum HBV DNA; reduced serum HBeAg; presence of anti-HBeAg; increased HBeAg seroconversion	Xu et al (2013); Yao et al (2007); Xu et al (2005)

Table 1 (continued)

Complex	Species	Disease targeted/model	Observations	References (authors, year)
HBsAg fused to Fc of mIgG2a	Mouse	HBV model	Higher specific CD8 activation; elevated CD4 response	Hong et al (2011)
Specific mIgG2a and mIgG1/FrCasE virus	Mouse	Model of HIV maternal transmission	Reduced adolescent mortality from the virus; ADCC and CTL activations are involved	Michaud et al (2010)
Human IgG mAb/CD4 binding site of gp120	Mouse	HIV model	Higher anti-gp120 titer; induction of neutralizing antibody	Hioe et al (2009); Visciano et al (2008)
Engineered specific hIgG1/P. falciparum	Mouse (humanized)	Plasmodium berghei	Protection from lethality; FcR-binding/FcγRI are critical	McIntosh et al (2007)
Chicken polyclonal serum/IBDV	Chicken	Chicken Bursal Disease	Protection against viral challenge; immunization	Ivan et al (2005); Giambone et al (2001); Jeurissen et al (1998); Haddad et al (1997)
IgG/HBsAg	Mouse	HBV model	HBsAb seroconversion in HBV transgenic mice	Zheng et al (2001)
Non-neutralizing IgG fraction/SIV gp120	Rhesus monkey	SIV	No protection, FcγRIIB signaling, anti-inflammatory gene expression	Polyanskaya et al (2001)
Matrixed rat anti-DHBV/DHBV	Duck	DHBV protection	Reduced viral DNA and DHBsAg in serum	Wen et al (1994)
IgG/anti-gp120 v3 loop	Human	HIV	Positive anti-HIV proliferation of CD4 T cells	Berzofsky et al (1988)
Human polyclonal IgG/HBV HBsAg	Human	In vitro antibody production	Enhanced CD4 T-cell activation; enhanced antibody production; requires Fc	Celis et al (1987); Celis and Chang (1984); Celis et al (1984)
Rabbit IgG/human or bovine albumin	Mouse	Vaccine efficacy	Enhanced model antigen destruction in vivo	Terres and Wolins (1959a, 1961)

IC-pulsed DCs, MO4 inoculation failed to establish the tumor (Rafiq et al, 2002). In addition, specific antibody targeting HER2/neu antigen expressed by a whole-cell vaccine (3T3-neu/GM) was shown to enhance antigen-specific CD8⁺ T-cell activation and memory development and protected the hosts from the tumor challenge (Wolpoe et al, 2003; Kim et al, 2008). Loading antibody against myeloma cell surface syndecan-1, a heparan sulfate proteoglycan expressed on cancer cells, induced HLA A2.1-mediated antigen presentation to MHC allele-matched donors, resulting in cytotoxic T-cell activation (Dhodapkar et al, 2002). Here, antisyndecan-1 antibody served as a facilitator of overall antigen presentation by DCs. The ensuing cytotoxicity elicited from human HLA-A2.1-restricted CTLs was directed toward other tumor antigens, such as MAGE-3 and NY-Eso-1 (Dhodapkar et al, 2002).

FcγRs play an important role in IC-mediated tumor immunity. In the syndecan-1 experiment, it was found the antibody coating did not alter the level of uptake of tumor cells yet blocking of FcγRs significantly reduced antigen-specific T-cell proliferation (Dhodapkar et al, 2002), suggesting that the regulatory role of FcγRs is mostly at steps beyond opsonization, primarily at DC maturation. Preferential induction of tumor immunity can be achieved by fine-tuning the signaling intensity ratios of activating vs inhibitory FcγRs. This concept was proposed by Ravetch et al (Nimmerjahn & Ravetch, 2005). For instance, a tumor-specific antibody (TA99) was found to have mIgG2a Fc that binds to the activating FcγRs with much higher intensity than the inhibitory FcγRIIB. This preferential binding, particularly to FcγRIV, endowed a strong antitumor effect

(Nimmerjahn & Ravetch, 2005). The signaling of FcγRs in IC-mediated immune therapy can lead to a vaccinal effect, especially under properly selected FcγR engagement. In a recent paper, mice challenged with CD20-positive EL4 tumor survived in the presence of mIgG2a CD20-specific antibody. This protection was lost in the absence of total or all activating FcγRs. As expected, those primed mice developed an immune response that rejected a subsequent challenge of neo-antigen CD20-positive EL4 tumor (DiLillo & Ravetch, 2015). When the lymphocytes from primed mice or from mice with a DC-specific deletion of FcγRIV were transferred into new hosts, the former transfusion showed a better tumor resistance than the latter in the naïve mice, suggesting the role of this receptor in mediating the vaccine effect (DiLillo & Ravetch, 2015). When the concept was tested in a humanized mouse system with the Fc region of mouse CD20-specific mAb replaced with the human counterpart and a total replacement of mFcγRs with hFcγRs in the host, it was found that mutations in the Fc that allowed better binding to hFcγRIIIa and IIa, as compared to IIIa alone protected the host to the best extent (DiLillo & Ravetch, 2015). Overall, as in the case of antipersistent viral infections, the selective pairing of IgG FcγR subtypes in vivo may represent the most promising direction in IC-mediated cancer therapy. Lastly, intracellular tumor antigens that are often shielded from immune recognition can become stimulatory in the presence of ICs. NY-seo-1 is a tumor antigen considered to be strictly intracellular. However, in combination with 5-fluorouracil, which likely stresses tumor cells for ensuing releases of intracellular contents, injection of mAb against NY-seo-1 resulted in significant

Pending issues*Precise mapping of Fc/Fc γ R subtype affinities*

Although the work has started, a complete map of subtype Fc/Fc γ R interaction affinities is not yet available. This map will be essential for precision targeting in IC/Fc γ R engagement in immune therapy and vaccination.

How antigen presentation is regulated

As ligation of subtypes of Fc γ Rs results in native protein retention or MHC class I/II-restricted antigen presentation, it is critical to systematically, using subtype Fc/Fc γ R pairing, determine the routing of antigen processing. This will provide a guideline for optimal induction of desired immune responses.

tumor growth inhibition. This specific combination caused antigen epitope spreading, whereby other intracellular tumor antigens also induced T-cell activation (Noguchi *et al*, 2012).

Future prospective

The demand to employ IC technology in disease prevention and treatment makes it an urgent task to study their effects in both basic and clinical settings (Table 1). As one of the oldest technologies of immune therapy, the track records of safety and simplicity of ICs are valuable on this day of enormous breakthroughs at other directions. The downside of IC-based interventions is also clear: relative low clinical efficacy and often unpredictable clinical and experimental results. However, with growing understanding of Fc γ Rs and their preferential bindings to IgG subtypes, and modern biomedical engineering to steer the desired pairing, the future outcomes will likely change. In hindsight, if these understandings were built into vaccine designs at the time, several large-scale clinical trials might have seen different and likely more favorable effects. In the case of HIV prophylaxis, IC formation that results in the undesired cytokine profile and the existing antibodies targeting the adeno vector are two factors likely addressable via precision targeting of activating Fc γ Rs. In IC-based anti-HBV vaccination and immune therapy, first-hand monitoring of responder cells and their Fc γ R expression should be built into the protocols of next-stage clinical trials. At this time of personalized medicine, IC preparations tailored to individual Fc γ R polymorphism could be within reach as well. Addressing Fc γ R binding is only a mandatory starting point. Additional efforts will be needed to understand the precise antigen trafficking as a consequence of antibody binding, as well as to map inflammatory pathways and tissue targeting. As the desired immune protections vary from pathogen to pathogen, and entry portals are also vastly different, specific IC preparations can be made to induce CD4/CD8 and antibody responses for optimal protection. For instance, for antitumor and antiviral immunity that relies on cytolytic T-cell activation, an IC vaccine designed to target the “shallow” endocytic compartment will be highly ideal. Admittedly, IC-mediated immune regulations have not been as intensely investigated as other classes of immune adjuvants. However, mechanistic research will be most crucial for future breakthroughs in IC-based human disease intervention and is the strongest assurance to maintain the continuing development of this old biomedical technology.

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Conflict of interest

The authors declare that they have no conflict of interest.

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