

Fc Receptor Targeting With Recombinant Immunoglobulins and Immunoglobulin Formulations

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1. Introduction

Manipulation of immunity for the purpose of controlling pathogenic responses or prevent diseases such as infections can be achieved by two different approaches: (1) an antigen- or epitope-based strategy, designed to influence the immune system by interaction with lymphocytes that express only receptors specific for such antigens (e.g., vaccines); or (2) the use of agents that decrease or increase immunity by acting via nonantigen specific receptors on lymphocytes (e.g., immune-suppressive drugs for the treatment of autoimmune diseases or immune stimulatory cytokines in cancer). Advances during the last two decades in particular have resulted in the understanding that noninfectious diseases such as autoimmunity and certain cancers can be influenced in a manner depending on antigen-specific recognition. This finding, combined with advances in target discovery and validation catalyzed by the elucidation of the human genetic map, has resulted in an unprecedented world-wide effort in designing safer drugs or therapeutic strategies that give the concept of vaccination a new meaning.

1.1. Dual Role of Antigen-Presenting Cells (APC) in the Control of Immunity

APC are critical in the regulation of immune responses from at least two points of view. First, they present the major histocompatibility complex (MHC) class I- and II-restricted epitopes to specific T-cells. This event in itself, although necessary, is not sufficient in triggering a full-blown immune response. An additional prerequisite for induction of strong immunity is co-stimulation via nonantigen-specific receptors. Thus, the immune responsiveness is regulated by a set of complementary cognate and noncognate factors under APC control.

The immune response is essentially controlled by the T-cells that recognize epitopes presented by APC in the context of the appropriate MHC molecules. T helper (Th) cells recognizing MHC class II (MHC-II)-restricted epitopes fall into different functional categories depending on the cytokine profile they acquire during the differentiation process. More than a decade ago, when the seminal observation of functional dichotomy of Th cells was made (1), it was proposed that the Th cells fall into two categories:

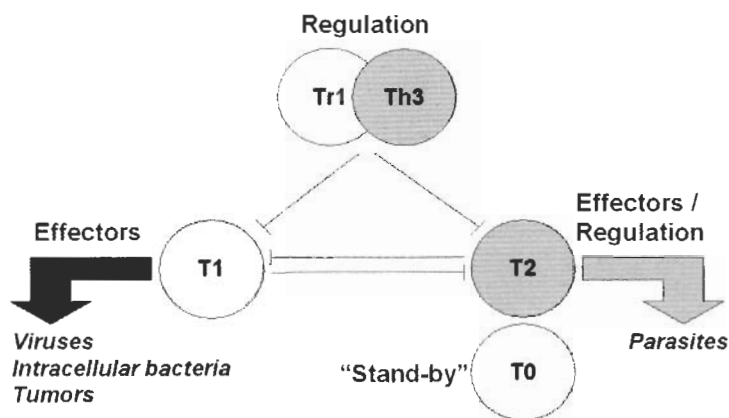


Fig. 1. Schematic representation of the functional organization of the T-cell compartment, as currently understood. Effector and regulatory T cells differentiate from naïve, competent precursors in the peripheral lymphoid organs depending on the presence of antigens, costimulation, and stochastic factors. They may transiently assume an ambivalent T_0 phenotype, consisting in production of limited levels, broad range cytokines ("stand-by" stage). From this stage, they could rapidly differentiate based on micro-environmental clues, to T2 cells (producing mostly IL-4, IL-5, and/or IL-13), T1 cells (producing IL-2, IFN- γ , TNF- β), Th3 (TGF- β), Tr1 (IL-10), or minute subsets such as CD4⁺CD25⁺ regulatory cells. T1 and T2 cells execute dual roles: T1 cells fight viral infections, certain bacterial infections, and tumors. In addition, they keep in check the differentiation and expansion of T2 cells. In contrast, T2 cells fight parasitic infections and they keep in check the differentiation and expansion of the T1 pool. Specialized, regulatory T cells such as Th3 and Tr1 are believed to be essential in maintaining under control the expansion of effector cells (T1 and T2) in general, facilitating a non-inflammatory status in basal conditions (chronic exposure to innocuous antigens) and preventing immunopathology during the response to pathogenic agents. Of note, the pleiotropism and versatility of this organizational structure, that is designed to mobilize as rapidly as possible an antimicrobial response but limit unnecessary immunopathology.

1. Th1 cells, which produce mainly interferon- γ (IFN- γ) and interleukin-2 (IL-2), and enhance the anti-viral response
2. Th2 cells, which produce IL-4 and are critical mediators of anti-parasitic immunity

Following more than one decade of intensive research in this area, it appears that the functional structure of the T-cell compartment is at least tripolar, rather than bipolar, as proposed initially (Fig. 1) and that it extends to MHC-I-restricted T cells (T_c) (2). A feature of Th1 and Th2 cells is that they are involved in both immune regulation and the effector arm of immunity. In addition to Th1 and Th2 cells, there are T regulatory cells, devoid of overt effector capability but endowed with potent immune modulatory functions via production of IL-10 (Tr1 cells) (3) or transforming growth factor- β (TGF- β) (Th3 cells) (4). T regulatory cells are mostly involved in maintaining the homeostasis of the immune response at mucosal sites, where a tight control of tolerance vs immunity must be maintained in order to allow effective immune defense without organ-associated immune pathology (5). It is currently believed that a failure of effective reciprocal regulation between different functional subsets of T cells is responsible for T1-mediated pathology (autoimmune diseases such as multiple sclerosis [MS] and type 1 diabetes) or T2-mediated diseases (asthma, allergies).

The induction of T-cell responses followed by differentiation is governed by specific rules that are believed to be essential to the process of self, nonself-discrimination. The system of MHC is a molecular-sorting mechanism that selects proteasome-processed peptides of potential significance for immunosurveillance. Mere exposure of T cells to peptides on MHC molecules expressed by APC in the presence of appropriate co-stimulation does not necessarily result in generation of effector cells. There are at least two additional homeostatic mechanisms involved in preventing immunopathology, independent of MHC-restricted recognition. Thymic negative selection ensures that the T-cell precursors colonizing the peripheral lymphoid organs do not recognize, with high affinity, most of the self epitopes expressed on MHC molecules by default (6). However, there is a set of T-cell epitopes that is not expressed in the thymus, and thus do not participate in central negative selection. Such epitopes may be tissue-restricted, cryptic, or developmental. In addition, the immune system is faced continuously with substantial exposure to innocuous nonself antigens associated, for example, with gut-flora or saprophytes colonizing the other mucosal areas. Thus, an additional mechanism ensures that full-blown autoimmune or inflammatory responses, respectively, are elicited only when supplemental conditions associated with pathogenic infection (7) and/or lesions (8) are present. Such a checkpoint is based on MHC and T-cell receptor (TCR)-independent recognition by APC of microbial-derived pattern motifs (e.g., carbohydrates, lipids, lipopeptides, or nucleic acids) (7), or triggered by endogenous mediators ("danger motifs") liberated following cellular injury (e.g., tumor necrosis factor- α [TNF- α], IL-1 β or heat-shock proteins [hsps]) (8). In addition, penetration of antigen beyond the mucosal-epithelial barrier that may be caused by, for example, virulent microbes contributes to enhanced immunity by mere "geographic" co-localization with cells capable of co-stimulation (9). It is believed that only recognition of MHC-peptide complexes, in context of simultaneous co-stimulation triggered by microbial or endogenous danger signals, results in full-blown differentiation of effector T cells. For example, IL-12, a critical factor for differentiation of IFN- γ -producing T cells via STAT-4-mediated activation (10), is produced by innate immune cells only in conditions of immunological or cellular stress. In stark contrast, innate immune cells associated with mucosal areas produce by default immune regulatory cytokines such as IL-10 and TGF- β that increase the threshold associated with induction of inflammation and assist in generation of regulatory T cells. Thus, continuous exposure to innocuous antigens in the absence of strong co-stimulation triggers nonpathogenic regulatory T cells rather than T-cell effectors, further increasing the stimulation threshold required for inflammation (5).

During the last few years, the dichotomy between the "danger-model" and microbial motif recognition model has been overemphasized (11,12) and questioned, fueled by the different nature (endogenous vs exogenous) of the pro-inflammatory mediators required for differentiation of T-cell effectors. For example, recent data brought into focus a new category of motifs—noncoding RNA-associated motifs—that are produced within cells infected with negative-stranded RNA viruses or certain DNA viruses (13). In contrast to previously defined microbial-associated motifs, such as CpG nucleotides, lipopeptides, and microbial carbohydrates, naturally occurring dsRNA motifs from infected cells are not borne by microbes and thus cannot be categorized strictly as microbial motifs. Instead, they are produced by the transcriptional machinery of the infected cell using nucleotides of endogenous origin, with critical participation from viral proteins such as RNA-dependent polymerases. However, such motifs cannot be categorized as strictly endogenous either, because their effect is dependent on the nucleotide composition (13), which is, in turn, determined by the genetic structure of the virus.

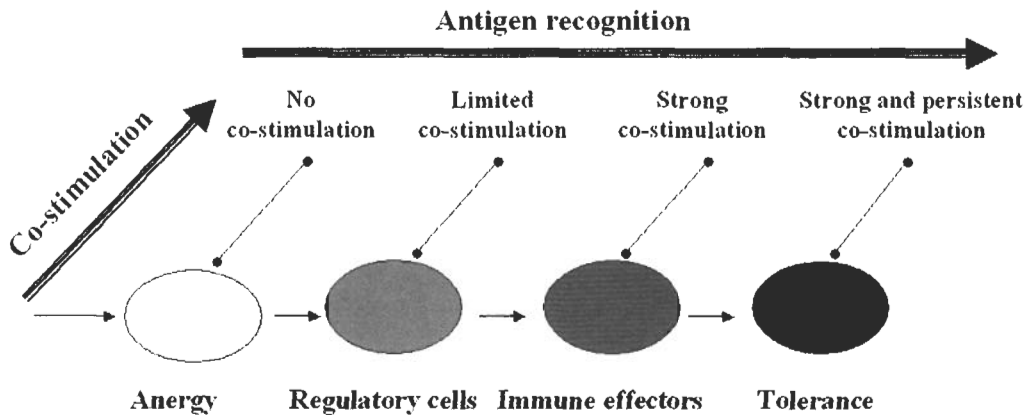


Fig. 2. Two categories of factors influence, essentially, the differentiation of T cells to functional/regulatory subsets. Exposure to antigen (peptides located on specialized MHC) or “cognate” interaction. The strength and persistence of cognate interaction has a decisive influence on whether the antigen-bearing material is largely neglected or effected upon. In addition, the co-stimulatory environment signaling a potential or real pathogenic process, dictates the functional profile of the differentiating T cells. In the presence of reduced “threat”-associated signals reminiscent of saprophytic or “normal” flora, the assumed T-cell profile will be largely regulatory. Upon exposure to pathogens, the T cells assume an effector phenotype. To limit uncontrollable expansion of effectors and thus fulminant immunopathology, excessive stimulation of T cells results in their death (“antigen-induced cell death”). Thus, the ability of T cells to decode different categories of signals is coupled to a complex but versatile functional organization.

Thus, neither the danger model nor the microbial motif-recognition model can accommodate this class of immune regulatory motifs. A unified perspective, as the one depicted in the Fig. 2, could integrate all these aspects if “endogenous,” “exogenous,” and “hybrid” motifs, such as dsRNAs, are all considered in fact “immune stress molecules” (ISM) with the ability to anticipate, prime, or signal pathologic processes associated with infection or other factors.

1.2. Targeting the APC: The Need

A prerequisite for the manipulation of the immune response in an antigen-specific manner is the use of molecules encompassing epitopes that are to be recognized by TCR. A method to circumvent the requirement for antigen processing within APC is the use of peptide epitopes. In addition, compared with recombinant proteins and live vectors, the use of peptides is currently more cost-effective from a production point of view. There is a substantial drawback, however, related to the use of peptides that consists of their unfavorable pharmacokinetics (PK) profile stemming from rapid elimination via the kidneys and/or peptidase-mediated degradation.

The PK profile of peptides, as limiting factor for the loading of APC *in vivo*, is dramatically revealed by comparing the immune responses triggered by injection of a T-cell epitope vs APC loaded *ex vivo* with dose-matched peptide. Whereas the adoptive transfer of mouse I-E^d dendritic cells (DC) pulsed *in vitro* with a Th peptide (homagglutinin [HA] 110-120 of PR8 strain of influenza virus) triggered detectable peptide-specific IL-4 and IL-2-producing T cells, direct injection of dose-matched HA

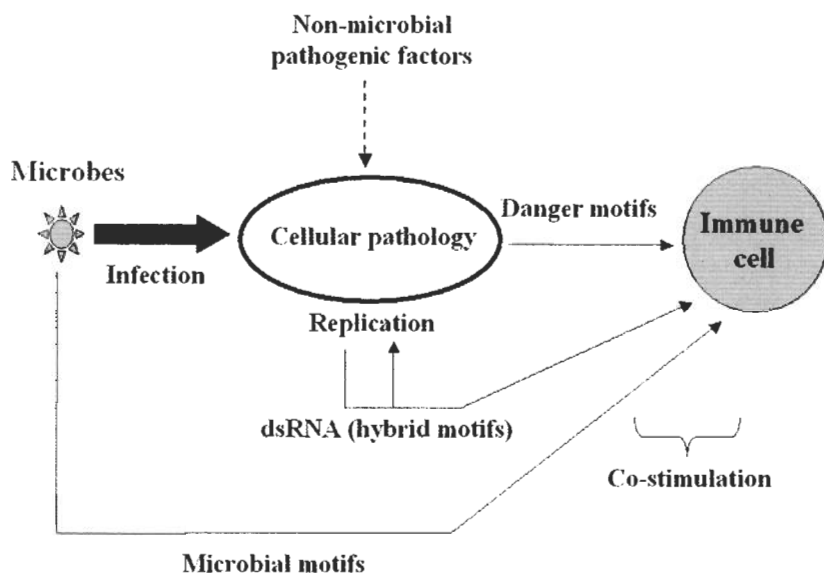


Fig. 3. The immune system decodes different categories of factors associated with potential or ongoing pathogenic processes. This results in the capability to pre-empt or modulate appropriate effector mechanisms. There are essentially three such categories of factors: (1) microbial-associated molecular motifs, present in the structure of the pathogen (such as peptidoglycans, lipopeptides, polysaccharides, endotoxin, certain species of DNAs); (2) mediators of strict endogenous origin (encoded in the host genome), produced subsequent to cellular injury (infectious or noninfectious); “danger motifs” such as $\text{TNF-}\alpha$, and heat-shock proteins; and (3) “hybrid” factors such as dsRNA that are produced by infected cells but encoded in the microbial genome.

peptide failed to prime immunity (Fig. 3). Failure to retrieve substantial numbers of APC loaded *in vivo* with peptide (*see* Section 2) strongly supports this interpretation.

In general, the literature supports the conclusion that whereas peptides are being considered effective agents to manipulate *in vitro* T-cell response, the *in vivo* efficiency in most of the systems is suboptimal, unless methods such as depot formulations or targeted delivery are used. Various targeted delivery strategies have been tested in the past: from microparticles that are able to deliver immunodominant epitopes to APC (14), to targeted administration of peptides into areas rich in APC (15), culminating with the use of recombinant vectors that deliver immunomodulatory peptides to APC, in a manner depending on ligand-receptor interaction (16). In the present chapter, we summarize our experience in preclinical models, with two novel categories of specialized targeting vectors: recombinant immunoglobulins that deliver immune modulatory peptides via $\text{Fc}\gamma\text{Rs}$ on APC and secondly, spray-dried lipid-based microparticles (SDM) bearing ligands for cellular receptors on APC.

2. Targeted Delivery of Epitopes to APC Via Recombinant Immunoglobulins

The main drawback of peptides as potential drugs derives from their poor PK profile. The ability of peptides representing T-cell epitopes to load endogenous MHC molecules depends on whether APC are exposed *in situ* to persisting, high concentra-

tions of peptide. On the other hand, mere prolongation of the half-life of peptides *in vivo* by chemical modification, is likely to interfere with the on/off rate of the peptide-MHC complex and does not necessarily target the epitope to APC. Thus, a molecular-targeting procedure that troubleshoots the drawbacks described earlier, may transform peptides into more effective drugs. Such a strategy should be based on vectors with the following properties:

1. The vector molecule should be biocompatible, in particular devoid of intrinsic immunogenic effects. Thus, it would allow repeated administration of the epitope without build-up in deleterious antivector antibodies. In addition, use of such a vector will be compatible with treatment of inflammatory disorders or, with some modifications in the vaccination strategy (co-administration of adjuvant), could be applied in antimicrobial or tumoral vaccination.
2. The vector should be able to correct the poor PK of peptide epitopes. This depends largely on the molecular weight of the carrier, as well as on the relative resistance to proteolytic degradation.
3. The vector should be able to localize nearby or target preferentially the APC and display reduced affinity for non-APC "somatic" cells. This would optimize the efficiency, limit requirement for large doses, and minimize safety concerns.
4. Finally, such a vector should be able to promote, if possible, more effective loading of MHC on APC, ideally by targeting the epitope in cellular compartments where this process occurs with maximal efficiency.

Based on such considerations, various groups started to consider immunoglobulins as a choice as epitope-delivery vectors (17–19).

2.1. Control of APC Loading With Peptide Epitopes

Use of immunoglobulins to deliver T-cell epitopes to APC requires preservation of the conformation of the carrier, such that neither the half-life nor the Fc γ R-targeting ability of the molecule is altered. To achieve this aim, one could use the complementarity determining regions (CDRs) located within the variable regions of the heavy and light chains for targeted binding to specific antigens. The immunoglobulin molecule is able to accommodate extensive amino acid residue variability in CDRs, without deleterious impact on the functionality of the molecule. The length of CDRs varies, from less than 9 to approx 15 amino acids, with few exceptions. This allows replacement of at least one CDR/Ig chain (20) and in certain cases, of two of them (21) with epitopes, without substantial interference in the assembling of the molecule and its ability to bind to Fc γ Rs, via the constant domains C_H2 and 3 of the heavy chain. In addition, the original binding specificity of the Fab segment is abrogated, which is not, in itself, a drawback.

An alternative engineering strategy is the insertion of epitopes into a C_H (e.g., CH2) (22). This strategy interferes with the functional conformation of the Fc portion of immunoglobulin and may result in the inability to bind Fc γ Rs on APC. This can be compensated by using, as a scaffold, immunoglobulins that bind to receptors on APC, such as MHC-II or Fc γ Rs. The N-terminal end of the heavy chain is another area where T-cell epitopes were introduced (23). Finally, peptides could be chemically coupled to carbohydrate moieties on immunoglobulin, which allows processing-free presentation of peptides by MHC-II molecules on the surface of APC (24).

Several studies evaluated the efficiency of delivering MHC-II-restricted epitopes by IgG, to APC *in vitro*. It was clearly demonstrated, using transformed and primary APC

cell types, that FcγR-mediated internalization of a MHC-II-restricted epitope results in effective processing and presentation to specific T cells. In fact, on a molar basis, IgG-mediated internalization of peptide was a few orders of magnitude more effective in formation of MHC-class II-epitope complexes, as compared to nonengineered peptide (25,26). This results from the more optimal loading of nascent MHC-class II molecules in endolysosomes, where processing of the recombinant immunoglobulin occurs subsequent to FcγR-mediated internalization. However, the enhancement in the generation of MHC-peptide complexes by professional (differentiated or immortalized) APC was 100–1000-fold more substantial—as compared with nonactivated APC harvested from central or peripheral lymphoid organs.

In addition to differential cellular handling, other factors may influence the immunomodulatory potency of peptide epitopes in or out of context of IgG vectors, subsequent to *in vivo* administration. More recently, when T-cell lines or hybridomas specific for defined MHC-II-restricted epitopes were generated and characterized, it became possible to evaluate the *in vivo* APC loading and subsequent generation of MHC-II-peptide complexes.

This allowed us to dissect a complex of factors responsible for the efficiency of epitope presentation subsequent to *in vivo* delivery by using IgG backbone or more conventional means. It was clear that on molar basis, delivery of peptides within IgG vector was roughly 2 orders of magnitude more potent than peptide alone, in forming MHC-peptide complexes *in vivo* when measured indirectly, by the ability of retrieved APC to stimulate, *ex vivo*, a co-stimulation-independent, antigen-specific T-cell clone. One of the major questions was, however, whether elevating the injection dose of nonengineered peptide could restore the potency of IgG-peptide molecule. Even 1000-fold molar excess of peptide in saline could not reach the APC-targeting efficiency of the IgG vector (*see* Table 1), suggesting that multiple factors limit the bioactivity of immune modulatory peptide epitopes *in vivo*, in an absolute fashion. A similar result was recently reported in a slightly different model (27). Because the PK of peptides is negatively influenced by the short half-life (owing to protease-dependent degradation and glomerular filtration) reducing the exposure time of APC, depot formulations may ameliorate the *in vivo* properties of peptide epitopes. As shown in the Table 1, use of oil-in-water depot-type emulsions was not able to parallel the APC-loading efficiency noted with Ig-based vector, although an improvement over peptide in saline was evident in regard to loading of APC in the draining lymph nodes. This modest effect could be explained by the fact that despite prolonging the exposure time of local APC to peptide, the cellular handling itself still remains a limiting factor. Strikingly, parenteral administration (subcutaneous [sc] or intraperitoneal [ip]) of Ig-peptide resulted in effective formation of MHC-peptide complexes on both draining lymph node and splenic APCs, suggesting excellent systemic exposure. The amount of MHC-peptide complexes on thymus-derived APC (expressed as equivalent percent loaded APC) was rather limited in all cases. *Ex vivo* experiments suggested that this may reflect an inherent deficiency of thymic APC in processing and presenting the epitope from this backbone rather than a more limited exposure of central lymphoid organs to antigen. Finally, this type of assay allows the estimation of the *in vivo* persistence of immunogenic/immune modulatory MHC-peptide complexes on APC, which is on the order of 1–2 wk on professional APC such as DC. This obviously has important consequences on the design of product profiles.

Table 1
IgG-Mediated Delivery of MHC-II-Restricted Epitopes: Published Studies

Type of construct	Reference	Nature of data
Peptide epitope inserted within CDR of IgG _H	Zanetti, 1992 (19)	Description of the general concept
	Zaghouani et al., 1993 (17)	More effective handling by APC ex vivo
	Brumeanu et al., 1996 (21)	Immunity to double epitope (T, B)-engineered IgG
Peptide epitope inserted within C _H of IgG	Lunde et al., 1997 (18)	In vitro APC processing
	Lunde et al., 2001 (30)	Targeting via MHC-II and sIgD
Peptide inserted at the N-termini of H chain	Zambidis and Scott, 1996 (23)	Induction of tolerance to foreign T and B epitopes
Peptide epitopes chemically coupled to IgG	Brumeanu et al., 1997 (32); Bona et al., 1998 (16)	APC handling and immune response

More recently, use of antibodies specific for sIgD on B cells or MHC-II on APC, further improved the ability of IgG vectors to deliver MHC-II-restricted epitopes inserted within the C_H region, to APC in vivo (*see* Table 2) (29,30). Such antibodies were orders of magnitude more potent than Fc-FcγR targeted constructs, although additional cumbersome steps of humanizing the construct are required in this case. In addition, it is important to note that in such cases, cross-linking of membrane-bound receptors may lead to effects beyond just epitope-loading of APC. Finally, insertion of peptides at the N terminus of the heavy chain (31) or just chemical coupling onto Ig carbohydrates (32) may allow accommodation of multiple epitopes or larger peptides, as opposed to manipulating just CDR.

In summary, use of advanced immunological techniques allowed the delineation of a matrix of factors responsible for the substantial increase in the efficiency of IgG vectors in targeting APC and resulting in formation of potentially immunogenic MHC-peptide complexes: (1) uptake by FcγR⁺ cells, which are essentially expressed by APC; (2) improved cellular handling owing to receptor-mediated internalization into endolysosomes and avoidance of rapid clearance via the glomerular filter and finally, (3) improved PK owing to protection from protease-mediated degradation. Thus, IgG vectors ameliorate important limiting factors associated with the PK of small peptides that represent T-cell epitopes.

2.2. Manipulation of T-Cell Responses Against MHC-II-Restricted Epitopes

A major question relates to the outcome of epitope expression on APC subsequent to use of various delivery vectors that correct the PK profile of peptides. The usefulness of a vector category is directly proportional to the ability to generate a broad, controllable spectrum of immune responses that could be applied as desired in various clinical circumstances. For example, use of replicating agents as epitope-delivery vectors which unavoidably encompasses or results in generation of "danger motifs," limits their usefulness to vaccination, immunotherapy of chronic infections/diseases, or cancer pur-

Table 2
IgG-Mediated Delivery of MHC-I-Restricted Epitopes: Published Studies

Type of construct	Reference	Nature of data
Peptide epitope inserted within CDR of IgG _H	Zaghouani, 1992 (40)	Ex vivo lysis by CTL of transfected target cells
	Kuzu et al., 1993 (41)	Immunity triggered by transfected cells
	Zaghouani et al., 1993 (39)	Lack of ex vivo presentation by APC to MHC-I restricted T cells
	Billetta et al., 1995 (42)	Immunogenicity of transfected B cells but not Ig-peptide
Polypeptide replacing CH2 and CH3	Wallace et al., 2001 (46)	Targeting via anti-FcγRI and ex vivo processing by APC

poses rather than to autoimmune or inflammatory diseases. In contrast, vectors that allow generation of antiinflammatory effectors and eventually tolerance, could be used in autoimmune diseases. In addition, with minimal modifications, such as the delivery in conjunction with T1-biasing adjuvants, the vector's use could be extended to antimicrobial or tumoral vaccination. In summary, epitope-delivery vectors that allow for the instruction of the resulting immune response are more promising from a commercial perspective.

From this point of view, the concept of IgG as an epitope-delivery vector has raised considerable interest. Such interest is amplified by the recent observations that in certain conditions, engagement of FcγR results in induction of IL-10 production by APC (33). Nevertheless, the *in vivo* Th-cell profile subsequent to use of Ig as delivery vectors was not explored in detail until recently. Our studies showed that injection of BALB/c mice with a recombinant IgG bearing Th- and B-cell influenza-virus epitopes within CDR3 and CDR2 of V_H segment, resulted in induction of T cell epitope-specific proliferative and antibody responses (28). Use of similar constructs to deliver self or self-modified epitopes such as multiple sclerosis (MS)-associated PLP-derived epitopes resulted in suppression of autoimmunity in the experimental allergic encephalomyelitis mouse model (34,35), in a manner dependent upon endogenous production of IL-10 (36). This immediately raised the possibility that formation of MHC-peptide complexes on APC subsequent to IgG-mediated delivery of epitopes, triggers an anti-inflammatory or regulatory immune response that can overcome T1-mediated pathogenic responses. The immune profile of the T-cell response to a foreign MHC-II-restricted epitope (HA 110-120 of PR8 influenza virus) was characterized by ELISPOT analysis and found to comprise IL-4 producing (T2), IL-10 (Tr1), and TGF-β-producing T cells. Subsequent to adoptive transfer of APC subsets *in vivo* loaded with Ig-peptide, it became evident that different types of cells are responsible for different aspects of the T cell profile (Fig. 4). Thus, monocytes were mostly responsible for the induction of IL-4 Th2 and IL-10 Tr1 cells, whereas DC were mostly capable of inducing Th3 cells. The Th1 response, however, was not significant. This observation reinforces that a pro-inflammatory immune response does not necessarily result when the antigen-loading of APC is increased and the nature of the epitope is foreign. Additional factors are

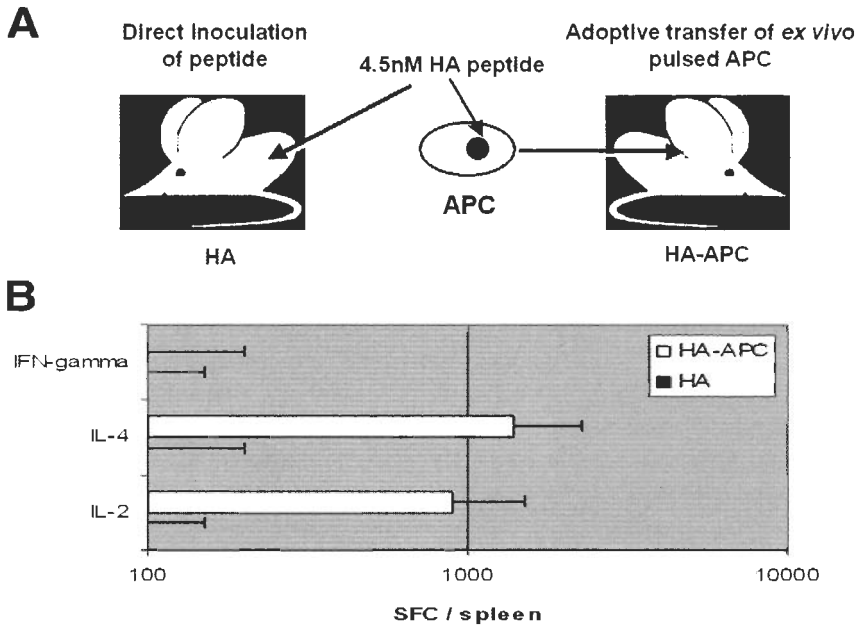


Fig. 4. The PK profile is a limiting factor for immune-modulatory peptides. The diagram describes a simple experiment that supports this conclusion: mice were injected with either 4.5 nM of an immune modulating peptide (HA 110-120, restricted to a mouse MHC-II allele), or alternatively, with APCs pulsed ex vivo with the same amount of peptide (**A**). The immune response developed by syngeneic mice was measured by ELISPOT analysis and represented in (**B**) as the average number/spleen of HA peptide-specific, cytokine-producing T cells (spot-forming cells [SFC]). In contrast to the mice inoculated with ex vivo pulsed APC (HA-APC), the mice injected with dose-matched peptide (HA) failed to develop a significant immune response.

needed, such as factors that stimulate the activity of APC, including IL-12 production. Finally, when the number of peripheral T cells is reduced by, for example, depletion procedures, effective Ig-mediated loading of APC with a Th epitope results in anergy rather than immune deviation (37). This observation raises the possibility that the ratio between the epitope-presenting APC and the specific T cells is critical to the outcome of immune modulation, an observation of potential practicality. Based on these observations, it was not surprising that Ig-mediated delivery of epitopes resulted in induction of immune unresponsiveness to Th- and B-cell epitopes in a distinct model, comprising bacteriophage-derived epitopes (23). Subsequent work from the same laboratory implicated the B cells as likely mediators of this effect (31). Our results demonstrate that obliteration of autoreactive T cells by anergy or peripheral deletion is not a prerequisite for suppression of autoimmunity such as EAE. Thus, multiple administrations of a mixture of IgG vectors bearing two dominant epitopes (MBP- and PLP-derived) into SJL mice with chronic progressive disease resulted in suppression of disease advancement; however, in mice that did not progress to a paralytic stage, the frequency of cytokine-producing autoreactive T cells was actually increased (Fig. 5). A closer look at the T-cell profile revealed T2 immune deviation because lymphocytes

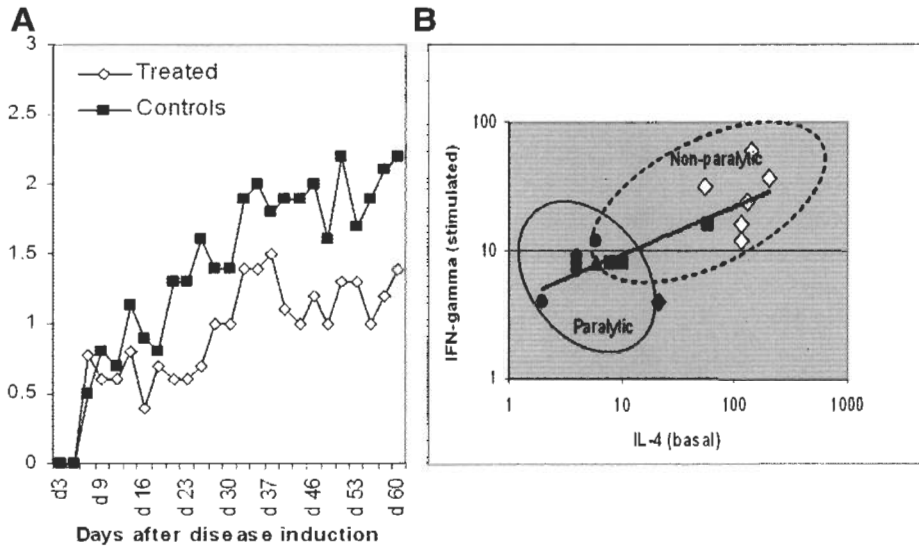


Fig. 5. In vivo epitope targeting of disease-associated epitopes to $\text{Fc}\gamma\text{R}^+$ APC results in suppression of autoimmune disease associated with expansion rather than diminution of autoreactive T-cell pools. Chronic-progressive encephalomyelitis was induced in SJL mice by injection of brain-homogenate in complete Freund's adjuvant. At various intervals after the induction of disease, a mixture of immunoglobulins carrying dominant epitopes (PLP- and MBP-derived) were administered to the mice (four times, at d 9, 14, 19, and 23, by sc injection; $150\text{ }\mu\text{g}/\text{construct}/\text{dose}$). (A) The mean clinical score ($n = 8/\text{group}$) representative for the degree of paralysis, was represented against time. A clinical score above 1.5 is associated with partial or complete paralysis of the hind-limbs. The T-cell reactivity against a major PLP-derived epitope included in the immunotherapeutic regimen was measured by ELISPOT analysis at d 60 after induction of disease (B). On the y axis, we represented the frequency (per million splenocytes) of IFN- γ -producing T cells, upon ex vivo peptide stimulation (the default production was nil). On the x axis, we represented the frequency of IL-4-producing T cell assessed in "default" conditions (incubation with media, without peptide). With diamonds, treated mice; other symbols, control mice. There was a correlation between expansion of cytokine-producing cells and protection from paralysis.

isolated from treated mice produced large amounts of IL-4 in a basal state but little IFN- γ , unless ex vivo stimulated with large concentrations of antigen.

In conclusion, Ig-mediated delivery of Th1 epitopes in vivo does not result in induction of Th1 immunity unless specific biasing adjuvants are used. Instead, depending on the ratio between the number of loaded APC to specific T cells, it results in anergy/tolerance in case of a high APC to T-cell ratio, or induction of Th2, Tr1, and Th3 responses when this particular ratio is more optimal. This creates the possibility of using Ig-mediated delivery of MHC-II-restricted epitopes for the immunotherapy of T1-controlled autoimmune diseases strongly associated with MHC alleles and specific antigens, such as type 1 diabetes and multiple sclerosis.

2.3. Induction of MHC Class I-Restricted Immunity by Targeted Delivery of Peptides

MHC-I-restricted T cells are instrumental in mediating antiviral and antitumoral immunity. In question is whether the incorporation of peptide epitopes within IgG vectors permits induction of MHC-I-restricted T cells. It was previously thought that for effective processing and presentation via MHC-I-restricted pathway, the epitope(s) should be produced inside the cell (e.g., subsequent to infection). During the last few years, however, accumulating evidence pointed to the possibility that in certain differentiation stages, APC can process and present exogenous nonreplicating antigens via the MHC-I-pathway (38). From an evolutionary standpoint, this alternative pathway may have stemmed from the need to complement intracellular immunity leading to rapid apoptosis of infected cells, and/or counteract immune evasion mechanisms deployed by microbes that avoid infection of APC.

Initial animal testing of an immunoglobulin carrying a nucleoprotein (NP)-derived MHC-I-restricted peptide within CDR3 (IgNP) showed that mouse APCs were unable to process the construct and present the peptide *ex vivo*, in contrast to Ig bearing an MHC-II-restricted peptide (39). Transfectomas expressing IgNP were able to process and present the NP-derived epitope (40), which suggested that when fed as protein, IgNP does not access the same cellular compartments accessible in the case of intracellular production and required for MHC-I processing and presentation. In addition, adoptive transfer of IgNP-expressing transfectoma to naïve mice resulted in generation of MHC-I restricted cytotoxic cells (41) with similar results obtained recently in a different model (42). The eventual interference of flanking residues with processing of the MHC-I-epitope was thus ruled out. Overall, the initial assessment was that Ig-mediated delivery of MHC-I-peptide does not result in immune response unless the protein is expressed within the APC, with implications toward gene-expression strategies.

There are two substantial drawbacks of these early experiments. First, the read-out used was conventional cytotoxicity. As more recent studies showed, there is a functional diversity at the level of Tc cells as well, with some of them capable of producing cytokines but largely devoid of conventional cytotoxicity (43). Also, it may be that the ability of APC to process and present MHC-I-restricted peptides depends largely on the type and degree of activation of APC. Indeed, more recently, it was shown that in certain differentiation stages, professional APC acquire the ability to cross-prime CD8⁺ T cells subsequent to effective processing of exogenous, nonreplicating antigen (44). A re-evaluation of the possibility to induce processing and presentation of MHC-I-restricted T cells by targeting nonreplicating agents via FcγR resulted in a few interesting recent studies. One of them (45) demonstrated that immune complexes are able to induce effective cross-priming, in a manner dependent on the engagement of FcγRs bearing activatory ITAM⁺ motifs. In addition, use of bispecific antibodies against an APC receptor and a tumor-associated antigen (Her-2) facilitated *ex vivo* cross-processing and activation of CD8⁺ T cells (46). Finally, in a more related system, engineered anti-FcγRI antibodies bearing MHC-I restricted tumor-associated epitopes within C_H segment were able to induce activation of specific T cells subsequent to *ex vivo* pulsing of APC (*see Table 3*) (47).

One possibility is that cross-linking of FcγR or, in general, activating receptors on APC, triggers activation of APC to a stage compatible with cross-processing. Using Fc-directed targeting of an MHC-I-restricted, viral-derived epitope via a recombinant

Table 3
Particle Engineering for Passive and Active Immunization

Excipients	In vitro dissolution ^a	Passive immunization		Active immunization	
		Peak lung concentration	Anti-viral activity	BA cell targeting	Immune response
DPPC, Ca ²⁺ , Lactose, Tyloxapol	Rapid (<5 min)	81 ± 22 ^b	95× ^c	0.9 ± 0.8 ^d	10–100× ^e
DPPC, Ca ²⁺ , HES	Slow (>15 min)	38 ± 6	2.5×	5.1 ± 0.7	100×

^aStability of the particles in saline, reflected by matrix dissolution and release of actives.

^bThe concentration of active (IgG) in pulmonary interstitial tissue of rodents, subsequent to pulmonary delivery (μg/mL, mean ± SEM).

^cFold decrease of the pulmonary influenza virus titer subsequent to respiratory delivery of specific IgG, compared with control mock-treated animals.

^dPercentage of cells from bronchoalveolar lavage (BA) that internalized IgG-loaded microparticles, subsequent to pulmonary aerosolization (mean ± SEM).

^eMagnitude of anti-IgG immune response subsequent to aerosolization (fold increase, over nonformulated IgG).

IgG bearing the nonself peptide within CDR3, we showed that mere loading of APC with antigen via FcγR results in presentation of the peptide in context of MHC-I. The APCs, thus loaded with antigen *ex vivo*, were able to induce an IL-4 Tc2 response upon adoptive transfer, but not a full-blown Tc1 response usually associated with cytotoxicity (Fig. 6). This may explain the earlier, negative assessment of the immunogenicity of such constructs. Interestingly, additional activation of APC by using a TLR-ligand polyA:polyU (pA:pU) resulted in acquisition of ability of APC to prime IFN-γ Tc1 cells.

Together, these results suggest a previous underestimation of the ability of APC to present MHC-I-restricted peptides from the context of FcγR-targeted IgG. Thus, rather than controlling the presentation, by modulating the degree of activation of APC, one could influence the nature of the effector cells triggered subsequent to epitope recognition (Fig. 7). This has obvious practical implications for the use of IgG as vectors in vaccination and immunotherapy.

3. Targeted Delivery of Immunoglobulin-Engineered Microparticles

One of the most spectacular advances in the area of biotechnology during the last couple of decades was associated with microparticle engineering and delivery of small drugs and macromolecules. If molecular vectors such as IgG can shuttle peptides to APC, microparticles could be used to improve bioavailability, optimize the therapeutic index, or target certain drugs to specific cells. Among those drugs, peptide epitopes and, in general, immune modulators are of considerable interest for vaccination or immunotherapy of inflammatory diseases and cancer. From this perspective, microparticle formulation of peptide epitopes can be viewed as an alternative to the use of molecular vectors, circumventing the need to design, scale-up, and purify expensive recombinant

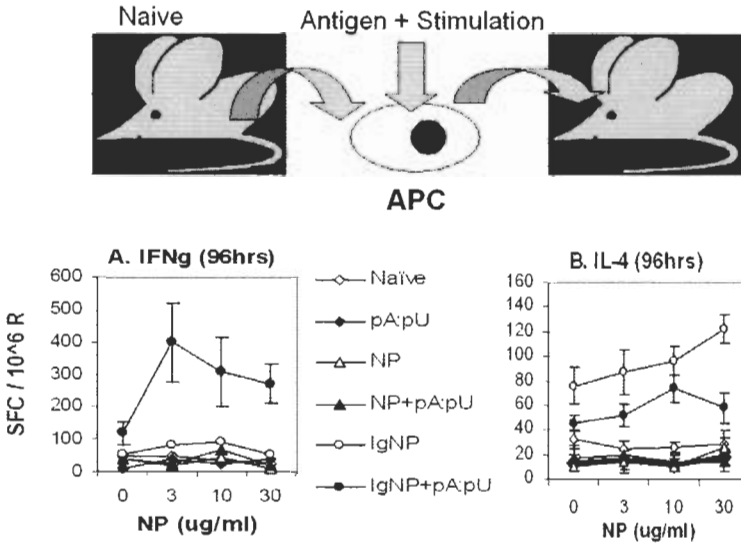


Fig. 6. Differential induction of Tc1 and Tc2 immunity to an MHC-I-restricted epitope delivered via recombinant IgG. The experimental protocol is represented in the top panel. APC were harvested from naïve mice and pulsed ex vivo with antigens (recombinant Ig carrying an influenza NP-derived epitope [IgNP]; or molar dose-matched peptide [NP]) plus or minus immune-stimulating motifs (dsRNA, pA:pU). Subsequently, the APC were adoptively transferred to naïve syngeneic mice and the T-cell response measured by ELISPOT analysis. The results were expressed as average frequency of SFC in spleen, per million responder cells: (A) IFN- γ ⁺ SFC and (B) IL-4⁺ SFC for various concentrations of NP peptide.

molecules. However, there are limitations as well because most microparticle formulations are usually compatible only with mucosal and/or a limited number of parenteral routes (e.g., sc) owing to safety concerns or nonspecific scavenging by the reticuloendothelial system.

Microparticle formulations may optimize the immune modulatory properties of peptides or antigens by one or more of following mechanisms:

1. Creating a depot effect (slow-release) and by this, increasing the time of exposure of local APC to peptides;
2. Resulting supramolecular complexes (peptide-peptide or peptide-matrix-peptide aggregates) that are more effectively dealt with by APC;
3. Facilitating receptor-mediated internalization of ligand-engineered microparticles with their content, into specific cells; and
4. With addition of immune stimulating co-excipients, instruction of magnitude and profile of resulting immune response to the peptide or antigen co-formulated.

A substantial amount of work has been focused on designing biocompatible microformulations, such as liposomes, able to dock onto specific cells in a manner dependent on receptor–ligand interaction. Such vesicles, denominated immunoliposomes (48), were originally designed as a lipid-based structure (mostly phosphatidylethanolamine) co-formulated with IgG monoclonal antibodies (MAbs) coupled to palmitic acid. Studies carried out two decades ago showed that such liposomes were able to dock onto antigen⁺ but not antigen[−] target cells, opening an avenue in microparticle engi-

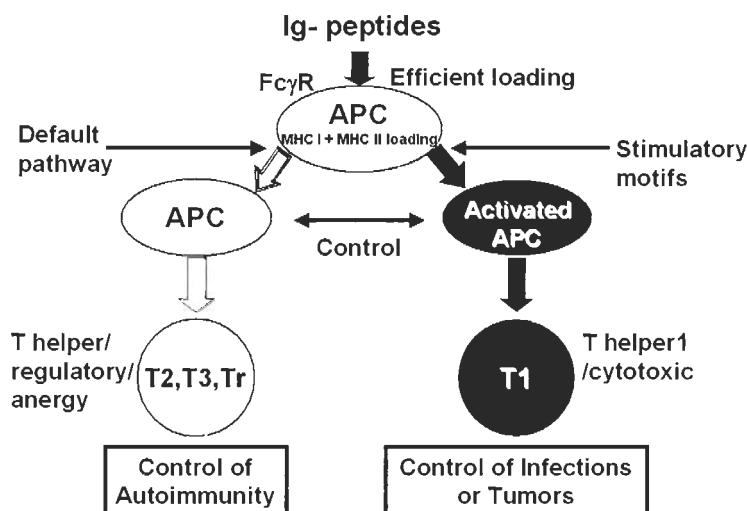


Fig. 7. In vivo manipulation of magnitude and quality of the T-cell response. Effective in vivo loading of APC by Fc γ R-targeted delivery of epitopes is compatible with both MHC-I and -II-restricted presentation. In the absence of immune-stimulating factors, however, the default pathway consists in generation of T2, T3, and Tr responses with potential application in the immunotherapy of autoimmune diseases. In contrast, when the effective loading of APC is paralleled by strong activation resulting in enhanced co-stimulation, the result is differentiation of T1 cells with potential applicability in viral infections and cancer.

neering and drug delivery. Subsequently, it has been shown that antibody-coupled immunoliposomes were effective in mediating specific intracellular delivery of cytotoxic drugs, with a major challenge: the potential inactivation of the drugs in the liposomes (49). Nevertheless, these early observations stimulated more work in the area of immunoliposome-mediated delivery of cytotoxic drugs, with the overall aim of ameliorating the efficacy and safety profile (50).

Interestingly, increasing the number of antibodies per liposome, although resulting in enhanced cellular internalization, diminished the specificity of the immunoliposome (51). Efforts were made, in parallel, to optimize immunoliposome formulations by exploring alternative lipid excipients, carbohydrates as minor excipients and various antibody-anchoring strategies (52). At the level of targeting molecules (the antibody), it was demonstrated that immunoglobulins recognizing foreign, virus-derived antigens on infected cells could be used to specifically target drugs to infected cells (e.g., the herpes simplex virus [HSV] system) (53). More recently, with the understanding that a potential complicating interaction limiting the targeting efficiency of immunoliposomes occurs between the Fc portion of formulated antibodies and Fc γ R on reticuloendothelial cells, effort has been put into designing such particles with single-chain or antibody fragments devoid of Fc (54,55). In contrast to the effort in designing immunoliposomes for delivery of therapeutic or diagnostic compounds to tumor cells expressing a wide variety of receptors (from folate receptors to Her-2/Neu), considerably less work was done in the field of using immunoliposomes for specific targeting of antigens or peptides. A notable exception is represented by studies involving targeting of M cells, critical APC involved in antigen translocation via mucosa (56,57), although in such

cases microbial derived compounds such as cholera toxin subunit B were used as targeting agents. One potential drawback of conventional liposomal technologies is the limited compatibility with mucosal and, in particular, respiratory delivery owing to their nature (suspensions in saline or liquid vehicle).

With the emergence of alternative technologies to create targeted particles, the opportunity to create targeted structures that could be delivered to or via the mucosal area increased considerably. Some efforts in this direction are summarized next.

3.1. The Challenge of Delivering Immunoglobulins by Solid Aerosols

There are two potential methods to target epitopes to mucosal-associated lymphoid cells by the airway route:

1. Delivery of molecules formulated within aerosols, capable of rapidly releasing the payload and allowing specific internalization into specialized cells; or
2. Delivery of metastable aerosols that interact in a receptor-ligand specific manner with target cells and allow the internalization of the payload.

Immunoglobulin delivery to or via the respiratory tract is appealing from the point of view of active or passive local or systemic immunotherapy. Recombinant immunoglobulins could carry disease-associated epitopes for active immunoprophylaxis or therapy, as described earlier. In contrast, passive immunization comprises delivery of specific antibodies, able to recognize microbial-associated antigens or mediators involved in disease processes.

If the aim is to deliver immunoglobulins in a fashion that maximizes the exposure of mucosal-associated lymphoid tissue or, in general, the pulmonary interstitial tissue, then airway delivery may be more effective than systemic administration (e.g., iv injection). In order for this to be accomplished, a formulation technology that preserves the functional structure of antibodies and results in optimal aerodynamic characteristics is warranted. Spray-dried lipid-based microparticles (SDM; major excipient dipalmitoylphosphatidylcholine, DPPC) containing human IgG could be formulated accordingly (58). Bioactive IgG formulated in such particles (SDM), retain their functionality upon in vitro or in vivo release. There are multiple formulation challenges associated with delivery of large molecules such as immunoglobulins: (1) The stability of microparticles: The design needs to be more refractory to water. Inclusion of high molecular-weight excipients such as hydroxyethylstarch or of cationic divalent ions (Ca^{2+} or Mg^{2+}), facilitates this task, (2) The aerodynamic profile: the density of microparticles should be ideally limited. (3) The kinetics of immunoglobulin release: this factor is negatively impacted by making the particles more stable. A slower in vivo release is associated with more substantial expelling of loaded microparticles by mucociliary escalator, early after airway delivery. In addition, internalization by airway phagocytes is increased, owing to interaction of IgG associated with the matrix with the Fc γ R on cells. This limits the amount of immunoglobulin released within airways and able to penetrate into the pulmonary interstitial tissue. Thus, as shown in Fig. 8, there is a trade-off among the structural characteristics of microparticles, important for the delivery of immunoglobulins to or via the respiratory tract. Increasing the kinetics of release to minimize clearance of loaded particles implies making them less stable/aerodynamic and vice versa. Potential strategies to ameliorate this problem are: (1) use of a major excipient that is less prone to scavenging by bronchoalveolar macrophages (such as DPPC); or (2) use of strong surfactant detergents, approved for human use, such as tyloxapol, that promote particle dissolution but do not interfere

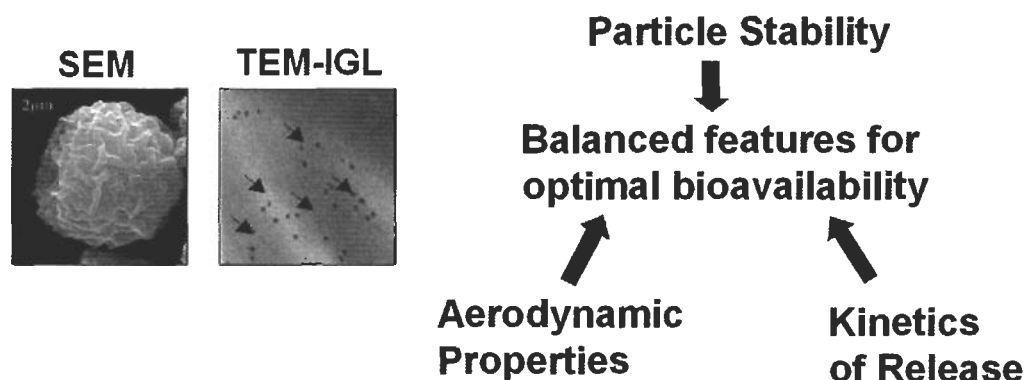


Fig. 8. Engineering microparticles for optimal delivery of macromolecules via the respiratory tract requires achieving a balance among different parameters. The electron micrographs depict spray-dried lipid microparticles (scanning and transmission electron micrographs [SEM, TEM]), loaded with IgG. The samples for TEM were prepared by immune gold-labeling with tagged anti-IgG reagents. In order to maximize the bioavailability of macromolecules delivered by microparticles, the following conditions must be achieved: (1) increased kinetics of release (which limits shelf stability owing to the association with increased hydrophilic nature); and (2) proper aerodynamic properties, allowing delivery to the bronchoalveolar region. Thus, a compromise must be achieved between stability of the particles and the other features required for optimal bioavailability, thus limiting the choice of excipients and engineering technologies that are in fact compatible with the task of microparticle-mediated delivery of macromolecules.

negatively with the stability and aerodynamic parameters. This last possibility may reduce the formation of protein-lipid-protein aggregates that, owing to their inherent immunogenicity, negatively interfere with the efficacy and safety profiles.

A parameter of interest in case of immunoglobulin delivery is the local vs systemic biodistribution. Results obtained in rodent models of aerosol delivery using particles optimized for aerosol delivery of immunoglobulins show that airway delivery achieves peak concentrations of Ig in the pulmonary interstitial tissue that are 50 times higher than those achieved by iv injection. In fact, less than 1% of the immunoglobulin delivered by injection localized in the lung tissue, compared with approx 40% of the immunoglobulin delivered via airways. Interestingly, although intra-airway depot effect is impossible to achieve with this generation of microparticles, the lung interstitial tissue itself facilitates slow-release into the systemic circulation. The peak concentration of Ig is achieved at 2–3 d after airway delivery, with approx 25% of the antibody delivered via airways being translocated slowly into the blood. Thus, the peak concentration of IgG achieved by airway administration is only 1% of that achieved by iv injection, but the slow release from the pulmonary tissue results in prolonged detectable levels. By subtraction, it results that approx 15% of the total amount of immunoglobulin delivered to the airways were cleared within the pulmonary interstitial tissue, where the mucosal lymphoid tissue is located. In addition, pulmonary interstitial tissue is drained via lymph mainly into mediastinal lymphoid nodes. Thus, in conclusion, airway delivery of immunoglobulin targets at least 10 times more efficiently the mucosal-associated lymphoid tissue of the respiratory tract, as compared with conventional intravascular administration (Fig. 9).

These results have implications for active and passive immunotherapy with immunoglobulins aimed for lung-associated molecular targets. There are complex factors influencing the bioavailability of such macromolecules that must be taken into account (some of them specific for Ig) beyond the delivery component. Optimized formulations, compatible with solid aerosol delivery, could achieve significant loading of pulmonary interstitial and lymphoid tissue in a manner that is practical in conditions of permissive airways. For example, if only 50% of a 10-mg dose of a dry-powder formulation containing 70% IgG reaches the bronchoalveolar area, this would result in approx one-half mg of immunoglobulin actually targeted to pulmonary-associated lymphoid tissue.

3.2. Receptor-Mediated Targeting of Spray-Dried Microparticles to APC

An alternative to using recombinant molecules to deliver disease-associated epitopes to APC is to employ microparticles loaded with antigens that are able to deliver the immune modulatory payload to specific cells. The difference is that the targeting agent and the active are brought together in a noncovalent fashion, via a biocompatible matrix. As described in detail earlier, the interest in immunoliposomes was channeled specifically in the area of targeted delivery of cytostatic or cytotoxic compounds to, with some exceptions, M cells, specialized APCs in the mucosal epithelial layer.

A significant practical improvement in microparticle targeting to the respiratory tract is represented by use of solid aerosols in the form of dry powder, obtained by spray drying. If formulated with certain lipids, such as DPPC or Di-Stearoyl Phosphatidyl Choline (DSPC), the dry particles quickly hydrate and transition into structures that may be reminiscent of liposomes *in vivo* (59). Thus, APCs associated with the mucosal respiratory tract could be accessed in a more practical and effective fashion, as opposed to *iv* delivery or airway administration of liposomes in saline. There are three critical parameters that should be taken into account in this context: (1) the intrinsic immunologic properties of excipients that may impact the activity of innate and immune response, as well as the overall outcome; (2) the specific design of particles, to ensure receptor–ligand-mediated targeting; and (3) the function of the targeted APC in the induction/regulation of the immune response.

Microparticle excipients such as DPPC, which are poorly phagocytosed by macrophages and are part of the normal composition of the lung surfactant, are desirable because they allow for enhanced targeting specificity upon additional engineering of the vector, as well as a higher capability of manipulating the immune response via supplementation with immune modulating co-excipients. Even in conditions when the excipients are separately immunologically inert, formulation of antigens in solid aerosols modifies their immune properties. This is owing to the fact that during the formulation process (e.g., spray drying, emulsification, micronization, or lyophilization), there is a certain degree of conformational denaturation, combined with protein–matrix interaction. The immune system is handling microaggregates or nanoaggregates in a different fashion, as opposed to monomeric, dissolved antigen in saline. These aggregates are handled more effectively by specialized APC, with substantial impact on the T-cell response. Our studies using formulations of spray-dried antigens in an animal model demonstrated that even in the case of immune-inert excipients, the response to formulated antigens was elevated as represented by an MHC-II-restricted, T2-controlled increase in antibody titers (60). When T cells were disabled (e.g., in MHC-II or CD3 ϵ deficient animals), the increase in IgG1 response was obliterated. However, the APC were more able to present specific epitopes subsequent to *in vivo* administration

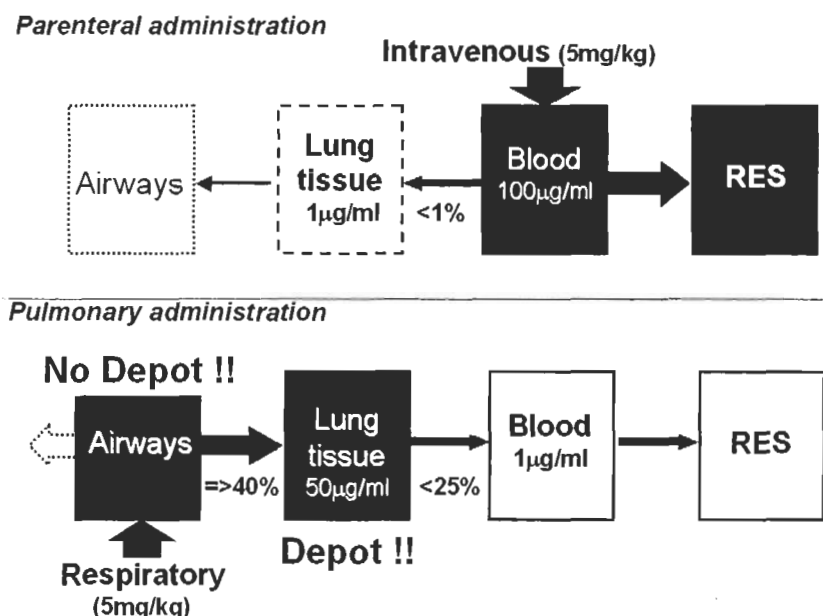


Fig. 9. Optimized technology to deliver IgG to the respiratory tract vs conventional parenteral delivery: differential biodistribution. Upper panel, iv administration of IgG in bolus; lower panel, IgG administration as dry powder (dose-matched). The biodistribution was assessed in rodents. Less than 1% of iv-injected IgG localized in the pulmonary interstitial tissue; most of it is internalized by the reticuloendothelial system (RES) including APC. In contrast, airway delivery of IgG by spray-dried microparticles results in approx 40% of immunoglobulin penetrating into the pulmonary interstitial tissue. There is no depot effect within the airway owing to active macrophage scavenging and mucociliary escalator expelling loaded microparticles. In contrast, there is a pronounced depot effect within the interstitial tissue. Less than 25% of IgG is slowly released into the systemic circulation (peak blood concentration achieved at 2–3 d). The peak IgG concentration in the lungs achieved by airway administration was roughly two orders of magnitude higher than the one resulting from iv injection. The opposite was true for the peak concentration in the blood.

of formulated antigen, supporting the concept that antigen handling is more effective. In addition, the engagement of B cells by formulated antigen was more effective, suggesting exposure of additional epitopes and/or multivalent interaction of protein aggregates with immunoglobulin receptors on lymphocytes (Fig. 10). Overall, in the absence of specific immune-stimulating adjuvants, the default immunity to formulated antigen consists of T2-dependent immunity while co-formulation with T1-driving agents, such as IL-12, reprograms the differentiation of lymphocytes *in vivo* (60). Thus, mere formulation of antigen in spray-dried lipid microparticles, without ligand engineering, results in amplification of immunity to a certain extent and in a given direction.

Specific cell-targeting capabilities of spray-dried microparticles may further amplify the immune properties of antigens, *in vivo*. One limitation in this concern is the rapid reorganization of the dried-lipid excipient subsequent to interaction with the respiratory mucosa. During this process, ligand, antigens and soluble co-excipient could be lost and the targeting ability of the complex diminished. Consequently, it is clear that microparticles designed to achieve higher bioavailability by having more rapid release-kinet-

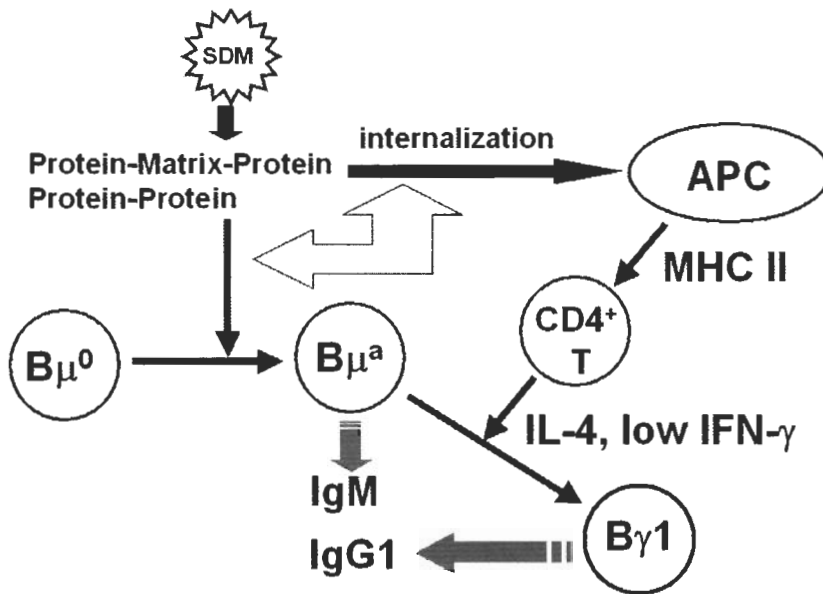


Fig. 10. Mechanisms responsible for the immunogenicity of spray-dried lipid microparticles loaded with antigenic material. Upon interaction with the mucosal environment, protein-protein, and protein-matrix-protein micro and nano-aggregates are released. Such aggregates have dual effects. First, they are more effectively internalized by APC, resulting in more effective processing and presentation of resulting epitopes in context of MHC-II molecules. Secondly, such aggregates interact with B-cells localized in the mucosal-associated respiratory tract, resulting in more effective surface receptor cross-linking and activation of IgM-producing B-cells (from resting $B\mu^0$ to activated $B\mu^a$ stage). The increased activation of $CD4^+$ T2 cells in the absence of immune-stimulating factors (T1-biasing adjuvants) promotes B-cell expansion and differentiation to IgG1-producing cells. This model based on studies carried out in wild-type and genetically manipulated mice, explains the increased immunogenicity of antigens formulated in spray-dried lipid microparticles.

ics profile, are not optimal for cellular targeting (*see* Table 3). In contrast, additional stabilization of the antigen-matrix-ligand structures facilitates a receptor-mediated delivery of the epitopes. The SDM-mediated technology is compatible with the design of microparticles fit for either one or the other of these tasks. As summarized in the Table 3, and previously reported (58–61), more stable, compact SDM structures, incorporating chelating ions and high-molecular-weight carbohydrates, as opposed to low-molecular-weight carbohydrates and surfactant detergents, can facilitate receptor–ligand mediated co-internalization of the ligand, the active, and the matrix into phagocytic cells. Importantly, immunoglobulin-ligand-engineered particles mediated a more effective handling of the antigen (influenza virus-derived) by APC *in vivo*, with a beneficial impact on the overall immune response (61). Strikingly, the APC isolated from the pulmonary interstitial tissue or by bronchoalveolar lavage, were able to stimulate peptide-specific T cells *ex vivo* (61). Finally, targeted delivery of influenza virus antigens remained compatible with induction of antibody responses, suggesting that the increased Th response compensated for the more limited exposure of B cells to antigen.

The ability of the mucosal-associated APC, targeted with antigen, to prime T cells *in vivo*, is a prerequisite for such a delivery strategy to be successful. In contrast to separate molecules, microparticles cannot usually penetrate the airways into the submucosal space, thus at least two contrasting delivery pathways could be exploited: first, as described earlier, rapid-release particles facilitating penetration of individual molecules into the interstitial tissue; or alternatively, ligand-mediated targeting of the microparticles to airway-associated APC such as M cells or bronchoalveolar APC (macrophages or DC). We previously showed that airway APC isolated by lavage can be targeted by IgG-engineered spray-dried microparticles and subsequently display an *ex vivo* priming capability (61). Combined with the increased immunity via such APC-targeted microparticles, our data suggest that airway APC may be involved in the priming of T-cell responses. Supported by other studies (62,63), this may occur owing to retrograde trafficking of microparticle-loaded APC or exocytosis of processed antigens followed by more effective handling by APC associated with the lymphoid structures in the lung. It is difficult to reconcile, however, the previously described immunosuppressive role of bronchoalveolar macrophages (64) with the putative involvement in priming a T-cell response subsequent to microparticle-mediated targeting. Based on these published data, we propose the following, testable model with practical implications in the area of microparticle-mediated delivery of antigens, or immune-modulating peptides. In this model, the default function of airway APC is to maintain immune homeostasis by producing regulatory cytokines such as IL-10, TGF- β , or prostaglandins as well as scavenge immunologically inert particles or saprophytes. The fate of these APC (macrophages mostly) would be clearance via the mucociliary escalator. In contrast, exposure of such airway APC to infection-related agents, or “danger-motifs,” abrogates their immune-suppressor capabilities, which may result in acquisition of the ability to traffic back into the interstitial tissue and further into T-cell dependent areas in the draining lymphoid organs, followed by priming of specific T cells. Thus, in this model, the airway APC are an intricate part of the active mucosal immunity, executing a dual role: maintenance of immune homeostasis and active surveillance/sampling of the extracorporeal environment. If this model proves to be valid, it will extend the known range of cellular immunity from intracorporeal to extracorporeal domain, with potential practical implications.

4. Conclusions

The area of APC targeting is rapidly evolving owing to the need to circumvent the poor PK profile of low molecular-weight peptide epitopes that are otherwise active *in vitro*. In addition, the intrinsic efficiency of immune-modulating peptides and antigens in general, may be enhanced by specific targeting to APC and particularly, subcellular compartments involved in antigen processing. Significant discoveries around the role of epitope-specific T cells in the pathogenesis of major inflammatory disorders, such as diabetes, MS, allergies, and asthma, further increased the interest in novel APC-targeting strategies. The initial interest in using APC targeting as a method to increase immunity to microbial and tumor-associated antigens expanded with the discovery that the resulting immune response could comprise MHC-I in addition to MHC-II-restricted T cells. Finally, a deep understanding of the functional organization of the APCs allows *in vivo* manipulation of the resulting T-cell profile irrespective of the targeting strategy

(e.g., recombinant molecules or microparticles). Thus, APC targeting of peptides or antigens creates the potential for an enhanced impact on immune response with co-administered modulating agents directing the immune profile when necessary.

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