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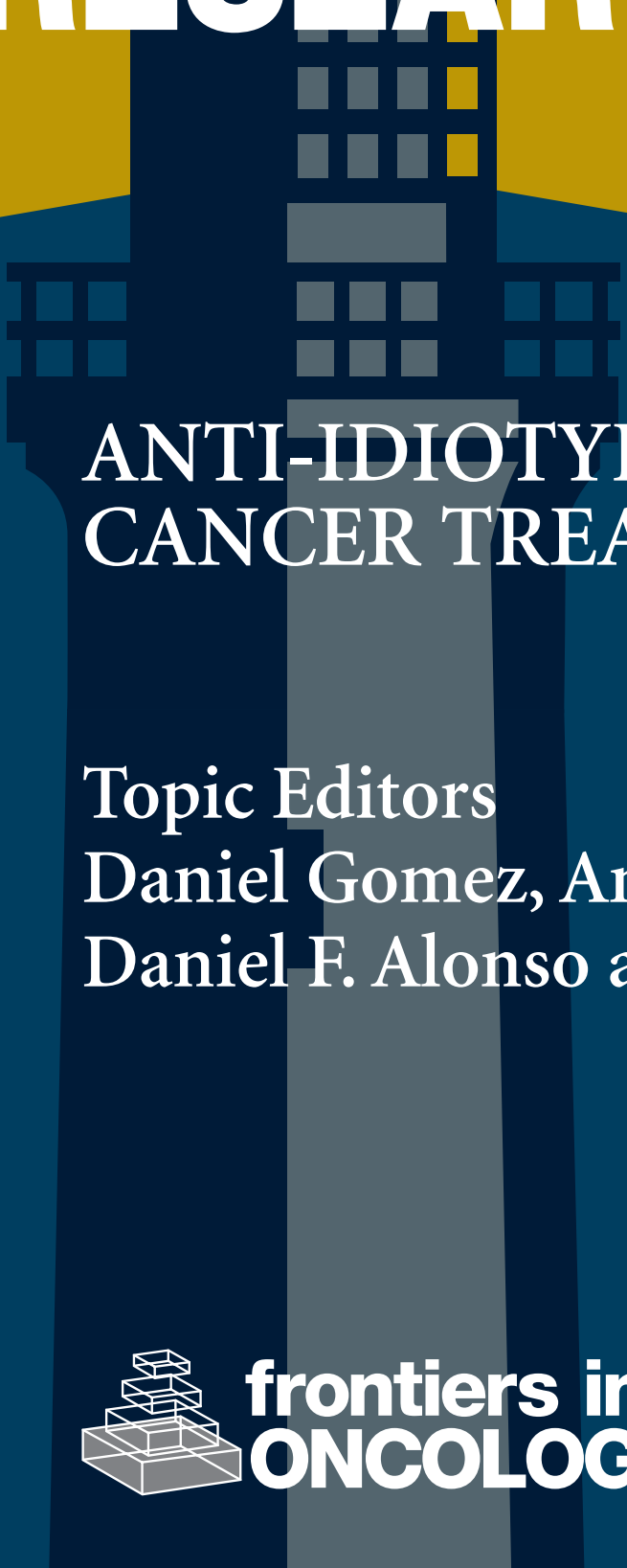

ANTI-IDIOTYPE ANTIBODIES IN CANCER TREATMENT

Topic Editors
Daniel Gomez, Ana María Vázquez,
Daniel F. Alonso and Amparo Macías

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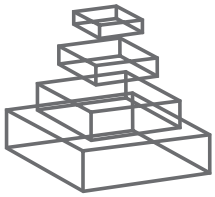

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ANTI-IDIOTYPE ANTIBODIES IN CANCER TREATMENT

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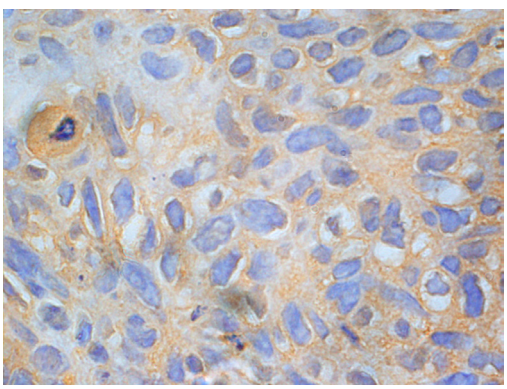

Immunoserological response (on brown) of sera from lung cancer patients treated with the anti-idiotype vaccine Racotumomab. 1000X (Courtesy of Valeria Segatori, MSc.)

Actively induced immunotherapy is one of the most promising fields in cancer research and numerous approaches are being studied to design effective cancer vaccines. Among the treatment strategies to develop an effective immune response against tumor associated antigens is the use of anti idiotype (Ab2) mAb as antigen surrogates. Several studies in animal models have demonstrated the efficacy of these vaccines for triggering the immune system to induce specific and protective immunity against tumors of different origin. In fact, anti-idiotypicmAbs have been used successfully in cancer therapeutics to overcome the poor immunogenicity of some tumor-associated antigens, in particular those of non-protein origin. In some clinical trials, including patients with different tumors, anti-Id specific humoral and/or cellular responses following immunization were associated with improved clinical outcome.

This number of Frontiers is dedicated to anti- idiotypic antibodies and their use in cancer treatment.

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EDITORIAL
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Anti-idiotype antibodies in cancer treatment

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Anti-idiotype antibodies (anti-Id Abs) are antibodies to idiotopes that are located in the variable region, including the antigen binding site, of another antibody. When the last is the case, these anti-Id Abs can act as surrogates of the original antigen. The capability of anti-Id Abs to modulate the immune response has been the basis for the development of anti-Id vaccines against different antigens, including tumor-associated antigens. Over the years, its use in cancer has been demonstrated as effective and promising. This book "Anti-idiotype antibodies in cancer treatment" resumes the latest findings in the field. The book starts with an opinion article by Gomez et al. (2012), whereas the authors discuss a method for prioritization of cancer antigens that paves the way to take more rational, informed decisions in vaccine development. Following, we will find a number of reviews that conform a complete updating on the subject. The first one by Kieber-Emmons et al. (2012) explore the concept of anti-Id Abs with its achievements and drawbacks. Following, Ladjemi (2012) focuses on recent achievements of use of anti-Id Abs as cancer vaccines in solid tumors. López-Requena et al. (2012) focus on the role of anti-Id vaccination in cancer management and on the current developments used to foster anti-idiotypic B and T cell responses. Vázquez et al. (2012a,b) deeply analyze the immunological mechanisms involved in the use of these antibodies, while Vázquez et al. (2012a,b) focus on racotumomab, an anti-Id vaccine already in Phase III clinical trials. Finally, Fredriksen et al. (2012) present a hypothetical model for how the APC-targeted vaccine molecules enhance Id-specific T and B cells. Next, the original article of Segatori et al. (2012) conveys preclinical research on racotumomab with or without chemotherapy, and explores the biological role of N-glycolyl gangiosides in a lung cancer mouse model. Two interesting clinical case studies are also part of this book. First, Llanos et al. (2012) report a maintenance treatment with chemotherapy and immunotherapy in a patient with non-small cell lung cancer. Also, Sampor et al. (2012) present results about the immune response to racotumomab in a child with relapsed neuroblastoma. The book closes with a very interesting article by Gómez and Ardigo (2012), analyzing the pharmaceutical perspective of the development of anti-Id Abs in cancer treatment, with a fresh point of view about the relationship between academy and industry. We as editors were very happy to work with such an excellent group of authors, putting together a book with good quality articles that shed light to the use of anti-Id Abs in cancer. Likewise, we hope it constitutes to the reader interesting material for their fields.

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OPINION ARTICLE
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Cancer antigen prioritization: a road map to work in defining vaccines against specific targets. A point of view

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The use of anti-idiotype antibodies as vaccines to stimulate antitumor immunity is a very promising pathway in the therapy of cancer. A good body of work in animal tumor models have demonstrated the efficacy of anti-Id vaccines in preventing tumor growth and curing mice with established tumors. A number of monoclonal anti-Id antibodies that mimic different human tumor-associated antigens (TAAs) have been developed and tested in the clinic, demonstrating interesting. In general terms, the antigen mimicry by anti-Id antibodies has reflected structural homology in the most of the cases, and amino acid sequence homology in a minority of them. The major challenge of immunotherapy using anti-idiotype vaccines is to identify the optimal anti-idiotype antibody that will function as a true surrogate antigen for a TAA system, and ideally will generate both humoral and cellular immune responses. Several clinical studies have shown enhanced patient's survival when receiving anti-Id vaccines, the true demonstration of efficacy of these vaccines will depend upon the results of several randomized Phase III clinical trials that are currently planned or ongoing (Bhattacharya-Chatterjee et al., 2002).

With the numerous antigens that can be used in immunotherapy the decision making process for researchers, hospitals, and companies, in whether or not invest resources in a specific antigen has been always a very complicated matter both for classic therapeutic vaccines and even more for anti-idiotype vaccines. Fortunately, in a recent work by the National Cancer Institute Translational Research Working Group (Cheever et al., 2009) was developed a method for prioritization of cancer antigens paving the way to take more rational, informed decisions. Such work aimed to develop a priority-ranked list of cancer vaccine target antigens based on predefined and preweigthed objective criteria. An additional aim was testing a new approach for prioritizing translational research opportunities based on an analytic hierarchy process (AHP), a structured technique and mathematical model for dealing with complex decisions. Antigen prioritization involved developing a list of "ideal" cancer antigen criteria/characteristics, assigning relative weights to those criteria using pairwise comparisons. The result of the criteria weighting, in descending order, was as follows: (a) therapeutic function, (b) immunogenicity, (c) role of the antigen in oncogenicity, (d) specificity, (e) expression level and percent of antigen-positive cells, (f) stem cell expression, (g) number of patients with antigen-positive cancers, (h) number of antigen epitopes, and (i) cellular location of antigen expression.

Some authors have enunciated the introduction of potential biases in the National Cancer Institute Pilot Project (Lang et al., 2009). These authors affirmed that the methodology used (AHP), is not well described, and is subject to several sources of possible bias, such as participant selection, number of antigens chosen for prioritization, errors in rank order, redundancy, and internal validity. First of all, we differ with Lang et al. in the fact that AHP is not well described, beyond being a very well known technique properly used in a variety of settings, including cancer clinical decisions (Katsumura et al., 2008; see also Dolan and Iadarola, 2008). Cheever et al. clearly described the method by citing the popular work of Busham and Rai (2004) and how AHP is used in a Web-based tool (Olson et al., 2007). AHP is a powerful tool, used widely in science, and although has had some detractors over the years, Forman and Gass (2010) carried out an in-depth paper discussing and rebutting the academic criticisms of AHP.

Anti-idiotype cancer vaccines development is limited by several factors, including funding concerns. The work of Cheever et al. in our opinion can also be used as a map to develop stronger and faster evidence or in case of failure, deciding when to stop a project. The associated lists of weighted criteria enumerated in Cheever's paper inform investigators as to what experimental evidence is required to advance antigens to higher priority levels, therefore, improving its chance to pass to translational research. Also, the lack of superb data, especially in the therapeutic function, could be multifactorial, including inadequate trial design or patient selection and inadequate vaccine formulation or regimens. These deficiencies can be overcome by more intelligent trial design based on assessment of past "productive failures."

Having that work as a reference, we analyzed the data available for the anti-idiotype vaccine Racotumomab (formerly known as 1E10) and its target antigen, *N*-glycolyl (NGc)-containing gangliosides and particularly NGcGM3 (reviewed in detail by Fernandez et al., 2010). As shown in **Table 1**, the antigen match all criteria considered at least in some proportion, having a cumulative score of 0.62. Interestingly, with this score NGcGM3 ranked within the top 15th cancer antigens selected by Cheever et al., with a score similar to the GD2 ganglioside, the Melan A/MART1 gene product, or the carcinoembrionic antigen.

At the time of Cheever's publication no cancer vaccine was yet approved by FDA. However, recent approval of sipeleucel-T for men with advanced prostate cancer targeting PAP antigen, gave us a valuable lesson on this matter (Bot, 2010). Interestingly, PAP ranked 26 out of 75 antigens in the ranking of cancer antigen pilot prioritization, confirming its capacity to somehow

Table 1 | Relevant characteristics and score for NGcGM3 ganglioside as a cancer antigen, according to the antigen prioritization criteria described by Cheever et al. (2009).

Criteria	Subcriteria for NGcGM3	Score (total weight of criteria)
Therapeutic function	Adequate data, controlled vaccine trial suggestive	0.27 (0.32)
Immunogenicity	T-cell and antibody responses elicited in clinical trials	0.17 (0.17)
Oncogenicity	Increased expression correlated with survival and advanced disease, but oncogenic function need to be clarified	0.03 (0.15)
Specificity	Overexpressed in cancer with little or no expression in normal adult tissues	0.05 (0.15)
Expression level and% positive cells	Highly expressed on most cancer cells in patients designated for treatment	0.02 (0.07)
Stem cell expression	Expression on most cancer cells but without information about putative stem cells	0.01 (0.05)
No. patients with antigen-positive cancers	High level of expression in many patients with a particular tumor type	0.04 (0.04)
No. antigen epitopes	Short antigenic segment with one or few epitopes	0.01 (0.04)
Cellular location of antigen expression	Expressed on the cell surface with little or no circulating antigen	0.02 (0.02)
Cumulative score for NGcGM3		0.62 (1.00)

"forecast" those antigens more likely to be translated to patients. Although the ranking is dynamic, given that priorities change as knowledge accrues from new studies, we must reinforce the idea that the associated lists of weighted criteria inform investigators as to what experimental evidence is required to advance antigens to higher priority levels, and even more, if the antigen of choice is relevant. Always perfectible, those criteria helped us to evaluate that NGcGM3 comprised most if not all the criteria. Therefore NGc-containing gangliosides are antigens worth investing in the acceleration of its translational research.

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REVIEW ARTICLE
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The promise of the anti-idiotype concept

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A basic tenet of antibody-based immunity is their specificity to antigenic determinates from foreign pathogen products to abnormal cellular components such as in cancer. However, an antibody has the potential to bind to more than one determinate, be it an antigen or another antibody. These observations led to the idiotype network theory (INT) to explain immune regulation, which has wax and waned in enthusiasm over the years. A truer measure of the impact of the INT is in terms of the ideas that now form the mainstay of immunological research and whose roots are spawned from the promise of the anti-idiotype concept. Among the applications of the INT is understanding the structural implications of the antibody-mediated network that has the potential for innovation in terms of rational design of reagents with biological, chemical, and pharmaceutical applications that underlies concepts of reverse immunology which is highlighted herein.

Keywords: anti-idiotype, carbohydrate mimetic peptides, idiotype network theory, cancer, tumor, mimotopes, antibody, vaccine design

INTRODUCTION

Scientific concepts can spring up now and then that captures the attention of scientific thought, only to be replaced by new ideas (Bornholdt et al., 2011). However, some concepts remain latent for years waiting to be rediscovered. Interestingly, this phenomenon has been modeled mathematically based on cooperative events in the evolution of ideas. The modeling suggests that systems with high innovation rates tend to contain a high degree of noise, along with many small domains of ideas that are constantly generated and replaced. In contrast, systems with low innovation rates tend to have low noise and a state that remains dominant for a long time until a single event replaces it (Bornholdt et al., 2011). Immunology seems to operate on two gears. At the system level, it is of a low innovation type with just a couple of theories slowly rising and/or falling over its lifetime of about a century. At the level of its bordering with molecular biology and cellular physiology, the avalanche of data spurs a much more intense flow of parallel concepts, e.g., mechanisms of antigen receptor repertoire generation, cytokine networks, suppression, lymphocyte population structure, etc. These are concepts that emerge often in loose relationship to each other as they address different domains of the immune system. Although of local importance, they often become fashionable and temporarily generalized, attempting to explain more than they can. This is due, at least in part, to the big theories having a hard time catching up because of their slower development. One such concept is the idiotype network theory (INT) brought forth by Jerne (1974, 1984). The INT postulates that a population of antibodies forms a hierarchical and dynamic network of interconnected elements that define the regulation of the immune system. The promise of the anti-id concept lies in (1) elucidating the immunological mechanisms associated with the regulation of the immune response, (2) defining how nature developed its own approach to reverse engineering which is applicable to vaccine design, (3) their use as vaccines and immunotherapeutics, and (4) their utility in understanding self-tolerance and control of lymphocyte homeostasis.

THE FOUNDATION OF THE STORY

The basis of the INT is the concept of the idiotype (Id). An Id is a shared characteristic between a group of B cells (immunoglobulin) or T cell receptor (TCR) molecules based upon the antigen binding specificity, and therefore structure of their variable region. The variable region of TCRs and immunoglobulins contain complementarity-determining regions (CDR) with unique amino acid structure that determines the antigen specificity of the receptor. The structure formed by the CDR is known as the idiotope. The term Id is often used to describe the collection of multiple idiotopes, and therefore overall antigen binding capacity, possessed by an antibody. Immunoglobulins or TCRs with a shared idiotope are the same Id. The antibody Id is determined by gene rearrangement, junctional diversity, palindromic nucleotides at sites of single-strand breaks, N-nucleotides, and somatic hypermutations. Inherent to the INT is the relationship between the combining site (paratope) for antigen and the expression of an Id (idiotope).

The Network Theory of Jerne postulates that the immune system functions as a regulatory network that is comprised of Ids (Ab1s) and their anti-Ids (Ab2s) in which B cells and other antigen-presenting cells (APC) provide for antigen processing (**Figure 1**). The inherent relationships of the network hierarchy activate both B and T cells through idiotypic network determinants that mimic the three-dimensional structure of the nominal antigen, and thereby

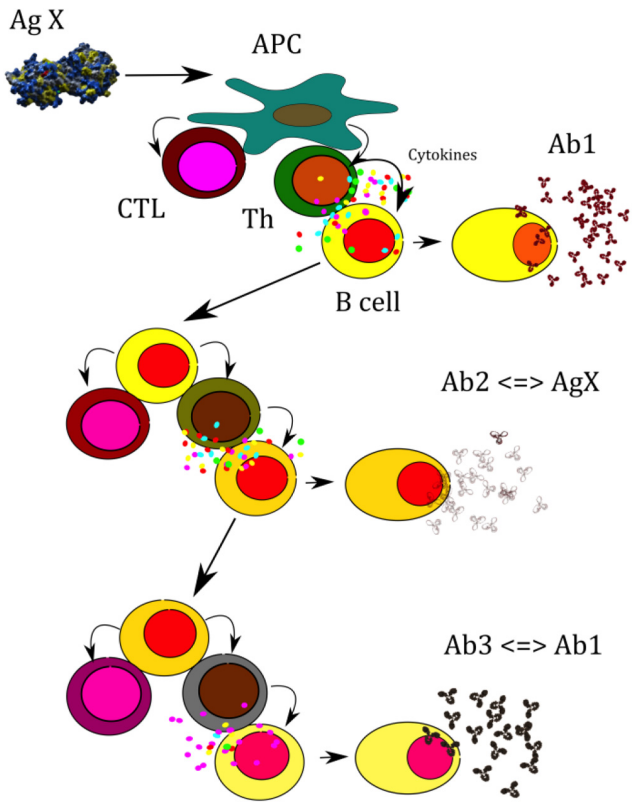

FIGURE 1 | A model for how conventional T–B collaboration could represent a cellular mechanism by which complementary Id$^+$ (Ab1), anti-Id (Ab2), and anti-anti-Id (Ab3) antibodies communicate in a T-cell-dependent manner. A central tenet of the network theory is that the interaction between Id$^+$ and anti-Id immunoglobulin has regulatory consequences in addition to generating cross-reactive immune responses to external antigens. The unification of these ideas is illustrated as adopted from a model presented by (Uner and Galvalchin, 2006). An antigenic determinate X is processed and presented by an antigen-presenting cell (APC) in association with major histocompatibility complex (MHC) class II molecules to an antigen-specific T- helper cell or, in association with class I MHC, to an antigen-specific cytotoxic T cell. Signals from the T-helper cell lead to the activation of B cells that recognize on their own an epitope on X and produce anti-determinate X reactive antibody (Ab1). Anti-X producing B cells, as APCs, present peptides of anti-X (idiotypic Y peptide) in association with class II to idiotype Y-reactive or specific T-helper cell and, in association with class I, to idiotype Y-specific cytotoxic T cells. Signals from the activated idiotype Y- reactive T cells lead to the activation of anti-idiotope Y-producing B cells (Ab2). Likewise, in this context extracellular Id$^+$ immunoglublin (Ab1) is endocytosed and processed by APC, resulting in Id-peptides that are presented on MHC Class molecules to Id-specific CD4$^+$ T cells. Anti-Y producing B cells, as APCs, present idiotypic Y peptide in association with Class II to idiotype Z-reactive or specific T-helper cell and, in association with Class I, to idiotype Z-specific cytotoxic T cells. Signals from the activated idiotype Z-reactive T cells lead to the activation of anti-idiotope Z-producing B cells (Ab3).

activate Ab1 precursors reactive with foreign or self-antigens. They may also be responsible for the stimulation and maintenance of memory T lymphocytes. Thus, MHC-restricted T cells appear to recognize immunoglobulin by the same rules as those that apply to recognition of proteins in general. Antibody Ab1, synthesized in response to a primary antigen, in turn elicits a secondary antibody Ab2. B cell clones recognizing idiotopes on Ab1 in the generation of Ab2s are a heterogeneous population displaying multiple specificities. Sometimes, immunization with Ab2 induces antibodies (Ab3s), which resemble Ab1s as induced by the original or nominal antigen.

This model suggests that conventional T–B collaboration can explain communication between complementary Id$^+$ and anti-Id antibody at the cellular level that integrates present and previous data on B cell regulation by Id-specific T cells (Jacobsen et al., 2010). Ab2 antibodies with antigenic properties have been recognized as two types: one that is the "internal image" binding to the antigen binding CDR, and another that binds close to the antigen binding Ab1 site. The first Ab2 termed by Jerne as Ab2 beta, the later as Ab2 gamma (Kohler et al., 1989). Ab2 alpha is defined as an anti-Id without internal imagery to the native antigen. A fourth kind of anti-Id has been described that binds to a framework region of VH (variable region heavy chain) families (Muller et al., 1991; Wang et al., 1995). This Ab2 is classified as Ab2 delta (Kohler, unpublished data). The role and potential of Ab2 delta in vaccine development is currently being explored. Ab2 alpha or Ab2 delta may exhibit a regulatory effect on the production of antibody bearing the Ab1 Id. **Figure 2** shows a flow diagram of idiotypic interactions and **Table 1** lists the different Ab2s.

The INT is perceived to have had better days and is considered to be a case in the rise and fall of a scientific paradigm in today's mainstay of immunological research (Eichmann, 2008). What initially appeared as an exciting new perspective of the immune system is now viewed as a scientific vagary, and is perceived to be largely abandoned (Eichmann, 2008). Literature searches of select keywords over the last three decades highlight a decline in publications in the respective topic areas except for anti-Id vaccines (**Table 2**). Yet it is this promise that has drawn skepticism, but remains exciting for some because anti-Ids continue to prove their potential in the clinic (Bhattachary-Chatterjee et al., 2000; Bhattacharya-Chatterjee et al., 2001; Maruyama et al., 2000; Li et al., 2002; Ruffini et al., 2005; Lee et al., 2007; Neninger et al., 2007; Ai et al., 2009; Fernandez et al., 2010; Hernandez et al., 2011; Inoges et al., 2011; Ng et al., 2012).

Perhaps the main weakness of the INT is its claim for generality while including a very restricted set of components and interactions in the immune system. With the buildup of data on its complexity, processes, and properties therein like memory, tolerance, and repertoire selection found better explanations in a less exciting, reductionist context. This made the speculations of the INT role in these processes at best overstated. Did it lose credibility altogether? No – only its level of generalization was corrected. So the "innovation" that brought about the paradigm shift did not replace the theory in its domain, but rather, expanded the field showing that there is much more to immune mechanisms than antibodies, B cell clones and their interactions. This is a promise fulfilled.

There are two aspects of the INT that not only survived its fall but even saw a major development becoming its most significant legacy. The first, is the role of the network in immune regulation as an important participant in the assortment of an improved clinical outcome (Abdeen, 2011). This role has been confirmed by the finding that "auto-anti-idiotypic" antibodies against the induced antibody (Ab1) arise during the immune response in

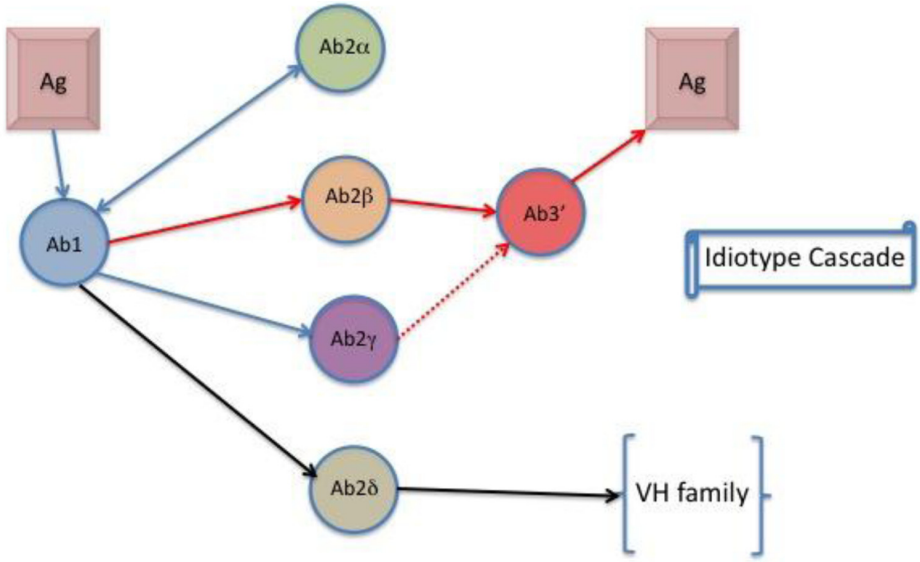

FIGURE 2 | The idiotype cascade. Ab1 binds to anti-idiotypic antibodies in ways associated with Ab2 properties. Ab2δ is a new designation that defines binding to framework regions of Ab2 associated with the Ab2 VH family.

Table 1 | Nomenclature of Ab2s.

Anti-Id class	Property	Reference
Ab2 alpha	Non-binding site idiotope	Jerne (1974)
Ab2 beta	Internal antigen image idiotope	Jerne (1974)
Ab2 gamma	Near antigen binding site idiotope	Kohler et al. (1989)
Ab2 delta	Non-binding site, VH-specific and outbred shared idiotope	Kohler, unpublished

mice (Kluskens and Kohler, 1974; Cosenza, 1976) and in humans (Stefanescu et al., 1993). The second, fundamentally proposes that antibodies can themselves function as surrogates of antigens and immunogens (McNamara et al., 1984; Hernandez et al., 2008, 2011) that redefines anti-Ids as network antigens (Kohler et al., 1989). In short, a promise that has wide sweeping implications is that anti-Ids can function as mimics of ligands and antigens, functioning as surrogates binding competitively to antigen-specific cell receptors. Thus, intrinsically, any antibody can function as a ligand surrogate by its very nature. This realization defines anti-receptor antibodies as surrogate ligands and indicates that this promise of the INT is still in the mainstream of immunological research but has morphed into the notion of approaches to targeted therapy (Goldenberg and Sharkey, 2012).

A major theoretical contribution is also the reassessment of the role of antibody polyspecificity. Molecular mimicry is now firmly considered as the basis of many autoimmune disorders where a foreign antigen shares sequence or structural similarities with self-antigens (Chastain and Miller, 2012; Cusick et al., 2012). Molecular mimicry in this context has typically been characterized at an antibody (B cell) or T cell level. Even if an epitope mimic can support a cross-reactive T or B cell response *in vitro*, its ability to induce an autoimmune disease *in vivo* will depend upon several factors including the appropriate presentation of the mimicry of host antigen expressed on the target tissue. In the case of T cell mimics, the ability to mimic an epitope to induce a proliferative response depends upon engagement of the MHC-peptide complex with the TCR (Davies, 1997). However, few investigators truly realize that peptides can be overlapping B cell and T cell epitopes and or simultaneously involved in the interaction with anti-idiotypic B and T cells, behaving as a regulatory idiotope (Perez et al., 2002). On the other hand, the different principles of repertoire selection makes T- and B-cell antigen receptors independent in their polyspecificity (Wucherpfennig et al., 2007) and, generally, only one of the epitopes will be triggering the process of mimicry but will recruit the help of the other because of mimicry. Indeed, the promise of understanding regulation is evident.

Despite that pathogen-associated animal models were often used to validate vaccination with anti-Ids, anti-Id vaccination has made it to the clinic for cancer. A number of monoclonal antibodies that mimic distinct human tumor-associated antigens as well as Id vaccines have demonstrated encouraging results in clinical studies for solid tumors (Bhattachary-Chatterjee et al., 2000; Bhattacharya-Chatterjee et al., 2001; Maruyama et al., 2000; Ruffini et al., 2005; Lee et al., 2007; Neninger et al., 2007; Fernandez et al., 2010; Hernandez et al., 2011; Ng et al., 2012). While the theoretical hypothesis is sound, trials have been limited and have not been tested prospectively. Several studies have provided proof-of-principle of biological efficacy of these vaccine types, clinical efficacy, and even clinical benefit in small studies conducted in humans (Bendandi, 2009). However, several randomized clinical trials have failed to achieve their main endpoints for reasons that might be unrelated to a vaccine (Bendandi, 2009; de Cerio and Inoges, 2009). While skepticism toward this type of approach is mounting, better-designed clinical trials might

Table 2 | Distribution of publications based upon keywords associated with the network theory.

PubMed keyword search	["1980/01/01" (date – publication): "1990/12/31" (date – publication)]	["1991/01/01" (date – publication): "2000/12/31" (date – publication)]	["2001/01/01" (date – publication): "2012/8/28" (date – publication)]
Idiotypic network	254	249	105
Idiotypic network theory	32	19	17
Anti-idiotype antibodies	4229	3326	2794
Anti-idiotypes	180	100	29
Anti-idiotypic vaccine	169	268	292
Idiotype	3432	1596	758
Regulatory idiotype	411	65	40
Idiotope	262	158	27
Idiotypy	62	14	2

prove to show efficacy for vaccinations that emphasizes idiotypy (Bendandi, 2009; de Cerio and Inoges, 2009). Still, the greatest challenge of immunotherapy by means of antibody-based vaccines, especially in the context of solid tumor therapy, is to identify the antibody that will function as a true surrogate-antigen generating both humoral and cellular immune responses to tumor cells.

It may seem worth mentioning here a recently debated aspect of immunoglobulin immunogenicity. The description of immunodominant regulatory T cell epitopes in the constant part of the immunoglobulin molecule (De Groot et al., 2008; Cousens et al., 2012) seems to add a balancing force to the immunogenic variable region with its T and B cell idiotopes. The net effect, though, should counterbalance the particular idiotopes not with the self-epitope found on the injected Ab2 molecule but with the combined amount of the identical constant region epitopes of the total endogenous immunoglobulin. For that matter, one can actually argue for a role of (and competition with) all immunodominant self-epitopes continuously presented. Obviously, a relatively negligible amount of immunoglobulin injected as an immunogen would hardly change the repertoire of self-epitopes presented and the "Tregitope" concept changes nothing in this case.

BIOLOGICAL VERSUS CHEMICAL MIMICRY

Lately, emphasis is being placed on using neutralizing antibodies as templates to reverse engineer immunogens by working back to reconstruct the neutralizing epitope by structure-based design technology with the intent to induce neutralizing antibodies by the mimicking immunogen (Sattentau and McMichael, 2010; Bundle et al., 2012; Lipinski et al., 2012). Id/Anti interactions are nature's own approach to reverse engineering. Antigen binding of antibodies is mediated by atomic interactions within complementary surfaces between antibodies (paratope) and antigens/determinants (epitope). The interaction between antibodies and antigenic determinants is determined not necessarily by a primary chemical structure, but by the stereochemistry of the antigen. Reviews over the years have discussed idiotypic relationships from a structural viewpoint (Kieber-Emmons et al., 1986; Kieber-Emmons et al., 1987a; Poljak, 1994). In most cases, Ids are associated, fully or entirely with the CDR of antibodies indicating that a binding site is both recognizing and being recognized. Antibodies are known to cross-react with non-structurally related molecules, and, paradoxically, the distinction between paratope and idiotope is not straightforward because mimicry is attributed to the CDRs of Ab2s and requires adjustment for the development of a rationale for immunomodulatory approaches using antibodies as immunogens (Kohler et al., 1989). But why should this interplay be structurally determined?

One line of evidence that suggests chemical mimicry can regulate biological mimicry stems from sequence analysis of the anti-Ids F6 and 4C11, which were defined as anti-Ids for phosphorylcholine (PC; Huang et al., 1988). Sequence and structure analysis suggested that the CDR2 of 4C11 was unique, displaying a positive and negative charge distribution that mimics the charge distribution of the PC head group that defines a correlate with function and structure (Kieber-Emmons et al., 1987b; Cheng et al., 1988). In fact peptides that mimicked PC were shown to compete with PC for antigen binding using a reverse engineering approach (Kieber-Emmons et al., 1987b). The crystal structure of anti-idiotypic monoclonal antibody 409.5.3 and its idiotypic Fab fragment complex (a feline infectious peritonitis virus neutralizing antibody) also illustrates the manner in which two Fabs interact by direct placement of their complementary CDRs (Ban et al., 1994). Analysis of the Ab1–Ab2 interface reveals that there is high degree of structural and chemical complementarity between the two as is observed in other antigen-antibody complexes.

Superimposed on structural complementarity though is the idea that antibodies are polyspecific. Polyspecificity is a basis for functional mimicry. Unlike the classical notion in the sense of a signal, switched from the legitimate target to an inappropriate self-target, here the context is rather of converging signals coming from a class of ligands carrying a common biological meaning (Cohn, 2008). For example, a common cross-reactivity between carbohydrates and intracellular hydrophobic determinants, points to a possible biological function of carbohydrate/protein mimicry (unpublished observations). It may overlap structural markers of dangerous change of the internal environment. Anti-Ids that mimic carbohydrate antigens are abound in description. The

premise for antigenic mimicry is thought to rely on the presentation of reactive groups such as hydroxyl groups that topologically correspond between the antigen and the anti-Id based on hydrogen bonds, which may or may not be supplemented by hydrophobic interactions.

Stereochemical similarity may determine an immunochemical likeness even between molecules belonging to different classes of compounds, for example, between peptides and polysaccharides (Luo et al., 2000; Cunto-Amesty et al., 2001a; Monzavi-Karbassi et al., 2007). Let us consider an antibody and an antigen it is specific for. Almost always other structures can be found that bind to the antibody, competing for the antigen. When such cross-reactive determinants also induce antibodies cross-reactive with that same antigen, they are defined operationally as mimics of that same antigen. This algorithm for defining mimics does not necessitate complete structural identity between the molecular interfaces in the antibody/antigen and the antibody/mimic complexes. Furthermore, due to the very nature of mimicry as an instance of antibody polyspecificity, it seems intuitively obvious that, in general, different mimics bind to the nominal antibody with diverse footprints. Thus, anti-Ids need not display an exact structural correspondence with the nominal antigen, let alone different stereochemical aspects from which the same epitope can be recognized by different antibodies (Luo et al., 2000; Cunto-Amesty et al., 2001a; James and Tawfik, 2003; Pashov et al., 2005; Monzavi-Karbassi et al., 2007; Talavera et al., 2009). Although this is the general principle, there are anecdotal examples of mimicry with a very high structural fidelity, especially in Id-anti-Id systems, proving that it is not only possible but also of not too low probability.

STRUCTURAL CHARACTERIZATION OF ANTIBODY RECOGNITION

The three-dimensional structures of several anti-Id antibodies, either alone or in complex with an idiotope have been determined (Garcia et al., 1989; Bentley et al., 1990; Ban et al., 1994, 1996; Evans et al., 1994; Chang et al., 2005). The first insight into the structural aspects of anti-idiotypic antibodies comes from Ab1 (FvD1.3), Ab3 (Fv E5.2) antibodies in the lysozyme system (Fields et al., 1995; Braden et al., 1996). The study showed that the Id-anti-Id interaction involves all six CDRs of each molecule although the interaction between E5.2 HCDR1 and D1.3 is achieved only through bridging water molecules. In the D1.3 complexes with the lysozymes, the conformation of D1.3 LCDR3 in complex with E5.2 is dependent on the electrostatic nature of the residue in contact with the L3 backbone.

The anti-Id antibody mimics the lysozyme by a strong topological similarity in hydrophilic interactions and by making a comparable number of Van der Waals contacts to the combining site of the Ab1. This mimicry is well exemplified by the patterns of hydrogen bonding: six of the 14 protein–protein interface hydrogen bonds in the Ab1-anti-Id Ab2 complex are superimposable with hydrogen bonds in the Ab1-lysozyme interface, suggesting fidelity in hydrogen bonding as a basis for cross-reactivity and mimicry. Not perfect fidelity but close. Interestingly, the solvent structure of the Id-anti-Id antibody complex is observed to contribute to the mimicry of the lysozyme in the context of recognition.

The mimicry of E5.2 for lysozyme does not however extend to the topology of the non-polar surfaces of E5.2 and lysozyme, which are in contact with D1.3 as revealed by a quantitative analysis of the contacting surface similarities between E5.2 and lysozyme. It was concluded that the anti-idiotypic antibody E5.2 mimics lysozyme in its binding interactions with D1.3. Validating these observations, E5.2, used as an immunogen, induces an anti-lysozyme response (Fields et al., 1995). This observation underlies an essential part of the INT; that hydrogen bonding is an important element that provides directionality to defining the degree of fidelity which is often overlooked in deciphering and discussing the structural basis for mimicry.

SEQUENCE RELATIONSHIPS THAT DEFINE MIMICRY

Bruck et al. (1986) described one of the earliest observations of shared sequence homology between antibodies and antigens. Importantly, this system also taught, in a series of papers, how to develop peptides and small molecules from antibody structure (Williams et al., 1988, 1989, 1990, 1991; Kieber-Emmons et al., 1997). Even before the work of Bruck et al., insight as to the sequence relationships between antigens and antibodies was emerging (Vasta et al., 1984). To estimate the minimal structural requirements for cross-reaction of idiotypic determinants, Vasta et al. (1984) determined the capacity of monoclonal antibodies specific for the Id of the PC-binding myeloma protein TEPC-15 for cross-reactivities with the PC-binding, acute-phase protein C-reactive protein (CRP), and the hemagglutinin from the horseshoe crab *Limulus polyphemus* (limulin), which binds sialic acid and PC. Human CRP displays calcium-dependent binding to a variety of autologous and extrinsic ligands, and aggregates or precipitates, but which binds with highest affinity to PC residues. Neither CRP nor limulin showed significant overall sequence homology to vertebrate immunoglobulins.

However, CRP, limulin, and TEPC-15 VH shared short stretches of homology (8–10 amino acids) that mapped to a stretch comprised of the CDR2 and third framework region of the TEPC-15 VH. These results suggested either evolutionary convergence forced upon molecules of diverse evolutionary histories because of steric requirements of binding the same ligand, or a conservation of primitive combining site gene segments in evolution. Further studies showed that CRP displays the same idiotope as an antibody that shares its specificity for PC (Swanson et al., 1991), whereby the shared epitope on TEPC15 and CRP was composed of similar charged residues. The mechanism by which one molecule has evolved, or was obtained by chance, similar amino acid sequences or the homologous three-dimensional crystal structure of immunodominant epitopes remains a mystery.

B CELL INTERACTIONS

The *in vivo* and *in vitro* results involving anti-Ids and PC hapten suggest that idiotope antigens can function like nominal antigens to induce antigen-specific B cell responses, providing a mechanistic view for priming and boosting primed B cells (Huang et al., 1986). The mechanisms of thymic-dependent B cell activation induced by idiotope and nominal antigen are similar in that MHC-restricted T and B cells interactions require cognate recognition (Huang et al., 1986). An important take home message was

suggested that the combined use of idiotope and nominal antigens in an immunization protocol might provide the maximal protective immunity. This translates the primary observation embedded in anti-Id vaccination protocols into the present day use of diversified prime and boost strategies to enhance anti-tumor immunity (Grosenbach et al., 2001; Monzavi-Karbassi et al., 2003; Nolz and Harty, 2011).

Mimicry is a powerful concept to develop tools for delineation of the mechanisms whereby antigens affect lymphocyte function (Weissberger et al., 1983; Shenk et al., 1984). Antibody specificity is determined by a limited number of residues. This fact has prompted the synthesis of small peptides based on CDR sequences, which retain binding properties and functions of the intact antibody. Studies also suggest that peptides derived from CDRs may act likewise effectors of the innate and adaptive immune response opening a new scenario about their interplay with the cellular immune response (Westerink et al., 1995; Gabrielli et al., 2009).

Because B1 cells can strongly activate T cells and induce T helper type 1 (Th1) cell differentiation in the context of antigen presentation, we have been testing how carbohydrate mimetic peptides (CMPs) mediate T cell responses. We have shown that immunization of mice with a CMP reactive with anti-GD2antibodies (GD2 is a tumor antigen expressed typically on cells of neuronal origin), induce GD2 reactive IgM antibodies (Wondimu et al., 2008). This CMP also induces a DTH response to GD2-positive D142.34 cells, while no response was observed against the GD2-negative expressing cell line B78.H1. The anti-GD2 IgM induced by CMP plays the role of an initiating factor for a DTH response perpetuated by T cells cross-reactive with CMP and an unknown antigen on the tumor cells line, which have been stimulated during the priming with CMP. This observation suggest that the dual character of a CMP carrying a T cell epitope but also mimicking unrelated carbohydrate epitope, provides for long-term IgM responses by promoting other aspects of cooperation between particular B cell subpopulations and CMP-specific T cells (Cunto-Amesty et al., 2003).

We further demonstrated that CMPs direct the generation of tumor-associated carbohydrate antigens (TACA) reactive antibodies in immune deficient *Xid* mice that generally fail to respond to T independent antigens (Cunto-Amesty et al., 2001c). Depending on formulation, CMPs can target repertoire compartments inaccessible to native TACA in these mice. Therefore, we hypothesize that CMPs, peptides derived from anti-Id CDRs and anti-Ids can stimulate B cell compartments and activate effector cells that bridge innate and adaptive immunity (Pashov et al., 2010).

TARGETING B CELL IDIOTYPES AS A SPECIAL CASE

B cell malignancy is usually derived from a single expanded B cell clone, which expresses an immunoglobulin with a unique Id (Shaffer et al., 2002). Therefore, anti-B cell antibodies targeting Ids are especially useful to probe the biology of B cell malignancies. The use of anti-B cell antibodies targeting Ids is also a model for therapeutic modality targeting receptors because the expression and signaling of the membrane bound immunoglobulin constituting the B cell receptor (BCR) is critical for cell survival and proliferation (Choi and Kipps, 2012; Kenkre and Kahl, 2012). Anti-Ids function as anti-receptor antibodies in this case whereby they directly recognize the tumor-associated Id (antigen) to mediate both antibody-dependent cellular cytotoxicity and signaling-induced cell death (Tutt et al., 1998). Lymphoma has been the model for the clinical utility of "anti-Id" therapy (Hsu et al., 1997; Ruffini et al., 2002), serving as a tumor-specific antigen for therapeutic vaccine development. Immunization of lymphoma patients with their own tumors generates humoral and cellular immune responses to their lymphomas (Neelapu et al., 2006). However, the clinical impact of an Id directed immune response is still under evaluation (Mahaseth et al., 2011) and often reviewed (Bendandi, 2009; Brody et al., 2011; Inoges et al., 2011; Hollander, 2012).

Structurally, tumor-specific Id might be considered in the context of a privileged target for vaccine therapy. The main goal of any biological therapy of tumors is the selectivity of the agent used with immunotherapy representing the protypical approach. In the case of lymphoma, the tumor-specific antigen is the unique variable region of the immunoglobulin produced by the malignant clone and anti-Ids use is based on their ability to detect highly restricted or "private" Ids therein. "Private" Ids are speculated to be associated with CDR regions while so-called "public" Ids might be related to framework residues. Such "private" Ids are reflected in somatic mutation, which might be relatively unique to an individual. Consequently, a major obstacle in production of Id vaccines derives from its patient-specific nature that requires the generation of a custom-made product. Anti-Id/Id interactions are also known to be mediated by framework residues (Brown et al., 1991), suggesting that framework residues affect Id expression (Corti et al., 1994) as originally proposed (Kieber-Emmons and Kohler, 1986; Kieber-Emmons et al., 1987a). However, because the focus is on developing both humoral and cellular immune responses to B cell Ids, CDRs are more likely targets for T cells. T cell lines generated from lymphoma patients actively immunized with Id protein were shown to specifically recognize CDR-derived peptides (Baskar et al., 2004). Synthetic peptides corresponding to hypervariable regions of immunoglobulin heavy chain have been described to be specifically stimulated by $CD4^+$ and $CD8^+$ T cells to proliferate and secrete proinflammatory cytokines in an MHC-associated manner (Baskar et al., 2004).

However, the plasticity of the BCR repertoire and the structural similarities among BCR and TCR allow antibodies to effectively mimic TCR binding to MHC (Polakova et al., 2000). Because a large number of HLA-binding idiotypic peptides can be identified among antibody hypervariable sequences, such peptides may spontaneously induce a type I MHC class I- as well as class II-restricted memory T cell response (Hansson et al., 2003). Early studies suggested that antigen-binding receptors on T lymphocytes and IgG antibodies with the same antigen-binding specificity as the TCRs display shared or identical Ids (Binz and Wigzell, 1975). Such shared Id might be associated with framework residues. While some reports associate CTL responses to framework residues (Trojan et al., 2000; Gricks and Gribben, 2003) framework peptides might play a more fundamental role in regulation in which Tregs induced by a shared Id epitope can systemically suppress T cell responses against Id-derived and immunodominant foreign epitopes *in vivo* (Warncke et al., 2011).

T CELL INTERACTIONS

In addition to inducing antibodies, Ids/anti-Ids also induce cellular responses. Such studies suggest that T cells need to be integrated into idiotypic regulation (Jacobsen et al., 2010). The ability to prime T cells derived from normal HLA-matched donors, rather than patients, may have direct application to current strategies, designed to generate allogeneic tumor-specific T cells for adoptive transfer (Weng et al., 2011a,b). MHC-restricted T cells appear to recognize immunoglobulins by the same rules as those that apply to recognition of proteins in general (Eyerman et al., 1996). In this context it is easy to rationalize that an Id^+ B cell presents Id peptides to Id-specific T cells. It follows that an Id^+ B cell primarily will be regulated by a limited set of T cells specific for highly expressed germ-line (maybe) Id^- and to a lesser extent by a diverse set of T cells specific for a multitude of Id-peptides derived from somatically mutated (maybe) anti-Id Ab. As for the anti-Id B cell, the converse is expected to hold true. Thus, complementary Id^+ B cells and anti-Id B cells are anticipated to be regulated by partly overlapping sets of Id-specific T cells whose Id-peptide/MHC class II ligands are expressed to different levels by the two complementary B cells (Jacobsen et al., 2010). More importantly, observations that T cells are activated by Id peptides associated within the CDRs imply that T and B cell epitopes do overlap and such peptides function as regulatory (Perez et al., 2002). Id-specific T cell clones can recognize and respond to idiotypic determinants on B cells (Eyerman et al., 1996; Osterroth et al., 2000; Wen et al., 2001). Id-reactive T cells are MHC-restricted and recognize idiotypic determinants in the form of peptide fragments in the context of MHC class II molecules presented on APCs. This type of binding suggests that a conformational Id was processed and presented to T cells in a manner that maintained its structure. It is known for sometime that the overlapping topology of T and B cell epitopes within synthetic peptides does not necessarily impair B cell immunogenicity (Harris et al., 1996).

In terms of autoimmunity, molecular mimicry is defined as the theoretical possibility that sequence similarities between foreign and self-peptides are sufficient to result in the cross-activation of autoreactive T and B cells (Ang et al., 2004). Apart from Ab2s inducing antibodies, there is evidence for differences among the Ab1-Ab2-Ab3 cascade induced by protective and non-protective anti-Id attributed to cellular responses (Raychaudhuri et al., 1990). Among various anti-Ids typed serologically as an internal image Ab2 of the mouse mammary tumor virus tumor-associated antigen gp52, only one induced protective immunity and was effective in immunotherapy. The DNA sequence of the variable regions of six anti-Ids was determined. Search for amino acid sequence homologies between the Ab2s and gp52 showed the strongest similarities in sequence in the CDR2 of the light chain for the protective Ab2 with a T cell epitope on gp52. This finding was the first to raise the question of where the short peptides, which carry T cell-defined epitopes, are located and their relationship with the tumor antigen.

In more recent studies, Ab2s with known amino acid sequence displayed similarity with peptides from a corresponding tumor antigen (carcinoembryonic antigen, CD55, and human high molecular weight melanoma-associated antigen), but differed from the tumor antigen peptides by the presence of side chains known to mediate stronger binding with MHC (Spendlove et al., 2000; Kawano et al., 2005; Ullenhag et al., 2008). In particular in the CD55 system amino acid homology was identified between three CDRs of the anti-id and three regions of CD55 (Spendlove et al., 2000). Anti-anti-idiotypic (Ab3) polyclonal antibodies raised against the Ab2 showed specific binding to these peptides. The antibodies were also found to bind synergistically to combinations of these peptides, indicating cooperatively between the peptides in stabilizing antibody binding (Spendlove et al., 2000). These findings contribute to identifying the mechanism by which a human anti-idiotypic antibody is able to mimic a tumor-associated antigen and stimulate anti-tumor B and T cell responses.

A more direct approach is proposed to use anti-Ids and monoclonals to target antigens directly to APC (Durrant et al., 2011). One approach entering the clinic stimulates anti-tumor immunity using monoclonals genetically engineered to express tumor-specific T cell epitopes to enhance T cell activation to eradicate tumors (Durrant et al., 2011). This work is an off-shoot of early ideas on antigenizing antibodies (Zanetti, 1992). However, natural regulatory T cells might control the specificity of T cell-mediated anti-Id immunity (Warncke et al., 2011). Tregs induced by a shared Id epitope can systemically suppress T cell responses against Id-derived and immunodominant foreign epitopes *in vivo* (Warncke et al., 2011). Collectively, these results further highlight the promiscuity of peptide sequences were a single antibody or TCR can be activated by a few crucial residues (Polakova et al., 2000). Consequently, again choosing the correct anti-Id is a challenge.

STRUCTURAL CONSIDERATIONS IN THE DESIGN OF MIMICS

In 1986, we suggested that the ultimate goal for Id vaccines was to prepare peptide vaccines derived from idiotypic sequence regions mimicking antigenic structures (Kieber-Emmons et al., 1986). We have accomplished this in an infectious model, being the first to do so (Westerink et al., 1995) and defined many of the paradigms associated with using such peptides (Cunto-Amesty et al., 2001b). We have applied lessons learned from the network theory to develop peptides that mimic TACA (Monzavi-Karbassi et al., 2007), bringing one of them into the clinic in a phase I safety study in breast cancer subjects (Monzavi-Karbassi et al., 2007) and now moving into a phase II trial of high risk breast cancer subjects to prevent recurrence of breast cancer.

Much like anti-Ids, peptide mimics may elicit anti-polysaccharide responses, but fail to elicit the Ids and isotypes observed in the protective response to the microbial antigen (Harris et al., 2002). Functional antibodies depend not only on the host's ability to mount an immune response, but also on its ability to mount the correct immune response. Whether an antibody response is protective or not depends on both the fine antigenic specificity that may be associated with particular Ids and epitope binding characteristics, and the isotype, determining antibody effector function. And herein lies the problem with mimics; the immune response is only assayed after a choice is made as to which mimic is to be followed. So what lessons can be learned about choosing the correct mimic?

In the first instance, the judicious choice of peptides for testing antibody responses against should be based on the peptide interaction with both the heavy and light chain in order to induce antibodies with similar antigen-specific properties (Luo et al., 2000); as the combination of heavy and light chains will influence specificity (Kabat and Wu, 1991). Thus, both the variable and the constant region of the antibodies induced by a peptide mimic or mimotope must be considered when assessing the success of any immunization. One way to determine this is to use structural information of the antibody–antigen interactions, e.g., reverse engineering concepts.

FIDELITY OF MIMICRY

We have previously reviewed the structural concepts and approaches used in vaccine design applications that illustrate the value and limitations of using chemical (peptide libraries which are mimics of a ligand) and immunological information to define novel peptide immunogens that function as mimotopes to generate immune responses targeting TACA (Pashov et al., 2005) and glycans on the human immunodeficiency virus (Pashov et al., 2007). In this context we showed early on that concepts associated with pharmacophore design (now considered reverse engineering) could be used to define CMPs applied to vaccine design (Luo et al., 2000; Cunto-Amesty et al., 2001a). We demonstrated that a structure-assisted vaccine design approach, whereby small molecules, defined in crystallographic databases, could be used to theoretically define peptide mimetics emulating the three-dimensional interaction scheme of a native carbohydrate antigen (Luo et al., 2000; Cunto-Amesty et al., 2001a). More importantly it was shown that virtual screening led to motifs being observed experimentally (Luo et al., 2000). We have also shown that by using this approach, an immunogenic peptide can be designed *de novo* (Cunto-Amesty et al., 2001a) and have shown that CMPs reactive with lectins and antibodies can induce antibodies with the same functionality as lectins and antibodies (Monzavi-Karbassi et al., 2005).

To generate sustained immunity to TACAs, we have developed immunogens based on CMPs – a strategy whose clinical promise is supported by our preclinical studies (Monzavi-Karbassi et al., 2007). CMPs can induce anti-tumor cellular responses, including CMP- and TACA-reactive Th1 $CD4^+$, and tumor-specific $CD8^+$ cells that may compensate for low-titer humoral responses (Monzavi-Karbassi et al., 2001, 2004). Most of all, unlike TACAs, CMPs can prime for memory responses to TACAs (Monzavi-Karbassi et al., 2003), suggesting that the CMPs facilitate cognate interactions between B cells and T cells, which is something that TACAs do not facilitate, but anti-idiotypic antibodies and peptides should and can do.

The question remains of how to enhance the ability of TACA mimetic peptides to induce TACA-specific antibodies with higher titers and association constants. We tested the hypothesis that improving the hydrogen bond pattern through amino acid substitutions in a CMP, to be coincident with that for the carbohydrate ligand, will enhance the ability of CMPs to elicit anti-TACA antibodies with high titers and association constants. Based on anti-Id/Id crystal structures, highly directional bonds represent an important set of interactions to establish a basis for mimicry because they mainly confer the specificity in binding of the peptide and the carbohydrate antigen.

MIMICS FOR GD2 ANTIGEN

In previous studies, we made use of the crystal structure of the Fab fragment of ME36.1 has been determined (Pichla et al., 1997), showing that its CDRs form a groove-shaped binding site. Molecular modeling has placed a four-residue sugar, representative of GD2, in the antigen-binding site showing much of the interaction with GD2 contributed by heavy chain interaction. Based upon hydrogen bonding schemes with the GD2 antigen, we used conformational and energy analysis to define potential binding modes of a CMP in the crystallographically defined ME36.1 binding pocket (Monzavi-Karbassi et al., 2007). Molecular modeling of the CMP in the ME36.1 binding site indicates that the CMP only shared two hydrogen bonds with the GD2 antigen when binding to ME36.1. This is in contrast to the seven hydrogen bonds formed between GD2 and the monoclonal antibody ME36.1.

Based upon hydrogen bonding schemes with the GD2 antigen, we wanted to determine if we could modify this CMP to increase the level of GD2 antigenic mimicry. Based upon conformational studies we surmised that removing the first three residues of the CMP would result in a peptide with a binding mode with ME36.1 with an increased number of hydrogen bonds in common with the way the GD2 antigen binds to ME36.1 (Monzavi-Karbassi et al., 2007). The redesigned CMP shared five hydrogen bonds in common with GD2 in binding to ME36.1. Computer-based binding studies indicate that the topographical binding mode of this redesigned CMP overlaps that of GD2 in the ME36.1 combining site. Our studies indicate that the redesigned peptide represents a more faithful mimic of ganglioside binding the monoclonal antibody ME36.1 than its original homolog based upon hydrogen bonding of ME36.1 to GD2. Immunization with this redesigned peptide resulted in enhanced antibody responses to GD2 and to tumor cells expressing GD2.

Although structural analysis may raise the confidence that the isolated peptide will have functional value, the induction of cross-reactive immune responses remains the ultimate proof of mimicry. To test if the increase in the level of GD2 mimicry translates into an improved GD2 reactive response, mice were immunized twice with versions of the CMP peptide (P10 original: GVVWRYTAPVHLGDG; P10s WRYTAPVHLGDG; synthesized as MAPs) and then bled 7 days after the boost (Monzavi-Karbassi et al., 2007). Immunization with MAP-P10s induced serum IgM antibodies superior in GD2 binding than serum antibodies induced by P10 (Monzavi-Karbassi et al., 2007). Serum IgM antibodies were also more reactive with the GD2-positive human WM793 cells, suggesting that an improved level of GD2 mimicry lends to an improved antibody response against GD2 (Monzavi-Karbassi et al., 2007). These results validated the hypothesis that mimetics can be more faithful in their mimicking potential. Such results confirm that stereochemically peptides and carbohydrates can bind to the same antibody-binding site, and that peptides can structurally mimic salient features of carbohydrate epitopes binding to a receptor fulfilling a promise of the anti-Id concept with these CMPs being tested in phase I trials.

SUMMARY

The regulation of immune responses is still in the mainstream of immunology research. However, the paradox of today's immunology is the lack of correspondence between the progress in basic science and the success of clinical applications. Many clinical trials in cancer currently ongoing aim to either stimulate an anti-tumor immune response or thwart immune suppression. Among those clinical applications are immunotherapeutics that make use of antibodies in some way. Moreover, antibodies and B cells are still considered beyond their effector roles, in terms of regulation and control of the mechanisms of tolerance.

Far from being refuted, concepts derived from INT have the potential to fuel new ideas and therapeutic approaches. Recent studies, reviewed here, confirm that idiotypy concepts hold promise in several aspects. First, although the basic immune phenomena are now known to have their origins in complex molecular and cellular interactions, idiotypic control exists and has undoubtedly its place in the overall immune dynamics, especially in selection of the antigen receptor repertoires. Second, studying idiotypic phenomena unveiled a mechanism of immune system's natural "reverse engineering" of antigens. A stimulating exercise in system immunology, INT provides also intellectual tools to understand how the immune system preserves structural information. Third, as an ultimate proof of validity, these concepts of decoupling of structural information from its carrier lead to applications in the development of vaccines and immunotherapeutics. Finally, INT helps understand the difference between molecular interactions of variable specificity and immune recognition as a function of the entire system. Thus, it contributes to the construction of an essential immunological paradigm.

On the one hand, the INT tackles the idea that immunoglobulin/immunoglobulin recognition mechanisms play a role in self-tolerance and control lymphocyte homeostasis. Although we know now that this is not the main mechanism of tolerance, signals, generated in the process, clearly control lymphocyte differentiation and homeostasis. Idiotypic interactions are known to participate in B cell repertoire selection, at least early in life and in restricted B cell compartments (Elliott and Kearney, 1992; Dietrich et al., 1993), contributing to dominant self-tolerance. On the other hand, the INT defines a paradigm of surrogate ligands and by extension – mimic-based immunogens. In principle, the level of innovation in INT is actually high, spawning some "high turnover" ideas and some others that form the mainstay of immunological research.

The INT might have been too innovative. Probably it came too much ahead of its time, followed by a heap of new data on structures and mechanisms in immunology that greatly expanded our view. The latter served for a kind of reductionist revenge instead of reassessment and development of the idea (except for isolated attempts, e.g., Varela and Coutinho, 1991). The skepticism in INT utility, that thus ensued, was the largest factor in its rise and fall. An interesting example is the perceived role of diverse T cell subpopulations in anti-cancer responses – a major shift of the emphasis, despite monoclonal antibodies being at the front line of some cancer therapies. Perhaps antibodies have not failed, but we do not know how to appropriately apply them and have not fully grasped the lessons learned from them. That very skepticism in INT is to blame, at least, to some extent for this situation. More importantly, only few appreciated the role of mimicry of antibodies in forming the idiotypic network (Varela and Coutinho, 1991; Coutinho, 1995). This may have contributed also to the premature fall of this beautifully speculative concept. Not unlike suppressor T cells which came back as Tregs, we anticipate that the rising interest in systems biology sooner or later will lead to a reassessment of the role of antigen repertoire networks at the systems biology level.

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Anti-idiotypic antibodies as cancer vaccines: achievements and future improvements

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Since the discovery of tumor-associated antigens (TAAs), researchers have tried to develop immune-based anti-cancer therapies. Thanks to their specificity, monoclonal antibodies (mAbs) offer the major advantage to induce fewer side effects than those caused by non-specific conventional treatments (e.g., chemotherapy, radiotherapy). Passive immunotherapy by means of mAbs or cytokines has proved efficacy in oncology and validated the use of immune-based agents as part of anti-cancer treatment options. The next step was to try to induce an active immune protection aiming to boost own's host immune defense against TAAs. Cancer vaccines are thus developed to specifically induce active immune protection targeting only tumor cells while preserving normal tissues from a non-specific toxicity. But, as most of TAAs are self antigens, an immune tolerance against them exists representing a barrier to effective vaccination against these oncoproteins. One promising approach to break this immune tolerance consists in the use of anti-idiotypic (anti-Id) mAbs, so called Ab2, as antigen surrogates. This vaccination strategy allows also immunization against non-proteic antigens (such as carbohydrates). In some clinical studies, anti-Id cancer vaccines indeed induced efficient humoral and/or cellular immune responses associated with clinical benefit. This review article will focus on recent achievements of anti-Id mAbs use as cancer vaccines in solid tumors.

Keywords: anti-idiotype, antibodies, cancer, vaccines, solid tumors

INTRODUCTION

Since the discovery of tumor-associated antigens (TAAs), researchers have tried to develop immune-based anti-cancer therapies. Thanks to their specificity, monoclonal antibodies (mAbs) offer the major advantage to induce fewer side effects than those caused by non-specific conventional treatments (e.g., chemotherapy, radiotherapy). Passive immunotherapy by means of mAbs or cytokines has proved efficacy in oncology and validated the use of immune-based agents as part of anti-cancer treatment options (Ferrantini et al., 2007; Weiner et al., 2009). The next step was to try to induce an active immune protection aiming to boost own's host immune defense against TAAs.

Active immunotherapy or vaccination is an antigen (Ag)-specific immune-stimulation. It consists in administering an Ag in the presence of an adjuvant. The role of the adjuvant is to induce a localized inflammatory reaction at the Ag administration site, making it more immunogenic. This adjuvant effect can be contained directly in the Ag, when it consists of an attenuated or dead infectious agent. Indeed, membranes substances (LPS) and DNA (CpG motifs) of infectious agents are able to activate the immune system. Currently, the artificial adjuvant most often used in vaccine preparations is aluminum hydroxide, while in mice the Freund's adjuvant is most used.

In oncology, unlike non-specific active immunotherapy, the goal of vaccination is to stimulate a specific anti-tumor response targeting a TAA (Foon et al., 1999). The scientific rationale of this approach was that the immune effectors generated would be tumor-specific while preserving surrounding normal tissues from a non-specific toxicity.

The initial orientation of preclinical studies naturally targeted tumor-specific neoAgs; either strictly specific of the tumor (mutations, gene rearrangements, idiotypes), or Ags with expression restricted to tumor cells. However, as most of TAAs are self antigens, an immune tolerance against them exists representing a barrier to effective vaccination against these onco-proteins. One promising approach to break this immune tolerance consists in the use of anti-idiotypic (anti-Id) mAbs, so called Ab2, as Ag surrogates. The presence of anti-Id Abs acting as the internal image of antigen epitopes (Ab2 β) and the ability of anti-Id Abs to modulate the immune response have paved the way for many therapeutic processes in different areas such as autoimmune diseases or cancer research.

Anti-Id cancer vaccines are able to induce humoral and/or cellular immune responses. Indeed, clinical benefit was observed in patients enrolled in clinical trials testing anti-Id vaccines in oncology, particularly in patients who developed an immune response against the vaccine itself (Foon et al., 1998, 2000; Samonigg et al., 1999; Wagner et al., 2001).

This vaccination strategy requires very little equipment and allows vaccination against Ags from non-protein origin (such as carbohydrates) that are difficult to purify. Moreover, in preclinical models, the anti-Id Abs are particularly effective in breaking immune tolerance to certain TAA (Bhattacharya-Chatterjee et al., 2000; Saha and Chatterjee, 2010; Ladjemi et al., 2011). HAMA (human anti-mouse antibodies) type responses can be induced

if the used anti-Id vaccine is from murine origin, but the techniques of Ab humanization (Losman et al., 1999) or the use of fully human Abs selected for example by phage display, can circumvent this problem.

In this review article, we will focus on recent achievements of anti-Id mAbs use as cancer vaccines in solid tumors. A first section will be dedicated to the concept of idiotypy and the anti-Id network theory first described by Jerne (1974). Indeed, according to this theory, the immune system is organized in Id and anti-Id network interactions able to regulate the immune response of the host against a given Ag. This particular feature of the immune system gave the idea to researchers to use the host's immune system to break immune tolerance to oncofetal TAAs. One main advantage of anti-Id cancer vaccines among other vaccine strategy is their ability to target Ags from non-protein origin; a special focus will be given on recent achievements on anti-Id vaccines mimicking TAA from carbohydrate origin. We will then discuss on the anti-Id vaccines mimicking TAA from protein origin which are currently evaluated in clinical development.

THE CONCEPT OF IDIOTYPY

IDIOTYPY AND ANTI-IDIOTYPIC ANTIBODIES

The notion of idiotypy followed the experimental observations described simultaneously by Kunkel et al. (1963) in humans and by Oudin and Michel (1963) in rabbits. The idiotype is composed of a set of antigenic determinants or idiotopes (Poskitt et al., 1991b). The idiotope can be located on the variable light chain (Pasquali et al., 1987), the heavy chain (Parhami-Seren et al., 1990) or can result from the interaction of the two chains (Bentley et al., 1990). Five amino acids may be sufficient to define a linear idiotypic determinant (Attanasio et al., 1993). It was estimated that there were potentially 15–20 idiotopes per Ab molecule (Novotny et al., 1986), some of them being directly involved in the Ag binding site while others can be located outside the paratope (Jerne et al., 1982).

An idiotope is called private if it is expressed by a given specific Abs in an individual. Whereas in turn, idiotopes common to Abs produced by different individuals within a population or shared by Abs of different specificity in the same individual, define a public idiotope (recurring or cross-reactive idiotope; Poskitt et al., 1991a). The idiotopes represent one of the two identified epitope classes for mAbs, the other class being formed by the allotopes. Unlike allotopes, which are mostly located on the constant domains of light and heavy chains, idiotopes are found only in the hypervariable regions of Abs. In addition, idiotopes are from somatic origin unlike allotopes which are derived from the germline. The Ab2 anti-Id Abs are directed against idiotopes present on Abs or on receptors expressed on B lymphocytes; they can bind to idiotopes located at the site of Ag recognition.

THE IDIOTYPIC NETWORK THEORY

Lindenmann (1973) and Jerne (1974) proposed theories describing the immune system as a network of interaction of Abs and lymphocytes. According to this hypothesis, Id and anti-Id network interactions would regulate the immune response of the host against a given Ag. The network theory is based on the fact that in the immune system the Ags are mimicked by idiotopes expressed by Abs and TCR (T cell receptors). According to this network concept (shown schematically in **Figure 1**) immunization with a given Ag will generate the production of Abs named Ab1 directed against this Ag. These Ab1 Abs can generate in turn the production of a series of anti-Id Abs directed against Ab1 Abs, so called Ab2. Some of these generated Ab2 Abs is able to mimic the three-dimensional structure of the starting Ag; the anti-Id Ab2 Abs constituting this subset are called Ab2 β. They are housed in the paratopes of Ab1 Abs and are able, when used as immunogens, to induce a specific immune response similar to that induced by the initial mimicked Ag.

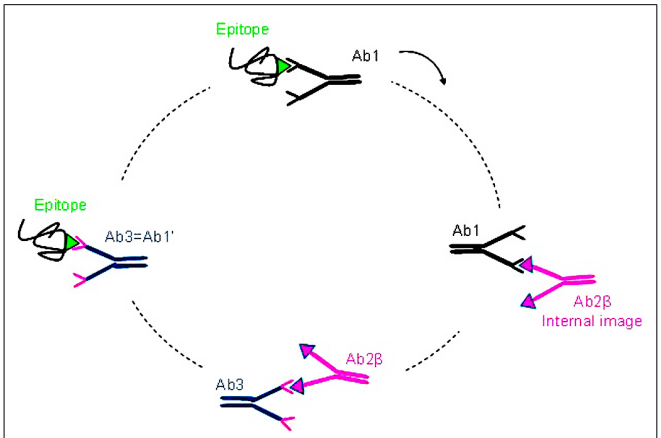

FIGURE 1 | The idiotypic network. Immunization with a given Ag (green) generates the production of specific Abs directed against this Ag, theses first generation of Abs is named Ab1 (black). These Ab1 Abs can generate in turn the production of a series of anti-Id Abs directed against the Ab1 Abs and called Ab2. Some of these Ab2 Abs are able to mimic the three dimensional structure of the starting Ag, the Ab2 β (pink). Immunization with these Ab2 β Abs may lead to the production of anti-anti-Id Abs (Ab3 Abs) including the Ab1′ Abs (blue) directed against the corresponding initial Ag recognized by the Ab1.

Indeed, the β subtype of anti-Id Abs express the internal image of the Ag recognized by the Ab1 Ab and can therefore be used as Ag surrogates. Immunization with Ab2 β Abs may lead to the production of anti-anti-Id Abs, the Ab3 Abs, part of which recognizes the starting Ag recognized by the Ab1 Ab. Because of this Ab1-like reactivity, the Ab3 Abs are also called Ab1′ Abs to indicate that they may differ from the Ab1 Ab in their other idiotopes.

There are four subgroups of Ab2 Abs (α, β, γ, and ε) which can be obtained by immunization with Ab1 Abs:

- Ab2 α Abs are specific to idiotopes associated with the structure of the Ab and therefore outside the binding site, their binding to the Ab1 do not prevent the binding of Ag to the Ab1 (Jerne et al., 1982).
- Ab2 β Abs recognize an idiotope located in the paratope of the Ab1 Ab. Their binding to the Ab1 is naturally inhibited by the Ag they mimic. It is this series of Ab2 Abs that Jerne named "internal image" (Jerne, 1974) and Lindenmann the "homobodies" (Lindenmann, 1973). Antigenic determinants of the Ab2 β Abs are similar in structure to the Ag that binds the Ab1 Abs. These Ab2 β are able to induce the production of Ab3 Abs which could bind the original Ag.

– Ab2 γ Abs recognize idiotopes close to the paratope of the Ab1 Ab and their binding to the Ab1 is inhibited by the binding of Ag, mainly because of steric hindrance.
– Ab2 ε Abs or "epibodies" have double features as they can bind both the epitope of the Ag and the idiotope of the Ab directed against this Ag (the Ab1; Bona et al., 1986).

Of all Abs that can be produced against a given Ab1 Ab, the one representing the internal image of the Ag would constitute the best candidate to induce anti-tumoral immune response. In a preparation of polyclonal Ab2 Abs, only a subset of these Abs is able to mimic the Ag (the Ab2 β Abs). Thus, the induced fraction of Ab3/Ab1′ Abs will be much higher with monoclonal Ab2 β rather than with a polyclonal Ab2 Ab preparation (Herlyn et al., 1996). **Table 1** summarizes the clinical studies conducted with anti-Id mAbs as cancer vaccines in solid tumors.

ANTI-IDIOTYPIC VACCINES FOR GLYCOPEPTIDES CONTAINING TACA

A subset of TAA of carbohydrate nature have been identified and called tumor-associated carbohydrate antigens (TACA; Hakomori, 1989). TACA have been described to be expressed specifically on tumor cells as compared to normal tissues due to an aberrant glycosylation in tumor cells (Singhal and Hakomori, 1990). Many of TACA are expressed in fetal tissues and therefore belong to the group of the so-called oncofetal antigens. Moreover, TACA play an essential role for metastasis induction and tumor invasiveness. Number of TACA have been described, including the blood group related, mucin related TAA, the gobo series glycosphingolipids, and the gangliosides belonging to the group of sialic acid containing glycosphingolipids (Kobata and Amano, 2005; Guo and Wang, 2009; Cazet et al., 2010).

Gangliosides gained a privileged place as a target for cancer immunotherapy and recently in a study from the National Cancer Institute, 75 representative antigens to be targeted in cancer therapy were selected; among them four gangliosides GD2, GD3, fucosyl GM1, and *N*-acetyl-GM3 (Cheever et al., 2009). *N*-glycolyl (NGc) gangliosides received particular interest especially with the anti-Id murine mAb Racotumomab mimicking NGc-containing gangliosides (first known as 1E10 Ab).

1E10 Ab (RACOTUMOMAB)

Racotumomab, first known as 1E10 Ab, an anti-Id Ab2 γ murine mAb was generated after immunization of BALB/c mice with the P3 Ab1 IgM murine mAb (Perez et al., 2002; Lopez-Requena et al., 2003; Hernandez et al., 2005). Racotumomab have been used in several clinical trials and its safety and efficacy were assessed in different tumor localizations: melanoma, breast, and lung cancers. More recently, there was a specific interest on pediatric tumors expressing *N*-glycolylated gangliosides. A phase I study is indeed ongoing in patients with pediatric malignancies resistant to conventional treatment (NCT01598454) and the primary outcome will measure the higher safe dose level for ensuing clinical trials. Racotumomab has now reached the phase III clinical trials with possible indications in breast and lung cancer and a possible extent to pediatric tumors (Fernandez et al., 2010).

Racotumomab clinical evaluation in melanoma

A clinical trial with aluminum hydroxide-precipitated 1E10 Ab was conducted in 20 patients with advanced melanoma. 1E10 proved to be safe, well tolerated and able to induce specific immune responses against 1E10 itself and Neu-glycolyl-GM3 ganglioside (Alfonso et al., 2002).

Table 1 | Clinical studies evaluating anti-idiotypic Abs as cancer vaccines in solid tumors.

Anti-Idiotype vaccine	Mimicked antigen	Tumor type	Clinical phase	Reference
Racotumomab (1E10)	NGc-containing gangliosides	Breast cancer	I	Diaz et al. (2003), Guthmann et al. (2006)
		Lung cancer	I/II	Neninger et al. (2007)
			Ongoing II/III	Alfonso et al. (2007), Hernandez et al. (2008)
		Melanoma?	I	Alfonso et al. (2002)
		Pediatric tumors?	Ongoing I	
TriGem	GD2	Melanoma	I/II	Foon et al. (1995, 2000)
MK2-23	HMW-MAA	Melanoma	I/II	Mittelman et al. (1994, 1995)
BR3E4	EpCam	Colorectal cancer	I	Birebent et al. (2001, 2003)
3H1 (CeaVac)	CEA	Colorectal cancer	I/II	Foon et al. (1995, 1997)
			III	Chong et al. (2006)
105AD7	CD55	Colorectal cancer	I/II	Denton et al. (1994), Maxwell-Armstrong et al. (2001) Maxwell-Armstrong (2002)
11D10 (TriAb)	HMGF	Breast cancer	I	Reece et al. (2001, 2003)
		Colorectal cancer	II in association with 3H1	Posner et al. (2008)
		Lung cancer	II in association with 3H1	No data published yet
Abagovomab	CA-125	Ovarian cancer	I/II	Reinartz et al. (2004), Pfisterer et al. (2006)
			Ongoing II/III	Sabbatini et al. (2006)

Racotumomab clinical evaluation in breast cancer

The same formulation of 1E10-Alum used in melanoma was also evaluated a phase I clinical trial conducted in patients with stage III/IV breast cancer. Here again 1E10 Ab was safe, well tolerated and induced specific humoral immune responses both against 1E10 itself and NeuGc-GM3 ganglioside (Diaz et al., 2003). The toxicity and immunogenicity of 1E10 was investigated in a prolonged vaccination regimen in 19 patients with high-risk or metastatic breast cancer. 1E10 immunization induced a humoral response directed against NeuGc-GM3 ganglioside in all patients; moreover five patients developed specific T-cell responses (Guthmann et al., 2006).

Racotumomab clinical evaluation in lung cancer

A phase I clinical trial was conducted to evaluate the toxicity and humoral immune response elicited by aluminum hydroxide-precipitated 1E10 vaccine in nine patients with small cell lung cancer (SCLC). No evidence of serious adverse effects was found. Most of the patients developed specific antibody responses against both 1E10 Ab and NeuGc-GM3 ganglioside (Neninger et al., 2007). Although this study was not designed to evaluate the therapeutic efficacy of this anti-Id vaccine, a prolonged survival was observed in several patients. However, due to the small number of patients enrolled in this clinical trial, it did not allow to make a correlation between the induced immune response and clinical outcomes.

1E10/Alum formulation was also assessed in a clinical trial with stages IIIb and IV non-small cell lung cancer (NSCLC) patients. No evidence of unexpected or serious adverse effects was reported (Alfonso et al., 2007). Patients that developed IgG and/or IgM Abs against NeuGc-GM3 showed longer median survival times (Hernandez et al., 2008). Moreover, NeuGc-GM3-specific Abs were able to induce complement independent necrosis of tumor cells (Hernandez et al., 2011). A phase II randomized trial is now ongoing in NSCLC patients to confirm the clinical effect of 1E10 MAb vaccine and to evaluate the correlation between the immune responses patients' survival (NCT01240447). A prospective randomized III study is also running in patients with advanced NSCLC, primary outcome will measure overall survival (OS; NCT01460472).

TRIGEM Ab

Other clinical trials were performed with anti-Id mAbs mimicking gangliosides (Lutzky et al., 2002). Different doses of the anti-Id mAb TriGem mimicking disialoganglioside GD2, which is highly expressed in melanomas, were administered to patients. An initial study showed that of the 12 treated patients, one has a complete response and six showed an arrest of tumor progression (Foon et al., 1998). A second study conducted on 40 patients showed a complete response in one patient and stable disease in 12 of them (Foon et al., 2000). Furthermore, the data obtained suggest that this anti-Id mAb could have a favorable impact on disease progression and survival (Foon et al., 2000).

ANTI-IDIOTYPIC VACCINES AS SURROGATES FOR SPECIFIC ONCOGENE PRODUCTS

We will detail in this section the use of Ab2 Abs mimicking specific TAA from protein origin as cancer vaccines in different types of solid tumors: melanoma, lung cancer, colorectal carcinoma, breast cancer, and ovarian carcinomas.

MELANOMAS

The murine mAb MK2-23 mimicking the TAA HMW-MAA (high molecular weight-melanoma-associated antigen), was used to treat patients with advanced melanoma. The authors observed a regression of metastases (Mittelman et al., 1994) and an increase in overall survival correlated with the induction of an anti-HMW-MAA humoral response (Mittelman et al., 1995). Even though this was a retrospective study, a multivariate analysis showed that the development of anti-HMW-MAA antibodies was the most important variable for predicting survival. Wang et al. (2005) demonstrated that MK2-23 (mimic HMW-MAA)-IL-2 fusion protein is useful to implement active specific immunotherapy in patients with melanoma, because it bypasses the requirement for KLH conjugation and adjuvant administration.

Other clinical applications of various anti-Id Abs mimicking the HMW-MAA were conducted in small clinical trials. Among them, the Melimmune, a mixture of two murine mAbs, has proved effectiveness to induce Ag-specific humoral and cellular immune responses (Saleh et al., 1998). A more recent study has shown that this vaccine was able to induce specific cytotoxic T lymphocytes directed against the HMW-MAA (Murray et al., 2004).

LUNG CANCER

As described above (see Idiotypy and Anti-Idiotypic Antibodies), the main anti-Id cancer vaccine tested in lung cancer remains Racotumomab with promising data. Two other anti-Id mAbs mimicking specific TAA from protein antigens are also evaluated in lung cancer patients: 3H1 (CeaVac) and 11D10 (TriAb) mAbs mimicking, respectively the carcinoembryonic antigen (CEA) and the human milk fat globule (HMGF) protein. A phase II study was conducted with 3H1 and 11D10 anti-Id mAbs in patients with completely resected Stage II and Stage IIIA NSCLC (NCT00006470). The objectives were the evaluation of toxicity, humoral and cellular immune responses, and to determine the progression-free survival (PFS) and OS in theses patients; results of this study are not published yet.

COLORECTAL CANCER

During the past 20 years, studies on the use of anti-Id Abs have focused on three main tumor Ags (i) the epithelial cell adhesion molecule (EpCAM) associated with colorectal cancer (CRC), also known as GA733, CO17-1A, KS-14, or KSA, (ii) the CD55, also known as decay-accelerating factor (DAF) involved in the regulation of the complement cascade, and (iii) the CEA known for its particularly strong expression in 95% of CRC, 70% of lung adenocarcinomas, and 50% of breast cancers.

BR3E4

BR3E4, an Ab2 anti-Id mAb, was produced in rats against the murine mAb CO17-1A (Ab1; Herlyn et al., 1987; Maruyama et al., 2000). In a phase I clinical trial, BR3E4 was administered to 45 patients with CRC as intact IgG or as F(ab′)2 coupled to KLH (Birebent et al., 2001). This study demonstrated that there was a trend for the KLH group to induce higher immune response rates (18/21 and 5/15 patients with anti-anti-Id Abs and T cells,

respectively) as compared to the group of patients immunized with the intact IgG (15/23 and 3/15 patients positive). However, clinical responses were rare as this study was undergone on stage IV CRC patients with liver metastasis (Birebent et al., 2001, 2003).

3H1 (CeaVac)

An anti-Id murine Ab2 mAb, called 3H1 or CeaVac, mimicking an epitope of CEA was developed and very early suggested to be a potential cancer vaccine in patients with CEA-positive tumors (Bhattacharya-Chatterjee et al., 1990).

In a first clinical trial, the safety and immunogenicity of different doses (1, 2, or 4 mg) of aluminum hydroxide-precipitated anti-Id mAb were evaluated in 12 patients with advanced CRC (Foon et al., 1995). The vaccine was safe and well tolerated. Moreover, 3H1 was able to break immune tolerance to CEA in these patients with CEA-positive tumors (Foon et al., 1995). In fact, 9 of 12 patients developed anti-3H1 humoral responses and more interestingly all nine patients generated specific anti-CEA antibody responses. Moreover, 7 of 12 patients demonstrated idiotype-specific T cell proliferative responses and four also showed T cell proliferation to CEA (Foon et al., 1995). This 3H1 formulation was evaluated in a phase Ib trial in 24 patients with advanced CEA-positive CRC (Foon et al., 1997). The safety and immunogenicity of the vaccine confirmed the previous study. Even though the clinical benefit of 3H1 vaccine could not be proved in this study, since overall median survival (11.3 months) was comparable to other phase II data with advanced CRC patients treated with a variety of chemotherapy agents, including irinotecan, 3H1 vaccine promisingly induced considerably less toxicity (Foon et al., 1997).

3H1 anti-Id mAb is now evaluated in phase II and III clinical trials either alone or in combination with the anti-Id mAb 11D10 (TriAb) mimicking HMGF protein.

A phase III controlled randomized clinical trial was conducted on 630 patients with untreated metastatic CRC (Chong et al., 2006). The patients received 5-fluorouracil (5-FU) and leucovorin (LV) plus either 3H1 or placebo. The addition of 3H1 to 5-FU and LV did not result in increased toxicity but was not shown to improve overall patient outcomes (Chong et al., 2006). Anti-CEA antibody responses were observed in 70% of patients treated with 3H1; these patients had improved survival (median survival not reached) as compared to patients with a negative CEA response (median survival: 8.3 months; Chong et al., 2006).

The data of the first phase II clinical trial evaluating the combination of 3H1 and 11D10 anti-Id mAbs in patients with CRC metastatic to the liver were published (Posner et al., 2008). Vaccinations consisted of four biweekly treatments of 3H1 and 11D10, then monthly for 2 years, then on every other month for 3 years. The primary endpoint of the study was to investigate the proportion of patients recurrence-free at 2 years. The vaccine was well tolerated but did not improve 2-year recurrence-free survival when compared with the expected value of 40% reported for hepatic resection alone (Posner et al., 2008).

105AD7

The cancer vaccine 105AD7 is a human anti-Id mAb that mimics the CD55 TAA on CRC cells. This anti-Id mAb was produced by fusion of a mouse/human heteromyeloma cell line with lymphocytes from a patient previously injected with mouse mAb 791T/36 for tumor immunoscintigraphy (Austin et al., 1989). Phase I studies in patients with advanced CRC demonstrated the safety and the immunogenicity of 105AD7 anti-Id mAb (reviewed by Maxwell-Armstrong, 2002). Immunization with 105AD7 induced T-cell immune responses in 83% of patients with a permissive haplotype (Durrant et al., 2000). In a phase I clinical trial, the cancer vaccine induced an increase in median-free survival for immunized patients versus unimmunized patients (12 vs. 4 months respectively; Denton et al., 1994). These results were not confirmed in a randomized double-blind phase II survival study (Maxwell-Armstrong et al., 2001). The reasons for lack of efficacy are unclear but with half of patients receiving only one or two doses of 105AD7, it seems to be insufficient to improve survival. A further survival analysis was then conducted but at 2-year follow-up, the vaccine did not improved OS (Maxwell-Armstrong, 2002).

BREAST CANCER

Studies on the use of anti-Id Abs as cancer vaccines in breast cancer have focused on three main tumor Ags (i) the HMGF protein with 11D10 (TriAb), (ii) gangliosides with 1E10 (Racotumomab), and (iii) HER2 receptor (still in preclinical development).

The use of 11 D10 was evaluated in conjunction with autologous stem cell transplantation in patients with metastatic breast cancer (Reece et al., 2001, 2003). Immunization with 11D10 anti-Id mAb induced specific humoral and T-cell immune responses in the majority of patients (Reece et al., 2003). Moreover, patients with the most vigorous immune responses had a significant improvement in PFS (Reece et al., 2001)

The data on the use of anti-Id Abs mimicking HER2 as cancer vaccines for breast cancers are preliminary and still in the preclinical development.

Baral et al. (2001) have immunized C57BL/6 mice with the murine anti-Id mAb 520C9-6b mimicking a human epitope of HER2 Ag. The results of this preclinical study have shown that immunization with 520C9-6b could induce anti-HER2 Abs in vaccinated mice suggesting that this antibody could be used as a surrogate Ag of HER2 to induce a humoral and cellular response in patients with HER2-positive tumors. More recently, the same group developed and characterized a murine mAb 6D12, which mimics a specific epitope of HER2, the one recognized by trastuzumab. Immunization of C57BL/6 with 6D12 in combination with the adjuvant vaccine QS21, has led to the development of specific humoral responses. In addition, mice immunized with 6D12 were protected against a syngeneic graft of a lethal dose of the same cells (Mohanty et al., 2007; Pal et al., 2007). Moreover, immunization of transgenic mice tolerant to HER2 Ag with 6D12-pulsed dendritic cells (DC) could reverse Her-2/neu unresponsiveness and result in the induction of HER2/neu-specific humoral and cellular immune responses and protection against tumors expressing HER2/neu (Saha and Chatterjee, 2010).

Two human scFv fragments, named 40 and 69, and a llama anti-Id single domain antibody (sdAb), named VHH 1HE, capable of mimicking the epitope of HER2 Ag recognized by trastuzumab were selected by phage display. These anti-Id scFv and VHH fragments induced an anti-HER2 antibody response in BALB/c mice

(Coelho et al., 2004; Alvarez-Rueda et al., 2009). Moreover, vaccination with anti-Id scFv fragments was able to reverse HER2 immunological tolerance and to protect HER2-tolerant mice from developing spontaneous mammary tumors (Ladjemi et al., 2011). Such vaccination elicited specific humoral and T-cell responses (Ladjemi et al., 2011).

OVARIAN CANCERS

The use of the anti-Id mAb ACA-125 (or Abagovomab) mimicking the tumor antigen CA-125 (cancer Ag 125), over-expressed in ovarian tumors has been reported in several clinical trials. It has been shown, in preclinical and phase I clinical studies, that Abagovomab could induce humoral and cellular immune responses against CA-125 without the occurrence of toxicity related to this treatment (Wagner et al., 2001). In another phase Ib/II study, 119 patients with advanced ovarian cancer received 10 injections of Abagovomab; a CA125-specific humoral response was induced in 50% of patients. A positive correlation was also observed between the development of a specific humoral response and OS (Reinartz et al., 2004). It should be noted that preclinical data suggest that use of a fusion protein formed by the Abagovomab and IL-6 may induce a more robust CA125-specific humoral response (Reinartz et al., 2003). Other phase I clinical trials evaluated Abagovomab in recurrent ovarian cancer (Pfisterer et al., 2006) or primary peritoneal tumors (Sabbatini et al., 2006) and confirmed the encouraging results obtained with this anti-Id mAb. The efficacy of Abagovomab is currently evaluated in a phase III randomized, double-blind, placebo-controlled clinical trial for patients in complete remission after a stage III-IV CA-125 positive ovarian, fallopian tube or primary peritoneal cancer (Grisham et al., 2011; Pfisterer et al., 2011). The primary outcome will measure recurrence-free survival; safety, time course of immune response, and OS will be measured as secondary outcomes. Preliminary immunogenicity data for weeks 10 and 22 showed that 68 and 69% of patients were positive for Ab3 Abs (Grisham et al., 2011).

CONCLUSION

Immunotherapy has nowadays an important place in oncology treatment. The major advantage of this type of strategy is the specific targeting of tumor Ags, which implies less toxicity and side effects compared to conventional therapies. Today, 12 therapeutic Abs were approved by FDA for the treatment of cancers. Nevertheless, the high number of patients treated by therapeutic Abs and the experience taken from the several clinical trials conducted in this field have pointed two major problems currently facing Ab-based immunotherapy: (i) the development of drug resistance by tumor cells and (ii) the need of repeated injections required to achieve a lasting therapeutic effect, which implies a high cost for this type of therapy. Cell-based immune therapy faces with technical difficulties as it is still hard to establish a cell-based immunotherapy because of the individualization of the procedure and technical conditions of relatively complex *ex vivo* culture. However, the technological progress that the sector is currently experiencing lets consider the cell-based immune therapy as a promising future therapeutic strategy.

Active immunotherapy or vaccination offers the main advantage of requiring fewer injections than for therapeutic Abs. More importantly, vaccines offer the establishment, theoretically, of a memory response that persists after the end of treatment and could prevent the occurrence of relapses. Nevertheless, this strategy is still in preclinical and clinical development. This delay, as compared to other immunotherapy strategies, could be explained at least in part by the fact that clinical trials currently conducted are not adequate with a vaccination strategy. Indeed, vaccines are tested in patients with advanced stages of disease with immune system already weakened by many cycles of chemotherapy already undergone. This implies that the clinical benefit of this type of therapeutic strategy is even more difficult to demonstrate. However, the increasing interest for anti-tumoral vaccination could accelerate the development of cancer vaccines and increase the number of vaccine candidates to be tested which implies a larger number of clinical trials and thus give rise ultimately to commercialization of vaccines cancer. In addition, with the recent approval of the first cancer vaccine sipuleucel-T by the FDA in 2010 for metastatic hormone-refractory prostate cancer, cancer vaccines are entering a new promising era. In fact, sipuleucel-T increased OS in a randomized phase III trial conducted in patients with advanced prostate cancer (Higano et al., 2009, 2010).

Overall, research on anti-Id cancer vaccines has greatly evolved over the past decades even though there is yet a lot to do in this field. This vaccination strategy requires very little equipment and allows vaccination against Ags from non-protein origin (such as carbohydrates). Anti-Id cancer vaccines present the advantage to address to the entire population (regardless of HLA) as compared to protein or peptide-based vaccines. Moreover, they are capable of inducing an immune response more robust, at least in theory, since it is formed of humoral but also cellular component. These advantages allow foreseeing a bright future for this type of vaccine strategy. However, although most of anti-Id cancer vaccines proved safety, tolerability, and immunogenicity, the clinical benefit remains to be proved. This proof of clinical benefit will be perhaps provided by the promising anti-Id mAbs Racotumomab and Abagovomab, which are now evaluated in phase III clinical trials.

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Idiotypes as immunogens: facing the challenge of inducing strong therapeutic immune responses against the variable region of immunoglobulins

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Idiotype (Id)-based immunotherapy has been exploited as cancer treatment option. Conceived as therapy for malignancies bearing idiotypic antigens, it has been also extended to solid tumors because of the capacity of anti-idiotypic antibodies to mimic Id-unrelated antigens. In both these two settings, efforts are being made to overcome the poor immune responsiveness often experienced when using self immunoglobulins as immunogens. Despite bearing a unique gene combination, and thus particular epitopes, it is normally difficult to stimulate the immune response against antibody variable regions. Different strategies are currently used to strengthen Id immunogenicity, such as concomitant use of immune-stimulating molecules, design of Id-containing immunogenic recombinant proteins, specific targeting of relevant immune cells, and genetic immunization. This review focuses on the role of anti-Id vaccination in cancer management and on the current developments used to foster anti-idiotypic B and T cell responses.

Keywords: idiotype, lymphoma, vaccines, cancer, idiotypic network

Immunoglobulins (Ig) are glycoproteins formed by two identical heavy and two identical light polypeptide chains. The N-terminal ends of each pair of heavy-light chains consist of two variable (V) immunoglobulin (Ig) domains (V_L and V_H) that form a unique surface for antigen binding. V regions are generated during B cell ontogeny by the so-called VDJ rearrangement of the germ-line Ig genes. This genetic rearrangement allows for the tremendous initial diversity of human Igs in naïve B cells, a critical feature of the immune system, which is further increased and reshaped by somatic hyper-mutation of V regions in antigen-stimulated mature B cells.

The association of the two V domains generates the idiotype (Id), a distinctive structure and a unique collection of antigenic determinants called idiotopes. Idiotopes derive mainly from the CDR regions of the Ig V domains, frequently found to be of a conformational nature and derived from somatic mutations (**Figure 1**). Despite being self-proteins Ids can be immunogenic. For this reason Ids have been exploited as therapeutic immunogens in cancer treatment in two well-defined and clearly distinct contexts: (i) directly as a tumor-specific target on membrane Ig-positive malignant B cells as a consequence of their clonotypic origin, and (ii) as surrogate of tumor-associated antigen (TAA) to induce specific immune responses (**Figure 2**).

In the first case the Id itself represents the immunogen and the target on the surface of the malignant B cell. Because of their clonotypic origin the Id expressed by malignant B cells represents the only true example of tumor-specific antigen (Anderson et al., 1984). Therapeutic strategies that target the unique structure of the Id of each malignant clone constitute therefore a powerful tool for the treatment of B cell malignancies (Bendandi, 2009; **Figure 2A**). These strategies are essentially based on the generation of an anti-Id antibody and/or T cell-based immune response by means of protein or DNA-based vaccines. The success of these approaches to eradicate established tumors or prevent their development has been largely demonstrated in animal models.

The second relevant context focuses on the use of a defined Id to induce, through a mechanism of molecular mimicry, a specific immune response against a TAA. In this case the system entails the selection of an anti-idiotypic antibody (Ab2) generated against the Id of an Ab1 specific for the TAA. The Id of the Ab2 is, in turn, used to induce an anti-idiotypic anti-Ab2 response (Ab3) that will not only recognize the immunizing Id of Ab2, but very frequently also the antigen for which the Ab1 is specific (Lopez-Requena and Burrone, 2009). The Id of Ab2 is said to carry the "internal image" of the antigen, in other words to mimic its structure, and therefore able to replace the latter for inducing the immune response (**Figures 2B,C**).

Antigen mimicry is, however, a still not-completely understood phenomenon. The concept is based on the idea that if both the antigen and the Id of the Ab2 bind to the antigen-combining site of Ab1 (paratope), then the paratope of the Ab2 would structurally resemble the antigen (**Figure 2B**). While in some examples structural homology between antigen and the Ab2 Id has been demonstrated to a certain extent (Pride et al., 1992; Chatterjee et al., 1998; Spendlove et al., 2000; Chang et al., 2005), this is hardly the case when it comes to non-protein antigens (Talavera et al., 2009). Nevertheless, irrespective of the molecular basis of mimicry, the fact is that some Ab2 induce antibodies that bind to the antigen. The strategy of active immunotherapy with anti-idiotypic vaccines

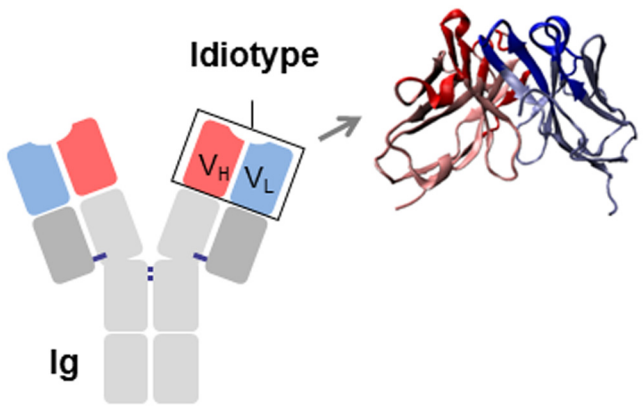

FIGURE 1 | Schematic representation of an immunoglobulin (Ig). The idiotype (Id), constituted by the variable regions of the heavy (V_H) and light (V_L) chains, is shown. CDRs are highlighted in red (V_H) and blue (V_L).

has been widely explored in cancer (Bhattacharya-Chatterjee et al., 2002; Lopez-Requena and Burrone, 2009).

Thus, an Ig Id can be conceived and used as an antigen to induce anti-Id responses in two different frameworks: (i) the Id itself as a target antigen on tumor cells, as in the case of B cell lymphomas (**Figure 2A**), and (ii) the Id as a mimic of a tumor antigen, with the anti-Id response ultimately intended to target the latter (**Figures 2B,C**). There is, however, an important distinction between the two cases. While the Id expressed on a tumor cell is a self-antigen, and therefore immunological tolerance is expected, this is not the case when it is used as a mimic of another antigen. In addition, Ab2 are frequently obtained in mice, for which the whole Ig is a foreign antigen for the patient. The response against the Id can thus be favored, and the constant region can act as a xenogeneic carrier. Nevertheless, in cases when the mouse antibody is not highly immunogenic immune-stimulating compounds are required to enhance the response (McCaffery et al., 1996).

Despite encouraging results at the preclinical level, Id-based immunotherapy has given disappointing outcomes in patients. Immunization with lymphoma Ids or anti-idiotypic antibodies mimicking tumor antigens has been demonstrated to be feasible and safe in clinical trials, despite proof of clinical efficacy is still awaited (de Cerio et al., 2007). As with the latter strategy (Giaccone et al., 2005), initial phase III clinical trials using Id vaccines in lymphoma patients did not meet their primary end points (Bendandi, 2009; Kannan and Neelapu, 2009; Rezvani and de Lavallade, 2011), highlighting the need for deeper research in both, the identification of predictors of the immune response in patients (Inoges et al., 2011a) and the design of clinical studies (Bendandi, 2009; Inoges et al., 2011b). Also, the improvement of vaccination strategies is an important issue under intense investigation (Bhattacharya-Chatterjee et al., 2002; Hollander, 2009; Rezvani and de Lavallade, 2011). The last issue will be the focus of this review.

IMMUNE-STIMULATING CARRIERS AND ADJUVANTS

In 1975, Herman Eisen reported the first case of induction of active immunity to a tumoral Id in a murine plasmacytoma model (Eisen et al., 1975). Vaccination with the idiotypic protein, obtained and purified from plasma, was efficient to protect animals against subsequent tumor challenge. However, the possibility of establishing an anti-Id response in patients with Ig-secreting tumors was low, and attention turned to lymphomas where tumor cells display membrane bound Ig but secrete little. Pioneering studies in several murine lymphoma models demonstrated that the low immunogenicity of idiotypic protein can be efficiently overcome when Id was linked to a strong immunological carrier such as keyhole limpet hemocyanin (KLH). Using this approach, induction of high titers of anti-Id antibodies and protection against tumor challenge was demonstrated (Campbell et al., 1987, 1990; Kaminski et al., 1987; George et al., 1988), even in animals with established lymphoma (Campbell et al., 1988), suggesting that, although nominally self-antigens, idiotypic determinants can become immunogenic when administered in a context that allows overcoming T cell tolerance. The experience gained in murine studies led to the first clinical trials of patients with low-grade, follicular lymphoma, primarily those in first remission following chemotherapy (Kwak et al., 1992). While this study reported the induction of an Id-specific humoral response, neither the activation of Id-specific T cells nor clinical efficacy were described. A phase II clinical study conducted by the National Cancer Institute first demonstrated clinical efficacy upon immunization with Id protein coupled to KLH and co-administered with granulocyte-monocyte colony stimulating factor (GM-CSF). The study described the clearance of residual tumor cells and long-term disease-free survival in follicular lymphoma patients in first complete remission after standard chemotherapy (Bendandi et al., 1999). The correlation between Id-specific immune response and *in vivo* control of minimal residual disease was also found in a similar study conducted in Europe (Barrios et al., 2002). More recently, clinical benefit associated to KLH-conjugated Id vaccines in lymphoma patients was reported in small phase II trials (Inoges et al., 2006; Redfern et al., 2006; Timmerman et al., 2009). However, clinical phase III studies aimed at obtaining regulatory approval for Id vaccines failed to reach their primary endpoints (Bendandi, 2009;Schuster et al., 2011a). Many factors have been taken into account as possible predictors of vaccination efficacy and induction of a clinically relevant immune response. In a recent double-blind multi-center controlled phase III trial in patients with follicular lymphoma, the outcome of vaccination with the patient-specific hybridoma-derived Id was dependent on the tumor Ig isotype, with IgM being significantly more effective than IgG (Schuster et al., 2011b). Despite the existence of Ids with an "intrinsic" ability to generate a syngeneic immune response irrespective of the format (Lopez-Requena et al., 2007b), the IgM isotype has been associated with immunogenicity in contrast to the IgG (Reitan and Hannestad, 1995, 2001, 2002), which was shown in turn, to contain epitopes in the Fc region able to activate regulatory T cells (De Groot et al., 2008). With the aim of improving vaccine efficacy in patients, the possibility that Id-KLH cross-linking with glutaraldehyde may damage critical immunogenic epitopes, has prompted researchers to look for alternative reagents. Murine and patient-derived human Id-KLH vaccines generated using maleimide-based conjugation were found to be superior to glutaraldehyde conjugation to generate anti-Id immune responses (Kafi et al., 2009).

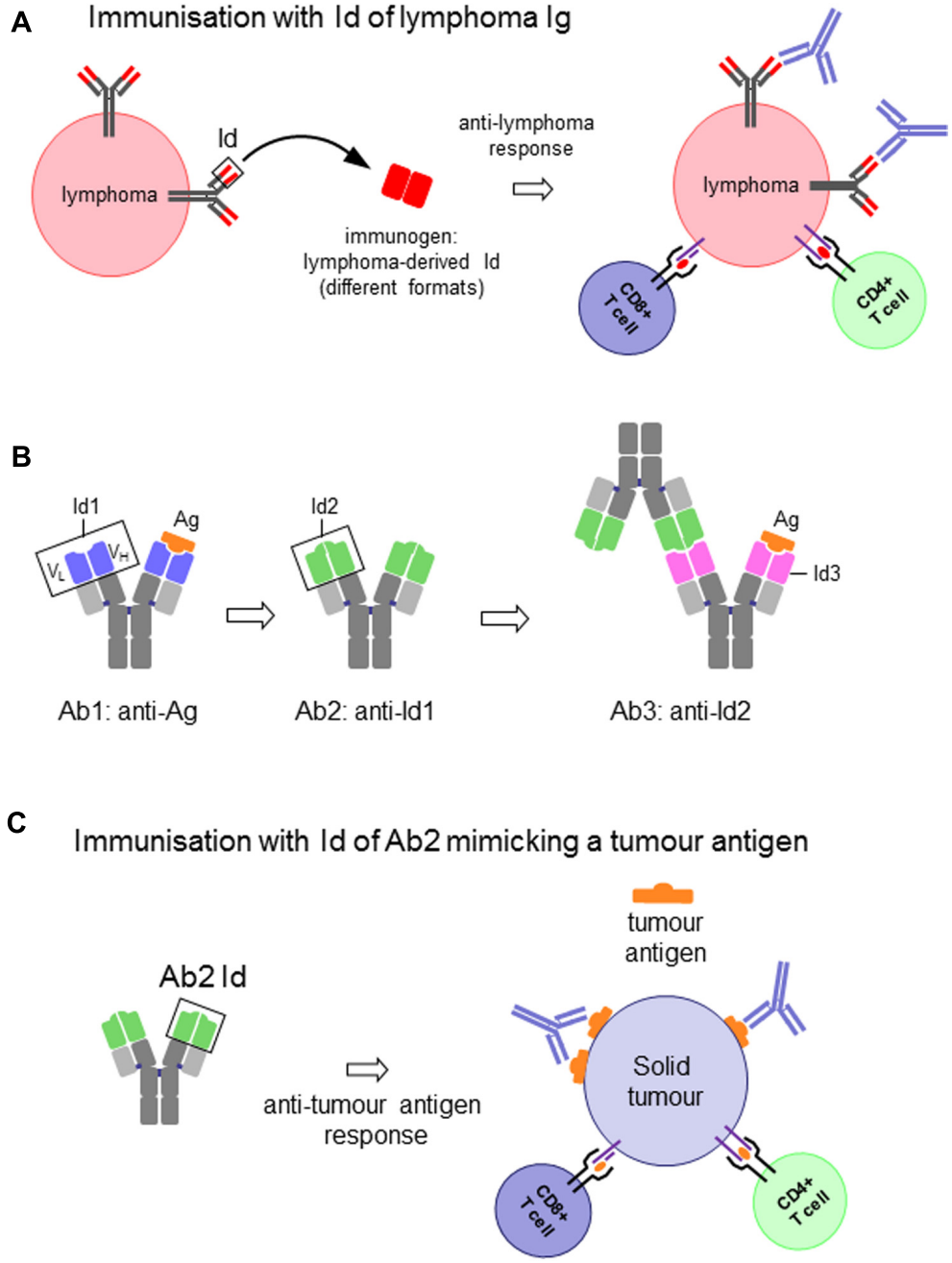

FIGURE 2 | (A) Idiotype (Id) of an immunoglobulin (Ig)-expressing lymphoma cell as vaccine to induce anti-lymphoma immune response. **(B)** Schematic representation of the antigen-specific antibody (Ab1), the antigen (Ag)-mimicking anti-Ab1 anti-idiotypic antibody (Ab2), the anti-Ab2 anti-idiotypic antibody (Ab3) and their interactions. **(C)** Id of an Ab2 as vaccine to induce antigen-specific immune response to solid tumors.

Conjugation to KLH has also been used in the context of antigen-mimicking anti-idiotypic vaccines. As examples, immunization of melanoma patients with the mouse anti-idiotypic antibody MK2-23, mimicking the high molecular weight-melanoma associated antigen (HMW-MAA), was significantly more efficient in inducing anti-anti-idiotypic antibodies when conjugated to KLH and administered in association to *Bacillus Calmette-Guérin* (BCG; Mittelman et al., 1995). Similarly, the murine anti-idiotypic antibody 3H1, which mimics a specific epitope of carcinoembryonic antigen (CEA), when conjugated to KLH and emulsified in Freund's adjuvant was found to be able to induce effective anti-CEA immune responses in animals (Saha et al., 2006b). Finally, immunization with KLH-coupled R24, a mouse monoclonal antibody (mAb) specific for the disialoganglioside 3 (GD3), which is over-expressed on transformed melanocytes, induced an anti-idiotypic cascade leading to the identification of

an anti-anti-idiotypic mAb able to mediate cytotoxicity on human melanoma cells and to inhibit tumor growth in xenografted mice (Ramos et al., 2011).

Apart from KLH, GM-CSF has been extensively tested in numerous protein immunization studies (Tao and Levy, 1993;Chen et al., 1994; Kwak et al., 1996), which altogether demonstrated the capacity of this cytokine to improve vaccine efficacy in terms of ability to induce Id-specific responses. The immunostimulatory properties of GM-CSF have been recently compared to those raised by CpG or IFN-α. This study demonstrated that these two last compounds are better immune adjuvants as, in contrast to GM-CSF, their administration induced efficient protection in a murine myeloma model upon vaccination with Id-KLH protein (Hong et al., 2012). Similarly, CpG co-administration significantly improved the cellular anti-CEA response in transgenic mice expressing human CEA and vaccinated with the CEA-mimicking murine 3H1 mAb (Ab2). In a CEA-transfected murine colon carcinoma cell model, the vaccine was effective in inducing protective anti-tumor immunity (Saha et al., 2006a). Other cytokines that have been explored in animals, as Id-fusion proteins, include IL-2 (Chen et al., 1994; Wang et al., 2005) and IL-4 (Chen et al., 1994).

Alum, QS-21, and BCG are adjuvants that have been used in the clinics in anti-idiotypic vaccine formulations with different antigen-mimicking murine Ab2 mAbs (Mittelman et al., 1995; Foon et al., 1999; Birebent et al., 2003; Giaccone et al., 2005; Grisham et al., 2011;Soriano et al., 2011). An interesting case is the Id of 1E10 mAb, which mimics a tumor-associated type of ganglioside (Alfonso et al., 2002; Diaz et al., 2003; Hernandez et al., 2005) and, when administered in patients as an anti-idiotypic vaccine (racotumomab) in alum, induces a set of antibodies that bind the ganglioside but not the 1E10 Id (Alfonso et al., 2002; Diaz et al., 2003; Hernandez et al., 2008), and a ganglioside-specific cellular response (Guthmann et al., 2006).

IMMUNOGENIC RECOMBINANT PROTEINS

Compared to hybridoma technology, the molecular rescue of the tumoral Id from patient's lymphoma cells is a less-time consuming alternative. Reverse transcription-polymerase chain reaction (RT-PCR) with family-based V gene primers allows identification and isolation of V_L and V_H region sequences directly from biopsy materials. The tumor-associated idiotypic V_L and V_H chains can then be cloned and assembled into different formats [single chain Fv (scFv), Fab, or full-length Ig] for expression in mammalian, bacteria, insect, or plant cells (Bird et al., 1988; Gurunathan et al., 2000). As example, recombinant human and murine Id in scFv format produced in plants, an appealing option that allows rapid production and recovery of the recombinant protein, were found to be highly immunogenic without the need of KHL cross-linking, both in presence and in absence of adjuvant (McCormick et al., 2008). scFv molecules can be also fused to immune-stimulating molecules. A fusion protein consisting of 38C13 murine B cell lymphoma scFv and tetanus toxin fragment C (FrC), produced in *E. coli* as inclusion bodies or using a cell-free protein synthesis system, induced anti-Id antibodies and was as effective as the IgM-KLH Id protein in increasing survival after tumor challenge (Patel et al., 2011).

Up to now, the only recombinant fragment assayed in the clinics in B cell lymphoma patients is the tumor Fab-Id produced in *E. coli* (Bertinetti et al., 2006; Navarrete et al., 2011). In the case of an antigen-mimicking Ab2, the $F(ab')_2$ fragment from rat BR3E4 mAb, which mimics the colorectal carcinoma-associated epitope CO17-1A, was administered to patients coupled to KLH and showed to be more effective than the whole uncoupled IgG in inducing humoral and cellular responses (Birebent et al., 2001, 2003).

Chimeric antibodies have been assayed at the preclinical level to increase Id immunogenicity. The 1E10 mAb expressed as a chimeric mouse-human IgG1 and administered to mice in PBS alone, was immunogenic in syngeneic mice and the induced anti-idiotypic antibody response was dominant over the anti-xenogeneic human constant region response (Lopez-Requena et al., 2003). In contrast, chimeric antibodies containing the 38C13 murine B cell lymphoma Id required coupling to KLH for inducing an anti-Id antibody response. However, when fused to GM-CSF, IL-2, or IL-4, these recombinant molecules elicited anti-tumor immunity when administered without carrier or adjuvant (Tao and Levy, 1993; Chen et al., 1994). Similarly, a fusion protein consisting of the HMW-MAA-mimicking chimeric MK2-23 Ab2 mAb linked to IL-2 was effective in enhancing immunogenicity without the need of coupling to KLH (Wang et al., 2005). Recombinant molecules containing a single xenogeneic IgG domain have also been designed for Id immunization. A bivalent Id protein, obtained by fusing the BCL1 murine B cell lymphoma scFv to the CH3 domain from human IgG1 as dimerizing unit (Li et al., 1997), was effective in inducing anti-Id antibodies when administered either with or without adjuvant (Benvenuti and Burrone, 2001).

DNA VACCINES

Compared with Id protein vaccines, direct vaccination with DNA encoding the lymphoma Id is logistically easier to use and cheaper to manufacture. In principle, it can provide longer antigen expression to enable a sustained stimulation of both humoral and T cell-mediated immunity (Benvenuti and Burrone, 2002). As in the case of the Id protein, initial DNA vaccination studies in murine models showed that the tumor-derived V regions alone are poorly immunogenic, but the relatively easy manipulation of the antigen-encoding cassette has facilitated the testing of several different designs to augment immunogenicity. This has been achieved by genetically linking the Id sequence to a cytokine sequence such as GM-CSF (Syrengelas et al., 1996) or to different $CD4^+$ T cell epitope carriers. King et al. (1998) tested plasmid-encoded Id (scFv) as a vaccine in the murine A31 B cell lymphoma and 5T33 myeloma models. Initially, only low anti-Id antibody levels and poor tumor protection were achieved when the Id was used alone. However, when the scFv gene was genetically linked to the FrC of tetanus toxin, the majority of the animals tested were protected with significant enhancement of the anti-Id antibody response. Another bacterial molecule, the B subunit of *E. coli* heat labile toxin, when fused to the BCL1 murine B cell lymphoma scFv and administered as intra-muscular injection of plasmid DNA followed by electroporation increased the antibody response against the Id and promoted survival in mice challenged with the tumor. These

effects depended on the pentamerization of the fusion protein and its binding to the GM1 ganglioside (Chen et al., 2009). On the same line, in the 38C13 murine B cell lymphoma model, Syrengelas et al. (1996) showed that provision of a DNA construct encoding a constant region of human Ig linked to the Id gene was required for specific induction of anti-Id antibodies. Moreover, vaccination with an Id-GM-CSF fusion construct resulted in enhancement of both anti-Id antibody levels and tumor protection. The scFv-Id gene was also fused to a DNA encoding an immuno-enhancing peptide derived from IL-1β and demonstrated induction of tumor immunity that protected mice from tumor challenge (Hakim et al., 1996). An alternative fusion gene between a scFv and a protein derived from a plant virus was described as a further way to provide T cell epitopes and induce protective immunity in lymphoma and myeloma (Savelyeva et al., 2001).

Although scFv is a popular format for the design of recombinant Id-based vaccines (King et al., 1998), dimeric proteins are frequently constructed by fusing the scFv with a dimerizing unit (Benvenuti et al., 2000; Fredriksen et al., 2006). In fact, bivalency was reported to be important for efficient anti-Id antibody induction and T cell activation by a scFv-based vaccine (Fredriksen and Bogen, 2007). In the BCL1 model the fusion of the scFv to the xenogeneic CH3 domain from human IgG1 was found to efficiently induce anti-Id antibodies and protection upon tumor challenge (Benvenuti et al., 2000). The minimal immunizing unit that has been assayed is the heavy chain CDR3 (H-CDR3). A vaccine based on Id H-CDR3 fused to tetanus toxin FrC was protective in the 38C13 murine B cell lymphoma model (Iurescia et al., 2010).

One particularity of DNA vaccination is that the immunizing molecule is *per se* immunostimulatory. The non-immunogenic 1E10 mAb Id, in the context of a fusion protein with CH3 domain from mouse IgG1 (i.e., with no xenogeneic carrier), induced a clear anti-idiotypic response by gene gun DNA immunization. The adjuvant properties of bacterial DNA seemed to be responsible for this outcome, as when the same recombinant construct was administered through recombinant adeno-associated virus (rAAV) infection no response at all was detected (Lopez-Requena et al., 2007a). In a contrasting result, the xenogeneic human IgG3 hinge/CH3 were required for good antibody responses and tumor protection in the murine MOPC315 myeloma and A20 B cell lymphoma models, with a DNA vaccine encoding the respective dimeric scFv-Id fused to the MIP-1α chemokine, administered by intramuscular injection followed by electroporation. When the syngeneic counterparts were used, the vaccine failed to elicit protective immunity (Fredriksen and Bogen, 2007).

VIRAL VECTORS

Administration of naked DNA has the disadvantage that a large amount of the immunogen is degraded before entering the cells (Dupuis et al., 2000). The use of virus-based vectors can partially circumvent this problem allowing the efficient penetration of cells while mimicking natural infection (Chen et al., 1996). Concerning the employment of viral vectors as vehicle to Id delivery *in vivo*, preclinical studies were performed in two murine B cell lymphoma models (38C13 and BCL1) to address the vaccine efficacy of Id-encoding adenoviruses (Timmerman et al., 2001). It was demonstrated in both models that a single injection of Id-adenovirus provided protection from subsequent tumor challenge, which was equivalent, or superior to that obtained by standard Id-KLH protein vaccine. However, this protection was dependent on the inclusion of xenogeneic Ig constant region. The ability of recombinant adenovirus encoding a lymphoma Id gene to induce humoral and T cell-mediated anti-Id responses was evaluated also in the A20 murine B cell lymphoma model. A single vaccination with an adenoviral vector encoding a scFv derived from the lymphoma tumor Id coupled to the human IgG1 Fc (Ad.A20hFc) elicited a specific anti-Id antibody response and protection in challenged animals (Armstrong et al., 2002).

Among non-replicative viruses, adeno-associated viruses (AAV) have been extensively explored as transgene delivery vectors for their capacity to infect mainly non-dividing cells, such as muscle cells, and to induce a long-term expression of the transgene. The potentiality of these vectors for immunotherapeutical purposes has been intensely explored (Manning et al., 1997; During et al., 1998; Liu et al., 2000; Xin et al., 2002). Overall, these studies demonstrated the reliable capacity of recombinant AAV (rAAV) to induce specific immunity to foreign antigens upon intramuscular, subcutaneous, and oral administration. In this regard, we explored the possibility to induce anti-Id immune responses by means of rAAV-mediated vaccinations. We demonstrated that immunization with two recombinant vectors, encoding the BCL1 murine B cell lymphoma scFv (Id^{BCL1}) fused to the human IgG1 CH3 domain (Id^{BCL1}/CH3), induced significant anti-Id antibody titers one month from rAAV injection (Cesco-Gaspere et al., 2008). However, while a single intra-muscular injection of rAAV-Id^{BCL1}/CH3 produced efficient tumor rejection *in vivo*, anti-Id antibody titers were significantly lower compared to previous standard immunizations consisting of repetitive administration of plasmid DNA by gene gun. Interestingly, when biolistic and rAAV immunization were combined in a prime-boost vaccination regimen the anti-Id immune response was further improved with a concordant increase in tumor protection. The mechanism that drives the efficacy of the prime-boost phenomenon is not clearly identified. In most cases, the huge amount of antigen supplied by the recombinant virus, in this case rAAV, seems to guarantee a more robust expansion of the antigen-specific immune response primed by the initial vaccination with naked DNA.

TARGETING OF ANTIGEN-PRESENTING IMMUNE CELLS

Alternative ways to enhance immunogenicity of Ids have been investigated to overcome the need of chemical cross-linking and adjuvants that may have deleterious side effects. Among them, immunization with autologous dendritic cells (DCs) pulsed with the Id protein or *in vivo* targeting of antigen-presenting cells (APCs) were tested. The first approach produced encouraging results in different clinical trials (Hsu et al., 1996; Timmerman et al., 2002). A combination of sequential administration of pulsed DCs and of Id-KHL protein, was also found to be efficient in inducing both humoral and cellular anti-lymphoma Id immunity (Hsu et al., 2001). A more recent study reported the induction of protective immunity in two lines of transgenic mice by pulsing DCs with an anti-idiotypic antibody (6D12) mimicking Her-2/neu antigen (Saha and Chatterjee, 2010).

In an alternative setting, Id vaccines have been targeted to APCs by fusion with chemokines, CD40-ligand (CD40L) or antibodies specific for APC-restricted molecules (**Figure 3**). In a pioneering study, two chemokines, IP-10 and MCP-3, were N-terminally fused to the Ids from two different lymphoma models in the scFv format, and delivered as plasmid DNA or purified proteins (Biragyn et al., 1999). The fusions elicited strong immune responses that protected from tumor challenge in both models and with both chemokines. The adjuvant effect was supposed to be due to targeting of the antigen to professional APCs where the receptor for chemokines is expressed. This view was suggested by the requirement of a physical link between the scFv-Id antigen and the functionally active chemokine. Using a similar vaccine design, the same group demonstrated that the chemokine fusion facilitates the presentation of the tumor Id by APC via the MHC class II pathway (Biragyn et al., 2004). Other chemokines have been exploited as targeting unit for APC priming, such as MIP-1α and RANTES. The presence of a xenogeneic dimerizing unit (human IgG3 CH3 domain) was found to be essential in inducing protective immune response in mice immunized with a vaccine consisting of the tumor Id fused to MIP-1α or RANTES chemokines (Fredriksen and Bogen, 2007). A DNA vaccine consisting of the A20 murine B cell lymphoma Id in scFv format fused to the MCP-3 chemokine and administered in sites pretreated with cardiotoxin had a higher impact on overall survival of challenged mice than the scFv alone. The presence of the myotoxin, which recruited APCs by promoting sterile inflammation, was also advantageous for the efficacy of the vaccination (Qin et al., 2009).

Targeting of antigen to APCs can be achieved also by antibodies or scFv specific for APC-restricted molecules. A DNA immunogen encoding a bivalent tumor Id and an anti-MHC class II scFv antibody was able to induce in mice higher levels of anti-Id antibodies than in control animals that had received an unrelated targeting unit or in animals not expressing the specific class II allele. The immune response led to tumor protection in two murine models, the MOPC315 myeloma and the A20 B cell lymphoma (Fredriksen et al., 2006). It was demonstrated a superior priming capacity of APC derived from draining lymph nodes to activate antigen-specific T cells *in vitro* when animals were vaccinated with the MHC class II-specific plasmid. Concordantly, antigen-specific $CD4^+$ T cells in lymph nodes were found to be more activated in these animals, consistent with a targeting effect due to enhanced uptake of protein by APC and presentation to $CD4^+$ T cells. The same prototype construct, encoding a myeloma patient-derived Id, efficiently induced in mice specific anti-Id responses that were patient-specific and dependent on the targeting effect (Froyland et al., 2011).

CD40 has been also exploited as APC-targeting receptor. Fusion of a chimeric antibody containing the 38C13 murine B cell lymphoma Id to CD40L improved immunogenicity compared to administration of the antibody alone, to levels similar to those of an Id/GM-CSF fusion protein (Huang et al., 2004). *In vivo* targeting of $CD40^+$ APCs with a vaccine consisting on the A20 murine B cell lymphoma Id chemically conjugated to an anti-CD40 antibody induced more efficient tumor protection than a classic vaccination with Id-KLH plus GM-CSF. Monophosphoryl lipid A but not GM-CSF had a synergistic effect with the Id conjugated to the antibody (Carlring et al., 2012).

Recently, the 38C13 murine B cell lymphoma-derived scFv-Id was linked, in a diabody format, to a B cell targeting moiety constituted by an anti-CD19 scFv. The recombinant molecule was proven to bind to non-cognate B cells, which in turn stimulated Id-specific B cells. Animals vaccinated with this diabody developed potent Id-specific antibody and T cell responses comparable to

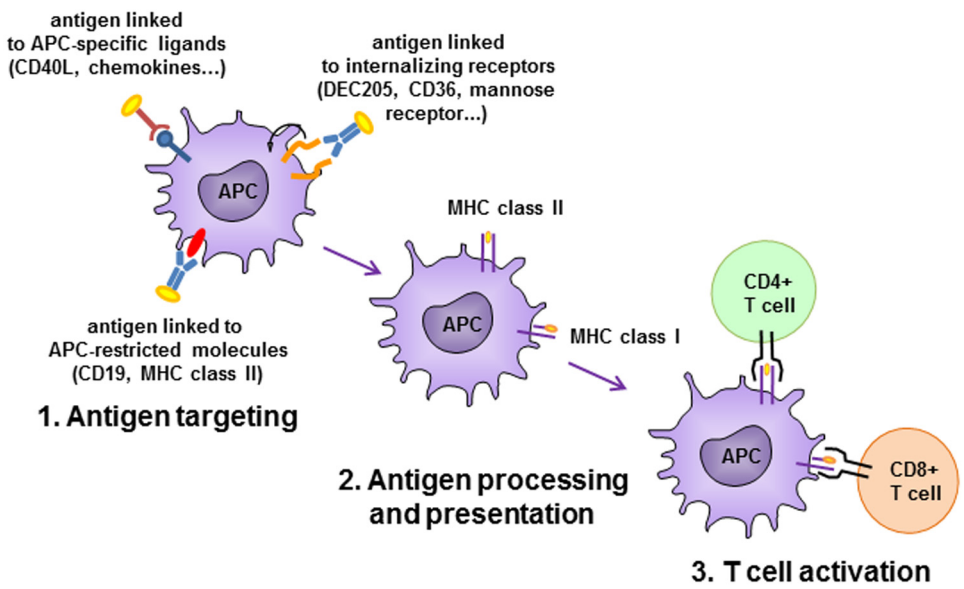

FIGURE 3 | Antigen-presenting cell (APC)-targeting strategies. Antigen is delivered to APC by antibodies specific to APC-restricted molecules or internalizing receptors, or by APC-specific ligands. Antigen is efficiently internalized, processed and presented to activate CD4+ and/or CD8+ T cells.

those induced by 38C13-KLH. Interestingly, such diabodies were produced in a cell-free protein expression system that allowed the preparation of proper amounts of vaccine in a few hours (Ng et al., 2012).

ANTIBODIES VERSUS T CELL RESPONSES

The evidences accumulated so far on tumor protection induced by anti-Id immunization do not allow to clearly define the main mechanism involved in the rejection of tumor cells. The understanding of this issue is of major relevance, since this may allow improving vaccine efficacy and prognosis of lymphoma patients.

In mouse models, protein-based vaccines were shown to work through an anti-Id antibody-dependent mechanism (Racila et al., 1995; Timmerman and Levy, 2000) or through the induction of effector T cells (Biragyn et al., 1999). In human studies, clinically relevant immune responses induced by immunization with Id-protein or Id-pulsed DCs were dependent on the induction of Id-specific $CD8^+$ T cells (Bendandi et al., 1999). Moreover, synthetic peptides derived from Ig framework region shared among lymphoma patients were able to induce cytotoxic responses (Trojan et al., 2000). These evidences indicated that malignant B cell can process and present class I-restricted peptides derived from Ig framework V regions. The study of Inoges et al. (2006) clearly reported the induction of a humoral and cytotoxic cellular immune response against Id in lymphoma patients immunized with KLH-coupled Id in association with GM-CSF.

Upon DNA immunization, the *in vivo* antigen synthesis and processing should favor the loading of antigenic peptides onto MHC class I molecules and the induction of cytotoxic T lymphocyte (CTL) responses only if the delivered DNA construct is expressed by APCs, which depends on the method of DNA delivery. However, by using a chimeric mouse/human IgG DNA vaccine delivered by intramuscular injection, it was shown that protection in the 38C13 murine B cell lymphoma model was not impaired by $CD4^+$/$CD8^+$ T cell depletion (Syrengelas and Levy, 1999).

Similarly, we demonstrated that anti-Id antibodies, were totally essential to confer tumor protection in the BCL1 murine B cell lymphoma model (Cesco-Gaspere et al., 2005), despite the fact that DNA was intradermally delivered by gene gun immunization, a method that allows expression of antigen also by dermal resident DCs (our unpublished observations). However, since we did not deplete any T cell subpopulation, a possible contribution of protective T cells in this model, in addition to antibodies, cannot be completely ruled out.

On the contrary, in the 38C13 as well as in the A20 murine B cell lymphoma models, protection was found to be dependent on $CD4^+$ and $CD8^+$ T cells, when animals were vaccinated with a DNA construct encoding the scFv-Id fused to two different chemokines (Biragyn et al., 1999). In addition, cytotoxic cell lines, with proliferative and cytotoxic activity against the A20 murine B cell lymphoma cell line, were efficiently generated from mice vaccinated with a scFv adenoviral vaccine (Armstrong et al., 2002). Interestingly, in some cases Id-specific $CD4^+$ T cells have been found to be unique players in tumor rejection. In the murine A31 B cell lymphoma and 5T33 myeloma models, protection induced by DNA vaccination with a scFv fused to potato X virus protein was clearly compromised upon *in vivo* depletion of $CD4^+$ T cells (Savelyeva et al., 2001). Another study performed on this issue showed that tumor protection against B lymphoma cells was induced in absence of B cells, antibodies and $CD8^+$ T cells in a T cell receptor (TCR)-transgenic mouse model specific for an MHC class II-restricted peptide of the λ2 Ig light chain derived from the MOPC315 plasmacytoma (Lundin et al., 2003).

Considering clinical trials, a positive correlation between anti-Id antibody response and overall survival was found in patients with follicular lymphoma receiving the Id-KLH plus GM-CSF vaccine. In contrast, the T cell response was not associated with clinical outcome, although this was attributed to the variability of the cellular assays (Ai et al., 2009). In a trial with an Id vaccine in the Fab format, the cellular response correlated with higher progression-free survival in the group of patients receiving the vaccine as first line treatment, but not in those immunized after remission consolidation following chemotherapy (Navarrete et al., 2011). Previous studies had indicated an association between T cell responses and clinical benefit (Bendandi et al., 1999; Inoges et al., 2006). In any case, the induction of Id-specific CTLs is almost always a desired feature. The use of GM-CSF in current clinical vaccine formulations is indeed directed to achieve this goal. Several patients enrolled in clinical trials developed T cell responses against the immunizing Id (Mahaseth et al., 2011; Rezvani and de Lavallade, 2011). T cell specificities were mapped to peptides derived from the CDRs of V_H (Navarrete et al., 2011). Induction of V_H-CDR3-specific CTLs was also achieved when immunizing mice with a plasmid encoding this peptide fused to tetanus toxin FrC (Iurescia et al., 2010). In fact, DNA immunization is an excellent tool to induce cellular responses (Stevenson et al., 2011).

Another strategy to achieve cellular responses against the immunizing Id is vaccination with Id-pulsed DCs. In a small trial, autologous DCs incubated with the Id and KLH and re-administered to the patients activated T cells specific for the Id in half of them, although the relative contribution of Th1 and Th2 compartments in this response was not defined (Rollig et al., 2011). However, it has also been reported that immunization with DCs pulsed with conserved idiotopes shared by different Ids (i.e., peptides from J regions) can lead to activation of regulatory T cells and thus dampen the response against Id-specific idiotopes such as H-CDR3 peptides (Warncke et al., 2011). In another approach, a shift toward Id-specific Th1 response was achieved in the A20 murine B cell lymphoma model using a complex mixture containing: the Id DNA, MIP3-α chemokine, and a small interfering RNA to silence IL-10. This formulation induced CTL activation and protection after tumor challenge (Singh et al., 2011).

Furthermore, the anti-tumor effect of the MCP-3 chemokine-scFv DNA vaccine in combination with cardiotoxin was dependent on the cellular response, as it was abrogated after T cell depletion, but independent of the humoral response, as protection was also achieved in B cell-deficient mice (Qin et al., 2009).

In the case of human antigen-mimicking Ab2 used in the clinics, the Id is neither foreign nor strictly self, but syngeneic. It has been suggested that for T cell responses the presence of the human Fc could be advantageous (Durrant et al., 2001). The 105AD7 mAb, which mimics the complement regulatory protein CD55

Table 1 | Summary of some vaccine designs to foster anti-Id immune response discussed in the text.

	Id-vaccine design	Target	Reference
Carrier and adjuvants	Tumor Ig coupled to KLH + GM-CSF	Human lymphoma	Bendandi et al. (1999), Barrios et al. (2002), Inoges et al. (2006), Redfern et al. (2006), Timmerman et al. (2009)
	Tumor Ig coupled to KLH + CpG/IFN-α	5TGM1 murine myeloma	Hong et al. (2012)
Immunogenic recombinant proteins	Tumor scFv fused to TT-FrC	38C13 murine B cell lymphoma	Patel et al. (2011)
	Tumor scFv fused to human γ1 CH3	BCL1 murine B cell lymphoma	Benvenuti and Burrone (2001)
	Tumor Fab + GM-CSF	Human lymphoma	Bertinetti et al. (2006), Navarrete et al. (2011)
	Chimeric tumor Ig fused to GM-CSF, IL-2, IL-4	38C13 murine B cell lymphoma	Tao and Levy (1993), Chen et al. (1994)
DNA vaccines	Tumor Id H-CDR3 fused to TT-FrC	38C13 murine B cell lymphoma	Iurescia et al. (2010)
	Tumor scFv fused to TT-FrC	Murine A31 B cell lymphoma and 5T33 myeloma	King et al. (1998)
	Tumor scFv fused to the coat protein of Potexvirus	Murine A31 B cell lymphoma and 5T33 myeloma	Savelyeva et al. (2001)
	Tumor scFv fused to human γ1 CH3	BCL-1 murine B cell lymphoma	Benvenuti et al. (2000)
	Tumor scFv fused to B subunit of *E. coli* heat labile toxin	BCL-1 murine B cell lymphoma	Chen et al. (2009)
	Chimeric tumor Ig fused to GM-CSF	38C13 murine B cell lymphoma	Syrengelas et al. (1996)
Viral vectors	Tumor scFv fused to human γ1 CH3 in adeno-associated virus vector	BCL-1 murine B cell lymphoma	Cesco-Gaspere et al. (2008)
	Chimeric tumor Ig in adenovirus vector	Murine 38C13 and BCL-1 B cell lymphomas	Timmerman et al. (2001)
APC-loading/ targeting	Tumor scFv fused to IL-1β-derived peptide	38C13 murine B cell lymphoma	Hakim et al. (1996)
	Tumor scFv fused to IP-10 or MCP-3	Murine 38C13 and A20 B cell lymphomas	Biragyn et al. (1999)
	Tumor scFv fused to MCP-3 + cardiotoxin	A20 murine B cell lymphoma	Qin et al. (2009)
	Tumor scFv fused to human γ3 CH3-hinge and to MIP-1α or RANTES	Murine A20 B cell lymphoma and MOPC315 myeloma	Fredriksen and Bogen (2007)
	Tumor scFv fused to human γ3 CH3-hinge and to an anti-MHC class II scFv	Murine A20 B cell lymphoma and MOPC315 myeloma	Fredriksen et al. (2006)
	Tumor scFv fused to an anti-CD19 scFv (diabody)	38C13 murine B cell lymphoma	Ng et al. (2012)
	Chimeric tumor Ig fused to CD40L	38C13 murine B cell lymphoma	Huang et al. (2004)
	Tumor Ig chemically conjugated to an anti-CD40 antibody	A20 murine B cell lymphoma	Carlring et al. (2012)
	Tumor Ig-loaded DCs	Human lymphoma	Hsu et al. (1996), Timmerman et al. (2002)

Id, idiotype; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; GM-CSF, granulocyte macrophage–colony stimulating factor; CpG, oligodeoxynucleotides containing unmethylated CG dinucleotides; IFN-α, interferon alpha; scFv, single-chain variable fragment; TT-FrC, tetanus toxin fragment C; IL-2, interleukin 2; IL-4, interleukin 4; Fab, antigen binding fragment; H-CDR3, heavy chain complementary-determining region 3; IL-1β, interleukin 1 beta; IP-10, interferon inducible protein 10; MCP-3, monocyte chemotactic protein 3; MIP-α, macrophage inflammatory protein 1 alpha; RANTES, regulated and normal T cell expressed and secreted; CD40L, CD40-ligand; DCs, dendritic cells; MHC, major histocompatibility complex.

(Spendlove et al., 2000) was obtained by the heterohybridoma technique using lymphocytes from a colorectal cancer patient administered, for diagnostic purposes, with an anti-tumor mouse mAb (Austin et al., 1989). Vaccination of colorectal (Robins et al., 1991; Ullenhag et al., 2006) and osteosarcoma (Pritchard-Jones et al., 2005; Ullenhag et al., 2008) patients with 105AD7 mAb in alum alone or with BCG in the first immunization (Ullenhag et al., 2006), either failed to induce an anti-anti-idiotypic antibody response (Robins et al., 1991; Ullenhag et al., 2006) or induced a weak response against CD55 (Pritchard-Jones et al., 2005), but it was able to activate both anti-Ab2 and anti-CD55 T cell responses (Ullenhag et al., 2006, 2008).

In one study B lymphocytes from a patient with colorectal cancer treated with a mouse mAb, specific for the epithelial-cell adhesion molecule (Ep-CAM), were immortalized with Epstein–Barr virus to isolate anti-idiotypic antibodies (Steinitz et al., 1988). A pool of these Ab2, given in alum either unconjugated or conjugated to immunogenic peptides from the *Bordetella pertussis* toxin subunit S1, induced humoral and cellular responses against the antibodies and the Ep-CAM antigen (Fagerberg et al., 1995). In another study immunization with the antigen or the Ab2 mAb with GM-CSF as adjuvant was compared. While both immunogens generated specific T cell responses, the Ab2 mAb did not induce anti-Ep-CAM antibodies (Mosolits et al., 2004b). The induction of antigen-specific T cell responses was later confirmed in a trial where the recombinant antigen, the Ab2 mAb and a combination of both, also with GM-CSF, were tested (Mosolits et al., 2005). Interestingly, in a preclinical study with Ep-CAM-expressing transgenic mice, the Ab2 protein vaccine was more efficient than the Ab2 DNA vaccine for the anti-anti-idiotypic antibody response. In the genetic strategy, fusion to GM-CSF but not the presence of a xenogeneic Fc region, enhanced the antibody response against the Ab2 (Mosolits et al., 2004a).

CONCLUSION

Although the Id of Igs is nominally a self-antigen and consequently poorly immunogenic, strategies intended to induce anti-Id immune responses have successfully demonstrated the feasibility of breaking tolerance against Ids, both in animal models and in the human context. The employment of adjuvants, carriers, viral vectors, or the direct recruitment of immune-related cells has allowed translating Id targeting by the immune system into therapeutic approaches for Ig-expressing tumors (**Table 1**). Treatment of several other malignancies can also involve Id-based vaccination, taking advantage of antigen mimicry by Ids. Solid evidences of clinical benefit are however still awaited, and more work is needed to unveil the mechanisms and factors that impact on the generation of therapeutic anti-Id immune responses.

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Anti-ganglioside anti-idiotypic vaccination: more than molecular mimicry

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Surgery, chemotherapy, and radiation therapy are standard modalities for cancer treatment, but the effectiveness of these treatments has reached a plateau. Thus, other strategies are being explored to combine with the current treatment paradigms in order to reach better clinical results. One of these approaches is the active immunotherapy based on the induction of anti-tumor responses by anti-idiotypic vaccination. This approach arose from Jerne's idiotypic network theory, which postulates that B lymphocytes forms a functional network, with a role in the establishment of the immune repertoires, in the regulation of natural antibody production and even in the establishment of natural tolerance. Due to the large potential diversity of the immunoglobulin variable regions, the idiotypes repertoire can mimic the universe of self and foreign epitopes, even those of non-protein nature, like gangliosides. Gangliosides are sialic acid-containing glycolipids that have been considered attractive targets for cancer immunotherapy, based on the qualitative and quantitative changes they suffer during malignant transformation and due to their importance for tumor biology. Although any idiotype could be able to mimic any antigen, only those related to antigens involved in functions relevant for organism homeostasis, and that in consequence has been fixed by evolution, would be able not only to mimic, but also to activate the idiotypic cascades related with the nominal antigen. The present review updates the results, failures and hopes, obtained with ganglioside mimicking anti-idiotypic antibodies and presents evidences of the existence of a natural response against gangliosides, suggesting that these glycolipids could be idiotypically relevant antigens.

Keywords: idiotypic network, anti-idiotypic antibody, ganglioside, vaccine, natural antibodies

GANGLIOSIDES

Gangliosides are glycosphingolipids present in the outer leaflet of the plasma membranes, where significantly contribute to the surface properties of cells (Sorice et al., 1997). These glycolipids are involved in various cellular functions, including signal transduction (Bremer et al., 1986; Fujitani et al., 2005), regulation of cell proliferation and differentiation (Hakomori, 1970; Sakiyama et al., 1972; Critchley and Macpherson, 1973; Yogeeswaran and Hakomori, 1975), cell–cell recognition (Feizi, 1985), adhesion (Cheresh et al., 1986), and cell death (De Maria et al., 1997, 1998; Kirschnek et al., 2000; Colell et al., 2001). Gangliosides function as the Ca^{2+} binding co-factor in synaptic transmission (Svennerholm, 1980), and as the co-factor of membrane adenylate cyclase (Partington and Daly, 1979). Furthermore, they interact with, or are the receptors of different bioactive molecules, such as bacterial toxins (Simpson and Rapport, 1971a,b; Van Heyningen et al., 1971; Holmgren et al., 1973, 1980a; Kato and Naiki, 1976; Ledley et al., 1977), glycoprotein hormones (Lee et al., 1976, 1977; Mullin et al., 1976; Holmgren et al., 1980b), type 1 interferon (Besancon and Ankel, 1974; Vengris et al., 1976; Ankel et al., 1980), fibronectin (Kleinman et al., 1979), lymphokines (Higgins et al., 1978; Miura et al., 1979; Poste et al., 1979), and serotonin (Woolley and Gommi, 1965).

The processes accompanying malignant transformation, like oxidative stress, hypoxia, and angiogenesis, as well as the metastasis formation, induce the expression on cell membranes of potential targets for cancer immunotherapy. It has been widely documented that the expression patterns of membrane gangliosides suffer quantitative and qualitative changes during neoplastic transformations (Brady et al., 1969; Hildebrand et al., 1972; Fishman, 1974; Keranen et al., 1976; Portoukalian et al., 1976). Normal melanocytes express predominantly GM3, while GD3 increases when these cells suffer an oncogenic process (Ravindranath et al., 1991). GD3 plays a role in the regulation of cell growth and the induction of angiogenesis by the tumor cells (Hedberg et al., 2000; Zeng et al., 2000; Fredman et al., 2003). Melanoma GD3 carries a ceramide portion with a long chain fatty acid, in contrast with the GD3 expressed in the normal tissues that carries a shorter chain fatty acid (Nudelman et al., 1982). Fucosyl GM1 is a unique structure that is found in small cell lung cancers (SCLC) with a very limited expression in normal tissues (Krug et al., 2004; Tokuda et al., 2006). GD2 is highly expressed on neuro-ectodermal tumors (Heimburg-Molinaro et al., 2011) and in sarcomas (Kailayangiri et al., 2012). This ganglioside is also a cancer stem cells marker and promotes tumorigenesis (Battula et al., 2012).

A very interesting case is the one of *N*-glycolylated (NeuGc) gangliosides, since these glycolipids are not naturally expressed in humans due to a genetic deletion in the gene that codes the CMP-*N*-acetyl hydroxylase, enzyme that catalyzes the conversion of *N*-acetyl to *N*-glycolyl sialic acid (Irie et al., 1998; Irie and

Suzuki, 1998; Olson and Varki, 2003). However, both direct and indirect studies have indicated that NeuGc is overexpressed in several human tumors (Devine et al., 1991; Kawai et al., 1991; Marquina et al., 1996; Malykh et al., 2001). The most accepted theory for this phenomenon is the incorporation of NeuGc from dietary sources. Free sialic acids from the medium can be taken up into cells via pinocytosis. The content of the resulting pinocytotic vesicles and endosomes would eventually be delivered to the lysosome, where a sialic acid transporter then delivers the molecules into the cytosol (Bardor et al., 2005). Also endogenous synthesis from glycolyl-CoA is a possibility (Malykh et al., 2001). The explanation for the differential expression of these antigens (Ag) in human normal and tumor tissues is that the rapidly growing tumor tissues might be more efficient at scavenging NeuGc. Furthermore, hypoxia induces the expression of sialin, a sialic acid transporter on tumor cells, and enhances the incorporation of the non-human sialic acid from the external milieu (Yin et al., 2006).

The gangliosides are not only attractive targets due to their over-expression on tumor cells membranes but also because of their importance for tumor biology. The metastatic capacity of the cells is strongly affected by the gangliosides expressed on the cell membranes: disialogangliosides GD2 and GD3 participate in the anchoring of the melanoma and neuroblastoma metastatic cells to the extracellular matrix proteins (Cheresh et al., 1986; Fredman et al., 2003). Comparing the ganglioside pattern expressed by the primary tumor or the metastatic cells of a melanoma patient, gangliosides expression was higher in the last ones, especially GD1. There were also abundant *O*-acetylation of GM2, GD3, and GD2, which were absent in the primary tumor. GM2 is also strongly expressed in prostate metastasis (Zhang et al., 1998), where is found in the areas of tumor cell-to-tumor cell contact indicating a role in cellular interactions and adhesion (Fredman et al., 2003).

Furthermore, gangliosides actively shed from tumors are inserted into the plasmatic membrane of surrounding cells, affecting the function of lymphocytes (Miller and Esselman, 1975; Lengle et al., 1979; Whisler and Yates, 1980; Ladisch et al., 1983; Gonwa et al., 1984), monocytes (Ladisch et al., 1984), natural killer cells (Diatlovitskaia et al., 1985), and antigen-presenting cells (Caldwell et al., 2003; Bennaceur et al., 2006). Gangliosides have been found to shift the cytokine profile from Th1 toward the Th2 in affected cells (Crespo et al., 2006). Negative modulation of CD4 molecule on T lymphocytes has been described for both the *N*-acetylated (Sorice et al., 1995) and the *N*-glycolylated variants (de Leon et al., 2006) of GM3 ganglioside. Gangliosides have been reported to block the nuclear translocation of NF-κB in human monocytes and dendritic cells (Caldwell et al., 2003). GD3, isolated from the polar lipid fraction of ovarian cancer-associated ascites, was shown to be an inhibitory factor that prevents innate immune activation of natural killer T cells (Webb et al., 2012). GM2 inhibits immunoglobulin production by B cells (Kimata and Yoshida, 1996). In this way tumor released gangliosides reinforce tumor evasion by blocking the immunological surveillance.

Despite the fact that gangliosides are poorly immunogenic, due to their self and glycolipidic nature, several reports show the presence of naturally occurring antibodies, not only in cancer patients, but also in healthy individuals, suggesting that anti-ganglioside reactivity is fixed in the natural antibodies repertoire.

NATURALLY OCCURRING ANTI-GANGLIOSIDE ANTIBODIES

Natural antibodies are considered humoral mediators of innate immunity and recognize antigens highly conserved throughout evolution (Cojocaru et al., 2009). It has been proposed that natural auto-antibodies and auto-reactive T cells in healthy individuals may be directed to a specific and limited set of self-molecules; this selective autoimmunity has been termed the immunological homunculus (Cohen, 2007). Due to the limited number of mutations in the genes encoding the variable region of these antibodies, the repertoire of these immunoglobulins is highly conserved within species (Cojocaru et al., 2009). Several authors have described their capacity to bind foreign antigens but also self and altered self-antigens, which may be or not of protein nature (Cohen, 2007). These antibodies recognize epitopes associated with pathogens, such as phosphorylcholine of Gram-positive bacteria, lipopolysaccharide (LPS) of Gram-negative bacteria, and various molecules expressed by parasites (Ochsenbein et al., 1999; Baumgarth et al., 2005). For this reason they are considered as a first, quick anti-infection barrier that helps to guarantee the survival since the very beginning of the organisms' life. Among their targets have also been identified intracellular molecules, such as some nuclear (e.g., histones) and cytoskeleton (e.g., actin) proteins (Coutinho et al., 1995) and single-stranded DNA (Schwartz-Albiez et al., 2009). They also recognize peptides (e.g., amyloid beta peptide), plasma membrane glycoproteins (e.g., CD90; Schwartz-Albiez et al., 2009), oxidized lipids (e.g., phosphatidylcholine), and antigens expressed by apoptotic cells (Annexin IV; Chou et al., 2009; Baumgarth, 2011). Mounting evidence suggest that natural IgM antibodies, through this self-reactivity might contribute to critical innate immune functions involved in the maintenance of tissue homeostasis, like the clearance of apoptotic cells (Chou et al., 2009), reduction of atherosclerotic lesions (Hartvigsen et al., 2009; Cesena et al., 2012), and the reinforcement of mechanisms involved in the protection from the development of autoimmune disease (Werwitzke et al., 2005; Chen et al., 2009a,b; Silverman et al., 2009; Jiang et al., 2011; Stoehr et al., 2011).

Recently it has been described the existence of auto-antibodies against tumor-associated antigens, which can arise in the patients even before the symptoms become evident and that can be detected also in healthy donors (Zhang et al., 2003; Storr et al., 2006; Chapman et al., 2008). Some of these IgM isotype, germline antibodies have been isolated from cancer patients and have proved to be able not only to recognize tumor cells, but also to kill them by different mechanisms (Bohn, 1999; Hensel et al., 2001; Jakobisiak et al., 2003; Vollmers and Brandlein, 2005, 2006; Lutz et al., 2009). Many of the detected anti-tumor antibodies bind to carbohydrate repeated motifs, including sequences of sugars contained in gangliosides (Lutz and Miescher, 2008; Cojocaru et al., 2009).

Naturally occurring antibodies reacting with tumor-associated gangliosides have been detected in cancer patients but also in healthy donors. Antibodies against GM2 and GD2 were detected

in the sera of both melanoma patients and healthy individuals (Watanabe et al., 1982; Tai et al., 1985). Other authors have reported the existence in healthy donors of naturally occurring antibodies with reactivity against gangliosides like GM1, GD1a, GD1b, and GT1b (Mizutamari et al., 1994; Ravindranath et al., 1997; Lardone et al., 2006). Silent auto-reactive B clones have also been identified in cancer patients, from which human monoclonal antibodies (mAb) against gangliosides were generated. The mAb GMA1 reacted with the gangliosides GD3, GM3, and GD2 from melanoma and neuroblastoma cell lines and not normal tissues (Mukerjee et al., 1998). The human monoclonal IgM antibody 7c11.e8, also generated by fusing lymph node cells isolated from a surgical specimen of malignant melanoma reacted with GM4, GM3, and GD3. In the presence of human serum the antibody initiated a strong lysis of melanoma tumor cells in complement-dependent cellular cytotoxicity (CDCC) assays (Abdel-Wahab et al., 1993).

The presence of naturally occurring antibodies that recognize NeuGc acid present in tumor-associated glycoconjugates has also been described. It has been shown that normal human serum contains high levels of NeuGc-specific antibodies, which attract complement molecules to the surface of leukemic cells expressing NeuGc, but not other normal cells (Zhu and Hurst, 2002; Tangvoranuntakul et al., 2003; Nguyen et al., 2005; Padler-Karavani et al., 2008). Ravindranath et al. (2007), examining the sera of healthy volunteers between the ages of 18 and 90, reported that anti-ganglioside antibodies occurred naturally and that their levels decline after 50 years, which could be relevant since the cancer incidence increases with age.

Anti-ganglioside antibodies have shown to have anti-tumor cytotoxic capacities. It has been reported the ability of a murine anti-GM2 to induce apoptosis through caspase activation in lymphoma, melanoma, and lung cancer cells expressing the antigen (Retter et al., 2005). The binding of an anti-GD2 antibody to the ganglioside expressed in lung cancer cells induced apoptosis by the reduction in the levels of phosphorylation of FAK and activation of mitogen-activated kinase p38. Immunoprecipitation experiments showed a physical association of GD2 with integrins, which were associated with FAK inside the membrane. Antibody binding to ganglioside caused conformational changes in this complex, inducing the transmission of intracellular signals that mediated the apoptosis (Aixinjueluo et al., 2005). It has been also proved that anti-GD2 antibodies of healthy donors have cytotoxic capacity against neuroblastoma cells (Ollert et al., 1997).

The capacity of the mAb 14F7, a murine IgG highly specific for NeuGcGM3, to induce oncotic cell death to tumor cells expressing this antigen has been reported. This antibody induced a tumor cell death that was accompanied by cellular swelling, membrane lesion formation, and cytoskeleton activation (Carr et al., 2000; Roque-Navarro et al., 2008). Another antibody specific for *N*-glycolylated gangliosides is P3 mAb. This is an IgM, germline encoded that is able to induce complement-mediated cytotoxicity to NeuGc expressing tumor cells (Vázquez et al., 1995; Carr et al., 2002). It has been reported that naturally occurring anti-NeuGc in healthy humans were able to kill human leukemic cells that were exogenously fed with NeuGc by a complement-mediated mechanism (Nguyen et al., 2005).

These evidences suggest that the evolution has fixed an innate immunity against gangliosides in the natural antibodies repertoire, which could play an important role for tumor immune surveillance. Since the natural antibodies secreting B cells arise in the neonatal period, they could be connected and regulated by anti-Id interactions, according to Jerne's idiotypic network theory.

THE IDIOTYPIC NETWORK THEORY

In 1974, Neils Jerne published the Idiotypic Network theory, which gave a different view of the immune system organization and the recognition of the "self." According to classical clonal selection theory, the immune system was "antigen driven" and in the absence of an external antigen challenge the system should be passively inactive. In contrast, according to the network theory, the immune system consists of lymphocyte clones which are stimulated and regulated by the immunoglobulins produced by other clones within the network. Since a huge diversity of idiotypes (Id) is generated by random somatic rearrangements of genes, idiotype's complementary structures can be found not only on antigens but also on antibodies of different idiotypes. In Jerne's own words "the immune system of a single animal, after producing specific antibodies to an antigen, continues to produce antibodies to the idiotopes of the antibodies which it has itself made. The latter anti-Id antibodies likewise display new idiotypic profiles, and the immune system turns out to represent a network of idiotypic interactions" (Jerne, 1974, 1985).

This phenomena was extensively proved firstly by Kunkel and Oudin, who showed that ordinary antibody molecules that arise in an immunized animal are antigenic and induce the formation of specific anti-antibodies (Kunkel et al., 1963; Oudin and Michel, 1963). Later experiments further demonstrated that the recognition of self-idiotopes by B or T cells is an active physiological process controlling the suppression or expansion of the immune response (Eichmann and Rajewsky, 1975; Cazenave, 1977; Urbain et al., 1977; Bona et al., 1981).

This immune network is established in the neonatal period, thus this theory predicts that the immune systems has an autonomous activity, manifested by the presence of activated lymphocytes and antibody secretion, before any external immunization. This prediction was confirmed by studies on "antigen-free" animals, which contain in their spleen and peritoneal cavity activated B cells that secret IgM antibodies and T lymphocytes that perform as effector cells, help or suppress the antibody production (Hooijkaas et al., 1984; Pereira et al., 1986).

Thus, a network of idiotypically interacting immune cells is formed, that has a dynamic equilibrium between the idiotypes, anti-Id, and the normal self-constituents of our body, influencing the shaping of the B- and T cell repertoires, and controlling autoreactive clones.

In the 1980s, there was an interesting debate between the proponents of the network paradigm and those of the clonal selection theory and several experiments were performed that provided evidences about both ideas (Cohn, 1981, 1986; Langman and Cohn, 1986; Cazenave, 1988; Behn, 2007).

The establishment of collections of antibody-producing hybridomas, derived from normal, unimmunized mice at different stages of ontogeny, provided proof for the existence of idiotypic

connectivity (Holmberg et al., 1984a). Matrices of idiotypic complementarities were established, that allowed to estimate the degree of connectivity within different B cell populations (Holmberg et al., 1984a,b, 1986a,b,c; Kearney and Vakil, 1986). High levels of connectivity were observed within collections of fetal or neonatal origin (Holmberg et al., 1984b). However, within collections from the adult lymphocyte population the degree of connectivity was 10- to 100-fold lower (Holmberg et al., 1986c). These experiments suggested that high idiotypic connectivity is not an intrinsic property of any collection of IgM antibodies, but a distinctive property of part of the perinatal antibody repertoire.

Then, Varela and Coutinho formulated the concept of second-generation immune networks (Coutinho, 1989; Varela and Coutinho, 1991), which tried to combine the two competing paradigms. They adopted the view that the immune system is formed by two compartments of B and T lymphocytes: a majority of small resting cells constituting 80–85% of the total population and a set of large activated cells making up the other 15–20% (Pereira et al., 1985, 1986), this last being the predominant in the neonatal period. The specific response to a foreign antigen would be mainly caused by the activation of resting lymphocyte clones, which are only poorly connected to the network, thus forming the peripheral part of the system. The fraction of highly connected cells forms the actual network, a compartment of naturally activated lymphocytes. The immune network includes, in addition to V-regions, all other molecules of the somatic self. This pool of connected cells may be responsible for maintenance of normal network dynamics and prevention of auto-aggression.

The idiotypic network hypothesis predicts that due to the huge diversity of immunoglobulin variable regions, and since each antibody will bind its nominal antigen and also other immunoglobulins, within the immune network the universe of external antigens is mimicked by idiotypes. According to this concept, immunization with a given antigen will generate the production of antibodies against this antigen termed Ab1. This Ab1 can generate a series of anti-Id antibodies against Ab1 termed Ab2. The particular anti-Ids which fit into the antigen binding site of the Ab1, can induce a specific immune responses against the nominal antigen. Then, a practical consequence of the idiotypic network theory was that the idiotopes could be used to mimic any existing antigen and used as surrogate antigens. Immunization with Ab2 can lead to the generation of anti-anti-Id antibodies (Ab3) that recognize the corresponding original antigen identified by the Ab1 (**Figure 1**). Several such Ab2 have been used to trigger the immune system to induce specific and protective immunity against tumor antigens (Miller et al., 1982; Jerne, 1985; Lee et al., 1985; Raychaudhuri et al., 1986).

Although Jerne, in his original network hypothesis, and later Coutinho with the second generation networks outlined the importance of naturally occurring idiotypic complementaries, must of the studies using anti-Id as vaccines are focus on the great mimetic capacity of idiotypes, no in activating network related properties, like immune regulation and natural immune surveillance. Beyond the functional mimetic capacity, those anti-Id antibodies related with antigens connected and regulated though networks due to their importance for organisms' homeostasis, could be able to activate natural antibodies secreting B cells. Their antigens, especially self-antigens, could be the suited ones to get targeted through the idiotypic vaccination. This could be the case of gangliosides.

ANTI-GANGLIOSIDE IDIOTYPIC VACCINES

Anti-idiotype antibodies that mimic ganglioside have been utilized as active specific immunotherapy in patients with different tumors. Chapman and Houghton (1991) generated in syngeneic mice the anti-Id antibody BEC2 against the anti-GD3 mAb R24. In studies in rabbits this antibody demonstrated its ability to mimic GD3, inducing a specific antibody response of IgG and IgM isotypes. In clinical trials in melanoma patients treated by surgery and with high risk of recurrence (McCaffery et al., 1996) and in patients with SCLC with limited disease (Grant et al., 1999) BEC2 demonstrated to be immunogenic and to induce anti-anti-Id response when administered with the adjuvant BCG. However, it induced specific anti-GD3 antibodies only in a low percentage of patients (Chapman and Houghton, 1991; McCaffery et al., 1996; Grant et al., 1999). The conjugation of BEC 2 to KLH did not increase but reduced the magnitude and frequency of the anti-GD3 response. When anti-GD3 antibodies were induced, they were detected only by ELISA, not by TLC immunostaining (Ritter et al., 1991) or by flow cytometry against GD3-positive melanoma cell lines, suggesting that these anti-GD3 antibodies had a relatively low avidity for cell surface GD3. A phase III trial with 515 patients with limited-disease SCLC after a major response to chemotherapy and chest radiation was performed with BEC2/BCG. This trial failed to show any survival advantage for vaccinated patients. Only one-third of the patients elicited an anti-GD3 response. Among vaccinated patients, a trend toward prolonged survival was observed in those who developed the humoral response ($P = 0.085$), so that it was suggested that the induction of higher titers of antibodies in a larger proportion of patients could make an impact on median survival (Giaccone et al., 2005).

Another trial that targeted a ganglioside, utilized a vaccine composed of an anti-Id mimicking GD2 injected with the adjuvant QS21, a preparation called TriGem. The anti-Id mAb, called 1A7 is a functional mimic of a specific epitope in the ganglioside GD2. In preclinical studies in mice, rabbits, and monkeys the immunization with 1A7 antibody induced a specific IgG response against the ganglioside, capable of causing the lysis of GD2-positive cells on ADCC assays (Sen et al., 1998).

Foon et al. (1998) initiated a clinical trial in patients with advanced melanoma, which were given anti-Id mAb 1A7 with the adjuvant QS21. All sera showed an anti-anti-Id response mainly of the IgG1 isotype. The purified Ab3 from all patients inhibited the binding of the Ab1 to a GD2-positive cell line and to purified GD2. In addition, sera specifically reacted with tumor cells expressing GD2 and were positive in ADCC studies. One patient had a complete clinical response and 6 patients, of a total of 12 enrolled in the trial were stable from 9 to 23 months. In a similar trial, 47 patients with advanced melanoma received 1, 2, 4, or 8 mg doses of TriGem. Hyperimmune sera from 40 of the 47 patients showed an anti-anti-Id response of IgG isotype that specifically bound purified GD2. One patient had a complete response that persisted at 24 months, and 12 patients were stable

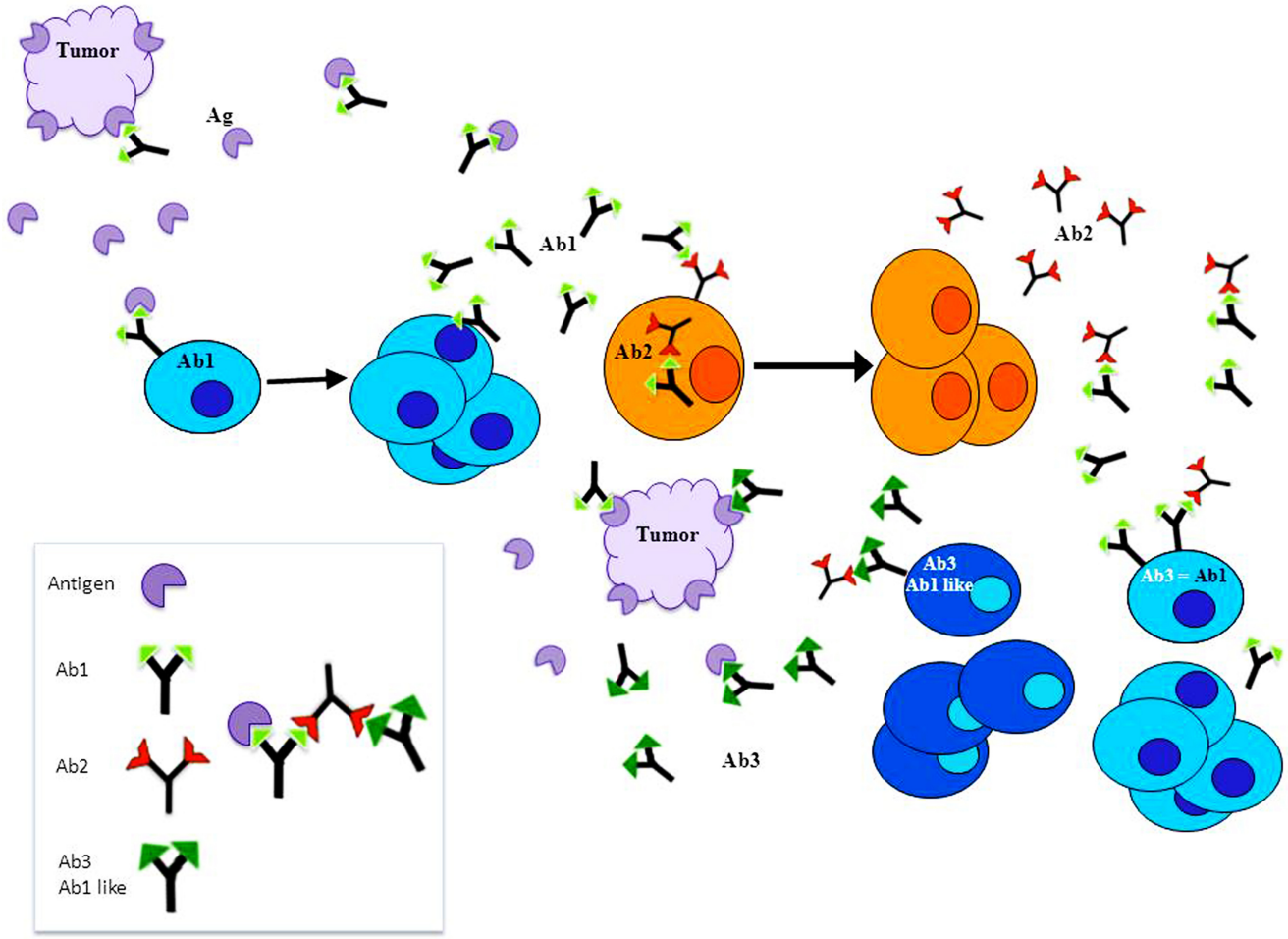

FIGURE 1 | According to the idiotypic network theory, idiotype's complementary structures can be found not only on antigens but also on antibodies of different idiotypes. The immune system after producing specific antibodies to an antigen (Ab1), continues to produce antibodies to the Ab1 idiotopes (Ab2). The particular Ab2 which fit into the antigen binding site of the Ab1, can induce a specific immune responses against the nominal antigen. Thus, the idiotopes could be used as surrogates of any existing antigen. Immunization with Ab2 can lead to the generation of anti-anti-Id antibodies (Ab3) that recognize the corresponding original antigen identified by the Ab1. Several Ab2 have been used to induce specific and protective immunity against tumor antigens.

from 14 to 37 months (median, 18 months). These results showed that this vaccine had minimal toxicity, induced a strong response against GD2 and seemed to have a favorable impact on the reduction of disease progression and survival of patients (Foon et al., 1998, 2000).

In 2003, Basak and colleagues generated Ab2 against the anti-GD2 mAb ME361. These Ab2s induced a specific DTH response in mice against melanoma cell lines that express this ganglioside. Furthermore, these antibodies were able to induce proliferative responses in cells from a melanoma patient confronted with human melanoma cells expressing GD2 *in vitro*, demonstrating the ability of these antibodies to induce cellular responses against carbohydrate antigens (Basak et al., 2003).

Several evidences have shown that tumor antigen-specific antibodies Ab1, used in preclinical experiments or for diagnostic and/or therapeutic purposes, may contribute to anti-tumor effects by triggering the idiotypic cascade and inducing a tumor antigen-specific immune response. The triggering of the idiotypic cascade has been reported to be associated with a favorable clinical response to antibody-based therapy in patients with neuroblastoma, colorectal carcinoma, ovarian carcinoma, and non-Hodgkin lymphoma (Koprowski et al., 1984; Saleh et al., 1993; Cheung et al., 1994, 2000; Schultes et al., 1998). GD2 ganglioside-specific antibodies have been induced in patients with neuroblastoma treated with anti-GD2 ganglioside antibodies.

Treatment with the anti-GD2 monoclonal antibody 3F8 (Ab1) at the time of remission prolonged the survival of children with stage 4 neuroblastoma. Among 34 patients treated with this antibody at the end of chemotherapy 14 were alive, and 13 (1.8–7.4 years at diagnosis) were progression-free (53–143 months from the initiation of 3F8 treatment) without further systemic therapy at the moment of the report. This long-term progression-free survival and survival correlated significantly with the induction of Ab3 anti-GD2 response (Cheung et al., 2000). These results reinforce

the importance of GD2 as a tumor target, and the connectivity capacity of anti-ganglioside antibodies.

Our group has developed a vaccine preparation featuring a murine anti-Id mAb related to the NeuGc-containing ganglioside antigen model. This Ab2, named 1E10 (Vázquez et al., 1998), was generated from the immunization of BALB/c mice with P3, an idiotypic antibody (Ab1) that recognizes NeuGc-containing gangliosides, sulfated glycolipids, and antigens present in different human tumors including those from the lung. This Ab1 is highly immunogenic in the syngeneic model, inducing an anti-Id response in the absence of adjuvant or carrier protein. Furthermore, the Ab1 P3 was able to activate NeuGcGM3 related idiotypic cascade, since antibodies against NeuGcGM3 (Ab3, Ab1 like) were detected in chickens immunized with this Ab1. The detection of Abs with this specificity in animals immunized with an Ab1 suggested that the elicited Ab2s behaved as a ganglioside surrogate inducing a specific Ab3 response against this antigen.

Preclinical data published by our group suggest that P3 and 1E10 mAb could be able to activate idiotypic networks, involving both B and T cells. Lymph node cells from BALB/c mice immunized with P3 mAb proliferated *in vitro*, in a dose-dependent manner, not only in response to P3 mAb but also to 1E10 mAb, suggesting the existence of a naturally occurring B/T cell idiotypic network (Perez et al., 2002). Phase I clinical trials have proven the safety and immunogenicity of 1E10 Id vaccination in melanoma, breast, and lung cancer patients (Alfonso et al., 2002, 2007; Diaz et al., 2003; Neninger et al., 2007; Hernandez et al., 2008). In all the cases, 1E10 idiotype proved to be immunodominant, since the induced anti-anti-Id response was significantly higher than the anti-isotypic response. Similar results were obtained when monkeys and chickens were immunized with 1E10 mAb (Hernandez et al., 2005), suggesting that 1E10 mAb Id immunodominance is not a species-depending property. High titer antibody responses to NeuGc-containing gangliosides were measured in the sera of cancer patients and were confirmed by TLC immunostaining. Interestingly, a fraction of non-suppressible anti-NeuGc-containing ganglioside Abs was demonstrated after the adsorption of the patients' sera with 1E10 mAb, suggesting that 1E10 Id vaccination was activating natural anti-NeuGcGM3 responses (Hernandez et al., 2008). The antibodies that recognize both 1E10 and the ganglioside (Id^+Ag^+) and the ones that recognize the ganglioside but not the immunizing Ab3 (Id^-Ag^+), recognized and induced the death of tumor cells expressing NeuGcGM3 by an oncotic necrosis mechanism. Those patients who developed IgG and/or IgM Abs against NeuGcGM3 showed a longer survival time. We hypothesize that 1E10 Id vaccination could be activating an existing idiotypic cascade related with *N*-glycolylated gangliosides, which would amplify

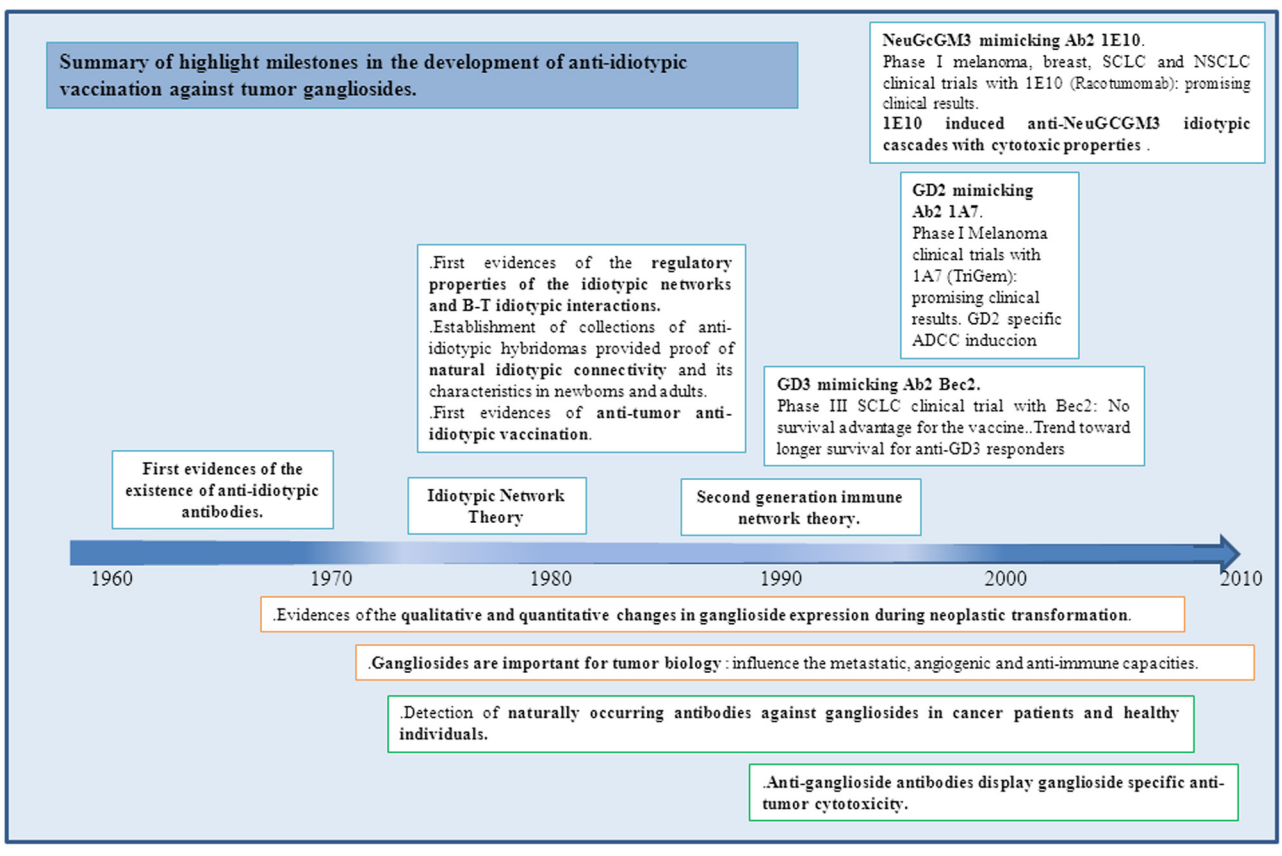

FIGURE 2 | Timeline of highlight milestones in the development of anti-idiotypic vaccination against tumor gangliosides.

the antigen-specific immune response to a tumor-associated neo-self antigens. This therapeutic concept goes beyond the classical concept of antigen mimicry. A randomized, double blind phase II clinical trial is ongoing to evaluate the clinical effect of 1E10 mAb vaccine in NSCLC patients and to define the value of the Abs induced by the anti-Id treatment as real predictors of clinical outcome. For a chronological representation of the principal milestones in the development of anti-Id vaccines against tumor expressed gangliosides see **Figure 2**.

CONCLUDING REMARKS

At present, most of the anti-Id vaccine approaches are based and study the mimetic capacity of the anti-Id antibodies, without searching for their immunoregulatory or natural anti-tumor potential. The use of anti-Id antibodies as immunogens could offer the possibility not only to generate Ab3 antibodies against their own idiotopes, but also to inducing a cascade of Id–anti-Id interactions leading to an amplified and long lasting immune response against the nominal antigen. This immunization could also involve T cells in the response against glycolipidic antigens. The expansion of natural antibodies repertoire by idiotypic vaccination could even participate in the lysis of tumor cells by the activation of evolutionarily fixed anti-tumor mechanisms. A naturally occurring antibody response against ganglioside, which has shown to carry anti-tumor properties, exists in healthy individuals and cancer patients. The idiotypic vaccination could be an optimum way to activate the idiotypic B and T cell cascades involving the natural responses against these antigens.

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Racotumomab: an anti-idiotype vaccine related to *N*-glycolyl-containing gangliosides – preclinical and clinical data

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Neu-glycolyl (NeuGc)-containing gangliosides are attractive targets for immunotherapy with anti-idiotype mAbs, because these glycolipids are not normal components of the cytoplasmic membrane in humans, but their expression has been demonstrated in several human malignant tumors. Racotumomab is an anti-idiotype mAb specific to P3 mAb, an antibody which reacts to NeuGc-containing gangliosides, sulfatides, and other antigens expressed in tumors. Preparations containing racotumomab were able to induce a strong anti-metastatic effect in tumor-bearing mice. Different Phase I clinical trials have been conducted in patients with advanced melanoma, breast cancer, and lung cancer. The results of these clinical trials demonstrated the low toxicity and the high immunogenicity of this vaccine. The induced antibodies recognized and directly killed tumor cells expressing NeuGcGM3. A Phase II/III multicenter, controlled, randomized, double blind clinical trial was conducted to evaluate the effect of aluminum hydroxide-precipitated racotumomab vaccine in overall survival in patients with advanced non-small cell lung cancer. The clinical results of this study showed a significant clinical benefit in the patients who were treated with the anti-idiotype vaccine.

Keywords: anti-idiotype vaccine, cancer, immunotherapy

Actively induced immunotherapy is one of the most promising fields in cancer research and numerous approaches are being studied to design effective cancer vaccines. An approach to generate an effective immune response against tumor-associated antigens involves the use of an anti-idiotype monoclonal antibody (Ab2 mAb). The use of Ab2 as a vaccine is a consequence of Jerne's idiotypic network theory, which postulates that, due to the large potential diversity of immunoglobulin variable regions, the idiotype repertoire can mimic the universe of self and foreign epitopes (Jerne, 1974). Thus, properly selected anti-idiotypic antibodies could act as tumor-associated antigen surrogates.

Studies in animal models have demonstrated the efficacy of these vaccines for triggering the immune system to induce specific and protective immunity against tumors of different origin (Bhattacharya-Chatterjee et al., 2001; Mohanty et al., 2007). In the clinical setting anti-idiotypic antibodies are also being actively evaluated for the treatment of malignant diseases (de Cerio et al., 2007). In fact, several studies have validated the capacity of anti-idiotype mAbs to mimic different kind of antigens in cancer patients, inducing humoral and cellular responses against the nominal antigens. One of these antibodies designated 3H1 mimics a specific epitope on the carcinoembryonic antigen (CEA), a tumor-associated antigen expressed on most gastrointestinal adenocarcinomas. This anti-idiotype mAb was used in Phase I clinical trials in advanced colorectal cancer patients and demonstrated specific active immunity to CEA, inducing anti-CEA antibodies and T cell proliferative responses. The level of the immune response correlated directly with the time to progression and survival. More recently these researchers performed a Phase III clinical trial with 630 patients with advanced colorectal cancer combining 5-fluorouracil and leucovorin with either 3H1 anti-idiotype mAb or placebo. Although the addition of 3H1 did not improve the overall patient outcome, again there was an improvement in the survival of the patients that developed strong anti-CEA responses (Foon et al., 1995, 1997, 1999; Bhattacharya-Chatterjee et al., 2000; Chong et al., 2006).

Mittelman et al. (1990, 1992) demonstrated the capacity of the anti-idiotype mAb MK2-23 to mimic a high molecular weight human melanoma antigen (HMW-MAA) and to induce an anti-tumor response in melanoma patients, which included the reduction in the size of metastatic lesions in some of the patients. In these studies the survival of the patients who developed anti-HMW-MAA antibodies was also significantly longer.

An anti-idiotype antibody that has shown to activate T cell responses against the nominal antigen is the 105AD7. This antibody has both amino acid and structural homology with the complement regulatory protein CD55, a glycoprotein that protects the cells from the attack by complement and which is overexpressed in osteosarcoma and colorectal cancer cells. Some of the osteosarcoma patients vaccinated with 105AD7 elicited an IFN-gamma T cell response against the vaccine, and TNF-alpha and GM-CSF responses not only to the vaccine, but also toward the

native antigen. This antibody was also injected in colorectal cancer patients, where again it induced proliferative responses and TNF-alpha and GM-CSF secretion against the nominal antigen. These researchers have not seen correlation between the induced T cell responses and patient's survival, probably due to the small number of patients included in the studies (Robins et al., 1991; Ullenhag et al., 2006, 2008).

Abagovomab, an anti-idiotype mAb directed against CA125, has shown to induce active immune response against this tumor-associated antigen in a Phase Ib/II clinical trial in advanced ovarian cancer patients. In this study, those patients who developed anti-anti-idiotype (Ab3) response had a significant longer overall survival (Reinartz et al., 2004). On the basis of these encouraging results, a Phase II/III pivotal study (MIMOSA study) to evaluate abagovomab as a maintenance therapy in ovarian cancer patients with no residual disease after frontline therapy was conducted (Pfisterer et al., 2011a). In this study was enrolled 888 patients, and 593 women were treated with abagovomab and 295 women were assigned to treatment in the placebo arm. Unfortunately, the results of this study showed that abagovomab maintenance treatment after debulking surgery and successful platinum and taxane first-line chemotherapy did not prolong progression-free survival in advanced ovarian cancer patients (Pfisterer et al., 2011b).

Also, anti-idiotype mAbs have been used to mimic non-protein antigens in patients with different tumors. One example of these kind of antigens are the gangliosides which are sialic acid-containing glycosphingolipids normally expressed in the membrane of eukaryotic cells (Wiegandt, 1985), but their expression patterns change during oncogenic transformation (Hakomori, 1985; Irie and Ravindranath, 1990).

The anti-idiotype mAb 1A7, which functional mimics the ganglioside GD2, (Sen et al., 1998) has been used as an anti-idiotype vaccine (TriGem) in patients with advanced melanoma (Foon et al., 1998, 2000). The immunization with this anti-idiotype vaccine elicited a strong anti-GD2 antibody response that specifically reacted with tumor cells expressing GD2. Although the studies included a small number of patients, the results suggested that TriGem vaccine may have a role in preventing disease progression and in increasing survival time for patients with advanced malignant melanoma (Foon et al., 2000).

Another antibody used as an anti-idiotype vaccine in cancer patients is the mAb Bec2, which mimics GD3 ganglioside (Chapman and Houghton, 1991). This antibody was studied in melanoma and small cell lung cancer (SCLC) patients, where it induced specific anti-GD3 antibodies, but only in a low percentage of patients (McCaffery et al., 1996; Grant et al., 1999). Furthermore these antibodies were hardly detectable by TLC immunostaining or flow cytometry (Ritter et al., 1991). Despite the low frequency of anti-GD3 antibodies induction in the first trials, a Phase III clinical trial with 515 SCLC patients with a major response to chemotherapy and chest radiation was performed with this antibody. Only one third of the patients elicited an anti-GD3 response. Although this trial failed to show any survival advantage for vaccinated patients, a trend toward prolonged survival was observed in those patients who developed the humoral response (Giaccone et al., 2005).

NeuGc-CONTAINING GANGLIOSIDES ARE ATTRACTIVE TARGETS FOR IMMUNOTHERAPY OF TUMORS

In particular the Neu-glycolyl (NeuGc)-neuraminic acid variant of gangliosides is widely expressed in most mammalian tissues, but is rarely found in normal human cells. The lack of expression of this type of sialic acid in humans is due to the inactivating mutation in the CMP-Neu5Ac hydroxylase gene, enzyme responsible for Neu5Gc biosynthesis of Neu5Gc-containing molecules (Chou et al., 1998; Irie and Suzuki, 1998; Olson and Varki, 2003). However, the presence of NeuGc-neuraminic acid residues has been reported in different human tumors, detected by antibodies and by chemical analysis (Higashi et al., 1984, 1988; Hirabagashi et al., 1987; Miyake et al., 1990; Devine et al., 1991; Marquina et al., 1996; Malykh et al., 2001; van Cruijsen et al., 2009; Scursoni et al., 2010), where they are known to be immunogenic (Nguyen et al., 2005). Additionally, recent experimental data suggest that NeuGcGM3 ganglioside is relevant for tumor progression (de León et al., 2006).

The most accepted theory for this phenomenon is the incorporation of Neu5Gc from dietary sources. Free sialic acids from the medium can be taken up into cells via pinocytosis. The content of the resulting pinocytotic vesicles and endosomes would eventually be delivered to the lysosome, where a sialic acid transporter then delivers the molecules into the cytosol (Bardor et al., 2005). The explanation for the differential expression of these antigens in human normal and tumor tissues is that the rapidly growing tumor tissues might be more efficient at scavenging Neu5Gc from dietary sources. Furthermore, it has been proposed that the preferential expression of NeuGc in cancers is closely associated with tumor hypoxia. Hypoxic culture of tumor cells induces expression of a sialic acid transporter, sialin, and enhances the incorporation of non-human sialic acid from the external milieu (Yin et al., 2006).

RACOTUMOMAB ANTI-IDIOTYPE ANTIBODY

We first generated a mAb, named P3, which distinguish between NeuGc and NeuAc gangliosides (Vázquez et al., 1995; Moreno et al., 1998). This mAb also reacted with human melanomas, breast and lung tumors (Vázquez et al., 1995; Alfonso et al., 2002; Alfonso et al., 2007; Neninger et al., 2007). Then, by immunizing Balb/c mice with P3 mAb we obtained an anti-idiotype mAb, named first 1E10 and now racotumomab. This IgG1 Ab2 mAb, was able to block the binding of the Ab1 mAb to NeuGcGM3 (Vázquez et al., 1998).

Our hypothesis was that racotumomab could act as a surrogate of NeuGc-gangliosides generating autologous antibodies in the immunized species which react with the Ab2 idiotype and with the NeuGc-containing gangliosides.

PRECLINICAL DATA

We tested the hypothesis using different animal models with a different NeuGc-sialic acid expression: mice and monkeys that express NeuGc-glycoconjugates (Chou et al., 1998; Hayakawa et al., 2001) and chickens, which do not express NeuGc-glycoconjugates in their normal tissues (Fujii et al., 1982; Ledeen and Yu, 1982).

Animals received different doses of preparations containing racotumomab and when we evaluated the serological antibody

response we found that all animals of the different species developed Ab3 response. It is noteworthy that in monkeys and chickens where racotumomab is a xenogenic immunoglobulin the serological response against racotumomab was stronger when compared with other isotype-matched mAb used as a control (immunodominance of the idiotype determinants). Besides, the Ab3 antibodies generated by immunization with racotumomab mAb were characterized to share idiotopes with P3 mAb (Ab1), as evidenced by their capacity of inhibiting racotumomab binding to P3 mAb (Ab3 Id+; Vázquez et al., 1998; Hernández et al., 2005).

In contrast, when the antibody response against NeuGc-containing gangliosides induced by racotumomab immunization was studied, a completely different pattern of response was detected. In mice and monkeys, no specific humoral response against NeuGc-containing gangliosides was detected in the sera of racotumomab immunized animals. In the case of mice and monkeys, since NeuGc-containing gangliosides are normal tissue components (Ledeen and Yu, 1982), tolerance mechanisms could be avoiding the immune response against these antigens. These mechanisms are still unknown, but as it has been demonstrated for other self-antigen models (Fillatreau et al., 2002; Takahashi and Sakaguchi, 2003), one possibility is the existence of T and/or B regulatory cells that would modulate the antibody response against NeuGc-containing gangliosides.

On the other hand, most chickens in our study developed a specific Ab3 response against NeuGcGM3 and NeuGcGM2 gangliosides (Ab3 Ag+), due to the immunization with racotumomab (Hernández et al., 2005). In addition, Ag+Id-antibodies were produced in chickens immunized with racotumomab. This was demonstrated by the reactivity of hyperimmune sera to NeuGcGM3 ganglioside after the adsorption of hyperimmune chickens sera with racotumomab, which abrogate the antibody response against this Ab2 (Hernández et al., 2005). The mechanism responsible for the generation of these antibodies is still unknown. A natural immune network could be involved in the generation of this unusual antibody "parallel set".

Although these results, it is known that the induction of antigen-specific humoral immune response is not predictive of the biological effect induced by an anti-idiotype mAb. Thus, we studied the effect of different preparations containing racotumomab in different experimental tumor murine models.

In BALB/c mice, vaccination with several intraperitoneal doses at 14-day intervals of racotumomab coupled to keyhole limpet hemocyanin in Freund's adjuvant, significantly reduced subcutaneous tumor growth of F3II carcinoma cells and the formation of spontaneous lung metastases. The effect of racotumomab as a biological response modifier on lung colonization was evaluated in C57BL/6 mice injected with B16 melanoma cells. Interestingly, intravenous administration of uncoupled racotumomab antibody, 10–14 days after inoculation of tumor cells, dramatically reduced the number of experimental metastases in comparison with lungs from mice treated with an irrelevant IgG (Vázquez et al., 2000).

Later, we evaluated the anti-metastatic effect of racotumomab in Alum adjuvant (racotumomab-Alum) in the 3LL-D122 Lewis lung carcinoma model. Racotumomab-Alum immunization was tested in two different settings distinguished by the frequency of the immunizations, the amount of vaccine and the initiation of the vaccine schedule related to the tumor challenge. Independently to the immunization schedule, racotumomab-Alum promoted a significant reduction of spontaneous lung metastases. The therapeutic effect was associated to the increment in the number of T cells infiltrating metastases, a reduction of new blood vessels formation, and the increment of apoptotic tumor cells in lung nodules. It is noteworthy that active immunization with the mAb in Alum does not induce measurable antibodies to racotumomab molecule, the NeuGcGM3 or tumor cells, which may suggest a different mechanism which has to be elucidated (Diaz et al., 2009).

More recently, preclinical studies were carried out with the main objective to determine the anti-tumor effect of racotumomab-Alum vaccine coadministered with low dose of cyclophosphamide in a murine mammary carcinoma model, based on their potentially shared antiangiogenic properties and/or a complementary proapoptotic effect by racotumomab-Alum vaccine. The results of the study showed that the combination enhanced the efficacy of chemotherapy or immunotherapy alone in the F3II carcinoma model, both when the mAb was obtained from mice ascites fluid or from bioreactor supernatant (Fuentes et al., 2010; Machado et al., 2011).

CLINICAL DATA

Taking in account the preclinical results together with the results of the toxicology studies, and after the approval of the Ethical Committees of the hospitals and the Regulatory agencies, different Phase I clinical trials were performed in advanced melanoma (Alfonso et al., 2002), breast cancer (Díaz et al., 2003; Guthmann et al., 2006; Soriano et al., 2011), and SCLC and non-SCLC (NSCLC; Alfonso et al., 2007; Neninger et al., 2007), with the main goals of evaluate the safety and the immunogenicity of racotumomab vaccine. All the patients had previously received the oncospecific treatment established in the Oncological Therapeutic Standards. In most of these clinical trials the patients were injected intradermally with 10 doses of 1 or 2 mg of racotumomab-Alum as base treatment: the first four or five doses at 2-week intervals and the remaining six every 28 days. Reimmunizations were administered if the patients had a favorable clinical status. The results of these Phase I clinical trials evidenced that racotumomab vaccine was well tolerated. Toxicity consisted mainly of local reaction at the injection site with erythema and induration that disappeared in a few days (adverse events grade I/II according to NCI-CTC criteria).

Racotumomab-Alum vaccine resulted very immunogenic, a strong, and specific response against NeuGc-containing gangliosides was detected in the sera of most of the immunized patients by ELISA and TLC-immunostaining (Alfonso et al., 2002; Díaz et al., 2003; Guthmann et al., 2006; Neninger et al., 2007). One interesting finding was the detection of a high level of binding of patient's hyperimmune sera to NeuGcGM3 ganglioside, after the complete abrogation of the reactivity against racotumomab mAb by adsorbing the patient sera with this Ab2 (Hernández et al., 2008). Also, antibodies able to react with lung carcinoma tissue sections were detected in the sera of immunized lung cancer patients (Alfonso et al., 2007; Neninger et al., 2007).

The finding that in most of the patients treated with racotumomab-Alum vaccine we detected a relatively high titer of

anti-NeuGcGM3 antibodies of both IgM and IgG isotypes is a relevant result taking into account that it is difficult to obtain an IgG antibody response against these antigens (Livingston, 1995). Even the use of anti-idiotype antibodies as protein mimicries of gangliosides does not guarantee the induction of this kind of response. Previously, it was reported that most of the melanoma patients immunized with 1A7 mAb able to mimic GD2 ganglioside developed specific IgG antibodies against this ganglioside (Foon et al., 2000). In contrast, when melanoma and SCLC patients were treated with the anti-idiotype Bec-2 mAb, the percentage of patients that developed anti-GD3-specific antibody response was low, mainly of IgM isotype. The presence of these antibodies was detected by ELISA, but could not be confirmed by TLC immunostaining or flow cytometry (Ritter et al., 1991; McCaffery et al., 1996; Grant et al., 1999). The differential induction of antibody responses against gangliosides could be dependent on their different expression in normal tissues. In fact, studies previously reported showed the relation between the level of ganglioside expression in human and murine normal tissues and their immunogenicity (Chen et al., 2000; Lunn et al., 2000; Bowes et al., 2002). The strong antibody response against NeuGcGM3 induced in patients by the racotumomab-Alum vaccine can be explained because NeuGc-containing gangliosides are not self-antigens in humans (Chou et al., 1998; Irie and Suzuki, 1998; Olson and Varki, 2003).

Although these studies were not designed to evaluate the therapeutic efficacy of the vaccine, a prolonged survival was observed in several patients. In particular, the clinical study performed in NSCLC patients stage IIIb/IV showed encouraging clinical benefit. This clinical trial study included 34 stage IIIb and 37 stage IV NSCLC patients. These patients were treated with the anti-idiotype vaccine, after received standard chemotherapy and radiotherapy, in a compassionate-use basis study. Patients received five bi-weekly injections of 1 mg of racotumomab-Alum, other 10 doses at 28-day intervals and later the patients who maintained a good performance status continued to be immunized at this same time interval. No evidence of unexpected or serious adverse effects was reported, although the patients were repeatedly injected with racotumomab-Alum and even when some of them received more than 15 doses of this vaccine. The overall survival of the patients who entered the study was superior to the one reported (González et al., 2007) for a group of more than one hundred advanced NSCLC patients receiving standard oncospecific treatment in our country (9.93 vs. 4.53 months). In this study, the survival of the patients who started racotumomab-Alum with at least disease stabilization after the end of standard therapy and with a PS1, was significant greater (11.50 months) compared with those patients with progressive disease and/or a PS2 (6.50 months). To assess whether this advantage in survival could be due to the vaccination with racotumomab, a comparison was performed with the median survival time calculated for a subgroup of the control patients with a PS1 who achieved partial response or stable disease after the oncospecific therapy, and there was a statistical significant difference between the two groups (11.50 vs. 5.70 months). Thus, the treatment with the racotumomab-Alum vaccine seems to prolong survival of this group of patients.

Immunological studies performed in 20 of these patients suggested an association between the induction of antibodies against NeuGcGM3 and longer survival times (Hernández et al., 2008). Then, one question we wanted to address was if the generation of NeuGcGM3 specific antibodies in patients could have some biological effect in tumor cells expressing this ganglioside.

Our results from flow cytometry studies, where the sera of patients were incubated with a cell line expressing NeuGcGM3 and the effect on their viability was studied using the propidium-iodine exclusion assay, showed that sera of most of the patients not only recognize the cells, but also directly killed the cells, by a mechanism independent of complement activation. When the cells were examined by scanning electron microscopy it was shown that the cytotoxic hyperimmune patient's sera produce large lesions on cell's membranes (Hernández et al., 2011). These results contributed to reinforce the therapeutic potential of anti-idiotype vaccines and the importance of NeuGcGM3 as tumor target.

Then, a Phase II/III randomized, multicentric, double blind, clinical study was performed in advanced NSCLC patients looking to the proof of concept with significant clinical benefit in the patients who were treated with the anti-idiotype vaccine in comparison with the placebo group (manuscript in preparation). Now, a Phase III multinational clinical trial is ongoing to confirm our clinical results in advanced NSCLC patients.

CONCLUDING REMARKS

Racotumomab-Alum vaccination has proved to be safe and highly immunogenic in patients with advanced melanoma, breast and lung cancer. Racotumomab-Alum vaccination seems to be effective to prolong overall survival in patients with NSCLC and preliminary data suggested a correlation between the induction of antibodies against NeuGcGM3 and increased survival times of vaccinated patients. The current ongoing Phase III clinical trial will give a definitive answer to the potential clinical benefits offered by this vaccine. Furthermore, studies will be required to determine the more efficient combination with chemotherapy or other immune interventions to prevail over the tumor-induced immunosuppression.

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Targeted DNA vaccines for enhanced induction of idiotype-specific B and T cells

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Background: Idiotypes (Id) are antigenic determinants localized in variable (V) regions of Ig. Id-specific T and B cells (antibodies) play a role in immunotherapy of Id^+ tumors. However, vaccine strategies that enhance Id-specific responses are needed. **Methods:** Id^+ single-chain fragment variable (scFv) from multiple myelomas and B cell lymphomas were prepared in a fusion format that bivalently target surface molecules on antigen-presenting cells (APC). APC-specific targeting units were either scFv from APC-specific mAb (anti-MHC II, anti-CD40) or chemokines (MIP-1α, RANTES). Homodimeric Id-vaccines were injected intramuscularly or intradermally as plasmids in mice, combined with electroporation. **Results:** (i) Transfected cells secreted plasmid-encoded Id^+ fusion proteins to extracellular fluid followed by binding of vaccine molecules to APC. (ii) Targeted vaccine molecules increased Id-specific B and T cell responses. (iii) Bivalency and xenogeneic sequences both contributed to enhanced responses. (iv) Targeted Id DNA vaccines induced tumor resistance against challenges with Id^+ tumors. (v) Human MIP-1α targeting units enhanced Id-specific responses in mice, due to a cross reaction with murine chemokine receptors. Thus, targeted vaccines designed for humans can be quality tested in mice. (vi) Human Id^+ scFv from four multiple myeloma patients were inserted into the vaccine format and were successfully tested in mice. (vii) Human MIP-1α vaccine proteins enhanced human T cell responses *in vitro*. (viii) A hypothetical model for how the APC-targeted vaccine molecules enhance Id-specific T and B cells is presented. **Conclusion:** Targeted DNA Id-vaccines show promising results in preclinical studies, paving the way for testing in patients.

Keywords: idiotype, vaccine, lymphoma, multiple myeloma, antigen-presenting cells

INDUCTION OF ANTI-Id ANTIBODIES TO SYNGENEIC Ig

In their classical studies, Oudin and Michel (1963) and Kunkel et al. (1963) immunized rabbits with human Ig and observed that the rabbits made antibodies specific for the injected Ig. The antigen recognized by the rabbit antibodies was called idiotype (Id). In hindsight, these observations are perhaps not so surprising since xenogeneic Ig variable (V) regions, where the Id is located, are expected to be immunogenic when crossing species barriers, due to inter species sequence differences between V regions.

Maybe more surprising, Eisen and colleagues demonstrated that immunization with a monoclonal Ig, myeloma protein M315, induced anti-Id antibodies even in syngeneic BALB/c mice (Sirisinha and Eisen, 1971). This important finding indicated that Ig V regions can be autoimmunogenic, as later formally demonstrated by Rodkey (1974). In order to elicit syngeneic anti-Id antibodies, Eisen and colleagues used an extensive immunization schedule, starting with M315 in complete Freund's adjuvant. Thus, syngeneic Ig appeared to be weak autoantigens in terms of eliciting anti-Id antibodies (Sirisinha and Eisen, 1971). Moreover, immunogenicity of various monoclonal Ig differed. In particular, an abundant Ig with germline-encoded V regions (T15) failed to induce anti-Id antibodies, presumably due to self-tolerance (Sakato and Eisen, 1975). Hence, somatic mutations and/or V(D)J junctional diversity appear to be required for sufficient "foreignness" of Id to be immunogenic in an autologous setting. These seminal observations of Sirisinha and Eisen (1971) have later been repeated with a large number of Igs with essentially similar results.

INDUCTION OF ANTI-Id ANTIBODIES IS DEPENDENT ON Id-SPECIFIC T CELLS

T cell deficient mice, either nude mice (Schrater et al., 1979) or neonatally thymectomized mice (Cosenza et al., 1977), do not produce anti-Id antibodies upon immunization with Ig. Thus, Ig is a T dependent antigen like most protein antigens. Consistent with this, Janeway et al. (1975) and Jorgensen and Hannestad (1977) found that immunization of mice with Ig induced Id-specific T cells that could help secondary hapten-specific B cells in adoptive cell transfer experiments performed essentially *ad modum* Mitchison (1971) and Rajewsky (1971). Jorgensen and Hannestad (1980, 1982) further found that elicitation of Id-specific T cells specific for the M315 monoclonal Ig was under the influence of MHC-linked immune response (Ir) genes. The Id determinant was localized to complementarity determining region (CDR) 3 of the $\lambda 2^{315}$ Ig light (L) chain (Jorgensen et al., 1983; Bogen et al., 1985). Bogen et al. (1986a,b) and Bogen and Lambris (1989) cloned T cells specific for this particular Id ($\lambda 2^{315}$)-determinant and showed that

these T cells were $CD4^+$ and recognized aa 91–101 of the $\lambda 2^{315}$ L chain presented on the MHC class II molecule I-E^d in BALB/c mice. Moreover, it was demonstrated that Ig requires antigen processing by the antigen-presenting cells (APC) for the Id-peptide to be presented on MHC class II molecules (Weiss and Bogen, 1989, 1991). Id-specific TCR transgenic T cells of this particular specificity indeed help anti-Id B cells in the presence of Id^+ Ig (Jacobsen et al., 2010).

IDIOTYPIC NETWORK THEORY: ANTI-Id (Ab2) AS A MIMICK OF ANTIGEN, AND AS INHIBITOR OF PATHOGENIC AUTOANTIBODIES

The observation that Ig is autoimmunogenic paved the way for the idiotypic network theory of Jerne (1974). Despite considerable criticism of the network theory over the years, there is much evidence to support an influence of Id in immune regulation – even though the mechanisms may not be exactly as suggested by Jerne (1974). In particular, immunization with anti-Id (Ab2) antibodies has been shown to induce anti-anti-Id (Ab3) antibodies that can bind the "original" Ag, a feature they share with Id^+ (Ab1) antibodies. Thus, anti-Id antibodies may function as a mimic of antigen. This principle may be of practical importance since antigens sometimes are poorly immunogenic, or difficult to obtain in sufficient amounts for immunization. In these cases, anti-Id monoclonal Ab (mAb) may be used as a surrogate antigen for vaccination purposes. This strategy has recently been successfully exploited by using anti-Id mAb as immunogen for induction of anti-anti-Id Ab that bind gangliosides on lung cancer (Hernandez et al., 2011) and melanoma (Ramos et al., 2011) cells. Another interesting function of anti-Id Ab is that they appear to block the function of pathogenic Id^+ autoantibodies as suggested in studies in type I diabetes (Oak et al., 2008). In either case, technologies to increase immunogenicity of Ig V region antigenic determinants by immunization, as described herein, is warranted.

IDIOTYPES AS TUMOR-SPECIFIC ANTIGENS

Another aspect of Id that has stood the test of time is their role as a target on malignant B cells for an immune attack. Lynch et al. (1972) found that when mice immunized with Id^+ M315 myeloma protein were later challenged with the Id^+ MOPC315 plasmacytoma tumor cells, the mice were protected against tumor development. Since plasmacytoma cells (an extramedullary form of multiple myeloma) secrete copious amounts of the Id^+ myeloma protein, anti-Id antibodies should be blocked by omnipresent myeloma protein and appear not important for protection. Rather, Id-specific $CD4^+$ Th1 cells have been shown to kill the tumor cells by a mechanism that involves IFNγ and tumor infiltrating M1 macrophages (Lauritzsen et al., 1994; Dembic et al., 2000; Corthay et al., 2005, 2009; Haabeth et al., 2011). Immunotherapy with anti-Id antibodies may be more important in B lymphomas that secrete little Ig but that express high amounts of surface Ig as targets for the anti-Id antibodies. Indeed, anti-Id antibodies have a therapeutic effect against B lymphomas in experimental models (George et al., 1987; Kaminski et al., 1987). However, phase III trials in humans have been negative in two cases (Levy et al., 2008; Freedman et al., 2009), while in a third study an improvement of disease-free survival in patients vaccinated in first remission was observed (Schuster et al., 2011).

IDIOTYPES ARE WEAK ANTIGENS: CONVENTIONAL AND NOVEL STRATEGIES TO INCREASE THEIR IMMUNOGENICITY

As reviewed above, there are two important reasons for efficient induction of anti-Id immunity: (i) use of anti-anti-Id mAb (Ab2) as mimic of antigen for vaccine purposes and (ii) induction of Id-specific T cells and anti-Id antibodies in therapy of multiple myeloma and Id^+ B lymphomas. However, immunogenicity of Ids is generally considered to be poor. The need for improving the immunogenicity of Id-immunization is underscored by two negative phase III trials on Id-vaccination of B lymphoma patients (Levy et al., 2008; Freedman et al., 2009), while a third phase III trial showed a marginal effect (Schuster et al., 2011). These trials employed vaccination with Id-keyhole limpet hemocyanin (KLH) conjugates and adjuvants.

The low immunogenicity of Id is probably related to B and T cell tolerance, the levels of which may vary with various individual Ids. As long as the sole object of Id-vaccination is to obtain anti-Id antibodies, and assuming that anti-Id B cells usually are not tolerant, the problem of T cell tolerance can be circumvented by conjugation of the Ig to a powerful carrier molecule, such as KLH, that contain a multitude of T cell epitopes that should fit polymorphic MHC II molecules of most individuals (Kaminski et al., 1987). Thus, Ig-KLH conjugates have become a "gold standard" for Id-vaccination of B lymphoma patients (Bendandi et al., 1999; Inoges et al., 2006; Redfern et al., 2006; Levy et al., 2008; Freedman et al., 2009; Timmerman et al., 2009; Schuster et al., 2011). However, the "carrier strategy" does indeed not solve the problem of lack of Id-specific T cell help – it just circumvents it. Ig-KLH is commonly delivered with adjuvants that increase the levels of anti-Id antibody responses. The empirical science of adjuvants has developed considerably during the last decade, and recent progress in the field of receptors of innate immunity, such as Toll-like receptors (TLRs), is likely to generate more powerful and clinically acceptable adjuvants. Given the poor effect of Id-KLH vaccinations in phase III trials, immunization strategies that elicit potent Id-specific T cells, and not only anti-Id antibodies, might be desirable. Apart from the low efficacy, the Id-KLH strategy for Id-vaccination is labor intensive. Id-KLH vaccination has for the most part been applied to patients with follicular B cell lymphomas. These lymphomas arise from germinal center B cells, and their B cell receptors (BCR) for antigen are usually marked by somatic mutations. Hence, lymphoma BCR of different patients are different, and Id-vaccines have to be prepared for each individual patient. Traditionally, this has involved fusion between lymphoma cells and non-secreting myeloma cells (rescue fusion) to obtain soluble lymphoma Ig for conjugation with KLH (Schuster et al., 2011). More recently, recombinant technologies have been applied to obtain lymphoma V region DNA sequences, and to express these as Ig used for preparation of Ig-KLH conjugates (Levy et al., 2008; Freedman et al., 2009). These strategies for generation of individual patient-dedicated protein Id-KLH vaccines are prohibitively time-consuming and costly.

There are a number of more recent innovative approaches to improve Id-vaccination. In one approach, Id-specific immune responses in mice could be enhanced by DNA vaccination with a single-chain fragment variable (scFv)-bacterial antigen (fragment C of tetanus toxin) fusion (King et al., 1998). In another approach, Id was fused with lysosomal-associated membrane protein 1 (Id-LAMP1), and integrated in recombinant vaccinia virus (rVV). Dendritic cells (DCs) infected *in vitro* with Id-LAMP1 rVV were used for immunization of mice, resulting in Id-specific T cell responses and tumor protection (Muraro et al., 2005). In an APC-targeting approach, but using protein rather than DNA, Id^+ scFv was fused with scFv specific for CD19 in a diabody format. Targeting of CD19 on B cells increased Id-specific responses (Ng et al., 2012). Finally, B lymphoma cells were generated that by gene targeting had their endogenous heavy (H) chain replaced by a human H chain. Such engineered lymphoma cells were used to immunize mice, and induced a T cell-mediated protection against wild-type B cell lymphoma (Selmayr et al., 2000). These studies have contributed interesting approaches for Id-immunization, but will not be discussed further as they are not examples of APC-targeted DNA Id-vaccines, which is the theme of the present paper.

In this review, it is considered that a combination of three elements could enhance Id-vaccination: (i) genetic construction of patient-specific Id-vaccines, (ii) targeting of these to APC, and (iii) delivery as DNA. Such a strategy could reduce the cost of preparing individual vaccines and improve anti-Id responses, particularly Id-specific T cell responses. Of these three elements, genetic construction of Id-vaccines, as well as delivery of Id-vaccines as DNA, was already reported in the nineties (Hawkins et al., 1993; Stevenson et al., 1995; Syrengelas et al., 1996; King et al., 1998). APC-targeted DNA Id-vaccines is more recent (Biragyn et al., 1999; Ruffini et al., 2004, 2010; Fredriksen et al., 2006; Fredriksen and Bogen, 2007; Schjetne et al., 2007; Qin et al., 2009;Froyland et al., 2011), and is the focus of the text to follow.

TARGETING ANTIGEN TO ANTIGEN-PRESENTING CELLS INCREASES IMMUNE RESPONSES

Given the poor immunogenicity and labor-intensive production of Id-vaccines, new vaccination strategies are warranted. It has been known since the eighties that targeting of antigen to APC increases both T and B cell responses (Kawamura and Berzofsky, 1986; Carayanniotis and Barber, 1987; Casten and Pierce, 1988; Baiu et al., 1999). These pioneering studies were done by chemical conjugation of antigen to antibodies specific for surface molecules such as BCR, MHC II, FcR, and complement receptors (Kawamura and Berzofsky, 1986; Carayanniotis and Barber, 1987; Baiu et al., 1999) on APC. However, chemical conjugation often results in different Ag:Ig ratios, therefore, chemical conjugates are fraught with batch to batch variation. This problem is solved by genetic fusion of antigen to APC-specific Ab, ensuring a defined fusion protein, as done by the authors and others in the late nineties (Biragyn et al., 1999; Lunde et al., 1999, 2002). This recombinant Ig strategy for APC has become very popular, e.g., in work targeting surface molecules on DCs such as DEC205 (Hawiger et al., 2001; Demangel et al., 2005; Kretschmer et al., 2006) and Clec9a (Lahoud et al., 2011).

APC-TARGETING OF T CELL EPITOPES INSERTED INTO THE IMMUNOGLOBULIN STRUCTURE

Together with Sandlie, Lunde and Bogen developed a recombinant Ig-based strategy for APC-targeting (Lunde et al., 1999). This strategy was based on the observation, described above, that Ig are endocytosed and processed by APC, and that CDR3 Id-peptides are displayed on MHC class II molecules for recognition by Id-specific $CD4^+$ T cells (Bogen et al., 1986b; Weiss and Bogen, 1991). Thus, if a CDR3 epitope could be excised from the Ig molecule by the antigen processing machinery, T cell epitopes engineered to replace loops between β-strands in constant (C) domains of Ig molecules should also be excised for MHC-presentation. This proved to be the case (Lunde et al., 1997). Later work demonstrated that many loops throughout the C-region, especially in the C_H2 domain, are suited for T cell epitope replacement (Flobakk et al., 2008). Indeed, multiple substitutions can be made within a single Ig molecule, suggesting the possibility of using recombinant Ig for multivaccine purposes (Rasmussen et al., 2012).

Importantly, since the T cell epitopes were inserted into the C-domains, the original V regions should be dispensable and therefore exchangeable with APC-specific V regions cloned from B cell hybridomas of appropriate specificity. Such dual-substituted recombinant Ig should target T cell epitopes to APC, resulting in improved T cell responses. To test this idea, V regions specific for IgD (Lunde et al., 1999; Rasmussen et al., 2001), MHC II (Lunde et al., 2002), and CD40 (Schjetne et al., 2007) were exchanged with original V regions, and T cell epitopes were inserted into C domain loops. Such APC-targeted recombinant Ig molecules, called Troybodies, had an enhanced ability ($\times 10^2$–10^4) to stimulate $CD4^+$ T cell specific for model T cell epitopes (Lunde et al., 1999, 2002; Rasmussen et al., 2001;Schjetne et al., 2007). A similar strategy was later used by Hawiger et al. (2001), who genetically attached antigen to the C-terminus of heavy chains of DC-specific anti-DEC205 mAb. This strategy has become popular and has been used with minor variations in a large number of studies.

TARGETING OF Id IN A scFv FORMAT TO ANTIGEN-PRESENTING CELLS

In 1996/1997, Bjarne Bogen had a sabbatical in the lab of Ron Levy at Stanford University. This experience made it evident that efficient induction of anti-idiotypic antibodies was important for immunotherapy of B lymphoma cells. In this respect, Troybodies (see above) were deficient because short Id-sequences introduced into the Ig structure, although stimulatory for T cells, did not generate conformational Id determinants recognized by B cells. Another problem of Troybodies was that short Id-sequences only would be presented by certain MHC molecules present in only a fraction of individuals in the population.

A solution to these two problems seemed to be to include patients' B lymphoma V regions into an APC-targeted vaccine molecule. This should allow induction of anti-idiotypic antibodies and possibly also induction of Id-specific T cells. Such an APC-targeted idiotypic vaccine should be bipolar, with APC specificity oriented in one direction and idiotypic V regions in the other direction. Moreover, similar to IgG antibodies, the molecule should be bivalent in order to increase avidity for APC. Bivalency for idiotypic antigen should also be beneficial, since bivalency

would engender cross linking of BCR of anti-Id B cells, probably inducing better anti-Id antibody responses. Finally, the molecule should be devoid of Fc-associated biological effector functions such as binding to Fc receptors and complement activation. This statement may appear surprising since binding to Fc and complement receptors could indeed result in a positive outcome (Fearon and Locksley, 1996). However, binding to such receptors could potentially result in absorption of vaccine molecules and thereby deviation from intended, optimal APC targets, potentially blurring the results.

HOMODIMERIC VACCINE MOLECULES THAT BIVALENTLY TARGET DIMERIC IDIOTYPIC ANTIGEN TO APC

Based on the deliberations made above, the APC-targeted Id-vaccine molecule was constructed as a homodimer, each chain of the homodimer being composed of the following units: a targeting unit specific for APC, a homodimerization unit and Id antigen expressed as scFv (**Figure 1A**). These units were connected by short linkers (**Figure 1B**). The molecules were genetically constructed by use of a shuttle vector where the targeting units, the dimerization unit and the Id scFv could be easily exchanged (**Figure 1B**). An overview of published molecules used for Id-vaccination is given in **Table 1**.

As for targeting units, the following have been published: anti-mouse MHC class II as scFv (Fredriksen et al., 2006; Froyland et al., 2011), agonistic anti-mouse CD40 as scFv (Schjetne et al., 2007), mouse MIP1α (CCL3) and RANTES (CCL5) binding CCR1,3,5 on APC (Fredriksen and Bogen, 2007), and human MIP1α (LD78β; Ruffini et al., 2010). For all these targeting units, negative controls not binding APC have been constructed, such as scFv specific for the hapten NIP (Fredriksen et al., 2006; Schjetne et al., 2007; Froyland et al., 2011), or chemokine versions where the structural integrity has been destroyed by introduction of a C11A mutation that disrupts a disulfide bond (Fredriksen and Bogen, 2007; Ruffini et al., 2010). The scFv targeting units have been expressed in the V_H-V_L order with maintenance of specificity (an exception has been anti-TLR2, used in other vaccine molecule studies not reviewed here, where reversal of orientation to V_L-V_H improved binding to TLR2; Tunheim et al., 2007). The different scFv used for targeting appeared to influence the level of secretion of fusion protein, in the order αNIP > αMHCII > αCD40. Concerning chemokines as targeting moieties, different chemokines have been expressed approximately to the same levels in secreted homodimeric fusion proteins, and maintained their binding characteristics and chemotactic properties.

As for homodimerization motif, we have used a shortened human γ3 Ig hinge with cysteines available for disulfide bond formation. The shortened hinge has been connected via a $G_3S_2G_3SG$ linker to human (γ3) or mouse (γ2b) C_H3 domains that associate non-covalently. Thus, the shortened hinge and the C_H3 domain should confer homodimerization. Covalent dimerization of proteins secreted by transfected HEK293 cells has been partial, as estimated by SDS-PAGE under reducing or non-reducing conditions, varying roughly from close to 100% homodimerization to about 50%. Degree of covalent dimerization appears to be influenced by targeting and antigenic units, but there are also other factors involved. The degree of non-covalent homodimerization under physiological conditions has not yet been tested.

As for idiotypic antigens we have used $scFv^{315}$ (from the BALB/c mouse plasmacytoma MOPC315; Eisen et al., 1968), $scFv^{A20}$ (from the BALB/c B cell lymphoma A20; Kim et al., 1979), and scFv of four human multiple myeloma patients (Froyland et al., 2011). ScFv have been in the V_H-V_L order, connected by a $(G_4S)^3$ linker. In general, we have experienced few if any problems in expressing these various Id scFv as part of homodimeric vaccine proteins secreted by transiently transfected HEK293 cells. Id scFv appeared to fold correctly in the vaccine molecule format since they bound anti-Id mAb (Fredriksen et al., 2006; Fredriksen and Bogen, 2007; Schjetne et al., 2007; Ruffini et al., 2010; Froyland et al., 2011).

TARGETED IDIOTYPIC VACCINE, TESTED AS PROTEINS, ENHANCE T AND B CELL RESPONSES

Both anti-MHCII-$scFv^{315}$ and MIP-1α–$scFv^{315}$ proteins were about ×1,000-fold more effective, on a molar basis, at stimulating Id-specific $CD4^+$ T cells from Id^{315}-specific TCR-transgenic mice (Fredriksen et al., 2006; Fredriksen and Bogen, 2007). These results are highly promising for *in vivo* immunization with proteins. Unfortunately, it has until now been cumbersome to produce sufficient amounts of purified vaccine proteins from transiently transfected HEK293 and stably transfected NS0 cells for extensive immunization experiments. However, a single injection of 100 μg of MIP-1α–$scFv^{315}$ vaccine proteins in PBS induced a 20% protection against a challenge with MOPC315 plasmacytoma cells on day 14 after immunization, compared to nil protection obtained with the non-targeted control (Fredriksen and Bogen, 2007). These results are encouraging, but more efficient protein production is needed for extensive investigations on protein vaccination to be carried out.

DELIVERY OF APC-TARGETED Id AS DNA VACCINES INDUCE STRONG ANTI-Id RESPONSES AND TUMOR PROTECTION

Given the problems in producing sufficient proteins for immunization, we resorted to perform DNA immunization. The rationale for this choice was the previous finding that Ig H and L chain genes, when injected intramuscularly, resulted in prolonged production of assembled and functional H + L mAb molecules that could be detected in serum (Tjelle et al., 2004). Production of mAb by muscle cells was dependent upon electroporation of the injection site, which enhances the number of DNA-transfected cells (Mathiesen, 1999), and thus protein production and secretion. The particular mAb produced and secreted by muscle, anti-MHC II ($I\text{-}E^d$) could be detected in serum of a mouse that lacked $I\text{-}E^d$ but not in a mouse that expressed $I\text{-}E^d$. Thus, muscle-produced mAb bound MHC II molecules *in vivo* (Tjelle et al., 2004).

On this basis, we considered it possible that injection of homodimeric vaccine plasmid, combined with electroporation, could result in secretion of vaccine fusion protein into extracellular fluid, followed by binding to surface molecules on APC. Experiments to test this were first done with anti-MHCII-$scFv^{315}$ plasmid, using anti-NIP-$scFv^{315}$ as non-targeted control (Fredriksen et al.,

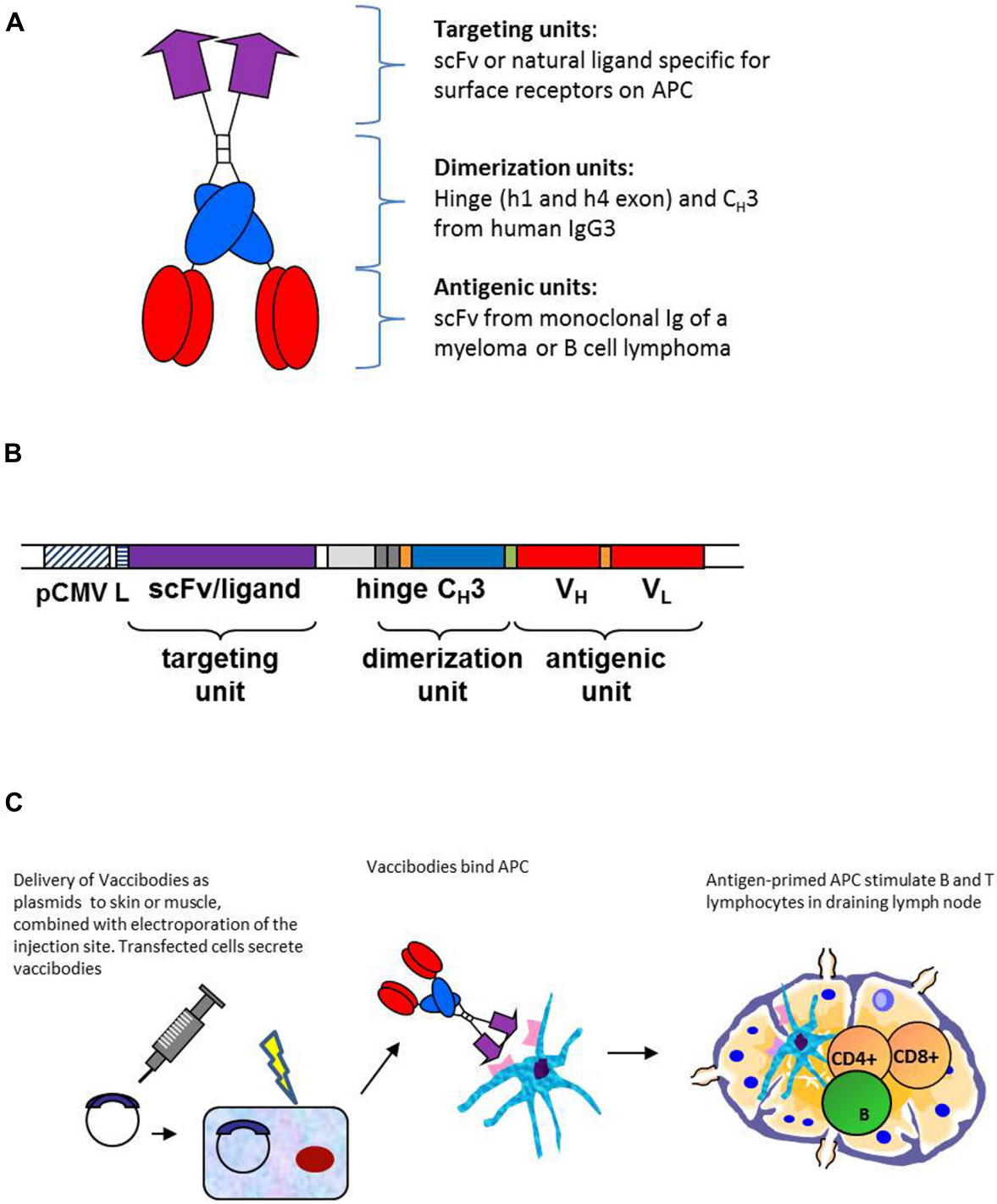


FIGURE 1 | Design, construction, and DNA delivery of APC-targeted vaccines. (A) The vaccine proteins are heterodimers. Each chain is composed of an N-terminal targeting unit [scFv($V_L + V_H$) or chemokines, *violet*], that bind surface receptors on APC, a dimerization unit composed of a shortened human Ig hinge and C_H3 domain (*blue*), and an antigenic unit corresponding to tumor-specific scFv from a B cell tumor (multiple myeloma, B cell lymphoma; *red*). As non-targeted controls we used vaccine molecule versions where the targeting unit was replaced with either scFv specific for the hapten NIP, or inactive (mutated) chemokine. **(B)** Gene construct. The targeting unit is inserted into the V cassette of the pLNOH2 vector. The dimerization unit, composed of h1 and h4 hinge exons and the C_H3 exon of human IgG3 (*blue*), is linked to the antigenic unit (*red*) and inserted together in the C cassette of pLNOH2. The $(G_4S)_3$ linkers (*orange*) and the GLSGL linker (*green*) are indicated. The gene is expressed from a CMV promoter (hatched) and a leader sequence (*striated*) of the pLNOH2 vector (*uncolored*). Upstream of h1 is an intronic sequence (*light gray*) Reproduced with modifications from *Mol. Ther.* 13: 776–785, 2006, with permission from the publisher. **(C)** Transfected cells secrete vaccine proteins that target antigen presenting cells. The APC travels to the draining lymph node where it meets $CD4^+$ T cells, B cells and $CD8^+$ T cells.

Table 1 | Efficiency of various targeting units in APC-targeted Id-vaccines*.

	Mouse αMHC class II (Fredriksen et al., 2006; Froyland et al., 2011)	Mouse αCD40 (Schjetne et al., 2007)	Mouse MIP-1α (Fredriksen and Bogen, 2007)¤	Human MIP-1α (Ruffini et al., 2010)	Mouse RANTES (Fredriksen and Bogen, 2007)¤
$CD4^+$ T cell responses *in vitro*	++	++ (+)	+++	++	++(+)
$CD4^+$ T cell responses *in vivo*	+++	++	+++	n.t.	++
Anti-Id antibody responses	+++	++	++	++	+
Protection against Id^+ tumors	60%	50–60%	70–80%#	n.t.	40–50%

Responses are qualitatively evaluated +++, ++, + compared to non-targeted controls. Since side by side comparisons were not done, and results were published separately (except MIP-1α and RANTES), exact comparison is impossible. The indicated refs should be consulted for details. Id-specific responses were mostly tested with scFv from the MOPC315 mouse myeloma, but also with scFv from the mouse A20 B lymphoma. In one study (Froyland et al., 2011) human MM scFv from 4 patients were tested. The results refer to vaccine molecules with a human dimerization motif (shortened hinge + C_H3). For the reference marked with ¤ besides the ref, equivalent mouse dimerization motif was tested in parallel (see also **Figure 2).*

#Antibody depletion experiments indicated that $CD8^+$ T cells conferred most of the protection against MOPC315 MM cells.

n.t., not tested.

2006). The results demonstrated that electroporation was needed to detect vaccine proteins in serum. Moreover, anti-MHC II ($I\text{-}E^d$) vaccine proteins were detected in mice lacking $I\text{-}E^d$ but were absent in mice expressing $I\text{-}E^d$, consistent with absorption on $MHC\ II^+$ APC. These results led to a model for how targeted DNA vaccines work. Briefly, transfected cells secrete vaccine protein that target APC, followed by drainage to lymph nodes for initiation of T and B cell responses (**Figure 1C**). The distinction from conventional DNA immunization, where the transfected cells themselves are thought to serve as APC, is evident.

Next, induction of anti-Id antibodies in serum was followed (Fredriksen et al., 2006). Anti-MHC II-$scFv^{315}$ induced a strong and rapid anti-idiotypic antibody response compared to non-targeted (anti-NIP) control. One immunization was sufficient for detection of anti-Id antibodies within 14 days. Antibody levels increased to day 60, then declined until day 170. Three immunizations spaced 21 days apart, increased antibody levels. A DNA dose-sparing effect of targeting was observed. These results were encouraging since M315 protein is poorly immunogenic and prolonged immunization schedules have been required to elicit anti-Id antibody responses.

Concerning *in vivo* T cell responses, MHC II-targeted DNA immunization was 100–1,000 times better at stimulation of Id-specific $CD4^+$ T cells, as revealed by immunizing TCR-transgenic mice and BrdU incorporation experiments (Fredriksen et al., 2006).

As for resistance to tumor challenge, mice immunized once with anti-MHC II-$scFv^{315}$ resisted a challenge with MOPC315 tumor cells while control mice immunized with non-targeted control were not protected. Similar results were obtained with DNA vaccines constructed for BALB/c B lymphoma A20, where vaccination also induced tumor resistance.

EXTENSION TO OTHER TARGETING UNITS

Two other mouse targeting units have been published: agonistic $scFv^{\alpha CD40}$ based on the agonistic anti-CD40 mAb FGK45 (Schjetne et al., 2007) and the mouse chemokines MIP-1α and RANTES (Fredriksen and Bogen, 2007). The former was indeed selected because agonistic targeting of CD40 could serve two functions: (i) activation of APC and (ii) loading with Id-peptide. Both effects were shown to be induced by vaccine molecules (Schjetne et al., 2007). Immunization with $scFv^{\alpha CD40}$–$scFv^{315}$DNA vaccines induced anti-Id antibodies and protection against tumor challenge.

MIP-1α and RANTES were selected because they are inflammatory chemokines and because previous experiments performed in our laboratory in surrogate systems employing mAbs to chemokine receptors had indicated that CCR1, 3, and 5 (to which MIP-1α and RANTES bind) could be promising targets (Schjetne et al., 2003). Moreover, chemotactic activity could cause APC to accumulate at site of injection, resulting in enhanced vaccine uptake and presentation. MIP-1α performed better than RANTES in assays for chemotaxis, induction of anti-Id antibodies and tumor protection. These results of the efficacy of the various targeting units are summarized in **Table 1**.

It should be emphasized that genetic fusion of other chemokines (MIP-3α and MCP3) to $scFv^{Id}$ has previously been successfully used as DNA vaccines by Biragyn and Kwak in A20, 38C13, and MOPC315 mouse tumor models (Biragyn et al., 1999; Ruffini et al., 2004; Qin et al., 2009; see below). In these studies, the fusion proteins had a monomeric form where the chemokine moiety was directly attached to scFv. Interestingly, when the chemokine-scFv DNA vaccine was combined with myotoxins that induce sterile inflammation with recruitment of APC at the intramuscular injection site, enhanced antitumor immunity was observed (Qin et al., 2009).

BIVALENCY AND XENOGENEIC SEQUENCES INCREASE IMMUNOGENICITY OF TARGETED BIVALENT IDIOTYPIC VACCINES

A comparison was done between bivalent and monovalent Id fusion protein with MIP-1α as targeting unit. The monovalent form was constructed *ad modum* Biragyn and Kwak, where the chemokine is directly attached to idiotypic scFv (Biragyn et al., 1999). Compared on a molar basis, the bivalent form had a higher chemotactic activity both *in vitro* and *in vivo*, was more efficient at stimulation of T cells *in vitro* and *in vivo*, had an increased ability to induce anti-Id antibodies, and induced a higher resistance to

tumor challenge. Thus, bivalency appeared to increase efficacy of the vaccine molecule in a variety of short-term and long-term assays *in vitro* and *in vivo* (**Figure 2**). A major part of the enhanced efficiency is likely to be due to bivalency of the targeting unit MIP-1α, and hence enhanced chemotactic activity and binding to APC.

A side by side comparison of vaccine molecules having human (γ3) or mouse (γ2b) C_H3 domains was undertaken, using MIP-1α-targeted vaccine molecules. While no influence was seen in short-term assays, such as chemotaxis or stimulation of Id-specific T cells *in vitro* or *in vivo*, a clear influence was observed in long-term *in vivo* assays such as induction of antibodies and resistance to an Id^+ tumor challenge (Fredriksen and Bogen, 2007; **Figure 2**). Thus, xenogeneic sequences in the homodimerization domain appeared to increase immunogenicity. However, vaccine constructs with mouse γ2b homodimerization domain appear to be less well secreted by transfected cells *in vitro*. If this is also the case upon DNA vaccination and electroporation, i.e., that transfected cells *in vivo* produce less vaccine protein, this could have contributed to the decreased immunogenicity of mouse γ2b-containing DNA vaccines. A possible explanation for these findings could be that xenogeneic C_H3 sequences are presented on MHC class II molecules. Xenogeneic sequences might be required for generation of sufficient help, since the T cell repertoire for syngeneic V regions is purged of T cells responding to germline–encoded sequences and is limited to recognition of Id-peptides expressing somatic mutations or V(D)J junctional sequences (Bogen et al., 1985, 1986a,b, 1993; Eyerman and Wysocki, 1994; Eyerman et al., 1996) reviewed in Bogen and Ruffini (2009). This explanation is consistent with the contribution of KLH to immunogenicity of Id in Id-KLH conjugates, by induction of KLH-specific T helper cells. Based on such results one may envisage that deliberate insertion of foreign promiscuous T cell epitopes, with ability to bind most MHC molecules in the species, could increase efficiency of the targeted vaccine molecules.

TARGETING SPECIFICITY INFLUENCES PHENOTYPE OF ELICITED IMMUNE RESPONSES

It is clearly of great importance to be able to direct the type of anti-Id immune responses elicited by Id-vaccination. For example, in B cell lymphoma, anti-Id antibodies appear to be important for tumor eradication (Syrengelas and Levy, 1999), while in multiple myeloma, Id-specific T cells seems to be the therapeutically most important arm of Id-immunity (Lauritzsen et al., 1994; Dembic et al., 2000; Corthay et al., 2005; Haabeth et al., 2011). Thus, Id-vaccines should elicit the kind of immune response suitable for the particular B cell tumor disease of the patient. Steering the phenotype of Id-immunity in the desired direction might be obtained by varying the targeting units of the bivalent idiotypic vaccine molecule. This has not yet been investigated fully, but available data suggest that targeting of Id-vaccines with $scFv^{\alpha MHC\ class\ II}$ induces high amounts of antibodies while targeting with MIP-1α induces more T cells (Fredriksen et al., 2006; Fredriksen and Bogen, 2007). It might be of particular merit to target particular subsets of APC, i.e., $CD8^+$ DCs, the latter being known for their ability to cross present antigen to cytotoxic CD8 T cells.

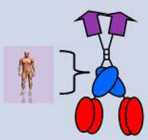
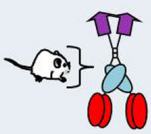
	Chemotaxis *in vivo*	Anti-Id antibodies	T cell responses	Tumor protection
Homodimer-Xenogeneic dimerization unit 	+++	+++	+++	70-80%
Homodimer-Syngeneic dimerization unit 	+++	+	++	10-20%
monomer 	+	+	++	10-20%

FIGURE 2 | Bivalency and xenogeneic sequences increase immunogenicity of vaccine molecules. Homodimers with human xenogeneic dimerization motifs were compared with homodimers with murine syngeneic dimerization motifs and monomers for induction of chemotaxis, Id-specific T and B cell responses, and protection against Id^+ tumor challenge. Vaccine molecules had MIP1-α as targeting unit and $scFv^{315}$ or $scFv^{A20}$ as antigenic unit (Fredriksen and Bogen, 2007).

A HYPOTHETICAL MODEL FOR ACTION OF APC-TARGETED BIVALENT VACCINE MOLECULES

The mechanism for why targeted vaccine molecules (called vaccibodies) improve T and B cell responses to Id is hypothesized in **Figure 3**. Briefly, targeting of vaccine molecules to APC such as DCs should result in efficient stimulation of $CD4^+$ T cells. Simultaneously, B cells should bind conformational determinants on the antigenic units, and process and present the antigen on their MHC class II molecules. Primed B cells will receive help from the $CD4^+$ T cells already stimulated by DCs, resulting in generation of plasma cells and antibody production. In addition, certain targeting units, such as MIP-1α, may by unknown mechanisms result in cross-presentation of Id on MHC class I molecules and generation of $CD8^+$ T cells (Fredriksen and Bogen, 2007; **Figure 3A**). A variant model may be considered where the vaccine molecules could bridge DCs and B cells, as in an APC B cell synapse (Batista et al., 2001). A "tug of war" between the APC and the B cell could result in Ag being displayed as peptides on MHC II molecules of both cells (**Figure 3B**). $CD4^+$ T cells could interact with both the APC and the B cell, either consecutively or simultaneously, in the latter case forming a three-member cellular complex.

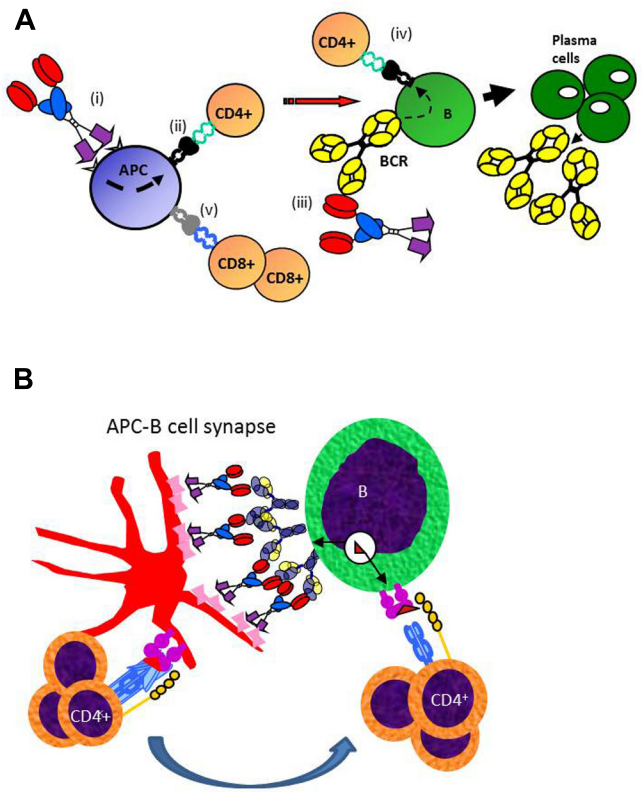


FIGURE 3 | Proposed mechanism for how APC-targeted homodimeric vaccine molecules efficiently stimulate both T and B cell responses. **(A)** (i) Vaccibodies bind to surface molecules on APC, such as dendritic cells and induce their maturation. (ii) Vaccibodies are processed and peptides from the antigenic unit are presented on MHC class II molecules of APC to naïve $CD4^+$ T cells that become effector T cells. (iii) B cells with a B cell receptor (BCR)-specific for conformational determinants on complete vaccibodies internalize vaccibodies; process them, and present antigenic peptides on their MHC class II molecules to the effector $CD4^+$ T cells. (iv) B cells receive help from the effector $CD4^+$ T cells and develop into plasma cells that secrete antibodies. Finally, antigen-specific antibodies and $CD4^+$ T cells cause the elimination of the antigen. (v) Vaccibodies with certain targeting units (MIP-1α) can induce presentation on MHC class I, and cross-priming of $CD8^+$ T cells is thought to be particularly important for viral infections and cancer. **(B)** A variant model where homodimeric vaccine molecules bridge APC and B cells, resulting in a "tug of war" for antigen. T cell could interact with MHC II-Ag complexes on both APC and B cells, as indicated.

EXTENSION TO HUMAN TARGETING UNITS AND HUMAN IDIOTYPES FROM PATIENTS

Homologous chemokines are expressed in mouse and man and indeed most mammal species. Therefore, since mouse MIP-1α gave encouraging results in mice (Fredriksen and Bogen, 2007), it was of particular interest to test if human MIP-1α could also function as a targeting unit. Moreover, if human MIP-1α bound to mouse chemokine receptors, a vaccine intended for human use could first be tested in mice prior to human application. Such studies could pave the way for application of APC-targeted Id-vaccines in humans.

In humans, there are two homologs of MIP-1α, LD78α and LD78β both sharing 74% homology with the mouse MIP-1α. The two variants are 96% homologous, however, while LD78α-targeted vaccine was unable to bind strongly to both murine and human chemokine receptors, LD78β-fusion vaccines bind both murine, human and macaque chemokine receptors. Furthermore, LD78β-targeted vaccines demonstrated increased ability to activate $CD4^+$ T cells and antigen-specific antibodies in mice models (Ruffini et al., 2010). Thus, the human vaccine product can be tested for functionality in both murine and non-human primate models before entering the clinic.

The overall aim of the studies reviewed herein is to develop Id-vaccines that work in patients. We therefore genetically constructed scFv from multiple myeloma cells obtained from bone marrow of four patients, and inserted these into vaccine molecules specific for mouse MHC II ($I\text{-}E^d$; Fredriksen et al., 2006). Mice DNA-immunized with these constructs produced antibodies that in ELISA bound the particular purified serum myeloma protein corresponding to the scFv used, but poorly or not at all to myeloma proteins from the other three patients. By this criterium, the scFv must correctly fold when produced and secreted by transfected mouse cells. Anti-idiotypic antibody titers were much higher in mice immunized with MHC II-targeted vaccine constructs compared with the non-targeted (NIP-specific) control (Fredriksen et al., 2006). The anti-idiotypic antibodies could be used to establish ELISAs specific for the myeloma protein of each patient (Froyland et al., 2011). These ELISAs were about 100-fold more sensitive than standard immunofixation for detection of myeloma protein in serum, and could be used for early detection of recurrence of disease.

FUTURE PERSPECTIVES: EXTENSION TO VACCINATION OF HUMANS WITH B CELL CANCERS

As reviewed above, scFv can be constructed from patients with B cell malignancies, and inserted into targeted bivalent DNA vaccines (Froyland et al., 2011). The ease and rapidity with

which the vaccines can be genetically constructed and produced as DNA are clear advantages for generation of individual patient-specific Id-vaccines. Mice immunized with MHC II-targeted constructs make anti-Id antibodies to human Id after a single DNA injection combined with electroporation (Froyland et al., 2011).

In future work, we plan to equip patient-specific scFv DNA vaccines with targeting units specific for human APC. Provided the targeting unit cross-react between human and mouse APC, which is the case for human MIP-1α (LD78β; see above), the DNA vaccine intended for human use can be tested in mice prior to human application. It should be noted that DNA vaccination with electroporation has been performed in humans without serious side effects (van Drunen Littel-van den Hurk and Hannaman, 2010; Vasan et al., 2011). However, the DNA vaccines tested so far have been of a non-targeted nature. In our long-term experiments in mice, we have yet to observe any detrimental side effects of targeted DNA vaccines.

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Conflict of Interest Statement: The authors are inventors on several patent applications, submitted via their employers (University of Oslo, Oslo University Hospital), related to the described vaccine concept (vaccibodies). Agnete Fredriksen is CSO and Bjarne Bogen is head of the scientific panel of Vaccibody AS. All three authors have shares in the company.

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Preclinical evaluation of racotumomab, an anti-idiotype monoclonal antibody to N-glycolyl-containing gangliosides, with or without chemotherapy in a mouse model of non-small cell lung cancer

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N-glycolylneuraminic acid (NeuGc) is a sialic acid molecule usually found in mammalian cells as terminal constituents of different membrane glycoconjugates such as gangliosides. The NeuGcGM3 ganglioside has been described as a tumor antigen for non-small cell lung cancer (NSCLC) in humans. Racotumomab is an anti-NeuGc-containing gangliosides anti-idiotype monoclonal antibody (mAb) (formerly known as 1E10) that has received attention as a potential active immunotherapy for advanced lung cancer in clinical trials. In this work, we have examined the antitumor activity of racotumomab in combination or not with chemotherapy, using the 3LL Lewis lung carcinoma as a preclinical model of NSCLC in C57BL/6 mice. Vaccination with biweekly doses of racotumomab at 50–200 μg/dose formulated in aluminum hydroxide (racotumomab-alum vaccine) demonstrated a significant antitumor effect against the progression of lung tumor nodules. Racotumomab-alum vaccination exerted a comparable effect on lung disease to that of pemetrexed-based chemotherapy (100 mg/kg weekly). Interestingly, chemo-immunotherapy was highly effective against lung nodules and well-tolerated, although no significant synergistic effect was observed as compared to each treatment alone in the present model. We also obtained evidence on the role of the exogenous incorporation of NeuGc in the metastatic potential of 3LL cells. Our preclinical data provide support for the combination of chemotherapy with the anti-idiotype mAb racotumomab, and also reinforce the biological significance of NeuGc in lung cancer.

Keywords: cancer immunotherapy, anti-idiotype antibody, N-glycolylneuraminic acid, NSCLC, mouse models

INTRODUCTION

N-glycolylneuraminic acid (NeuGc) is a sialic acid molecule usually found in mammalian cells as terminal constituents of different membrane glycoconjugates such as the GM3 ganglioside (NeuGcGM3). Gangliosides are a broad family of glycosphingolipids found on the outer cell membrane, involved in cell communication, regulation of the immune response, and cancer progression (Patra, 2008; Lopez and Schnaar, 2009). NeuGcGM3 has been described as a tumor antigen for non-small cell lung cancer (NSCLC) in humans (van Cruijsen et al., 2009; Blanco et al., 2012). The significance of NeuGc overexpression in human cancer is still under investigation. Considering that anti-NeuGc antibodies can be detected in several cancer patients, it was hypothesized that antibody-mediated inflammation could facilitate tumor progression (Varki, 2010). However, it is widely accepted that high titers of these antibodies can induce tumor cell death (Roque Navarro et al., 2008; Varki, 2010; Hernandez et al., 2011). In addition, experimental data indicated that growth-stimulating features of NeuGc on tumor cells can be explained by immune system down-modulation (De Leon et al., 2008).

Racotumomab is an anti-NeuGc-containing gangliosides anti-idiotype monoclonal antibody (mAb), formerly known as 1E10, that has received attention as a potential active immunotherapy for advanced lung cancer in clinical trials (Neninger et al., 2007; Alfonso et al., 2008). As an anti-idiotype antibody, racotumomab is the mirror image of the P3 mAb idiotype which specifically reacts against NeuGc antigens on cell surface (Vazquez et al., 1998).

Previously, we evaluated the antitumor activity of racotumomab in syngeneic mouse tumor models. Vaccination with several biweekly intraperitoneal doses of racotumomab coupled to keyhole limpet hemocyanin in Freund's adjuvant, significantly inhibited the formation of spontaneous lung metastases by F3II mammary carcinoma cells (Vazquez et al., 2000). Administration of low-dose cyclophosphamide together with subcutaneous immunization with aluminum hydroxide-precipitated racotumomab (racotumomab-alum vaccine) significantly reduced F3II primary tumor growth. The antitumor response was comparable to that obtained with standard high-dose chemotherapy in such breast cancer model, but without overt signs of toxicity. Interestingly, combinatory chemo-immunotherapy promoted $CD8^+$ lymphocyte tumor infiltration and increased tumor

apoptosis (Fuentes et al., 2010). In addition, intravenous administration of uncoupled racotumomab, as a biological response modifier, dramatically inhibited metastatic lung colonization by B16 melanoma cells (Vazquez et al., 2000).

In the present work, we have examined the antitumor activity of racotumomab-alum in combination or not with pemetrexed- or taxane-based chemotherapy, using the 3LL Lewis lung carcinoma in C57BL/6 mice as a preclinical model of NSCLC. It is a validated model for the NeuGcGM3 ganglioside, showing an increased expression of such specific antigen in disseminated nodules with respect to the primary tumor or *in vitro* cultured cells (Labrada et al., 2010). In this regard, we also obtained evidence on the role of the exogenous incorporation of NeuGc in the metastatic potential of 3LL cells.

MATERIALS AND METHODS

RACOTUMOMAB-ALUM VACCINE

Racotumomab was produced by the Center of Molecular Immunology (La Habana, Cuba). The mAb was purified from mouse ascites by good manufacturing practices, as previously described (Alfonso et al., 2002). Briefly, purification was performed by DEAE-exchange chromatography followed by affinity chromatography and size exclusion chromatography using a Sephadex G-25 column. The vaccine preparation was produced by mixing aluminum hydroxide as adjuvant with purified racotumomab at a final concentration of 1 mg/ml. Some experiments were carried out using a bioreactor-obtained mAb, as recently described by Machado et al. (2011).

TUMOR CELLS AND CULTURE CONDITIONS

We used the 3LL Lewis lung carcinoma, clone D122, a low immunogenic and high-metastatic cell line in syngeneic C57BL/6 mice (Eisenbach et al., 1984). Additionally, the X63 murine myeloma cell line, expressing high levels of NeuGcGM3 in its membranes, was employed. Tumor cells were maintained in Dulbecco's Modified Eagle Media (DMEM) culture medium (Gibco BRL, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum. Cells were subcultured twice a week using trypsin-EDTA, and cell viability was assessed using the trypan blue exclusion technique. The concentration of chemotherapy drug causing 50% growth inhibition (IC_{50}) was determined by the MTT colorimetric assay.

ANIMALS

Pathogen-free C57BL/6 mice (approximately 10 weeks-old, with an average weight of 25 g) were obtained from the Animal Care Division of UNLP (La Plata, Argentina). Up to 5–6 mice per cage were kept with water and food *ad libitum* in the animal house facility at Quilmes National University. Pooled sera from experimental or control groups were obtained, and frozen at −20°C in aliquots for further analysis. Experimental protocols were approved by the Animal Review Board and maintenance of animals was conducted under accepted international standards.

Ex vivo NeuGc PREINCUBATION

Tumor cells were harvested with trypsin-EDTA solution and resuspended in serum-free DMEM containing NeuGc (Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 100 μg/ml. After an incubation of 1 h at 37°C, 3LL cells were extensively washed and resuspended in fresh culture medium.

NeuGcGM3 DETECTION BY FLOW CYTOMETRIC ASSAY

We used the specific anti-NeuGcGM3 mouse IgG1 mAb 14F7 (Carr et al., 2000), produced by the Center of Molecular Immunology. Tumor cells were harvested with trypsin-EDTA solution, resuspended in serum-free DMEM, and 0.5–1 $\times 10^6$ cells per sample were incubated with 2 μg of 14F7, isotype control, or mouse sera (dilution 1:50) for 30 min at room temperature. Then, tumor cells were washed with phosphate buffered saline and incubated with R-phycoerythrin-conjugated goat anti-mouse immunoglobulins (DakoCytomation, Carpinteria, CA, USA) for 30 min at 4°C. A total of 5×10^4 events were analyzed per tube with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA), using the WinMDI 2.9 software.

PRIMARY TUMOR GROWTH AND SPONTANEOUS METASTASES

At day 0, groups of at least six mice were inoculated subcutaneously in the right flank with 3LL cells ($4–5 \times 10^5$ viable cells per mouse in 0.2 ml of DMEM). Primary tumor development was monitored by palpation. The largest perpendicular tumor diameters were measured with a caliper thrice a week, and tumor volumes were calculated using the formula $\pi/6 \times \text{length} \times \text{width}^2$. Animals were sacrificed by cervical dislocation at day 50 or when subcutaneous tumor volume exceeded 3,000 mm^3. Lungs were fixed in Bouin's solution and surface lung nodules were counted under a dissecting microscope, as described elsewhere (Alonso et al., 1996). Four doses of 50 μg of racotumomab-alum vaccine were administered s.c. in the interescapular area at 14-day intervals, beginning the day tumor cell inoculation (days 0, 14, 28, and 42). Control animals received only the saline vehicle. When tumors became palpable at day 10–12, mice received 3 weekly i.p. doses of pemetrexed (100 mg/kg) or docetaxel (20 mg/kg).

EXPERIMENTAL LUNG METASTASES

At day 0, groups of at least eight mice were injected into the lateral tail vein with control or NeuGc-preincubated 3LL cells (7.5×10^4 viable cells per mouse in 0.3 ml of DMEM). At day 21, animals were sacrificed and surface lung nodules were counted, as described above. Mice were vaccinated with racotumomab-alum at 50 or 200 μg/dose, receiving 2 doses before (days −14 and −7) and the third after (day +7) tumor cell inoculation.

STATISTICAL ANALYSIS

Statistical analyses were carried out using GraphPad Prism version 3.0 (GraphPad Software, La Jolla, CA, USA).

RESULTS

PROTECTION AGAINST SPONTANEOUS LUNG TUMOR FORMATION BY IMMUNIZATION WITH RACOTUMOMAB-ALUM

We first studied antitumor protection by the anti-idiotype mAb racotumomab against the formation of lung nodules. Tumors were induced in the flank of the mice by subcutaneous injection of 3LL cells and lung lesions were formed by spontaneous metastatic

spread. As shown in **Figure 1A**, growth of subcutaneous primary tumors was not affected by vaccination with biweekly s.c. doses of 50 μg of racotumomab-alum beginning at the day of tumor challenge. On the other hand, immunization significantly reduced the formation of lung tumor nodules (**Figure 1B**), indicating an increased NeuGcGM3 antigen expression in spontaneous metastatic lesions in comparison to the primary tumor, as previously reported (Labrada et al., 2010). Most experiments were performed using racotumomab produced in mouse ascites fluid. Similar antitumor activity was found using a vaccine formulation containing a bioreactor-obtained mAb within a dose range from 50 to 200 μg per dose, with a reduction of about 40–50% in lung nodule formation (data not shown).

COMBINATION OF ANTI-IDIOTYPE IMMUNIZATION WITH STANDARD CHEMOTHERAPY

We then explored the antitumor response induced by racotumomab-alum in combination or not with chemotherapy. As racotumomab, pemetrexed had no significant effects on subcutaneous 3LL primary tumors. Immunization with biweekly doses of the vaccine exerted a comparable effect on spontaneous lung tumor formation to that of weekly i.p. cycles of pemetrexed at a dose of 100 mg/kg (**Figure 2**). Combination of racotumomab-alum with pemetrexed was highly effective against lung nodules (see also **Figure 2**), although no significant synergistic effect was observed as compared to each treatment alone in the present experimental conditions. No antitumor effects were observed with taxane-based chemotherapy using weekly docetaxel at 20 mg/kg (data not shown). In all cases, chemo-immunotherapy protocols with pemetrexed were well-tolerated, not affecting body weight gain, food and water consumption or inducing other signs of overt toxicity.

ASSOCIATION OF NeuGc EXPRESSION WITH HIGHLY AGGRESSIVE EXPERIMENTAL LUNG TUMOR FORMATION

We asked whether antitumor activity of ractumomab was truly associated with expression of NeuGc-containing gangliosides in tumor cells in the present mouse lung-cancer model. We established a highly aggressive experimental disease by intravenous lung colonization by 3LL cells. Lung lesions progressed rapidly and control animals died about 25 days after challenge. In this experimental condition, preliminary experiments demonstrated no antitumor effects of racotumomab-alum, even starting immunization before tumor cell challenge. We hypothesized that during the rapid disease progression, tumor cells are not able to incorporate enough NeuGc from lung tissue, and immunization is not effective. Thus, we decided to perform an *ex vivo* preincubation of 3LL cells with purified NeuGc, following a method known to induce expression of NeuGc gangliosides in the cell membrane (Gabri et al., 2009). To confirm antigen expression, cells were analyzed by flow cytometry with a specific anti-NeuGcGM3 antibody.

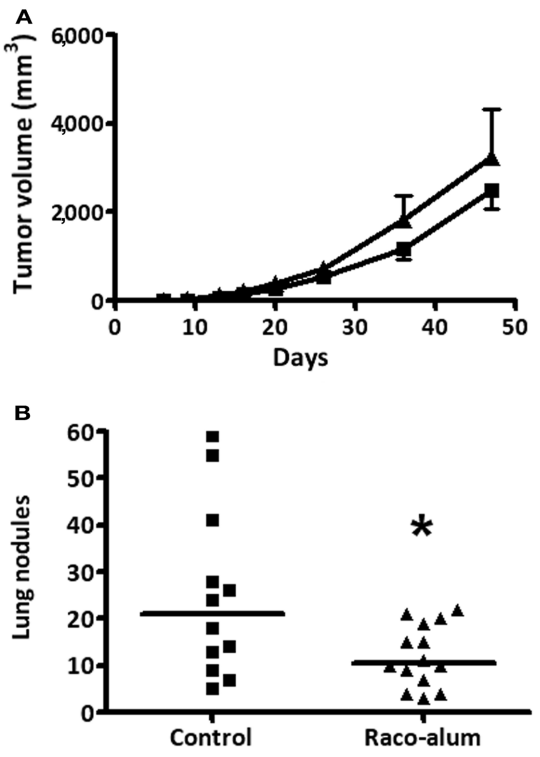

FIGURE 1 | Antitumor protection against spontaneous lung nodule formation by immunization with racotumomab-alum. Mice were inoculated subcutaneously with 3LL cells and immunized with racotumomab-alum at a dose of 50 μg, as described in "Materials and Methods." **(A)** Subcutaneous primary tumor growth. Data represent mean ± SEM. No significant effects were detected between control and treated animals. **(B)** Spontaneous lung tumor nodules. Immunization significantly reduced the number of lung nodules compared to control. Data points represent individual mice and horizontal lines indicate the median values. Data were pooled from two independent experiments with similar results. $^{*}p = 0.0377$ (unpaired *t*-test with Welch's correction).

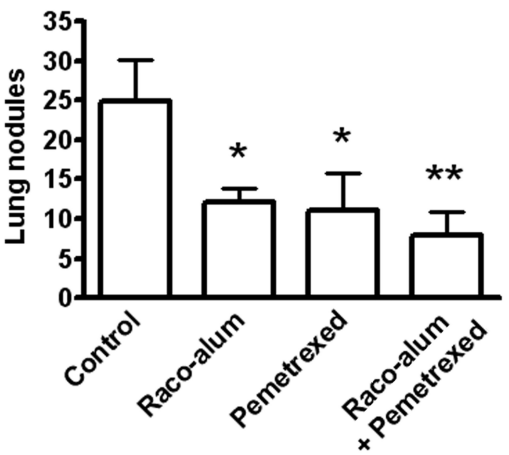

FIGURE 2 | Effects of racotumomab-alum immunization and chemotherapy on spontaneous lung nodule formation. Mice were inoculated subcutaneously with 3LL cells, and then immunized with racotumomab-alum (raco-alum) at 50 μg/dose and/or administered with pemetrexed (100 mg/kg), as described in "Materials and Methods." Data were pooled from two independent experiments with similar results. Results are shown as mean ± SEM. $^{*}p < 0.05$, control versus raco-alum or versus pemetrexed; $^{**}p < 0.01$, control versus raco-alum plus pemetrexed (ANOVA followed by Bonferroni's multiple comparison test).

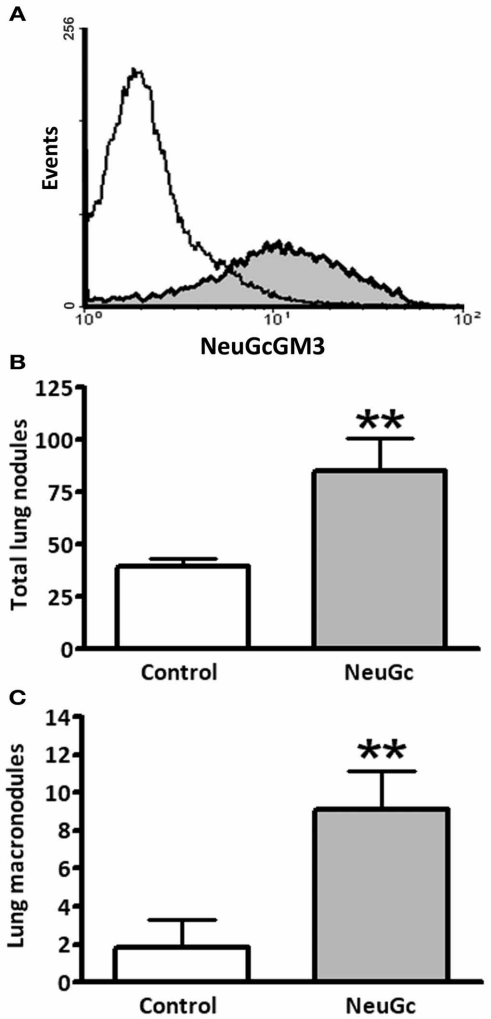

FIGURE 3 | Effect of exogenous incorporation of NeuGc on experimental lung tumor formation. 3LL cells were preincubated *in vitro* with purified NeuGc and then injected intravenously in mice, as described in "Materials and Methods." **(A)** Flow cytometryc analysis of 3LL cells after preincubation with purified NeuGc, resulting in an increase of NeuGcGM3 ganglioside in cell membrane, as detected by the specific 14F7 mAb. White and gray curves present results from control and NeuGc-preincubated cells, respectively. **(B)** Total lung tumor nodules experimentally formed by control or NeuGc-preincubated 3LL cells. **(C)** Experimental formation of lung macronodules (>2 mm in diameter) by control or NeuGc-preincubated 3LL cells. Lung lesions are shown as mean ± SEM. $^{**}p < 0.01$ (unpaired *t*-test with Welch's correction).

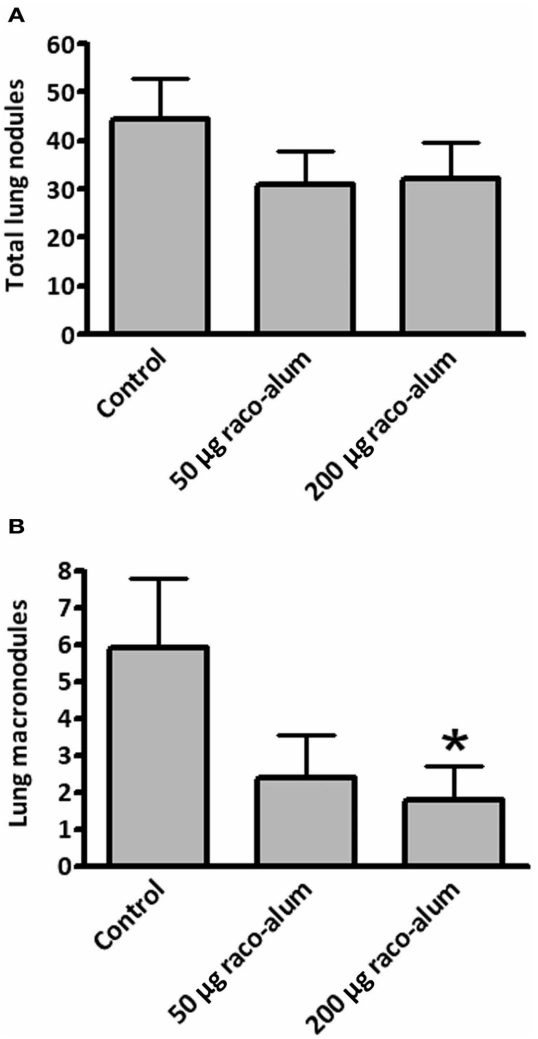

FIGURE 4 | Antitumor activity of racotumomab-alum vaccination in highly aggressive, NeuGcGM3-positive experimental lung tumors. Mice were vaccinated with racotumomab-alum (raco-alum) and then injected intravenously with control or NeuGc-preincubated 3LL cells, as described in "Materials and Methods." **(A)** Total lung tumor nodules. **(B)** Lung macronodules (>2 mm in diameter). Lung lesions are shown as mean ± SEM. $^{*}p < 0.05$, control versus raco-alum at 200 µg/dose (ANOVA followed by Bonferroni's multiple comparison test).

While a marked staining was detected in NeuGc-preincubated cells, control 3LL cultured cells were almost negative (**Figure 3A**). We also checked the *in vitro* sensitivity of NeuGc-preincubated tumor cells to chemotherapy. The IC_{50} for pemetrexed after a 3-day exposure of rapidly-growing 3LL cells was about 200 nM (193 ± 6 nM). A similar IC_{50} value was found in 3LL cells preincubated with NeuGc (192 ± 31 nM), suggesting no direct effects of target antigen expression on chemotherapy effectiveness.

Preincubation with NeuGc significantly increased the formation of experimental lung tumor nodules, particularly the macronodules of more than 2 mm in diameter (**Figures 3B,C**). A significant antitumor activity was obtained for macronodules by vaccination with racotumomab-alum at 200 µg per dose in this highly aggressive, NeuGcGM3-positive lung disease (**Figure 4**).

ANTIGEN-SPECIFIC IMMUNE RESPONSE IN MICE VACCINATED WITH RACOTUMOMAB-ALUM

Finally, we addressed the NeuGc-specific immune response in mice bearing subcutaneous 3LL tumors. Animals were challenged with 3LL cells preincubated or not with purified NeuGc, and tumors were allowed to progress. Then, the sera were analyzed by flow cytometry using the X63 murine cell line, in which NeuGcGM3 is the major ganglioside expressed on the cell

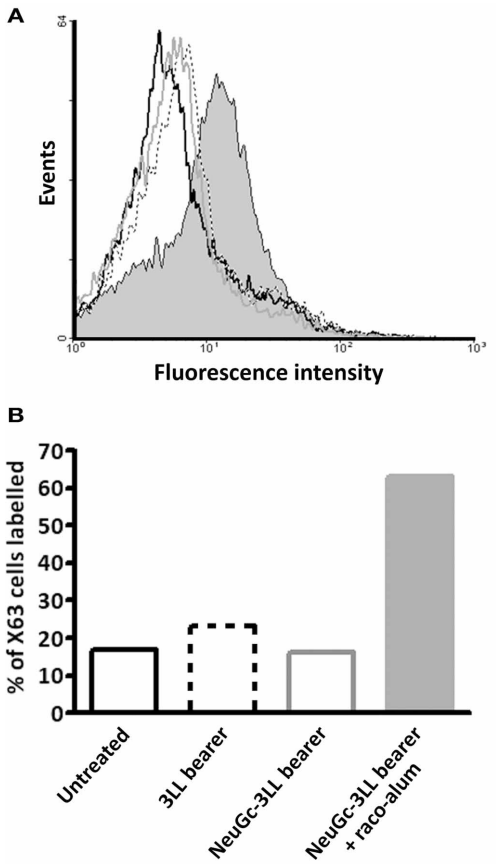

FIGURE 5 | NeuGc-specific humoral response elicited by racotumomab-alum vaccination. Mice were challenged subcutaneously with 3LL cells preincubated or not with purified NeuGc, and immunized with racotumomab-alum (raco-alum) at 50 μg/dose, as described in "Materials and Methods." After tumor growth, pooled sera from the different groups were obtained and analyzed by flow cytometry against X63 cells expressing NeuGcGM3 ganglioside. **(A)** Flow cytometric analysis. **(B)** Percent of recognized X63 cells. Data from healthy untreated mice (black line), mice bearing control 3LL tumors (dotted line), mice bearing NeuGc-enriched 3LL tumors (gray line), and mice bearing NeuGc-enriched 3LL tumors and immunized with raco-alum (filled bar).

membranes (Hernandez et al., 2008). As shown in **Figure 5**, no reactivity was observed either in mice bearing NeuGc-enriched or control 3LL tumors. On the contrary, the sera from tumor-bearing animals that were immunized with racotumomab-alum reacted brightly against X63 cells expressing NeuGcGM3 (see also **Figure 5**).

DISCUSSION

The development of cancer vaccines, aimed to enhance the immune response against a tumor, is a promising area of research. Preclinical studies would support the development of vaccination strategies with enhanced clinical efficacy (Palena et al., 2006). In this regard, mouse models are excellent tools to test efficacy of novel therapies and to understand the biological mechanisms that govern the generation of an effective antitumor response. One of the main challenges in developing mAb-based cancer therapies directed to glycolipids is to find attractive targets, with specific expression in tumors and a documented role during tumor progression. This is not easy to demonstrate experimentally in murine models, since mouse tumors often do not express similar glycolipids as human tumors do. Besides, human xenograft tumors can grow in immunodeficient athymic mice which are precluded for active immunotherapy studies.

In the present work, we obtained evidence suggesting that exogenous incorporation of NeuGc promotes the metastatic potential of 3LL lung cancer cells and that antitumor activity of the anti-idiotype mAb racotumomab is associated to NeuGcGM3 expression in tumor nodules. Previously, we reported that cultured mouse melanoma and mammary cancer cells are able to process and incorporate NeuGc from different sources such as fetal bovine serum, NeuGc-rich mucins or purified NeuGc, thus promoting the formation of blood-borne metastases (Gabri et al., 2009). NeuGc increased the adhesive properties of tumor cells and seemed to be involved in tumor nesting at distant sites. Similarly, the 3LL Lewis lung carcinoma model was consistent with an increased expression of NeuGcGM3 from subcutaneous primary tumors to spontaneous metastatic lung nodules (Labrada et al., 2010).

In most mammals, the synthesis of NeuGc is catalyzed by the cytidine monophospho-N-acetylneuraminic acid hydroxylase. However, the enzyme is inactivated due to a frameshift mutation in human beings (Irie et al., 1998), and also absent in both human and many mouse cancer cell lines (Segatori et al., 2012). Tumors seem to incorporate NeuGc from dietary sources or the tissue microenvironment. It is known that resistant cancer cells could overexpress NeuGc-containing gangliosides under hypoxic conditions by inducing the sialic acid transporter sialin (Yin et al., 2006).

The ganglioside NeuGcGM3 has been described in several human neoplasms, including NSCLC (van Cruijsen et al., 2009; Blanco et al., 2012), but is usually not detected in healthy human tissues and fluids (Tangvoranuntakul et al., 2003). This fact defines NeuGcGM3 as an interesting neoantigen target for immunotherapy (Fernandez et al., 2010). Assessment of NeuGcGM3 expression in about 200 samples of NSCLC by tissue microarray immunohistochemistry demonstrated a wide expression in more than 90% of cases. Moreover, based on the expression of CD83, which is a marker of mature dendritic cells, NeuGcGM3 appeared to be involved in tumor-induced dendritic cell suppression (van Cruijsen et al., 2009).

A biweekly immunization protocol with racotumomab-alum was highly effective against 3LL lung tumor nodules, either alone or in combination with chemotherapy cycles of pemetrexed (Alimta™). As described previously, the therapeutic effect of racotumomab-alum was associated to an increase of $CD4^+$ and $CD8^+$ T cell infiltration, a reduced angiogenesis and tumor cell apoptosis in lung nodules, although measurable antibodies were not detected against purified NeuGcGM3 by ELISA assay in C57BL/6 mice (Diaz et al., 2009). Here, we demonstrated that sera from tumor-bearing mice immunized with racotumomab-alum can recognize X63 cells overexpressing NeuGcGM3. It is important to note that sera from non-vaccinated animals bearing 3LL tumors (either with or without NeuGc enrichment) were

unable to react with X63 cells indicating that immune response to antigen-positive cells is not elicited by tumor progression itself.

In human NSCLC patients vaccinated with racotumomab, it was suggested a correlation between the induction of IgG or IgM against NeuGcGM3 and longer survival (Hernandez et al., 2008). Anti-NeuGcGM3 specific antibodies were capable of recognizing and killing tumor cells expressing the antigen, by a mechanism resemble the oncotic necrosis (Hernandez et al., 2011).

To our knowledge, this is the first preclinical report demonstrating the feasibility of the combination of the anti-idiotype mAb racotumomab with chemotherapeutic drugs such as pemetrexed, and thus providing a rationale for chemo-immunotherapy combinations in NSCLC. Our experimental data also contribute to reinforce the biological significance of NeuGcGM3 ganglioside as a target for cancer immunotherapy.

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CLINICAL CASE STUDY
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Maintenance treatment with chemotherapy and immunotherapy in non-small cell lung cancer: a case report

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A 53-years-old woman was diagnosed with lung adenocarcinoma state IV (synchronous pleural involvement) in April 2009. First-line systemic treatment included six cycles of Carboplatin, Paclitaxel, and Bevacizumab. Partial response was achieved. Maintenance therapy with Bevacizumab and Pemetrexed was given from September 2009 to February 2010. No response changes were observed. Immunotherapy was initiated, and then Pemetrexed was given with the same disease status. Both treatments were well tolerated. Immunotherapy toxicity included reaction at the site of injection grade 2. At present, the patient is still on this treatment. Given the poor prognosis of patients with advanced lung cancer, the combination of both treatments during the stable phase of the disease may improve progression-free survival.

Keywords: vaccines, lung cancer, immunotherapy, chemotherapy, concurrent review

CASE PRESENTATION

A 53-years-old woman otherwise previously healthy and a non-smoker, was diagnosed with lung adenocarcinoma, stage IV T 3 Nx M1a (TNM classification 7th edition) on April 2009.

On March 2009, she presented with functional class III dyspnea. A Chest X-ray showed veiling in the left side of the thorax. CAT scans were performed, and a solid lung mass in the left lower lobe, associated with pleural effusion and moderate lung collapse was seen contralateral pleural effusion was also evidenced.

Bronchoscopy showed extrinsic compression of the left lower lobe bronchus. Pleural biopsy by thoracoscopy, and pleurodesis with sclerosing agents were performed (talc).

Histological examination of the pleura revealed a proliferation of epithelial-like atypical cells arranged in glands, nests, and cords with moderate anisocytosis, anisokaryosis, macronucleoli, and scattered mitoses. Pleural fluid was positive for neoplastic cells.

Immunostaining techniques were performed against the following antigens: cytokeratin 7, cytokeratin 20, calrretinina, chromogranin, and TTF, which were positive for cytokeratin 7 and TTF. These morphological findings are related to moderately differentiated adenocarcinoma of pulmonary etiology. Cobas EGFR test was non-mutated.

First line chemotherapy with carboplatin AUC 6 + paclitaxel 200 mg/m^2 + bevacizumab 15 mg/kg every 21 days was started on May 2009. The patient received six cycles, and this regimen finished in September 2009. Tumor assessment showed partial response (RECIST). Maintenance therapy with bevacizumab 15 mg/kg + pemetrexed 500 mg/m^2 every 21 days was administered no significant toxicity was associated with these regimens. Bevacizumab was discontinued in February 2010 and the patient was included in a compassionate program including Racotumomab. Pemetrexed was administered together with immunotherapy, and the patient is still on treatment. Partial response was maintained (**Figure 1**). As for toxicity associated with the investigational regimen the patient exhibited a reaction at the site of injection of the vaccine grade 2. Adverse events related to pemetrexed were not different from expected, asthenia grade 2.

BACKGROUND

Non-small cell lung cancer (NSCLC) is about 85% of all newly diagnosed cases of lung cancer, and the leading cause of cancer related mortality worldwide. Despite some advances in therapy, the overall prognosis is not encouraging yet; as for all stages of this devastating disease, less than 20% of patients are alive 5 years after diagnosis, in the setting of metastatic disease, the median overall survival (OS) is below 1 year and 4–6 months without treatment (Fong et al., 2005; Jemal et al., 2010; Winter et al., 2011).

Conventional therapies for NSCLC such as surgery and radiotherapy are quite effective in the treatment of localized tumors; in the setting of progressive disease, chemotherapy is still the treatment of choice but, because of toxicity involving normal tissue, its use is often limited.

The introduction of first-generation chemotherapy (platinum based regimens including paclitaxel, docetaxel, gemcitabine or vinorelbine) has proven to have only limited activity. The response rate was 10–15%, with slight improvement in OS with median survival rates below 11 months, and 31–36% at 1 year (Winter et al., 2011).

Nowadays, many patients with advanced NSCLC will benefit from the individualized regimens based on the identifiable molecular characteristics of their tumors. The tumor molecular profile should help select the appropriate agents for a given patient (Kim et al., 2012).

One of the proposed treatment algorithms for advanced NSCLC in negative or unknown epidermal growth factor receptor (EGFR) mutation or anaplastic lymphoma kinase fusion protein (EML4-ALK) involves four to six of platinum-based combinations and currently remains one of the preferred approaches in the first-line setting (Schiller et al., 2002; Scagliotti et al., 2008; Gandara et al., 2009).

Second-line therapies have improved OS, but up to 50% of patients completing first-line treatment become ineligible for further treatment, mostly because of significant tumor progression or rapid decline in performance status (PS) ECOG. Therefore, many investigators studied the early use of second-line therapy in the form of maintenance therapy.

Maintenance therapy is defined in the absence of progression after a first-line platinum-containing regimen. Some investigators have studied prolonged platinum partner use from the first-line regimen called "continuation maintenance"; others have studied the use of a non-cross-resistant agent after induction, which has been termed "switch maintenance" (Gridelli et al., 2009a). The approved agents for maintenance therapy include, Pemetrexed, Docetaxel, Erlotinib, Bevacizumab, and Cetuximab. Although some randomized studies have shown a small but significant progression free survival (PFS) and OS benefit for the maintenance treatment, and guidelines recommend some approved drugs as first category, this therapy is not universally considered standard.

Moreover, a better understanding of the immune system regulation is essential, particularly how immune responses against cancer can be induced, which is mainly mediated by an adaptive cellular immune response and finally results in cancer cell recognition and destruction.

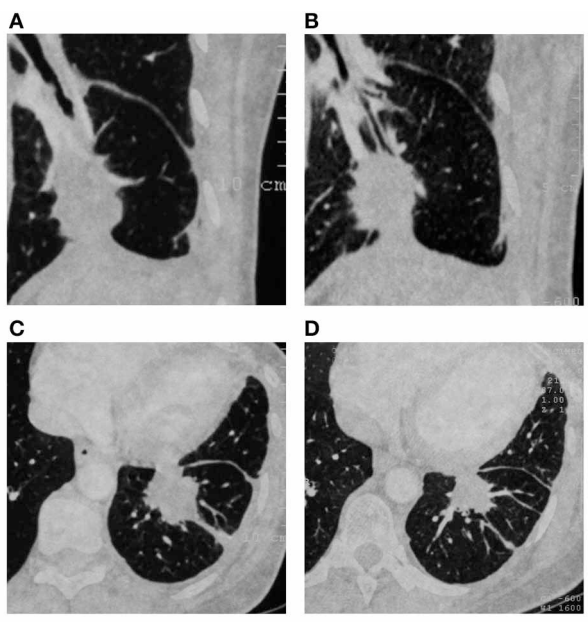

FIGURE 1 | Stable disease after maintenance treatment with Racotumomab and pemetrexed. (A,C) Computed tomography in March 2010 before treatment. **(B,D)** Computed tomography in February 2012, last assessment.

New approaches to improve immune responses and treat human malignancies have become increasingly refined. These therapies may prime the immune system to recognize the antigens expressed in tumor cells, but not in normal tissue thus being able to destroy these abnormal cells and leave the normal cells intact. The human immune system uses a complex coordinated set of cells and signaling molecules to either activate or inhibit immune responses to endogenous antigens, which are the most commonly expressed tumor antigens the cellular immune system can use to specifically target cancer cells.

Some preclinical studies have shown that immunotherapy is considerably effective against small tumor burdens, but seems unable to control large masses (Baxevanis et al., 2009). In advanced stage disease, the pre-existing immunity must have been insufficient for tumor eradication, although tumor-specific immune responses have been detected in some cases. This poor immunological response may include acquired or innate host tolerance to tumor-associated antigens, tumor development in an immunoprivileged site, or the expression of tumor-associated proteins suppressing the activity of cytotoxic T lymphocytes, among others.

Therapeutic anti-cancer vaccines are intended to cause or enhance an adaptive immune response to the tumor cells. Therefore, it is important to identify a specific anti-genic stimulus that will be recognized as immunogenic by the patient's immune system, and to create an efficient delivery system to generate a sufficiently good immune response to the antigen leading to a clinically relevant result (Thatcher and Heighway, 2010). In other words, vaccines include a tumor antigen source in order to be immunologically relevant, combined with some type of "adjuvant" to make these tumor antigens more visible to the immune system. It was hypothesized that antibody mediated inflammation could facilitate tumor progression, but high titers of these antibodies may kill tumor cells (Varki, 2010).

For a long time, lung cancer was not considered an immune-sensitive malignancy. However, increasing evidence that NSCLC may evoke specific humoral and cellular anti-tumor immune responses is available. With more knowledge about the link between the induced immune response and a resulting objective clinical response, lung cancer vaccines may be promising in sequence and/or combination with other anti-tumor treatment modalities such as chemotherapy to improve vaccination results (Ma et al., 2004). Both strategies have improved the immune response in both preclinical and human trials.

A number of promising vaccines based on different types of antigenic stimuli have been evaluated in clinical studies; e.g., different immunotherapeutic strategies in lung cancer include, active immunotherapy (vaccines, i.e., MAGE), passive immunotherapy [monoclonal antibodies (mAbs) i.e., Ipilimumab] and adoptive T-cell transfer among others (Kelly et al., 2010).

Gangliosides are a family of sialylated glycolipids that are typical components of the cell membrane. Some of them have been identified as tumor associated antigens capable of inducing an antibody response (Guthmann et al., 2004). For this reason, they are considered possible targets for cancer management, and have become the focus of many immunotherapeutic approaches.

N-acetyl GM3 is one of the most common sialic acid on the cell surface, abundant in normal serum and one of the most immunologically tolerated members of the family. Also, N- glycolyl-GM3 (NGcGM3) is relevant for tumor biology due to its high immunogenicity and expression in several human cancer cells like melanoma, breast and lung cancer but usually not detected in normal tissue. Such features make it an excellent target for immunotherapy (Guthmann et al., 2004; Fernandez et al., 2010; Machado et al., 2011).

Carbohydrate determinants undergo significant changes during malignant transformation. The only structural difference between N- acetyl- GM3 and N- glycolyl- GM3 is a single oxygen atom at the C-5 position of NGcGM3, catalyzed by the cytidinemonophospho-N-acetylneuraminic acid hydroxylase (CMAH). The absence of NeuGc-neuraminic acid in human cells is due to the inactivation of the gene by the enzyme responsible for NeuGc biosynthesis. Evidence suggests that its presence in human cancer might be derived from dietary sources or from an alternative metabolic pathway (Fernandez et al., 2010).

One of the approaches to target tumor- associated antigen-expressing cells is the Anti-idiotype vaccination. Anti-idiotypic mAbs have been used as cancer vaccines with encouraging results. This approach comes directly from Jerne's idiotypic network theory. This theory states that due to the huge diversity potentiality of the immunoglobulin variable regions, the idiotype repertoire may mimic the universe of self and foreign epitopes (Machado et al., 2011). Anti-idiotypic mAbs have proved to be able to mimic and induce Ag-specific Ab responses, even against non-protein tumor associated Ags like gangliosides (**Figure 2**) (Rabu et al., 2012). These anti–anti-idiotypic and anti-ganglioside mAbs may bind to tumor gangliosides and mediate complement-dependent cell lysis or Ab-dependent cell cytotoxicity, inhibit ganglioside-dependent survival cell functions, or block gangliosides release from tumor in patient sera, which are known to have immune suppressive activity (Hernández et al, 2011).

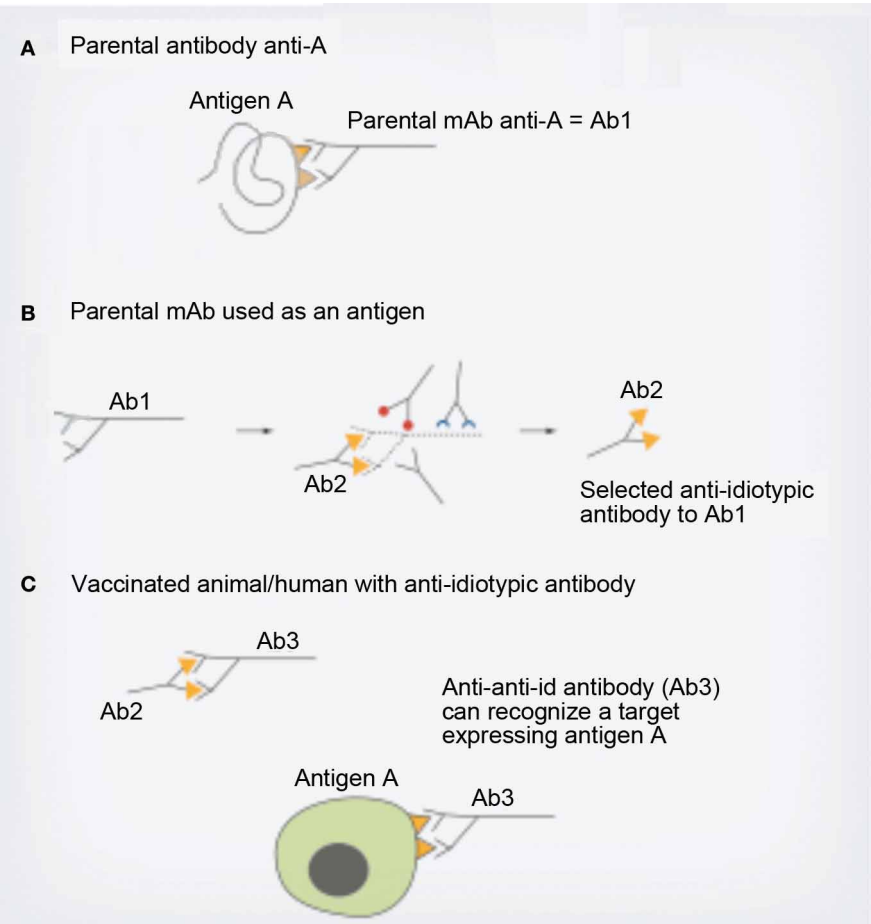

FIGURE 2 | Anti-idiotypic antibodies. An anti-idiotypic antibody (Ab2) recognizes the hypervariable idiotypic region of a parental antibody (Ab1). Due to molecular mimicry, an anti-idiotypic antibody may behave like the original antigen A, in particular when the antibody response (Ab3) it triggers when used as a vaccine is similar to the antibody response mounted against the original antigen A (referred to as anti-anti-Id+/anti-antigen+). Immunizing an animal with antigen A **(A)** raises Ab1. Animals immunized with Ab1 will mount a polyclonal antibody response, amongst which Ab2 may be selected as an anti-idiotypic antibody **(B)**. Patients or animals vaccinated with Ab2 may mount an Ab3 antibody response, which may both recognize and kill tumor cells expressing the antigen A **(C)**. mAb, Monoclonal antibody.

mAbs against gangliosides like NGcGM3 may bind to and mediate anti-proliferative or cytotoxic activities directly against target cells through different mechanisms. Because of the genetic variability and immune evasion capacity of tumors, mAbs with multiple effector mechanisms may be needed to achieve maximal anti-tumor effects.

Racotumomab (formerly known as 1E10) is a vaccine that contains a murine anti-idiotype mAb designed to mimic the NGcGM3 ganglioside. It is an anti-idiotypic IgG (Ab2-type-antibody) generated from the murine tumor model immunization, the F3II mammary carcinoma (BALB/mice), which reacts to the IgM mAb (Ab1-type-antibody), named P3 that recognizes gangliosides as antigens. Both Racotumomab anti-tumoral activity and preclinical toxicity were analyzed in the murine model mentioned above and in the B16 melanoma (C57BL/6 mice). The drug was both safe and effective in these models.

Tumor- specific expression of NGc-containing gangliosides in some human tumors suggests that the induction of an effective immune response against these targets might be useful in patients whose tumors express the antigen. Phase I clinical trials have proven the safety and immunogenicity of Racotumomab in melanoma, breast cancer and small cell lung cancer patients. High titer Ab responses to NeuGc-containing gangliosides were detected in the sera of these patients (van Cruijsen et al., 2009; Fernandez et al., 2010). NGcGM3 is widely expressed in more than 90% of NSCLC (van Cruijsen et al., 2009).

In a phase II compassionate—use study, 71 patients with advanced NSCLC, IIIB, and IV received standard chemoradiotherapy and then received five biweekly injections of 1 mg of Racotumomab intradermically, other 10 doses at 28 days intervals and continued to be immunized at this same time interval if they were in good PS ECOG. The vaccine was well tolerated; no serious adverse events were reported in this patient cohort. The most common adverse event was reaction at the injection site. OS from the time of initial vaccination was 9.93 months (95% CI, 8.61–11.25); 1 year survival rate was 34%, the median survival time of patients who entered the study with partial response or disease stabilization and with PS of 1 was 11.5 months (95% CI, 7.97–15.03 months), considered since the start of vaccination. Those with progressive disease or $PS = 2$ had a median OS of 6.5 months (95% CI, 4.31–8.69 months). A statistically significant correlation was observed between anti-ganglioside response and survival time in a subset of 20 NSCLC patients from this study. Non-responders ($n = 4$) had a median survival time of 6.35 months (95% CI, 4.97–9.67 months), whereas patients who developed IgG and/or IgM antibodies against NGcGM3 had a median survival time of 14.26 months (95% CI, 5.95–17.3 months; $P < 0.01$) (Kelly et al., 2010; Guthmann et al., 2004; Hernández et al., 2008; Gridelli et al., 2009b).

Immune approaches are unlikely to replace conventional cancer therapies but, in combination with other therapies, they may contribute to better results. Moreover, the complex interactions between cancer cells and host elements within the tumor microenvironment imply that targeting one aspect of tumor biology will have clear consequences in other elements involved in both tumor growth and progression.

It is well known that chemotherapy induces cell death by apoptosis. Recent evidence suggests that apoptosis may be highly immunogenic and its immunomodulatory potential is exerted by a variety of mechanisms. For example, chemotherapy may condition the tumor microenvironment by modulating the expression of tumor antigens, accessory molecules of T-cell activation or inhibition, and molecules involved in antigen processing and presentation; furthermore, it may manipulate systemic pathways of immune tolerance and regulation (Emens, 2010).

Some preclinical studies evaluating the combination of vaccines with other oncospecific treatments have been published, providing a rationale for chemoimmunotherapy combinations in the clinical setting (Fernandez et al., 2010). Preclinical models using the 3LL Lewis lung carcinoma in C57BL/6 mice as a model of NSCLC, have shown that the combination of Racotumomab with chemotherapeutics drugs such as Pemetrexed leads to satisfactory results (Segatori et al.).

On the other hand, Racotumomab and low-dose Cyclophosphamide in a mammary carcinoma model significantly reduced breast carcinoma growth in mice, and that response was comparable with the co-administration of the standard high-dose chemotherapy for breast cancer based on 60 mg/m (Winter et al., 2011) of Doxorubicin and 600 mg/m (Winter et al., 2011) of Cyclophosphamide, without toxicity signs (Fuentes et al., 2010).

The Center of Molecular Immunology from Havana, Cuba, where Racotumomab was developed, conducted a phase I study to assess the feasibility of combining the vaccine with the standard first line chemotherapy used in advanced NSCLC. Twenty patients were included and treated with cisplatin/vinblastine. The vaccination schedule was administered concomitantly with chemotherapy and continued beyond progression, until unacceptable toxicity or until the patient decreased PS to grade 3 or lower. Nineteen patients achieved control disease, median survival has not been reached and the mean survival was 12.94 months (Macias et al.).

The combination was considered safe and all the patients developed high antibody responses against Racotumomab during the vaccination schedule as well as IgM and IgG antibody response against NeuGcGm3 antigen as in the standard not concomitant vaccination schedule used in former clinical trials, suggesting that chemotherapy does not inhibit vaccine –mediated immune response (Macias et al.).

DISCUSSION

In advanced NSCLC, systemic chemotherapy and/or localized irradiation can produce objective responses and palliation of symptoms; however, these therapies are associated with a modest improvement in survival despite continuing advances.

To date, maintenance therapy with either a chemotherapeutic or a molecular target agent after standard first line treatment is one of the strategies that are continuing under investigation in several trials. This strategy has shown a substantially longer progression free survival, but the positive impact on OS is modest. This approach is associated with more frequent adverse events with the consequent impairment on quality of life. For these

reasons, this indication is controversial, and is not considered a standard of care in many centers.

Tumor vaccines alone in the treatment of solid tumors had not the expected impact on survival, but its combination with other treatment modalities such as chemotherapy may increase its effectiveness. Evidence suggests that irrespective of the potency of chemotherapy and the specificity achieved with immunotherapy, neither of these by itself has been enough to eradicate the disease. Clinical trials have evaluated the combination of immunotherapy and chemotherapy and have shown synergistic effect between them that could improve the therapeutic efficacy. Certain chemotherapeutic drugs have immunomodulatory activities, enhancing the efficacy of tumor cell vaccines and the immunotherapy response. On the other hand, vaccination may sensitize the tumor to subsequent chemotherapeutic agents and induce a dynamic phenomenon in the host immune response, which it could be modified by concomitant treatment by different ways.

The challenge is to combine both conventional treatment and immunotherapeutic strategies, in order to lower the tumor burden and prepare the host immune system to control minimal residual disease.

Racotumomab has acceptable safety outcomes and is able to induce specific humoral and cellular immune responses. These responses seems to be stronger in those patients with lower tumor burden, better PS and a good response to previous oncological treatments and, in this respect, vaccine therapy might be an appropriate (van Cruijsen et al., 2009).

Regarding Pemetrexed maintenance therapy, the first analysis has shown that Pemetrexed improves median progression-free survival vs. placebo (4.1 months from randomization vs. 2.8 months respectively). This analysis shows an improvement on median OS also (13.9 months from randomization vs. 11 months) (Paz-Ares et al., 2012a,b).

Here, we presented a case report of an advanced NSCLC patient who seems to have been benefited from maintenance therapy and/or immunotherapy. PFS and OS are higher than expected. Toxicities were not higher than described with these agents when used alone.

CONCLUDING REMARKS

Given the results presented in this case report we consider that the combination of chemotherapy and immunotherapy deserves further investigation.

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CLINICAL CASE STUDY
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Immune response to racotumomab in a child with relapsed neuroblastoma

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Immunotherapy targeting ganglioside antigens is a powerful tool for the treatment of high risk neuroblastoma. However, only treatment with anti-GD2 antibodies has been used in clinical practice and other options may be pursued. We report the use of racotumomab, an anti-idiotype vaccine against N-glycolyl neuraminic acid (NeuGc)- containing gangliosides, eliciting an immune response in a child with relapsed neuroblastoma expressing the NeuGcGM3 ganglioside.

Keywords: immunotherapy, monoclonal antibodies, neuroblastoma, ganglioside, racotumomab

INTRODUCTION

This is a 4-year-old female patient who presented with diffuse pain in lower limbs leading to walking problems and a 1-month history of intermittent fever and generalized pallor. Physical examination showed right eye proptosis and an abdominal mass. Laboratory tests showed anemia (hemoglobin 7.5 g %) and elevated LDH. The child was hospitalized at another center for evaluation. A CT scan of the abdomen revealed a large calcified abdominal mass originating from left adrenal gland. She was referred to our Hospital for further treatment. At our hospital, neuroblastoma cells were evident at a bone marrow examination. Malignant cells were positive for 1p deletion and showed MYCN amplification. Metaiodobenzylguanidine (MIBG) scintigraphy revealed multiple tumoral foci in skull, spine, and left upper quadrant of the abdominal mass. Urinary catecholamines determination revealed elevated norepinephrine levels and vanillyl mandelic acid (VMA).

Therefore, with a diagnosis of Stage 4 neuroblastoma belonging to the high risk group, chemotherapy was prescribed, including 5 cycles of a standard induction regimen (Matthay, 1999). Evaluation after induction chemotherapy showed progressive disease in the abdominal tumor and in the bone marrow.

A second line regimen including 3 cycles of topotecan and carboplatin was given. A repeated response evaluation revealed persistent bone marrow infiltration, progressive disease in the orbit with intracranial extension, thoracic and lumbar vertebrae as well as a persistent left heterogeneous retroperitoneal mass $10 \times 8 \times 8$ cm and high catecholamine levels. The patient had significant malaise and widespread pain. The disease was deemed refractory to conventional therapy and she was considered for experimental treatment.

BACKGROUND

Despite advances in the treatment of pediatric malignancies, cancer is the second most common cause of death in children over 1-year-old in developed countries. In Argentina, it accounts for the third leading cause of death in children preceded by accidents and congenital malformations (Scursoni et al., 2011). Children with primary multifocal, refractory or relapsed malignant solid tumors still have a very poor prognosis. On the other hand, most therapies are associated with significant toxicity, causing long-term morbidity.

Neuroblastoma is a cancer of the sympathetic nervous system accounting for about 12% of cancer-related deaths in children under 15-years old. It is a heterogeneous disease in which up to 50% of patients have a high-risk behavior characterized by widespread dissemination and poor long-term survival, even when using intensive multimodal treatments. Significant improved outcomes were published nearly a decade ago with the use of myeloablative therapy with stem-cell rescue, followed by differentiation treatment with isotretinoin (Matthay, 1999).

However, over 50% of patients receiving standard therapy relapse and ultimately die from the tumor. Consequently, the major obstacle to cure, once remission is achieved, is the chemotherapy-refractory disease that eludes the current methods for its detection. This failure has led to a resurgence of interest in alternative methods of disease eradication. Immunotherapy became a particular and hopeful option.

DISCUSSION

EXPERIMENTAL TREATMENT: IMMUNOTHERAPY

Our patient was eligible for our phase I study of the monoclonal anti-idiotype antibody racotumomab (formerly called 1E10), that targets NeuGc-containing gangliosides. In this case, biopsy specimens from the bone marrow showed marked positivity to the ganglioside antigen NeuGcGM3 (**Figure 1**) (Scursoni et al., 2011). As scheduled by the protocol, she received 3 intradermal applications of alum-adsorbed racotumomab at a dose of 0.15 mg each in the anterior left forearm. The drug was administered on an ambulatory basis every 14 days and the child presented only mild side effects such as localized painless erythema 3 cm in diameter at the injection site that appeared 8 h after application and disappeared within 24 h, without any treatment. No laboratory alterations or other evidence of toxicity was observed.

One month after the last monoclonal antibody dose, she had progressive disease in the orbital metastasis and complained of generalized bone pain and lower limb paresis. A nuclear magnetic resonance imaging (MRI) was performed revealing spinal cord compression. Because of that, local orbital and spinal radiotherapy was carried out for palliation. The patient died one month after this episode due to disease progression.

Along with follow-up laboratory results, serum samples were drawn to assess the induction of antigen-specific antibodies. The patient developed a positive anti-racotumomab IgG response (**Figure 2A**). The reactivity against iorC5, an isotype-matched murine monoclonal antibody was significantly lower than that to racotumomab, underscoring the immunodominance of racotumomab idiotype. Most interestingly, anti-NeuGcGM3 IgM antibodies were also induced. The anti-ganglioside response was observed two weeks after the second racotumomab immunization (**Figure 2B**), but faded away later and was not detectable one month after the third immunization. The anti-ganglioside response was NeuGcGM3 specific, as no significant reactivity was observed against N-acetyl (NeuAc) GM3 (**Figure 2B**).

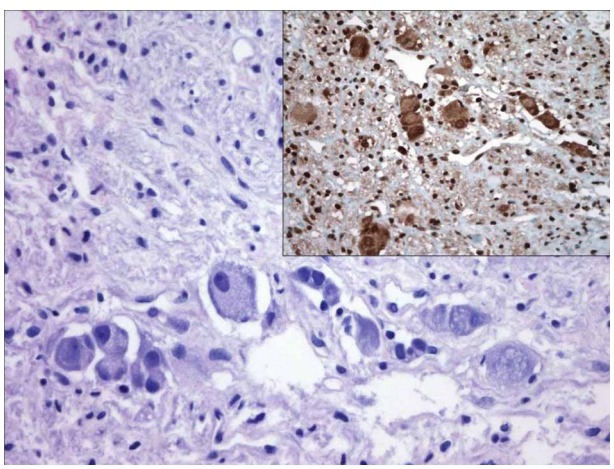

FIGURE 1 | Nests of maturing ganglion cells. Inset: positivity of these cells for 14F7 ganglioside.

IMMUNOTHERAPY IN NEUROBLASTOMA

Current conventional therapies against neuroblastoma have proven inadequate to treat advanced and refractory disease. The future success of immunotherapy against neuroblastoma should include the combination of treatment modalities for targeting minimal residual disease. Neuroblastoma is the archetypical pediatric tumor for the use of immunotherapy (Modak and Cheung, 2007). The most widely used immunotherapy for neuroblastoma consists of murine or humanized-murine chimeric monoclonal antibodies directed against GD2, a disialoganglioside expressed in tumors of neuroectodermal origin. However, GD2 is poorly immunogenic, so this antibody had to be combined with immunomodulators to elicit a significant response, which was also associated to severe toxicity (Gray and Kohler, 2009). This treatment is not available outside clinical trials, so it is not an option for patients with neuroblastoma in developing countries. There is good evidence that many children with neuroblastoma elicit an immune response against their tumor. Although these spontaneous immune responses are generally weak and fail to control tumor growth, they have the potential to

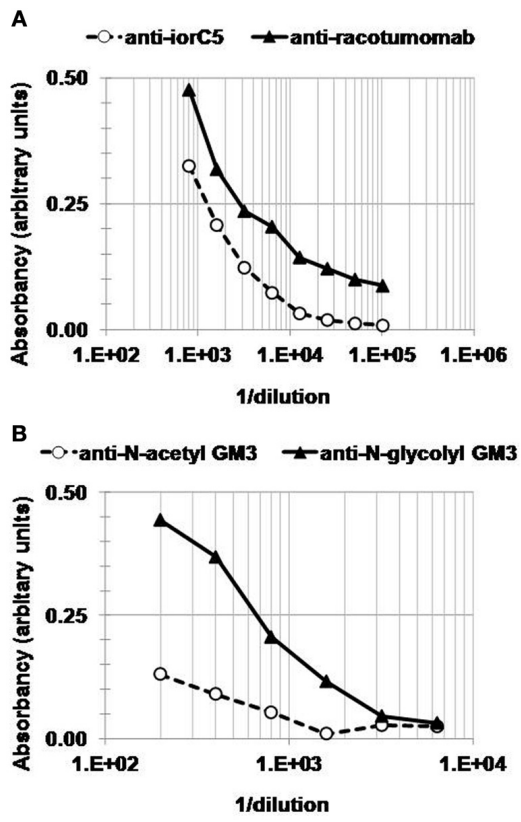

FIGURE 2 | Antibody response elicited by racotumomab immunization. Serum samples obtained two weeks after the second immunization were assessed for anti-mouse **(A)** and anti-ganglioside **(B)** antibodies. **(A)** Plates were coated with either racotumomab or an isotype-matched monoclonal antibody (iorC5). Overlaid titration curves show a significantly stronger reactivity toward racotumomab. **(B)** Plates were coated with N-glycolyl GM3 or N-acetyl GM3. No significant binding to N-acetyl GM3 was observed.

be manipulated to provide a highly specific therapy for minimally disseminated neuroblastoma. Many different immunotherapy approaches have emerged over the last decades, but only a small number of these have achieved the level of clinical studies with relevant but arbitrary clinical responses, providing evidence that the immune system is capable, under certain conditions, of controlling and eradicating the tumor (Gray and Kohler, 2009).

GD2 is uniformly expressed in neuroblastomas (Wayne et al., 2010). Its function is not fully established but is thought to play a major role in tumor cell attachment to extracellular matrix proteins. GD2 expression in normal tissues of adults and children is restricted to the central nervous system, peripheral nerves, and skin melanocytes (Modak and Cheung, 2007). Due to the relatively tumor-selective expression combined with its presence on the cell surface, GD2 is an attractive target for this kind of therapy and immunotherapy with the humanized monoclonal antibody 14.18 showed improved results combined with high dose therapy, autologous stem cell rescue, and differentiation therapy in a randomized trial (Modak and Cheung, 2007).

Active specific immunotherapy is a promising field in cancer research. NeuGc gangliosides, particularly the NeuGcGM3 ganglioside, have received considerable attention as a privileged target for cancer therapy (Fernandez et al., 2010). They are usually undetectable in healthy human tissues and fluids, but widely expressed in tumor tissues, including neuroblastoma and other pediatric solid tumors (Fernandez et al., 2010). Glycosidic chains of gangliosides contain at least one sialic acid residue. Sialic acid may have some variations, with the most common versions in mammals: Neu-Ac and Neu-Gc. Many of these glycolipid compounds are abundantly expressed in tumor cells, being regarded as promising targets for immunotherapy, as is the case of the NeuGcGM3 monosialoganglioside.

Clinical trials have been carried out with murine monoclonal anti-idiotype racotumomab to treat adult tumors such as melanoma, breast, and lung cancer, which express surface NeuGc gangliosides. These studies showed a correlation between development of antibodies against NeuGcGM3 and increased survival time (Guthmann et al., 2006; Oliva et al., 2006; Hernandez et al., 2008; Fernandez et al., 2010). Our group reported the expression of these gangliosides in 85% of the cases of neuroblastoma studied (Scursoni et al., 2011). Thus, a Phase I clinical trial is currently underway at our hospital in pediatric patients with refractory or resistant neuroblastoma and other neuroectodermic tumors using racotumomab. This vaccine has not been previously used in children, so we hereby present a case report of the first patient recruited in the study who received this vaccine.

This Phase I clinical trial is being carried out in Garrahan Hospital in pediatric patients diagnosed with cancer expressing NeuGc gangliosides and who have been previously refractory or resistant to conventional cancer treatments. The main objective is to evaluate the acute toxicity and maximal tolerated dose and, secondly, immunological and clinical response to treatment with racotumomab. This is the first time that this monoclonal antibody is used in children. Since this is a treatment directed to minimally disseminated disease, it is probable that very few patients with refractory disease would present a clinical response. Racotumomab was immunogenic and induced a strong anti-mouse IgG response with marked specificity to racotumomab idiotype. Furthermore, a transient IgM response specific for NeuGcGM3 was also observed after the second administration of racotumomab. No anti-ganglioside IgG response was detected, which is in line with the need for an extended booster regimen to elicit the antibody class switch (Guthmann et al., 2006). The immunodominance of racotumomab idiotype and the NeuGcGM3 specificity of the anti-ganglioside response have been described earlier for adult patients under a 1 mg racotumomab immunotherapy regimen (Guthmann et al., 2006). The present results, obtained from a 4-year-old infant receiving a 0.15 mg dose-level, suggest that a similar immunogenicity might be elicited in infants, thereby warranting further investigation of racotumomab in pediatric cancer immunotherapy.

CONCLUDING REMARKS

Interest in immunotherapy for pediatric cancer is increasing. However, most therapies are still experimental. The challenges for the next decade involve translating pre-clinical treatment outcomes into effective treatments for patients and making these treatments available in developing countries where toxicity is a severe problem.

Racotumomab vaccination proved to elicit an immune response with a favorable toxicity profile. A Phase I trial is accruing patients to fully characterize its toxicity profile and it may become an innovative alternative for treatment of neuroblastoma and other embryonal pediatric malignancies.

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Anti-idiotype antibodies in cancer treatment: the pharmaceutical industry perspective

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Active immunotherapy is an interesting field from the industry's perspective and in the last years, regulatory agencies and the medical community have showed renewed expectations and interest in cancer vaccines. The development of new immune therapies offers many challenges, and this is reflected in the small number of phase III trials showing clear benefits. Traditional concepts applied in clinical trials for the development of chemotherapeutic agents may be inadequate for immunotherapies and a new paradigm is emerging. It is possible that organized efforts and funding will accelerate the development of therapeutically effective cancer vaccines. This article reviews the attributes of cancer vaccines which make them attractive from the industry's perspective, and focuses especially in the characteristics of Racotumomab, an anti-idiotype antibody vaccine.

Keywords: lung cancer, Racotumomab, cancer vaccines, immunotherapy, pediatric tumor

There are different immunotherapeutic approaches in cancer, including passive and active immunotherapy, adoptive T cell transfer, and non-specific immunotherapy, amongst others.

Active immunotherapy is an interesting field because vaccines usually have a favorable side effect profile and are well-tolerated and can be used in combination with other therapies.

However, the development of these new immune therapies offers many challenges, and this is reflected in the small number of phase III trials showing clear benefits. Immune response may not always translate into clinical benefit, and for solid tumors, traditional criteria for evaluation of tumor response may not be appropriate or relevant (Tuma, 2006; Hoos et al., 2007; Schlom et al., 2007).

In the last years, the regulatory agencies and the medical community have increased their expectations regarding these therapeutic strategies. The FDA released in October 2011a guidance document for the industry addressing the challenges and particular issues with the development of cancer vaccines such as monitoring for immune response, disease progression/recurrence immediately or shortly after the start of the vaccine, delayed effects of the vaccines when evaluating time to event endpoints, etc (Guidance for Industry, 2011). This shows that in the development phases of vaccines and immunotherapies, some of the traditional concepts applied in oncology clinical trials for chemotherapeutic agents are at least controversial or inappropriate and a new paradigm is emerging for immunotherapies.

The NCI recently recognized the untapped potential of therapeutic cancer vaccines and set a pilot project for identification and prioritization of cancer antigens (Cheever et al., 2009). There is increasing interest in the cancer vaccine field, and it is possible that organized efforts and funding will accelerate the development of therapeutically effective cancer vaccines.

The successful development of a vaccine for cancer treatment is influenced by several factors. Some of them are related to the product, type of tumor, expression of the target, and also to the patient characteristics, such as performance status or stage of the disease, play an important role.

An anti-idiotype monoclonal antibody (mAb) is the mirror image of the original antibody formed against specific surface antigens. Thus, anti-idiotype antibodies can act as antigens, inducing a response against the original antigen.

Racotumomab is an anti-idiotype antibody used as a therapeutic vaccine. Although it is as mAb, it is administered in small amounts, intradermally, and acts as an active specific immunotherapeutic agent.

Racotumomab was formerly known as 1E10 anti-idiotype vaccine and is a good example of a candidate for development because it holds many positive characteristics:

- It has a well-defined antigen, expressed only in tumor cells: N-glycolil-GM3 is the target of this vaccine. It is a ganglioside which normally does not express on the surface of human cells, but appears on the surface of tumor cells (Irie et al., 1998; Muchmore et al., 1998). The differential expression of the target makes immune cross reactions unlikely, hence preserving normal cells and reducing the risks of toxicity and side effects.
- The target is expressed in several tumor types: it has been shown that several tumors express N-glycolil-GM3, such as non-small cell lung cancer (van Cruijsen et al., 2009), breast cancer (Vázquez et al., 1995; Moreno et al., 1998), melanoma (Alfonso et al., 2002), and several pediatric tumors of neuroectodermal origin (Scursoni et al., 2011, 2012). From the industry's perspective this is interesting because it allows a broad range of potential indications. Particularly in the case of non-small cell lung cancer, the expression of the target is greater than 70% (van Cruijsen et al., 2009). This provides two additional advantages: (1) the potential for combination of Racotumomab with other therapies used in more selected

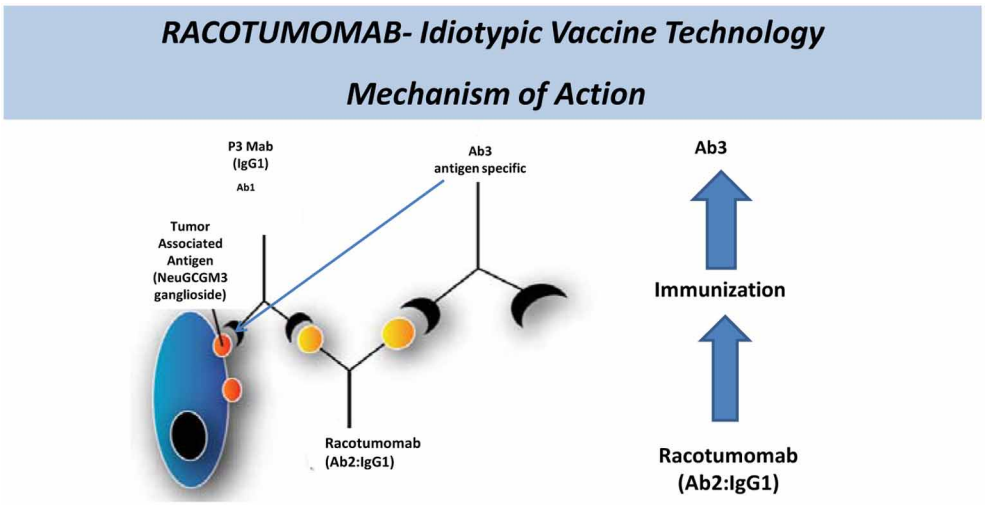

FIGURE 1 | Racotumomab—mechanism of action of an anti-idiotype antibody.

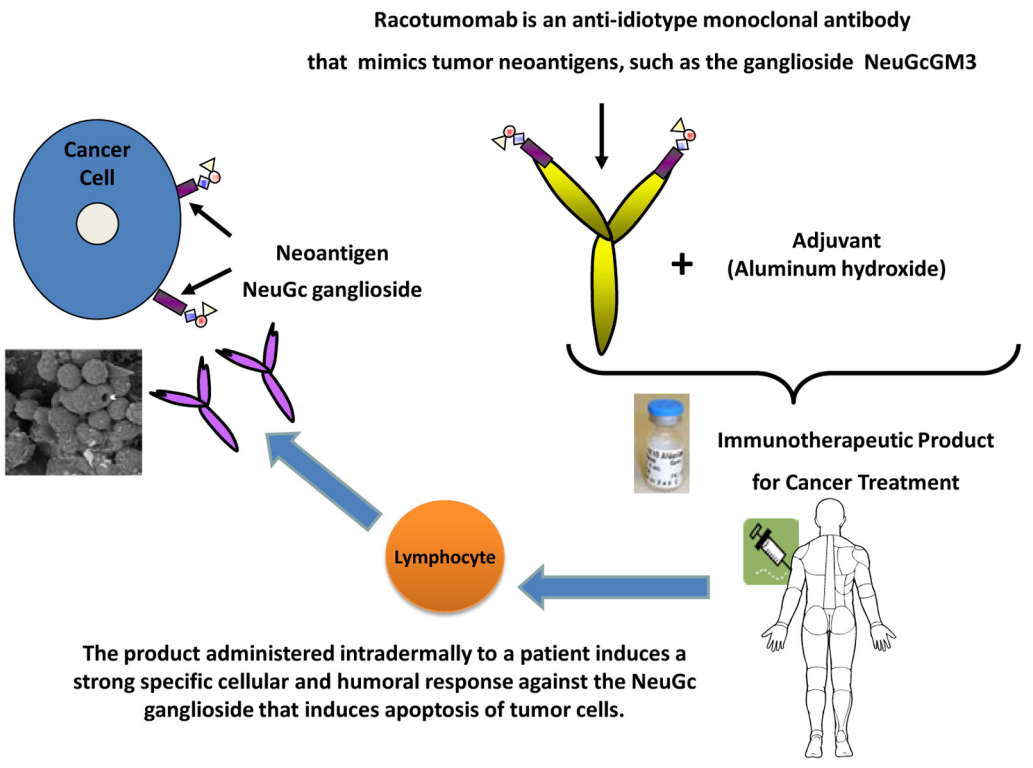

FIGURE 2 | Immune response to vaccination with Racotumomab.

patient populations (patient with specific mutations or histological types) without the need of prior evaluation of the presence of the target in the tumor and (2) even if the detection of the target ganglioside (N-glycolil-GM3) were needed, the fact that it is an immunohystochemical evaluation, makes it technically easy to perform, of low cost and widely accessible.

- It has an innovative mechanism of action (**Figure 1**): anti-idiotype antibodies are a useful strategy to elicit an immune response toward a ganglioside, which is a scarcely immunogenic molecule in itself.
- It is highly immunogenic (**Figure 2**) and shows clinical benefit: Racotumomab is a monoclonal antibody, but is used as a vaccine. Only a small quantity of Racotumomab (1 mg) is needed per dose. It has been shown that Racotumomab is able to elicit a strong humoral and cellular immune response (Alfonso et al., 2002; Díaz et al., 2003; Guthmann et al., 2004, 2006; Hernández et al., 2008, 2011) and that this response has a positive impact in patient survival (Guthmann et al., 2004; Alfonso et al., 2007; Neninger et al., 2007). In a proof of concept trial, patients with NSCLC treated with Racotumomab had a longer overall survival in comparison to a placebo group (final results submitted for publication).
- It can be used in a broad target population: despite the fact that more than 65% of all malignancies and more than 70% of the deaths associated to cancer occur in patients beyond 65 years old (Lynn et al., 2003), elderly patients continue to be underrepresented in clinical trials. The incidence of cancer is 10 times larger and death rate is 16 times larger in this group than patients below 65 years of age (Lynn et al., 2003). When the treatments evaluated in younger patient populations are approved and then used in the clinical setting and in elderly patients frequently the results obtained are not the same. Due to comorbidities and polimedication the risk of side effects, toxicity and complications is increased, and the tolerance to onco-specific treatments is reduced. Several studies have shown that patients more than 65 years old tend to have a greater risk of bone marrow depletion, neutropenia, infections, and neurotoxicity with the use of chemotherapy (Balducci, 2001, 2003; Balducci and Repetto, 2004; Belani, 2005; Gridelli, 2008). Cancer treatment in pediatric population presents a similar challenge. In addition, traditional cancer agents have impact in developing organs and systems and are likely to produce irreversible, negative changes.
- Well-tolerated and safe products such as Racotumomab continue to be very much needed in these specific patient populations. This is also very important for diseases with late diagnosis and no chance of cure, because in this context patients and their families tend to recur to alternative therapies which may be costly, ineffective and unsafe.
- The schedule of vaccination is comfortable and simple: it consists of five intradermal doses (1 every 2 weeks) followed by subsequent monthly intradermal doses.
- The administration of the vaccine is quick and easy: the route of administration of Racotumomab and the fact that this product does not require the safety precautions that are needed when manipulating chemotherapeutic agents, nor costly processing or time consuming infusions makes this treatment interesting, since it can be delivered by a nurse at home, at the pharmacy or at the clinic. Although in the clinical trials it is recommended that the patients remain 1 h in observation after the first 2 doses, no threatening immediate reactions have been observed so far in more than 700 treated patients and no further surveillance is required if the vaccine was well-tolerated. All these factors favor patient compliance to therapy and have an impact on quality of life.
- Good tolerance and feasibility of combination with standard therapies: preservation of the patient's quality of living is extremely important, especially when there are no chances of cure and palliative therapies are the only option. Racotumomab is mostly associated with mild to moderate injection-site reactions (local erithema, induration, and pain), which disappear within 24–48 h. Systemic reactions, such as flu like symptoms and chills are less frequent, reversible, and self-limited. A favorable safety profile allows administration of monthly booster doses during a long period of time to maintain the immune response beyond disease progression, possibilities of treatment combinations with a broad range of standard therapies (radiotherapy, chemotherapies, etc.,) and use in special populations such as elderly and pediatric patients. Especially in NSCLC Racotumomab could play a role in maintenance therapy, with an acceptable and easy administration schedule and a favorable safety profile, alone or in combination with other agents.

In summary, Racotumomab is a well-tolerated, immunogenic cancer vaccine which has shown to prolong survival in NSCLC and is currently being evaluated in a multinational, phase III trial.

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