



## Research Article

# Therapeutic Vaccination with Recombinant Idiotype and RN-Adjuvant<sup>®</sup> Cures Mice with Pre-Established A20 Lymphoma

Maurizio Bendandi<sup>1,2\*</sup>, Ascensión López-Díaz de Cerio<sup>3</sup>, Fernando Pastor<sup>4</sup>, Susana Inogés<sup>3</sup>, Mario Martínez Soldevilla<sup>4</sup>, Helena Villanueva<sup>4</sup>, Arianne González<sup>5</sup>, Patricia Rubio<sup>4</sup>, Franziska Jarczowski<sup>6</sup>, Victor Klimyuk<sup>6</sup>

<sup>1</sup>Department of Internal Medicine (Hem/Onc), Comprehensive Cancer Center, Wake Forest University Baptist Healthcare Center, Winston-Salem, NC, USA

<sup>2</sup>Department of Internal Medicine (Hem/Onc), W.G. Hefner VA Medical Center, Salisbury, NC, USA

<sup>3</sup>University of Navarra Hospital, Pamplona, Navarra, Spain

<sup>4</sup>Aptamer Unit, Center for Applied Medical Research, Pamplona, Navarra, Spain

<sup>5</sup>TiGenix, Madrid, Spain

<sup>6</sup>Icon Genetics GmbH, Halle, Germany

\*Corresponding author: Maurizio Bendandi, Hematology/Oncology - VA Healthcare Center, 3506 West Tyvola Rd, Charlotte, NC 28208, USA. Tel: +17043291300-81639/40; Email: maurizio.bendandi@va.gov; mbendand@wakehealth.edu

**Citation:** Bendandi M, de Cerio AL, Pastor F, Inogés S, Soldevilla MM, et al. (2017) Therapeutic Vaccination with Recombinant Idiotype and RNAdjuvant<sup>®</sup> Cures Mice with Pre-Established A20 Lymphoma. J Vaccines Immunol: JVII-120. DOI: 10.29011/2575-789X. 000020

**Received Date:** 10 November, 2017; **Accepted Date:** 21 November, 2017; **Published Date:** 28 November, 2017

## Abstract

Idiotypic vaccination for Follicular Lymphoma (FL) has shown clear proof of principle of biological and clinical efficacy, as well as of clinical benefit, in early-stage clinical studies. Clinical benefit, however, has been partially confirmed only by one of three randomized clinical trials. As a variable fraction of patients per trial fails to respond to idiotype vaccines, it remains paramount to improve their overall formulation. In pre-clinical models, current vaccination strategies provide a certain degree of protection, but have never shown curative potential. Previous studies have demonstrated the immune-stimulatory adjuvant effects of Toll-Like Receptor (TLR) ligands, such as synthetic double-stranded RNA. In this work, a new-generation TLR ligand adjuvant named RNAdjuvant<sup>®</sup> was evaluated. In order to study the effects of this novel adjuvant on the induction of anti-idiotypic immune responses, Balb/c mice with pre-established, palpable tumors were intradermally immunized with a recombinant A20 lymphoma idiotype vaccine incorporating RNAdjuvant<sup>®</sup>. Compared to idiotype vaccine formulations repeatedly tested in clinical trials, the new vaccine formulation enhanced a balanced immune response with special emphasis on Th1 response. Moreover, T-cell depletion studies indicated that CD8-positive T cells are indispensable for tumor rejection and improved survival. Crucially, we were able to document for the first-time tumor eradication in a substantial percentage of vaccinated mice bearing palpable A20 lymphoma.

**Keywords:** Idiotype; Immune Response; Lymphoma; Th1; Th2; Vaccine

## Introduction

Tumor vaccination aims at stimulating a specific immune response targeted to antigens in the tumor lesions, with the goal of thus achieving prophylactic or therapeutic results [1].

Arguably, the main challenges in developing effective vaccine protocols are the identification of tumor-specific antigens as well as that of effective and safe adjuvants to stimulate a robust and durable immune response [2]. Among the very few tumor-specific antigens described so far, one of the best characterized is the B-cell Lymphoma Idiotype (Id), which is contained in the variable region of the clonal tumor immunoglobulin and is both tumor- and patient-

specific [3]. Despite the extreme specificity of this antigen, the natural immune response against it, if at all generated, is inherently very weak, since it is directed against a self-tolerated antigen, in a context in which most vaccination strategies are implemented in an autologous setting [4]. Therefore, it is necessary to potentiate the immune response by the incorporation of adjuvants within the vaccine formulation [3]. Over the last two decades, in order to increase its immunogenicity, the Id has been typically conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH), and this conjugate (Id-KLH) has been administered to patients together with Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) as an adjuvant. This vaccine formulation has shown evidence of biological [5,6] and clinical [6,7] efficacy, as well as of clinical benefit [8,9].

Two prospective, independent studies have indeed demonstrated that patients who receive idiotypic vaccination and mount an Id-specific immune response feature an improved disease-free survival compared to those who either do not respond to vaccination [8] or are vaccinated with a control product [9]. At the same time, two other clinical trials failed to show the clinical benefit of idiotypic vaccination in different settings. While substantial differences in study design, clinical setting and vaccine formulation [3] may explain different study outcomes, one thing that both successful and unsuccessful clinical trials have in common is that a non-negligible, variable fraction of patients receiving the bona fide Id vaccine formulation failed to respond to it [3, 8-11]. As such, there is a pressing need to considerably improve the current vaccine formulation. KLH itself is a potent immunogen that can distract the immune system at the expenses of its ability to recognize the far less immunogenic Id, resulting in a powerful and specific immune response solely or mostly directed against the carrier protein, but not, or at least not always in a detectable fashion, against the tumor Id. Moreover, chemical conjugation of molecules (e.g. Id and KLH) with glutaraldehyde is random, meaning that on the one hand identical or different molecules can be conjugated, and on the other hand the number of conjugated molecules may considerably vary within the same vaccine formulation. This could affect, positively or negatively, the immunogenicity of each vaccine and of each vaccine dose. Also, Id-KLH needs to be co-administrated with GM-CSF on the first day of vaccination, while GM-CSF is administered alone during the following three days, with its inherent immunological and side effects.

RNAAdjuvant®, a novel, physically and chemically well-defined RNA-based adjuvant containing synthetic RNA with a fully-characterized sequence and a polymeric carrier, was recently developed. The RNA component consists of a single-stranded, non-coding, non-capped RNA sequence containing several poly U-repeats complexed with a polymeric carrier formed by a disulphide-crosslinked cationic peptide and protected against RNase

degradation. RNAAdjuvant® exhibits strong immunostimulatory properties and can be combined with recombinant proteins and peptides. It is able to elicit balanced immune responses comprising humoral as well as cellular effector responses and, importantly, memory responses [12]. When used in lymphoma mouse models, idiotypic vaccination often results in substantial degrees of protection from subsequent tumor challenge [13], but is unable to cure palpable tumors when used as a treatment. In this study, we show instead that the new, RNAAdjuvant®-based, recombinant Id vaccine formulation (rId+ RNAAdjuvant®) induces a more robust immune response than the conventional version (Id-KLH + GM-CSF) broadly used in clinical trials, that is that which has already shown an important clinical benefit [8,9]. The new vaccine formulation, that is rId + RNAAdjuvant®, without addition of GM-CSF and/or KLH conjugation [3], led to an increase in anti-Id IgG2a antibodies without decreasing IgG1 levels, thus showing the ability to elicit a balanced immune response. Finally, these findings were associated with the cure of a considerable fraction of treated mice and translated into a dramatically-increased overall survival of mice treated with rId+ RNAAdjuvant® when compared to that of mice treated with Id-KLH+ GM-CSF. In addition, CD8+ T cells were indispensable, as shown by the fact that *in vivo* CD8+ deletion considerably reduced the vaccine's therapeutic effect. To the best of our knowledge, there is no other Id vaccine formulation able to induce such a pronounced survival benefit in Balb/c mice with pre-established, palpable A20 lymphoma. In our opinion, this new Id vaccine formulation should be tested in clinical trials designed for patients with follicular lymphoma.

## Materials and Methods

### Mice and Cell Lines

Specific pathogen-free, 6-week-old female Balb/c mice from Harlan Laboratories (Barcelona, Spain) were housed in appropriate animal care facilities during the experimental period. Animal experiments were carried out in accordance with Spanish laws and under approval provided by the Ethics committee of animal experimentation at the University of Navarra. The A20 cell line is a Balb/c B-cell lymphoma cell line originally derived from spontaneous reticulum cell neoplasm (ATCC® TIB-208). Cells were cultured in RPMI 1640 medium (GIBCO, Bethesda, MD) supplemented with 10% heat-inactivated FCS (GIBCO, Bethesda, MD), 2mM L-glutamine (GIBCO, Bethesda, MD), and penicillin-streptomycin (GIBCO, Bethesda, MD) in a humidified atmosphere at 37°C and 5% CO<sub>2</sub>. For tumor inoculation, 200.000 A20 tumor cells in 100 µL of PBS were given subcutaneously (s.c.) in the right flank.

### Treatment

A20 lymphoma-bearing mice began the idiotypic vaccination protocols when tumors reached 5 mm of diameter, that

is approximately 10 days after tumor inoculation. The number of mice included in each group ranged between 10 and 30. All mice received eight vaccine doses over 4 weeks. Tumors were measured twice a week and the mice were sacrificed when/if the tumor volume reached 3500 mm<sup>3</sup>. The vaccines contained either recombinant, humanized A20 Id (hA20) alone, hA20 + RNAAdjuvant®, hA20-KLH (KLH was purchased from Biosyin, Fellbach, Germany) + GM-CSF (Pharmingen, San Diego, CA), RNAAdjuvant® alone or PBS. All vaccinations were administered by intradermal injection except those containing hA20-KLH, which were delivered s.c. according to the current clinical practice. Mice vaccinated with hA20-KLH + GM-CSF received one dose of GM-CSF on the following day, also to mimic the procedure employed in humans. Depending on the vaccine formulation actually employed, each vaccine dose contained: 50 µg of hA20, 50µg of KLH, 10.000U of GM-CSF or 10 µg of RNAAdjuvant®. RNAAdjuvant® is composed by a synthetic RNA strand and a polymeric carrier. The RNA component consists of a U-rich RNA sequence with several polyU-repeats as described in Patent WO2009/095226. The RNA component is complexed with a polymeric carrier formed by disulphide-crosslinked cationic peptide and is protected against RNase degradation (Patent WO2012/013326).

### Re-challenge experiment

All mice featuring a post-vaccine tumor immune rejection (6 mice treated with hA20 + RNAAdjuvant® and 1 mouse treated with hA20 alone) were re-inoculated with the same type of tumor cells 120 days after the beginning of treatment. In particular, 200.000 A20 cells in 100 µL of PBS were s.c. given in the right flank. Previously-untreated mice were included as a control group. Tumors were measured twice a week, and the mice were sacrificed when the tumor volume reached 3500 mm<sup>3</sup>. Prior to the rechallenge, as well as 2 and 3 weeks thereafter, serum was obtained to quantify anti-Id IgG1 and IgG2a humoral responses by ELISA.

### In-vivo T-cell depletion studies

Mice bearing A20 lymphoma started the idiotypic vaccination protocols when tumors reached 5 mm of diameter, that is approximately 10 days post tumor inoculation. Each group included 10 mice, each of which received eight doses of hA20 + RNAAdjuvant® (50 µg and 10 µg, respectively, per dose) over 4 weeks. To deplete T cells, mice were intra-peritoneally injected with 200 µg of depletion antibodies, (anti-CD4: GK 1.5; anti-CD8:

2.43 TIB-120, both from Aldevron, Freiburg, Germany) eight hours before each vaccination. Once again, tumors were measured twice a week and the mice were sacrificed when the tumor volume reached 3500 mm<sup>3</sup>.

### Humoral response assessment

Blood samples were taken retro-orbitally and analyzed for the expression of A20 Id-specific antibodies by standard ELISA [4]. The antibody titer was indicated as a reciprocal dilution limit.

### Antibody detection by cytometry

5x10<sup>5</sup> A20 lymphoma cells were incubated for 20 minutes with 100 µl of Fetal Bovine Serum (GIBCO, Bethesda, MD) at room temperature. Cells were washed and incubated for 1 hour with 20 µl of serum collected from the mice 1 week after the administration of the fourth dose of vaccine. Following incubation, the cells were washed and stained with anti-mouse IgG1-PE (Pharmingen, San Diego, CA). The cells were then washed and analyzed by using a FACS Calibur flow cytometer (Becton Dickinson, Bergen, New Jersey).

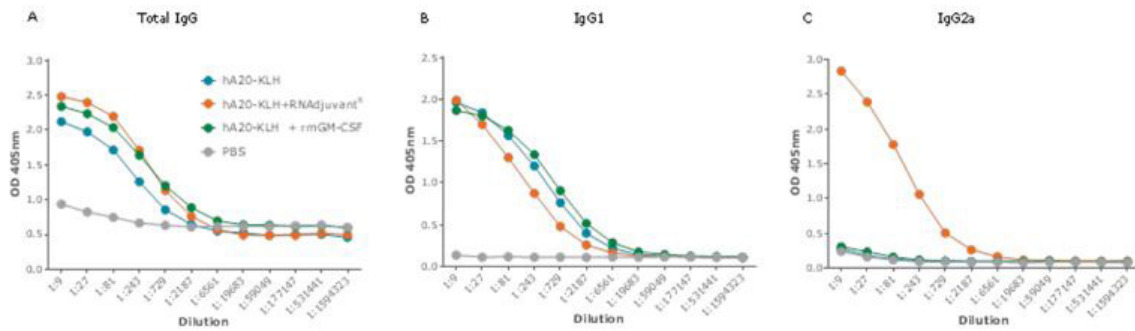
### Statistical Analysis

Mice survival was estimated according to the method of Kaplan and Meier and compared among groups using a log-rank test (GraphPad Prism 5.0c software).

## Results

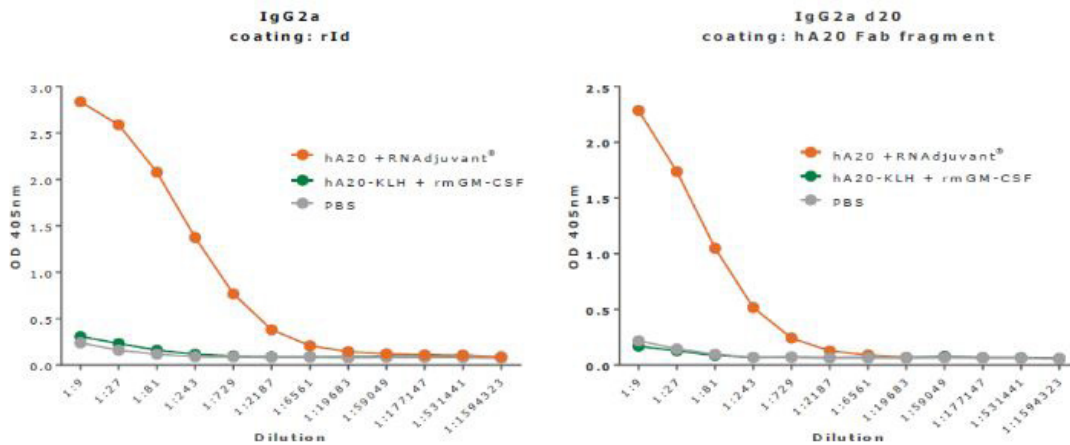
### RNAAdjuvant® Strongly Enhances the Immune Responses Induced by Id Vaccine (Id-KLH)

To characterize the immunostimulatory efficacy of RNAAdjuvant® in combination with an Id vaccine (Id-KLH), Balb/c mice were intradermally immunized with hA20 conjugated to KLH (hA20-KLH) either alone or in combination with RNAAdjuvant® or GM-CSF. Post-vaccine sera titers of anti-idiotypic antibodies were analyzed by ELISA at day 20. In this assay, the same hA20 that was used for vaccination functioned as antigen. Both vaccine formulations led to the induction of comparable titers of total anti-hA20 IgG antibodies (Figure 1A). However, vaccination with hA20-KLH, alone or in combination with GM-CSF, induced high IgG1 as well as barely detectable IgG2a titers, indicating a Th2-skewed immune response (Figure 1B, 1C).



**Figure 1:** RNAAdjuvant® enhances the anti-idiotypic immune response. Eight mice per group were injected once with hA20-KLH, hA20-KLH + RNAAdjuvant® or hA20-KLH + GM-CSF. Post-vaccine sera were obtained on day 20, pooled per group and used to detect anti-hA20 total IgG (A), IgG1 (B) and IgG2a (C) antibodies by ELISA.

In contrast, vaccination with hA20-KLH combined with RNAAdjuvant® resulted in significantly increased IgG2a titers without affecting the levels of IgG1 titers, which reflects the induction of a far more balanced immune response. As hA20 represents a humanized antibody consisting of murine variable and human IgG1 constant regions, the detected antibodies might have also been directed against the human component of the antigenic product. To verify that the vaccination with hA20-KLH + RNAAdjuvant® resulted in the induction of truly anti-idiotypic antibodies, a second ELISA using the single hA20 Fab fragment as antigen was carried out. Indeed, we once again detected high levels of post-vaccine, IgG2a antibodies, indicating that anti-idiotypic antibodies directed against the variable region of the tumor immunoglobulin were indeed induced by vaccination (Supplementary Figure 1).

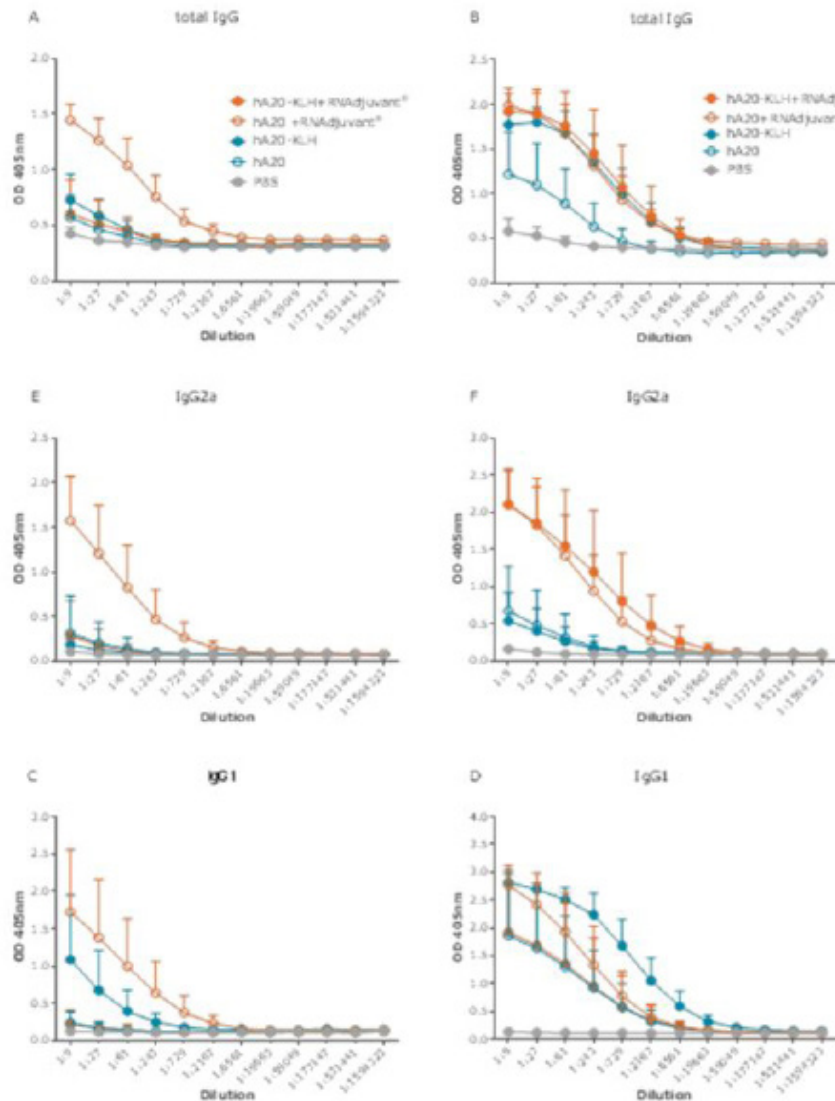


**Supplementary Figure 1:** The humoral response induced by vaccination with hA20 + RNAAdjuvant® is truly Id specific. The results do not change when the hA20Fab fragment is used to coat the ELISA plates instead of the humanized, rId-expressing, full immunoglobulin employed in the vaccine formulation.

### RNAAdjuvant® Can Replace KLH in the Id Vaccine Formulation

Next, it was studied whether KLH can be replaced by the RNAAdjuvant® in the vaccine formulation. Mice were immunized with: hA20alone, hA20-KLH, hA20 + RNAAdjuvant®, or hA20-KLH +RNAAdjuvant®. The induction of anti-Id antibodies was assessed after 10 and 24 days. Remarkably, RNAAdjuvant® accelerated the development of the humoral anti-idiotypic immune response (Figure 2).



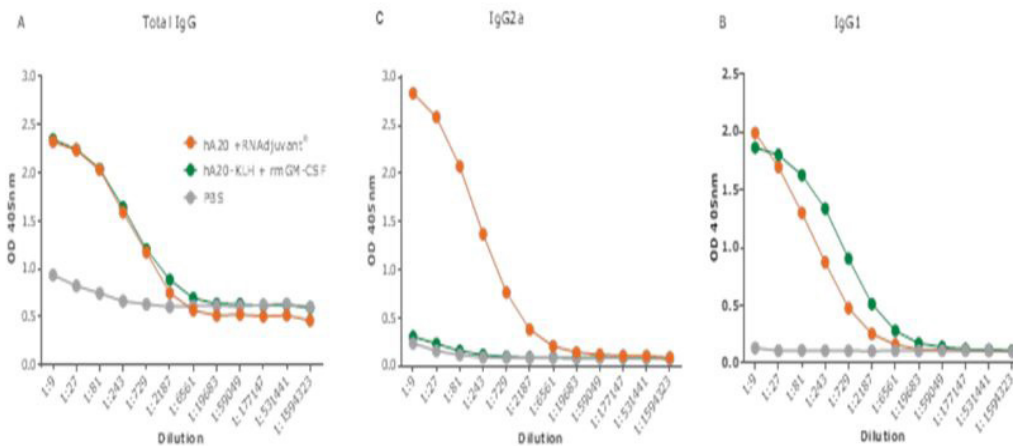


**Figure 2:** RNAAdjuvant® can replace KLH in the Id vaccine formulation. 8 mice were immunized twice (d0, d14) with unconjugated hA20 or hA20-KLH, either with or without RNAAdjuvant®. Post-vaccine sera were obtained on day 10 (A,C,E) and on day 24 (B,D,F) and used to detect anti-hA20 total IgG (A,B), IgG2a (C,D) and IgG1 (E,F) antibodies by ELISA. Besides not being in anyway inferior to KLH, RNAAdjuvant® clearly accelerates the appearance of the humoral anti-idiotypic immune response, particularly that consisting of IgG2a antibodies.

Moreover, vaccination with hA20 + RNAAdjuvant® strongly accelerated the onset of such a humoral response even when compared to vaccination with hA20-KLH administered with or without RNAAdjuvant® (Figure 2). At day 24, the combination of hA20-KLH plus RNAAdjuvant® induced the same IgG antibody titer as hA20-KLH alone. However, the combination of hA20 +RNAAdjuvant® induced a higher IgG antibody titer than hA20 alone. Furthermore, the IgG antibody titer induced by hA20 + RNAAdjuvant® was similar to that induced by hA20-KLH + RNAAdjuvant® (Figure 2). Therefore, when using RNAAdjuvant®, KLH can be omitted from the vaccine formulation. As shown in the previous experiment, in the absence of RNAAdjuvant®, post-vaccine anti-hA20 IgG2a antibodies were barely detectable, while anti-hA20 IgG1 antibodies were induced at levels comparable to those seen with vaccines containing RNAAdjuvant®. This is reflected by the detection of considerable amounts of total IgG and IgG2a titers, respectively, as early as 10 days after the first immunization (Figure 2). The induction of IgG1 antibodies was only slightly delayed by KLH, suggesting that KLH had a stronger impact on the delay of a Th1 immune response. Interestingly, the addition of KLH to the vaccine formulation resulted in a delayed induction of the anti-hA20 humoral immune response, even in the presence of RNAAdjuvant® (Figure 2).

### Replacement of both KLH and GM-CSF by RNAAdjuvant® Elicited a Balanced Immune Response

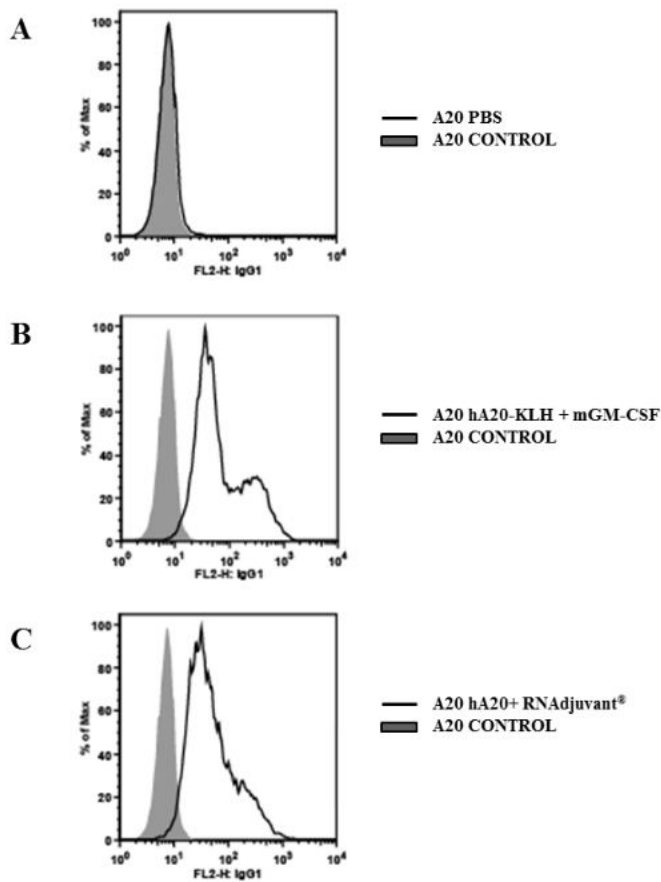
To investigate whether GM-CSF can be also replaced by RNAAdjuvant®, mice were immunized with either hA20 +RNAAdjuvant® or hA20-KLH + GM-CSF. The induction of anti-Id antibodies was assessed by ELISA. The presence of GM-CSF in the vaccine formulation did not enhance the Th1 immune response, as mice immunized with hA20-KLH + GM-CSF did not show any anti-hA20 IgG2a antibody production. Anti-Id IgG1 titers were slightly higher in the group of mice treated with the conventional vaccine formulation (hA20-KLH + GM-CSF) than in that of those immunized with hA20+ RNAAdjuvant®. Total IgG titers elicited against the Id were similar for both vaccine formulations, although the RNAAdjuvant® induced a more balanced response, with antibodies from Th1 and Th2 immune responses, while the hA20-KLH plus GM-CSF vaccine formulation failed to elicit any Th1-like immune response (Figure 3).



**Figure 3:** Replacement of both KLH and GM-CSF by RNAAdjuvant® induced a balanced immune response. The induction of anti-hA20 total IgG (A), IgG1 (B) and IgG2a antibodies is shown at day 20 post immunization with either the conventional idiotypic vaccine formulation (hA20-KLH + GM-CSF) or the unconjugated hA20 + RNAAdjuvant®.

### Id-Specific Antibodies Elicited by hA20+ RNAAdjuvant® Recognize A20 Tumor Cells

Next, we wanted to ascertain whether the antibodies elicited by the RNAAdjuvant®-based vaccination could recognize the native Id on the tumor cell surface. A20 lymphoma cells were incubated with post-vaccine sera from mice immunized with either hA20 + RNAAdjuvant® or hA20-KLH + GM-CSF. The binding of post-vaccine sera anti-Id antibodies to A20 tumor cells was detected by flow cytometry using a PE-labeled anti-IgG1 antibody (Figure 4).



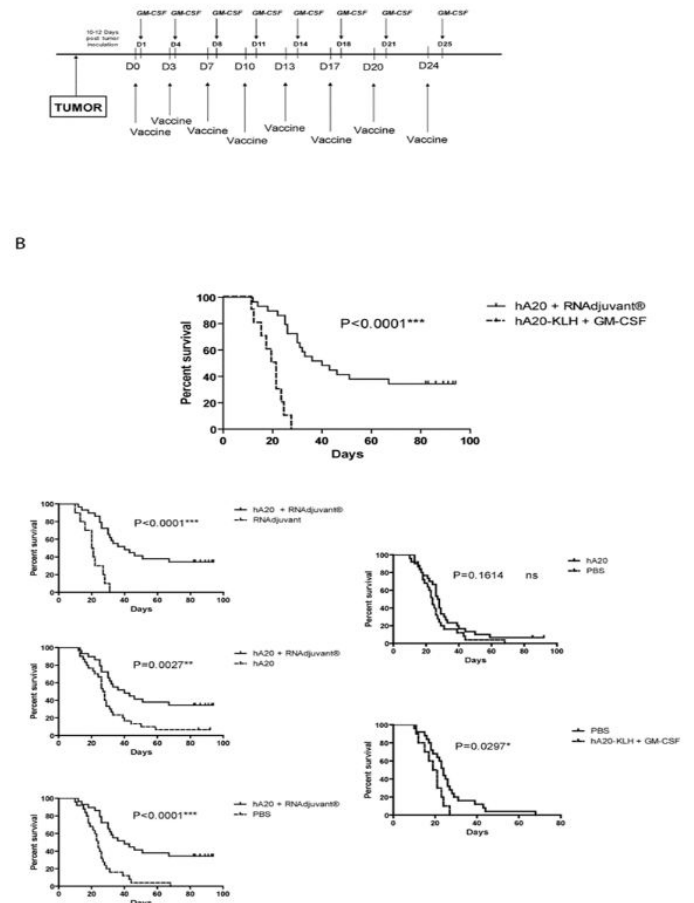
**Figure 4:** The anti-hA20 antibodies induced by the hA20 + RNAAdjuvant® vaccine formulation recognize naive A20 cells. After incubation of A20 lymphoma cells with serum collected from mice one week after the fourth dose of vaccine, these cells were stained with anti-mouse IgG1-PE and analyzed by means of a FACSCalibur flow cytometer. A20 cells were incubated with serum extracted from mice treated with PBS (A), hA20-KLH + GM-CSF (B), or hA20+ RNAAdjuvant® (C).

Anti-hA20 antibodies induced by immunization with either vaccine formulation was indeed detected on the tumor cells at similar rates, demonstrating that they were both able to recognize and bind to the native Id on the tumor cell surface.

### Treatment of Lymphoma Bearing Mice with The Id Vaccine Formulation Including RNAAdjuvant® Leads to Significant Improvement in Overall Survival

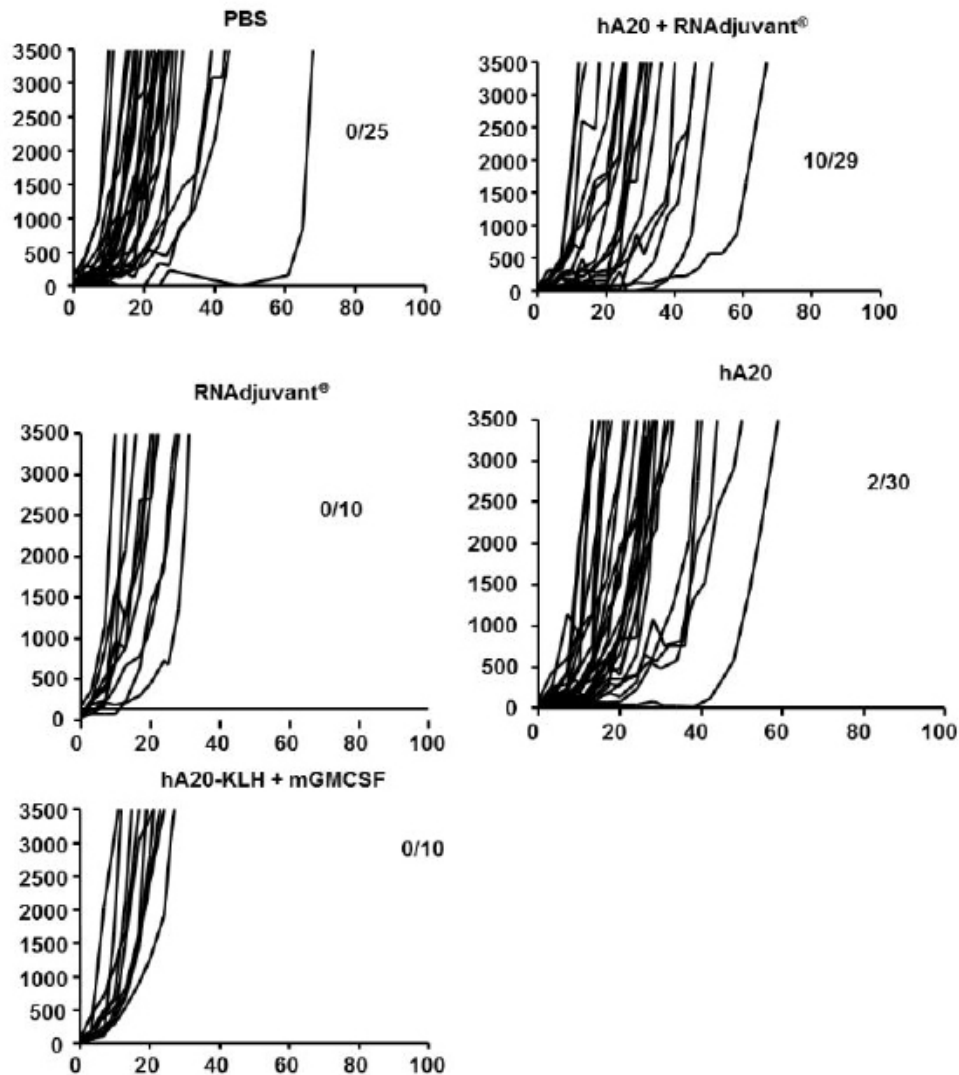
Therapeutic vaccination of A20 lymphoma-bearing mice

was started when tumors had reached 5 mm of diameter, that is approximately 10 days after tumor cell inoculation. The vaccination calendar is shown in (figure 5A).



**Figure 5:** Anti-tumor effect of the Id vaccine in combination with RNAAdjuvant®. Mice were s.c. injected in the flank with 2x10<sup>5</sup>A20 tumor cells. When tumors reached a diameter of 5 mm, the mice were vaccinated twice a week over four weeks (A) with hA20 +RNAAdjuvant®, hA20-KLH + GM-CSF, RNAAdjuvant®, hA20, or PBS. Mice survival (days) from the start of vaccinations is shown (B). The difference between control groups and the groups of mice immunized with hA20 + RNAAdjuvant® was highly statistically significant by long rank test. The experiment was repeated twice with nearly identical results.

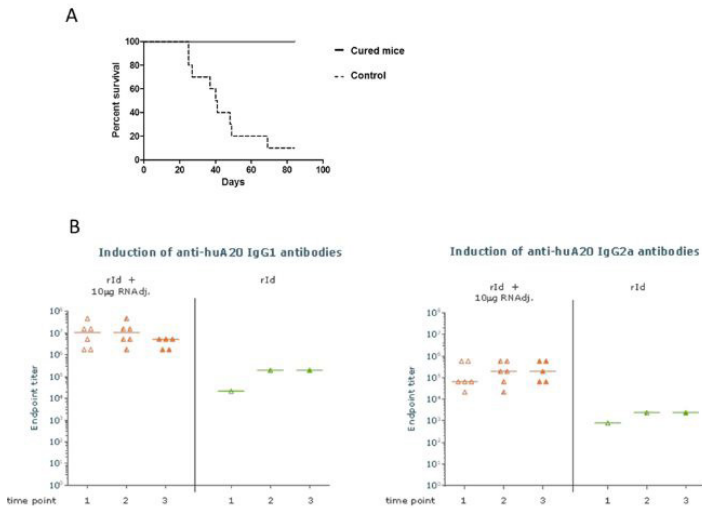
Under these conditions, the group of mice vaccinated with hA20 + RNAAdjuvant® showed a significant delay in tumor growth (Supplementary Figure 2),



**Supplementary Figure 2:** Anti-tumor effect of the Id vaccine formulation including RNAAdjuvant®. Mice (10-30 per group) were subcutaneously injected in the flank with  $2 \times 10^5$  A20 cells. When tumors reached a diameter of 5 mm, mice were vaccinated as in all other treatment experiments. The tumor volume of each mouse was measured every three days. The group of mice vaccinated with hA20 + RNAAdjuvant® showed an evident delay in tumor growth, as well as a remarkable cure rate (10/29 mice).

reaching a nearly 35% cure rate (Figure 5B). The overall survival of mice vaccinated with hA20 + RNAAdjuvant® was highly statistically superior to that of those vaccinated with hA20-KLH + GM-CSF ( $p < 0.0001$ ). All mice featuring an immune rejection of the tumor (6 mice treated with hA20 + RNAAdjuvant® and 1 mice treated with hA20 alone) were protected against an A20 lymphoma re-challenge performed 120 days after the beginning of the first treatment (Supplementary Figure 3A).



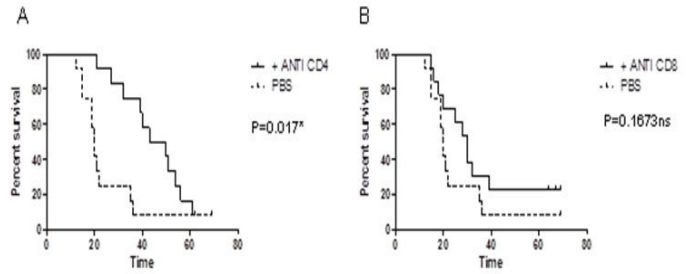


**Supplementary Figure 3:** Cured mice are protected from tumor re-challenge. In all mice featuring the immune rejection of the tumor (6 mice treated with hA20 + RNAAdjuvant® and 1 mouse treated with hA20 alone), the tumor was inoculated again 120 days after the beginning of treatment. Prior to the re-challenge, as well as 2 and 3 weeks thereafter, serum was obtained to quantify anti-hA20 IgG1 and IgG2a. A) Cured mice survival (days) from the second tumor inoculation is shown in comparison of that of mice who had never been vaccinated. B) Titers of anti-hA20 IgG1 and IgG2a in the serum of vaccinated mice obtained at different times are shown.

This result supports the notion of the induction of a strong, long-lasting, anti-tumoral memory response by the hA20 + RNAAdjuvant® vaccine formulation. Moreover, the balanced immune response in terms of post-vaccine IgG2a antibody production was confirmed (Supplementary Figure 3).

### The Anti-Tumor Effect of The Immune Response Generated Through Vaccination With hA20 + RNAAdjuvant® is CD8+ T-cell Dependent

In order to test whether the induction of a Th1 immune response by the RNAAdjuvant®-containing vaccine formulation activates a potent CD8+ cytotoxic, anti-tumor immune response with the potential of ultimately rejecting the tumor, anti-Id vaccination was preceded by CD8+ or CD4+ T-cell depletion. In a group of mice vaccinated with hA20 + RNAAdjuvant®, the anti-tumor effect was lost upon CD8+ T-cell depletion, resulting in an overall survival similar to that of the control group of non-vaccinated mice (Figure 6A).



**Figure 6:** CD8+ T lymphocytes are crucial for lymphoma control/regression by idiotypic vaccination with RNAAdjuvant®. Vaccination experiments were repeated with the integration of either CD8+ or CD4+ T-cell depletion and mice survival is reported in days elapsing between vaccination being started and tumor becoming palpable. CD4+ T-cell depletion does not prevent the reproducibility of the highly statistically-significant survival advantage for mice vaccinated with hA20 + RNAAdjuvant® over controls (A), while CD8+ T-cell depletion clearly does (B).

On the contrary, immunized mice previously depleted of CD4+ T cells did not show such an important reduction in overall survival. Taken together, these results indicate that, under this vaccination protocol, anti-Id CD8+ T cells play a pivotal role in tumor rejection.

### Discussion

If after two decades of clinical development no Id vaccine has yet succeeded at obtaining regulatory approval, it is because two completed trials [10, 11] failed to show vaccination superiority [3, 14, 15], while other two [8,9] achieved statistical significance in favor of vaccination, but for different reasons [3] were not sufficiently powered to make their conclusion worth of the ultimate goal. However, it also has to be recognized that, no matter their outcome, all such trials featured a variable number of patients who received the vaccine and yet failed to respond to it from an immunological standpoint [8-11]. Therefore, while considering new venues to clinically explore the potential of idiotypic vaccination as a safe, patient- and tumor-specific tool to eradicate lymphoma and/or preventing its reoccurrence, the improvement of vaccine formulation remains paramount. Currently, KLH and GM-CSF are the best immunogenic carrier and immunologic adjuvant, respectively, clinically tested in the Id vaccine setting [3]. However, KLH and GM-CSF have some disadvantages. KLH is a potent immunogen that, as we clearly show in this work, can indeed distract the immune system and skew its response towards a slower, Th2-based type.

Therefore, beside the well-known clinical problem of many vaccinated patients with detectable anti-KLH, but not anti-Id, immune response, our data invite to consider the very type of immune response (e.g. Th1, Th2 or both) elicited by novel versus old Id vaccine formulations. Also, KLH must be conjugated with the Id, and chemical conjugation of molecules with glutaraldehyde is random, meaning that on the one hand identical or different molecules can be ultimately produced, while on the other hand the number of conjugated molecules may vary among vaccines and vaccine doses [16]. This could negatively affect both vaccine immunogenicity and result reproducibility. Furthermore, Id-KLH needs to be administrated with GM-CSF. GM-CSF may have some secondary effects and is an agent not entirely deprived of clinical activity [17]. As such, any equally safe (or safer) and less complicated product able to replace either or both molecules might in itself represent a substantial improvement over what has been typically used in patients. Then, should the same product even prove superior in terms of immunological efficacy and clinical benefit in lymphoma mouse model, its potential use in humans would certainly warrant investigation in early-stage clinical trials.

The A20 lymphoma murine model has been used for decades to test idiotypic vaccination, with considerable success when it comes to protection of naive mice, but with no results in terms of consistent, reproducible cure/eradication of pre-established tumors [13]. Self-immunization of individual mice by the tumor cell line itself, as well as failure of the tumor to grow in individual mice are both postulated and anecdotal, but they represent the exception rather than the rule, as noted in this as well as in previous studies [13]. To the best of our knowledge, the fact that about 35% of mice with pre-established A20 lymphoma were both cured and protected from subsequent re-challenge by means of vaccination with hA20 + RNAAdjuvant® is unprecedented. Other immunotherapeutic strategies have also obtained positive results [18]. Yet, they are based on complex combinations of immunomodulatory agents delivered via intra-tumoral co-injections, not on a straightforward vaccine effect. As such, it remains to be seen whether such novel and exciting strategies, if clinically successful, might readily become available to patients in terms of logistical implementation outside clinical trials [18]. Our novel vaccine formulation stands out as being capable of eliciting a balanced, Th1- and Th2-like immune response, something not achievable by the Id-KLH + GM-CSF formulation used in the clinic, while showing that the induction of CD8+, but not CD4+, antigen-specific T cells is crucial in its ability to improve mice overall survival. While toxicology and a first-in-human study on RNAAdjuvant® are being carried out [19] in a different setting, it is encouraging to note that the very same mice that were unexpectedly cured of a tumor never before eradicated by vaccine therapy, and protected from subsequent re-challenge, did not die of any other cause. All in all, we believe that these data strongly warrant clinical exploration of the novel rId + RNAAdjuvant® vaccine formulation.

## References

1. Rosenberg SA (2001) Progress in human tumour immunology and immunotherapy. *Nature* 411: 380-384.
2. Mesa C and Fernandez LE (2004) Challenges facing adjuvants for cancer immunotherapy. *Immunol Cell Biol* 82: 644-650.
3. Bendandi M (2009) Idiotype vaccines for lymphoma: proof-of-principles and clinical trial failures. *Nat Rev Cancer* 9: 675-681.
4. de Cerio AL, Inoges S, Ai WZ, Villanueva H, Pastor F, et al (2013) Successful idiotypic vaccination following stem cell allotransplant in lymphoma. *Leuk Lymphoma* 54: 881-884.
5. Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller RA, et al. (1992) Induction of immune responses in patients with B-cell lymphoma against the surface-immunoglobulin idiotype expressed by their tumors. *N Engl J Med* 327: 1209-1215.
6. Bendandi M, Gocke CD, Kobrin CB, Benko FA, Sternas LA, et al. (1999) Complete molecular remission induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nat Med* 5: 1171-1177.
7. Barrios Y, Cabrera R, Yanez R, Briz M, Plaza A, et al. (2002) Anti-idiotypic vaccination in the treatment of low-grade B-cell lymphoma. *Haematologica* 87: 400-407.
8. Inoges S, Rodriguez-Calvillo M, Zabalegui N, Lopez-Diaz de Cerio A, Villanueva H, et al (2006) Clinical benefit associated with idiotypic vaccination in patients with follicular lymphoma. *J Natl Cancer Inst* 98: 1292-301.
9. Schuster SJ, Neelapu SS, Gause BL, Janik JE, Muggia FM, et al (2011) Vaccination with patient-specific tumor-derived antigen in first remission improves disease-free survival in follicular lymphoma. *J Clin Oncol* 29: 2787-2794.
10. Freedman A, Neelapu SS, Nichols C, Robertson MJ, Djulbegovic B, et al (2009) Placebo-controlled phase III trial of patient-specific immunotherapy with mitumprotimut-T and granulocyte-macrophage colony-stimulating factor after rituximab in patients with follicular lymphoma. *J Clin Oncol* 27: 3036-3043.
11. Levy R, Ganjoo KN, Leonard JP, Vose JM, Flinn IW, et al (2014) Active idiotypic vaccination versus control immunotherapy for follicular lymphoma. *J Clin Oncol* 32: 1797-1803.
12. Heidenreich R, Jasny E, Kowalczyk A, Lutz J, Probst J, et al. (2015) A novel RNA-based adjuvant combines strong immunostimulatory capacities with a favorable safety profile. *Int J Cancer* 137: 372-384.
13. Bendandi M, Marillonnet S, Kandzia R, Thieme F, Nickstadt A, et al (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. *Ann Oncol* 21: 2420-2427.
14. Bendandi M (2006) Clinical benefit of idiotype vaccines: too many trials for a clever demonstration? *Rev Recent Clin Trials* 1: 67-74.
15. Bendandi M (2008) Aiming at a curative strategy for follicular lymphoma. *CA Cancer J Clin* 58: 305-317.
16. Swerdlow RD, Ebert RF, Lee P, Bonaventura C, Miller KI (1996) Key-hole limpet hemocyanin: structural and functional characterization of two different subunits and multimers. *Comp Biochem Physiol B Biochem Mol Biol* 113: 537-548.

**Citation:** Bendandi M, de Cerio AL, Pastor F, Inogés S, Soldevilla MM, et al. (2017) Therapeutic Vaccination with Recombinant Idiotypic and RNAAdjuvant® Cures Mice with Pre-Established A20 Lymphoma. *J Vaccines Immunol*: JVII-120. DOI: 10.29011/2575-789X. 000020

---

17. Poire X, Kline J, Grinblatt D, Zimmerman T, Conner K, et al (2010) Phase II study of immunomodulation with granulocyte-macrophage colony-stimulating factor, interleukin-2, and rituximab following autologous stem cell transplant in patients with relapsed or refractory lymphomas. *Leuk Lymphoma* 51: 1241-1250.
18. Marabelle A, Kohrt H, Sagiv-Barfi I, Ajami B, Axtell RC, et al (2013) Depleting tumor-specific Tregs at a single site eradicates disseminated tumors. *J Clin Invest* 123: 2447-2463.
19. Meyer I (Phase I Safety and Tolerability Trial of Single and Repeat Doses of the RNA-based Adjuvant CV8102 Alone and in Combination with a Licensed Rabies Virus Vaccine in Healthy Adults. *ClinicalTrials.gov* Identifier: NCT02238756.