Anti-CD20 IgA can protect mice against lymphoma development: evaluation of the direct impact of IgA and cytotoxic effector recruitment on CD20 target cells

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ABSTRACT

Background
While most antibody-based therapies use IgG because of their well-known biological properties, some functional limitations of these antibodies call for the development of derivatives with other therapeutic functions. Although less abundant than IgG in serum, IgA is the most abundantly produced Ig class in humans. Besides the specific targeting of its dimeric form to mucosal areas, IgA was shown to recruit polymorphonuclear neutrophils against certain targets more efficiently than does IgG1.

Design and Methods
In this study, we investigated the various pathways by which anti-tumor effects can be mediated by anti-CD20 IgA against lymphoma cells.

Results
We found that polymeric human IgA was significantly more effective than human IgG1 in mediating direct killing or growth inhibition of target cells in the absence of complement. We also demonstrated that this direct killing was able to indirectly induce the classical pathway of the complement cascade although to a lesser extent than direct recruitment of complement by IgG. Recruitment of the alternative complement pathway by specific IgA was also observed. In addition to activating complement for lysis of lymphoma cell lines or primary cells from patients with lymphoma, we showed that monomeric anti-CD20 IgA can effectively protect mice against tumor development in a passive immunization strategy and we demonstrated that this protective effect may be enhanced in mice expressing the human FcαRI receptor on their neutrophils.

Conclusions
We show that anti-CD20 IgA antibodies have original therapeutic properties against lymphoma cells, with strong direct effects, ability to recruit neutrophils for cell cytotoxicity and even recruitment of complement, although largely through an indirect way.

Key words: Anti-CD20 IgA, lymphoma, FcαRI.

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** These two authors contributed equally to this manuscript.*

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The online version of this article has a Supplementary Appendix.

Introduction

Most of the tumor-directed, unconjugated antibodies employed in clinical trials are currently chosen from the IgG1 isotype, because of their long plasma half-lives[1] and their highly effective biological activities.[2] However, in spite of many successful results in treating cancers, clinical trials and research are generating a wealth of information about some functional limitations of therapeutic antibodies. Additional research is thus required and many efforts have recently been concentrated towards the development of so-called "third-generation" antibodies.

There have been preliminary studies on the development of antibodies of classes or isotypes other than IgG1, whose specific functions differ from those of IgG. IgA stands as the major Ig class at the mucosal surfaces. Human IgA displays a unique molecular heterogeneity, with various polymerization forms with specific distributions in body fluids. IgA-producing plasma cells found in the human spleen, lymph nodes and bone marrow secrete monomeric IgA (mIgA), mostly of the A1 isotype which is predominant in serum.[3] In contrast, the majority of mucosal plasma cells produce polymeric IgA (pIgA), with a relative increase in the proportion of IgA2 in rectal or colonic mucosa in comparison to the upper respiratory tract.[3] pIgA include a mix of dimers (dIgA), trimers or higher order polymers bound to the joining chain (J-chain). pIgA produced by the lamina propria plasma cells are transported by the polymeric Ig receptor (pIgR) from basolateral to apical surfaces of the mucosal epithelial cells where the pIgR is then cleaved and processed into the so-called secretory component and stays bound to the secretory IgA (sIgA) in the mucosal lumen.[4] It has been well established that sIgA are major players in protection against pathogens at mucosal sites, while the functions of serum IgA are still incompletely understood. Whether and how the IgA class activates complement has been a matter of controversy for years. It is generally presented as unable to recruit C1q and thus to activate the classical pathway,[5,6] but this issue is controversial.[7] Conversely, many studies have shown activation of the alternative pathway.[8,9] IgA has also been suggested to trigger the complement lectin pathway.[10] However, there is no report of complement recruitment by IgA against tumor targets. In contrast, several studies concerning the potential therapeutic effect of IgA have focused on its binding to the human FcαRI receptor (CD89), notably expressed on polymorphonuclear neutrophils (PMN)[11,12] i.e. on a population with potent antitumor properties.[13] FcαRI was demonstrated to promote endocytosis, phagocytosis, cytokine release and cellular cytotoxicity.[14,15] In several *in vitro* models, human IgA effectively triggered PMN-mediated lysis of target cells.[16-18] Moreover, studies with anti-EGF-R monoclonal antibodies showed a significantly stronger activity of IgA than IgG1 in recruiting PMN for antibody-dependent cellular cytotoxicity (ADCC), which resulted in greater tumor cell killing in whole blood assays.[19,20] Beyond these *in vitro* studies, *in vivo* antitumor effects of IgA are still unexplored because of the difficulties in developing relevant animal models, especially because mice do not express FcαRI.

Among therapeutic antibodies, chimeric anti-CD20 rituximab has become a gold standard for the treatment of many lymphomas and its action is probably the most widely studied. Rituximab binding to CD20 causes growth inhibition[21] and induction of apoptosis[22] in a subset of lymphoma cell lines. Nevertheless, various *in vitro* and *in vivo* experiments have reported that elimination of $CD20^+$ cells is mainly due to the γ1 constant chain of rituximab, which triggers complement-dependent cytotoxicity (CDC)[23,24] and recruits natural killer cells, leading to ADCC.[25,26] *In vivo* experiments in mouse models also showed that direct growth inhibition and apoptosis signaling by CD20 cross-linking were not sufficient to control $CD20^+$ grafted tumors.[27] In this study, we analyzed the therapeutic potential and mode of action of anti-CD20 IgA by comparison with IgG1. To this purpose, we generated chimeric CD20 antibodies of IgG or IgA class, each featuring the rituximab variable regions, and analyzed their capacity to kill CD20-expressing tumor cells through *in vitro* or *in vivo* assays.

Design and Methods

Cell lines and mice

Human B lymphoma cell lines, DHL-4, BL-2, Raji, and the T lymphoma cell line Jurkat were obtained from the American Type Culture Collection (Bethesda, MD, USA). The non-transfected EL4 thymocyte cell line derived from C57BL/6 mice and its hCD20-expressing variant (EL4-CD20) were kind gifts from Pr. H. Watier (Tours University). All cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 mg/mL streptomycin.

C57BL/6 mice (female, 6 to 8 weeks old) were purchased from Charles River (L'Arbresle, France). $RAG2\gamma c^{-/-}$ mice, kindly provided by Dr. James Di Santo (Institut Pasteur, Paris) and CD89 transgenic mice on a BALB/c background, previously described by Dr. M. van Egmond,[15] were used at 8 to 10 weeks of age. All procedures were conducted under an approved protocol according to European guidelines for animal experimentation.

The *Online Supplementary Design and Methods* section provides details about the production of the anti-CD20 chimeric antibodies and the various classical assays used with these antibodies for evaluating CDC (on cell lines of follicular lymphoma primary cells), cell proliferation, DNA synthesis, cell aggregation, and apoptosis.

In vivo *antibody-mediated killing model*

In vivo antibody-mediated killing (IVAK) assays[28] were performed, using fluorescent probes carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) and CellTrace Far Red DDAO-SE (DDAO-SE) (Molecular Probes). Cells were labeled with 2 μM DDAO/0.1 μM CFDA-SE (control cells) or 2 μM DDAO/2 μM CFDA-SE (target cells). Control and target cells were mixed at a ratio of approximately 1:1 and injected intraperitoneally (i.p.) (10^6 in 200 μL) into mice, followed by i.p. injection of antibody (2, 20 or 150 μg in 200 μL). Five hours later, mice were euthanized and peritoneal washings were harvested individually. Cells were fixed and analyzed by flow cytometry. Cytograms defined the target and control cell populations and ratios were calculated between the percentage of $CD20^+$ cells ($CFDA^{hi}$ $DDAO^{hi}$) and percentage of $CD20^-$ cells ($CFDA^{low}$ $DDAO^{hi}$). Ratios from individual antibody-treated mice were then normalized to PBS-treated mice, and percent killing was calculated as: [1 – (ratio individual $Ab_{\text{treated mouse}}$) / (mean ratio of the $PBS_{\text{injected group}}$)] * 100.

Syngeneic lymphoma therapy model

A syngeneic lymphoma model was established in C57/BL6 mice by tail vein inoculation of 8×10^3 CD20/EL4 cells in 200 μL.[24]

Twenty-four hours after inoculation of cells, mice received 20 μg of plasmid DNA encoding antibodies (pGTRIO-IgA2-βgal coding for anti-βgal IgGA2, pGTRIO-IgG1-Ritx-H for anti-hCD20 IgG1, pGTRIO-IgA2-Ritx-H for anti-hCD20 IgA2). DNA administration (in 2.5 mL saline buffer per mice) was through high-pressure "hydrodynamic" tail vein (HTV) injection.[29]

One and 4 days after HTV injection, serum IgG and IgA were titered by enzyme-linked immunosorbent assays using plates coated with goat anti-human IgA or IgG (Southern Biotechnologies).

To validate antibody specificity, sera from HTV-injected mice (or control anti-CD20 IgG or IgA monoclonal antibodies) were incubated with EL4 cells or EL4-CD20 cells for 30 min at 4°C, washed, incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG antibodies or FITC-conjugated anti-human IgA antibodies for 30 min at 4°C and washed. Specific CD20 staining was evaluated by flow cytometry.

Recruitment of leukocytes on lymphoma target cells by recombinant anti-CD20 antibodies

EDTA anticoagulated blood from healthy volunteers was mixed (4:1, v/v) with 6% dextran T500 (Pharmingen) and incubated for 45 min at room temperature. Supernatant was collected and residual erythrocytes were lysed. After washing, human leukocytes, containing potential effector cells, were adjusted to 10^7/mL in PBS/2% fetal bovine serum. Target DHL-4 cells were re-suspended at 10^6 cells/mL and labeled with 0.5 μM CFSE (Invitrogen). Cells were washed twice, adjusted to a concentration of 10^7/mL with PBS/2% fetal bovine serum and incubated with 20 μg/mL anti-CD20 antibodies at 4°C. Labeled target cells were then mixed with leukocytes at a 1:1 ratio. For inhibition assays, leukocytes were pre-incubated with 1 ng to 10 μg/mL of an anti-human CD89 monoclonal antibody (clone MIP8a, AbD serotec). DHL-4 and leukocytes were incubated for 30 min at 4°C, and then stained with ECD-conjugated anti-human CD45 antibody (Beckman) and phycoerythrin (PE)-conjugated anti-human CD16 (Beckman), washed and fixed. Cells were labeled with draq5 (Coger) just before acquisition. For each sample, 5000 images were analyzed using an ImageStream apparatus (Amnis). The percentage of DHL-4 cells associated with at least one CD16$^+$ effector corresponds to the formula: % of aggregated targets = (count of CFSE$^+$ cells bound with CD16$^+$ cells)/(total count of CFSE+ cells) * 100.

Results

Structural and functional characteristics of chimeric anti-CD20 antibodies

The integrity and purity of anti-CD20 antibodies were checked by sodium dodecylsulfate polyacrylamide gel electrophoresis and western blotting. Under reducing conditions, they all showed the expected bands at ~50-60 kDa for H chains and 25 kDa for L chains (*Online Supplementary Figure S1A*). Western blotting under reducing conditions using anti-human Cγ or Cα (*Online Supplementary Figure S1B*) confirmed the expected identity of these bands. Under non-reducing conditions, anti-CD20 IgA demonstrated bands of ~160 kDa and ~120 kDa, corresponding respectively to entire monomeric antibodies or only H chain homodimers (characteristic of the IgA2m(1) isotype in which H chains are not covalently linked with L chains).[30] The pIgA sample showed main bands over 300 kDa, corresponding to polymeric IgA (*Online Supplementary Figure S1C*). IgA polymerization was also evaluated by size exclusion chromatography, distinguishing two retention peaks (polymers in Peak 1, monomers in Peak 2) (*Online Supplementary Figure S1D*). Comparison of the monoclonal antibody profiles with that of human serum (mostly monomeric) IgA confirmed that our "mIgA2" sample mostly included monomers whereas "pIgA2" assembled in the presence of J chain showed a higher amount of IgA in a polymeric form.

Antigen specificity of the assembled antibodies was confirmed on h-CD20 transfected EL4 cells by indirect immunofluorescence using anti-κ chain antibody. All four variants readily bound h-CD20 transfected cells (*Online Supplementary Figure S1E*), while irrelevant control IgA did not (not shown).

IgA is more efficient than IgG1 for direct effector mechanisms

We investigated the direct effects of our antibodies and their abilities to inhibit proliferation of CD20$^+$ cell lines *in vitro*. Irrelevant control mIgA2 did not inhibit proliferation of any cell line. After 48 h of culture, growth of DHL-4 or BL2 cells was significantly reduced with all CD20-specific antibodies whereas Raji cell proliferation was unaffected (Figure 1A). Overall, IgA, and especially pIgA2, was more effective than IgG1 in inhibiting proliferation. The superiority of pIgA2 with regard to growth inhibition was observed regardless of the dose used. At a low dose (1 μg/mL), only anti-CD20 pIgA2 significantly inhibited proliferation (Figure 1B).

CD20-induced growth inhibition may result from either cell cycle arrest or apoptosis.[21] Since all antibody variants induced DHL-4 apoptosis similarly, as measured by annexin V staining (Figure 1C), the observed variation in growth inhibition was likely at the level of cell cycle arrest. Indeed, analysis after 24 h of culture of DHL-4 in the presence of a low concentration of anti-CD20 pIgA2 only induced minimal apoptosis but caused accumulation of cells in the G1 phase (to a greater extent than cells incubated in the presence of anti-CD20 IgG1) (Figure 1D).

We also checked that both IgG1 and IgA1 anti-CD20 antibodies induced cell aggregation of target cells cultured *in vitro* (*Online Supplementary Figure S2*).

Complement activation by anti-CD20 IgA partly results from late indirect C1q recruitment

CDC is considered a major property of anti-CD20 antibodies, alongside ADCC and direct signaling.[24,31] We evaluated our anti-CD20 antibodies for their capacity to trigger CDC against human lymphoma cell lines (Figure 2A). Surprisingly, all forms of IgA monoclonal antibodies showed strong cytotoxicity against DHL-4 (>80% cells killed in the presence of human serum). Serum heat inactivation abrogated lysis, highlighting its CDC-dependence. Similarly, IgA anti-CD20 monoclonal antibody triggered CDC of Raji cells. This IgA-mediated CDC was not restricted to cell lines established *in vitro* and could be confirmed by using primary cells from four patients with follicular lymphoma as the target cells (*Online Supplementary Figure S3*). A study on DHL-4 with serial dilutions of monoclonal antibodies showed that IgA-mediated CDC was dependent on the antibody concentration (Figure 2B) and that, irrespective of its state of polymerization, IgA was less effective than IgG1 for CDC. In these assays, none of the anti-CD20 antibodies induced lysis of cells from the BL2 cell line.

We further explored which pathway was involved,

since CDC-mediated tumor cell lysis by IgA has never been reported. We checked the cytotoxic capacity of anti-CD20 IgA in the presence of sera depleted of C1q, C2 or factor B. Unexpectedly, we observed an important decrease of IgA-mediated CDC with these sera (Table 1), with at least partial restoration upon complementation by the "depleted" C1q or factor B (Figure 3A), suggesting that both the alternative and the classical complement pathways were involved. To support this hypothesis, we measured recruitment of C1q on DHL-4 cells pre-incubated with mIgA2. These experiments were performed with recombinant C1q in the absence of serum, in order to avoid any indirect interaction between C1q and recombinant IgA. We found that after pre-incubation for 2 h with anti-CD20-mIgA2 or anti-CD20-IgG1 at 37°C, only a subpopulation of the mIgA2-treated cells were covered by C1q, whereas C1q was found on virtually all the IgG1-treated cells (Figure 3B). C1q binding did not occur at 4°C in the presence of anti-CD20-mIgA2. Dynamic evaluation of C1q binding after incubation of DHL-4 cells with anti-CD20 monoclonal antibodies showed that C1q binding increased over time for IgA, but that the increase was delayed in comparison with that of IgG1 (*Online Supplementary Table S1*). Unlike for IgG1, C1q binding in the presence of mIgA2 was restricted to annexin V^+ cells (Figure 3C). Taken together, these results showed that anti-CD20-IgA did not bind C1q directly but that their pro-apoptotic activity against tumor cells was through secondary recruitment of C1q on those cells engaged in apoptosis.

Anti-CD20 IgA protects animals from the development of lymphoma tumor

As both direct effects on cell growth and CDC were observed for anti-CD20-IgA *in vitro*, we sought to evaluate their anti-lymphoma activity *in vivo*. Short-term IVAK assays were used to measure *in vivo* cytotoxicity of anti-CD20 IgG1, mIgA2 and pIgA2 by injecting human lymphoma cells into the peritoneal cavity of RAG2$\gamma c^{-/-}$ mice (Figure 4A). Both mIgA2 and pIgA2 forms proved able to kill lymphoma cells, but the specific lysis always remained incomplete and below that achieved by IgG1.

We subsequently assessed the therapeutic potential of IgA by using long-term *in vivo* assays, i.e. the EL4

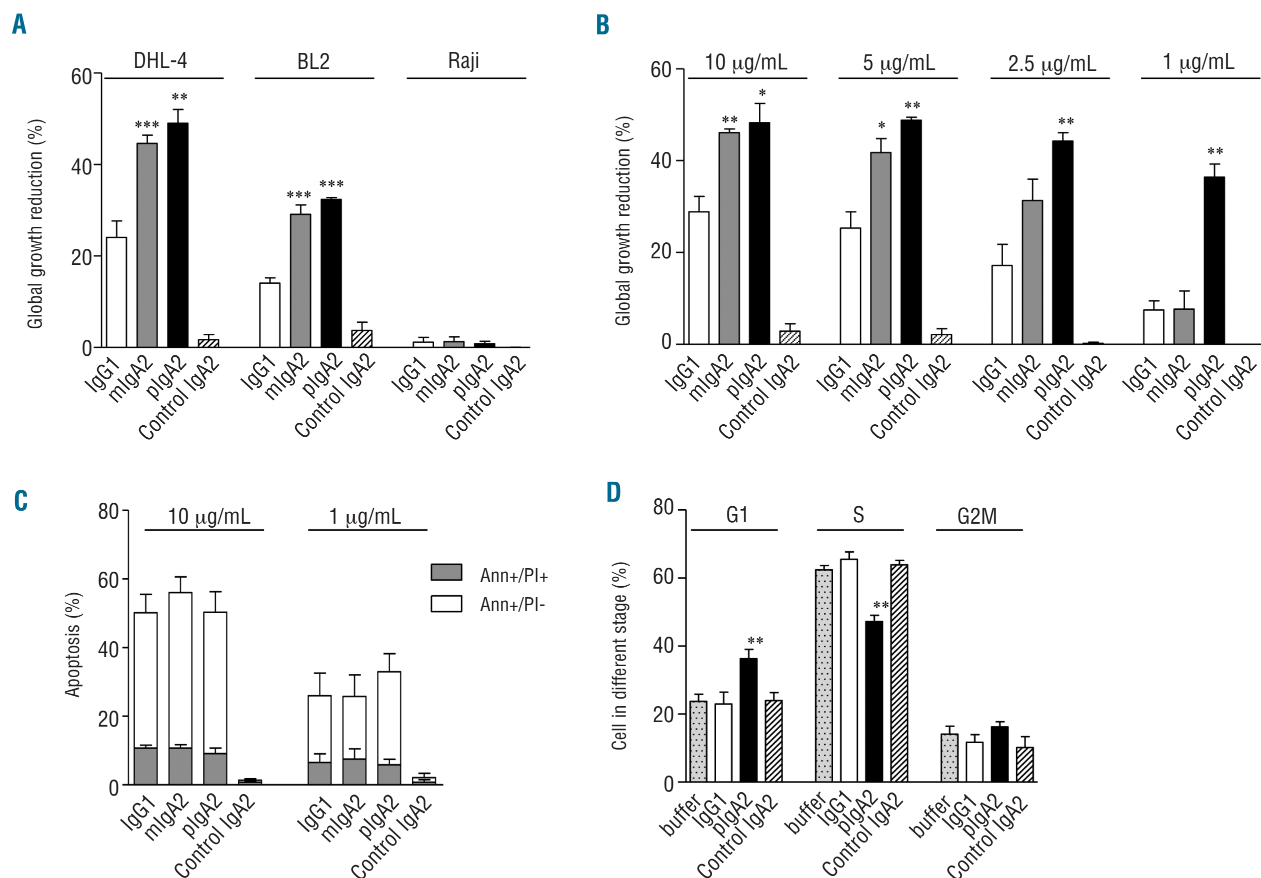

Figure 1. Direct effects of anti-CD20 monoclonal antibodies on human lymphoma B cells. Control IgA2 is an anti-βgal mIgA2. (A) Growth-inhibitory effect on lymphoma cell lines was measured by the MTS assay. After 48 h with 10 µg of anti-CD20 antibodies, amounts of viable cells were measured for three lymphoma cell lines. (B) Growth inhibition of DHL4 at 48 h was analyzed in the presence of serial dilutions of CD20 antibodies. (C) Apoptosis induction. After 5 h with 10 or 1 µg/mL of each anti-CD20 antibody or control, DHL-4 cells were stained with annexin V-FITC and propidium iodide (PI). The graph shows the percentages of early (Ann^+/PI^-) or late (Ann^+/PI^+) apoptotic cells. (D) DHL-4 cells were cultured with 1 µg/mL of anti-CD20 IgG1, pIgA2 or control. At 24 h, BrdU incorporation *vs.* DNA content (PI) showed the percentage of cells in G_0/G_1 ($BrdU^-/PI^n$), S ($BrdU^+$), or G_2/M phases (BrdU-/PI2n). Data are presented as mean ± SEM of four independent experiments (Asterisks indicate statistically significant differences between values observed for each form of IgA and IgG1 with an unpaired t-test : **P*<0.05, ***P*<0.01, *** *P*<0.001).

immunotherapy model developed in C57BL/6 by Gaetano *et al.*[24] In order to obtain strong infusion of therapeutic Ig, this assay was not performed by administering antibodies i.v., but using DNA administration: mice injected with tumor cells were thus treated through a protocol known to induce hepatic production of proteins encoded by non-integrative plasmids after HTV injection. This protocol of DNA injection rather than direct antibody administration was chosen because procedures for purifying IgA to a quality grade sufficient for prolonged *in vivo* administration are not yet available. Figure 4B shows human antibody serum concentrations in mice 1 and 4 days after DNA injection. The CD20 specificity of human antibodies present in mice sera after HTV injection was also checked by flow cytometry on target cells (*Online Supplementary Figure S4*). Plasmids were injected at the same concentration and only differed by the class of the encoded Ig, so that the Ig production rates were expected to be similar. However, IgA has a shorter half-life than IgG, resulting in a lower serum concentration on both days 1 and 4. Western blot analysis of serum samples after HTV injection showed that synthesized IgA2 were correctly assembled, mostly as monomers of ~ 120 kDa (with the expected loss of non-covalently linked L chains upon such analysis) (Figure 4C).

Injection of $8x10^3$ hCD20-EL4 in C57BL/6 syngeneic animals constantly yielded lethal tumors within 30-60 days. As for injection of rituximab,[24] IgG1 expression led to survival of the entire treated cohort. Surprisingly, anti-CD20 IgA2, despite its short half-life in serum, induced survival in over 80% of injected mice (Figure 4D).

CD89$^+$ effector recruitment can contribute to the therapeutic activity of IgA in vivo

One limitation of our lymphoma therapy model is the lack of a murine FcαRI/CD89 receptor homologue. Indeed, in most previous *in vitro* studies, the anti-tumor effects of IgA seemed to be mediated predominantly through an interaction with this receptor. We, therefore, initially tested the capabilities of antibodies to recruit human effector cells on target cells. CFSE-labeled DHL-4 cells were incubated with anti-CD20 antibodies and mixed with purified human leukocytes for analysis on a flow imager. Figure 5A shows a representative example of the images observed. An analysis of these images provided the percentage of target cells associated with one or more human CD16$^+$ effector leukocytes (Figure 5B), showing that monomeric IgA2 bound to target cells recruit leukocyte effector cells around B lymphoma targets with a higher efficacy than that of IgG (50%±4.8 of target aggregates for mIgA2 *versus* 40.5%±3.2 for IgG1). Conversely, in this context, the polymeric form of IgA appeared to be less effective with only 30.7% (± 4.9) of target cell aggregation with effector. It was confirmed that this phenomenon was CD89-dependent by pre-incubating human leukocytes with an inhibitory anti-human CD89 antibody,[32] then strongly reducing the percentage of DHL-4/CD16$^+$ effector aggregation (Figure 5C). We then performed additional IVAK assays using transgenic mice which expressed functional human CD89 receptor on their neutrophils. In such mice, mIgA2 was clearly more

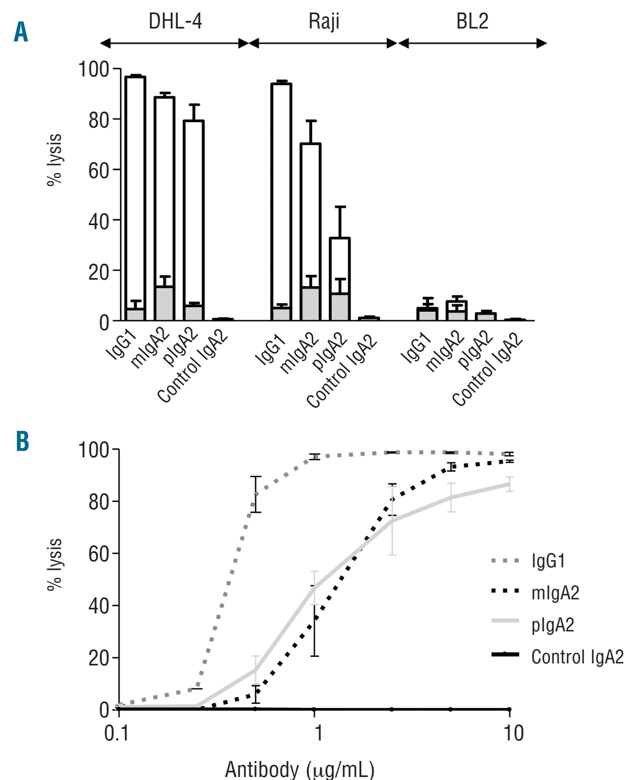

Figure 2. Antibody-complement dependent cytotoxicity against lymphoma cell lines. (A) CDC by anti-CD20 antibodies. DHL-4, BL-2 or Raji cells were incubated with monoclonal antibodies (10 μg/mL) in fresh (open bars) or heat inactivated (gray bars) human serum at 37°C for 4 h. CDC was evaluated by the percentage of propidium iodide-positive cells detected by cell cytometry. (B) Dose-dependence of CDC on DHL-4. The various anti-CD20 monoclonal antibodies were diluted from 10 to 0.1 μg/mL and incubated with DHL-4 for 4 h as above.

Table 1. Evaluation of CDC induced by anti-CD20 IgA in the presence of depleted human sera. DHL-4 cells were incubated with monoclonal antibodies (10 μg/mL) in either normal human serum (NHS), heat inactivated serum or specifically depleted sera (serum lacking C1q$^-$, C2$^-$ or factor B$^-$) at 37°C for 4 h. CDC was evaluated as the percentage of propidium iodide-positive cells. Data are presented as mean ± SEM of three independent experiments (asterisks indicate statistically significant differences between values observed for each depleted serum and NHS with an unpaired t test : **P*<0.05, ***P*<0.01, ****P*<0.001, ns: not significant).

	IgG1	mIgA2	dIgA2	Control mIgA2
Normal human serum	95.39±1.16	87.09±3.15	81.69±4.78	0.93±0.27
Heated NHS	15.6±1.12	12.75±3.25	18.8±2.5	2.31±2.18
C1q-	28.02±4.47***	15.17±5.21***	14.48±1.15***	0.33±0.33
C2-	68.28±6.53**	15.19±4.32***	23.46±27.89***	0.37±0.22
Factor B-	96.58±0.39^{ns}	63.05±4.71**	54.93±0.75*	0.13±0.06

effective at killing EL4-CD20 cells than in wild-type animals (Figure 5D). This result shows that anti-CD20 mIgA2 is able to recruit neutrophils *via* CD89 for specific lysis of target cells.

Discussion

Until now, only IgG monoclonal antibodies have been used for anti-cancer immunotherapy, thereby restricting the spectrum of therapeutic applications. In this context, new strategies could emerge from a better understanding of how other antibodies work and could potentially enable new effector activities and different pharmacokinetics to be exploited. The IgA class stands out as an interesting candidate for numerous reasons including its potential to recruit PMN[18] and its specific ability to target mucosal surfaces. The anti-viral and anti-bacterial properties of IgA have been widely studied and may lead to the emergence of new therapeutic candidates.[33,34] In contrast, no *in vivo* data are available regarding the potential use of IgA monoclonal antibody in cancer therapy. In this context we created monomeric and polymeric chimeric human IgA class variants of rituximab and sought to evaluate their therapeutic potential against CD20-expressing tumor targets.

Using *in vitro* assays, we found that all the cytotoxic pathways documented for rituximab were also active for the anti-CD20 IgA, including (somewhat surprisingly) CDC. Ligation and cross-linking of CD20 with antibodies

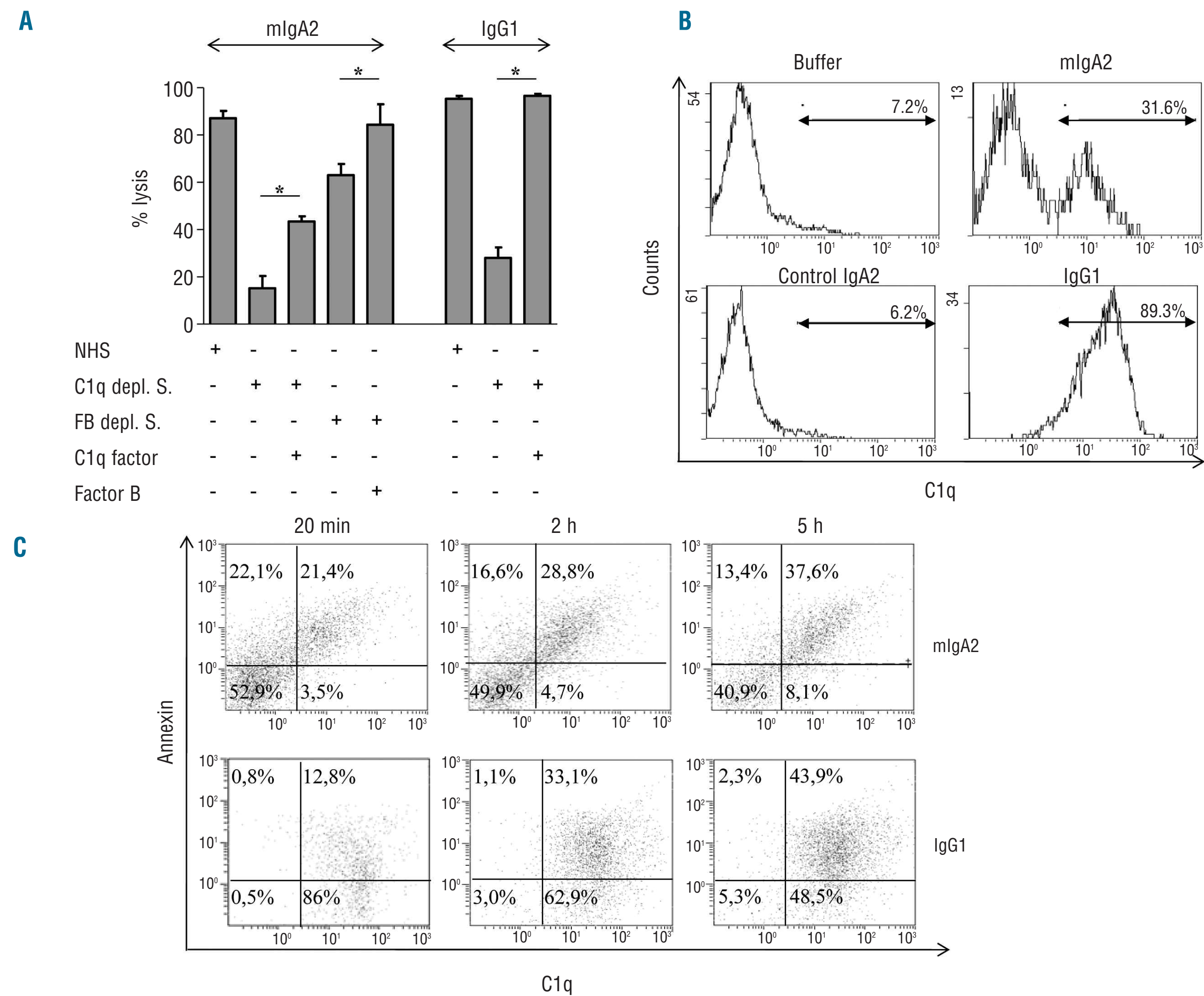

Figure 3. Complement activation by anti-CD20 IgA. (A) Complement depletion studies. DHL-4 cells were incubated with anti-CD20 mIgA2 or IgG1 (10 μg/mL) in normal human serum (NHS), heat inactivated NHS, C1q-depleted serum or factor B-depleted serum at 37°C for 4 h. In control experiments, human exogenous C1q (60 μg/mL) or factor B (300 μg/mL) was added to the depleted serum in order to complement the defect. CDC was evaluated by the percentage of propidium iodide-positive cells by cell cytometry. Data are presented as mean ± SEM of three independent experiments (asterisks indicate statistically significant differences between values tested by an unpaired t test: *P<0.05, **P<0.01). (B) Recruitment of recombinant C1q factor. DHL-4 cells were incubated with 20 μg/mL of anti-CD20-antibodies at 37°C for 2 h. Subsequently, cells were washed and incubated at 4°C for 1 h with 10 μg/mL of recombinant C1q. The attachment of C1q was then revealed by staining with anti-human C1q factor. (C) C1q recruitment and apoptosis. DHL-4 cells were incubated with 20 μg/mL of anti-CD20-antibodies at 37°C for 20 min, 2 h or 5 h. Cells were washed and incubated successively with C1q and anti-human C1q before staining with annexin V (the lower right dot plot appears under-estimated because many cells were completely negative and thus stuck on the horizontal axis).

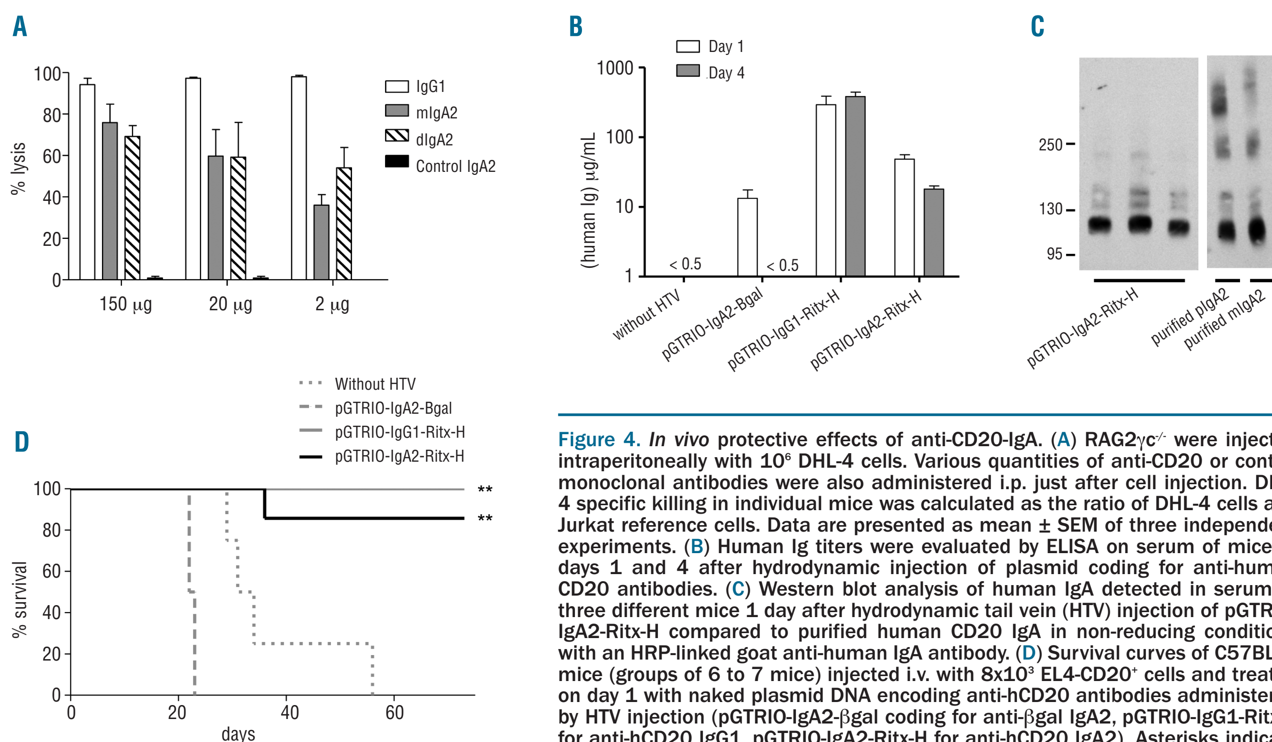

Figure 4. ***In vivo*** **protective effects of anti-CD20-IgA. (A) RAG2γc$^{-/-}$ were injected intraperitoneally with 10^{6} DHL-4 cells. Various quantities of anti-CD20 or control monoclonal antibodies were also administered i.p. just after cell injection. DHL-4 specific killing in individual mice was calculated as the ratio of DHL-4 cells and Jurkat reference cells. Data are presented as mean ± SEM of three independent experiments. (B) Human Ig titers were evaluated by ELISA on serum of mice at days 1 and 4 after hydrodynamic injection of plasmid coding for anti-human CD20 antibodies. (C) Western blot analysis of human IgA detected in serum of three different mice 1 day after hydrodynamic tail vein (HTV) injection of pGTRIO-IgA2-Ritx-H compared to purified human CD20 IgA in non-reducing conditions with an HRP-linked goat anti-human IgA antibody. (D) Survival curves of C57BL/6 mice (groups of 6 to 7 mice) injected i.v. with 8x10^{3} EL4-CD20^{+} cells and treated on day 1 with naked plasmid DNA encoding anti-hCD20 antibodies administered by HTV injection (pGTRIO-IgA2-βgal coding for anti-βgal IgA2, pGTRIO-IgG1-Ritx-H for anti-hCD20 IgG1, pGTRIO-IgA2-Ritx-H for anti-hCD20 IgA2). Asterisks indicate statistically significant differences between survival observed for each form of anti-CD20 antibody and anti-βgal IgA2 treatment with a log-rank test: *****P*<0.01.**

initiate signaling pathways and growth arrest or cell death. *In vitro* experiments have shown that the signal yielded by rituximab is enhanced by hyper-cross-linking using either anti-Ig antisera, anti-CD20 monoclonal antibody multimers or FcγR-bearing cells.[35] In our experiments, anti-CD20 IgA proved more effective for growth inhibition than IgG1 (Figure 1). By analogy to anti-CD20 multimers,[36] this effect might also be related to polymerization. Indeed, even anti-CD20 IgA produced in the absence of J-chain included IgA polymers (Figure 1D), and our 'pIgA' preparation assembled together with J chain was enriched in polymers and proved the most efficient for inducing growth inhibition of lymphoma cells. Anti-EGFR pIgA were also recently shown to be more effective than mIgA in receptor blockade and down-modulation as well as in growth inhibition of an EGFR^{+} cell line.[20] The T-shape of the IgA molecule instead of the Y-shape of IgG might also limit steric hindrance and increase the ability to cross-link targets expressed at the cell surface. Some studies with IgG have shown in mouse models that direct effects were not sufficient to control tumor growth when ADCC was absent.[27] IgA might, however, be of interest in cases in which mainly direct signaling is desired, especially for lymphomas displaying resistance to CDC or ADCC mediated by rituximab.

Contrary to what has already been described so far for IgA with regards to other anti-tumor therapy models, the anti-CD20 IgA triggered the complement cascade leading to target cell death in both lymphoma cell lines and primary follicular lymphoma cells (Figures 2 and *Online Supplementary Figure S3*). This was particularly true for DHL-4 cells in which anti-CD20 IgA2 induced CDC almost as efficiently as rituximab. In addition to the alternate complement pathway, we demonstrated that C1q unexpectedly contributed to CDC by anti-CD20-IgA. Unlike IgG, IgA does not recruit C1q directly; rather, the direct pro-apoptotic activity exerted by the antibody appears to induce deposition of C1q secondarily on the surface of target cells, thus leading to an accelerated lysis dependent on the classical complement pathway (Figure 3). This result is reminiscent of reports suggesting a contribution of the classical pathway to the clearance of apoptotic cells.[37,38] However, CDC by anti-CD20-IgA does not depend only on the indirect recruitment of C1q, and we found that CDC of mIgA2 against DHL-4 cells was also dependent on factor B and the alternative complement pathway. Sensitivity to C2-depletion also suggests that the lectin pathway might be involved. While not described previously and less complete with anti-CD20 IgA than with IgG, this IgA-mediated CDC might be of interest for therapeutic applications. With regards to the usually strong CDC induced by rituximab, it is also worth noting that the benefits of massive complement recruitment in patients have recently been questioned,[39] especially considering the secondary effects that this mechanism has.[40]

In addition to the *in vitro* analysis of various effects of IgA against target cells, we tested the *in vivo* therapeutic efficacy of anti-CD20 IgA in an immunotherapy mouse model of grafted T-cell lymphoma expressing human CD20 (Figure 4).[24] The therapeutic monoclonal antibodies were obtained by a gene therapy protocol yielding strong hepatic production of the antibodies. In this experiment, the levels of expression of IgA were much lower than those for IgG, probably because of the shorter half-life of the IgA class in serum. However, while injection of the

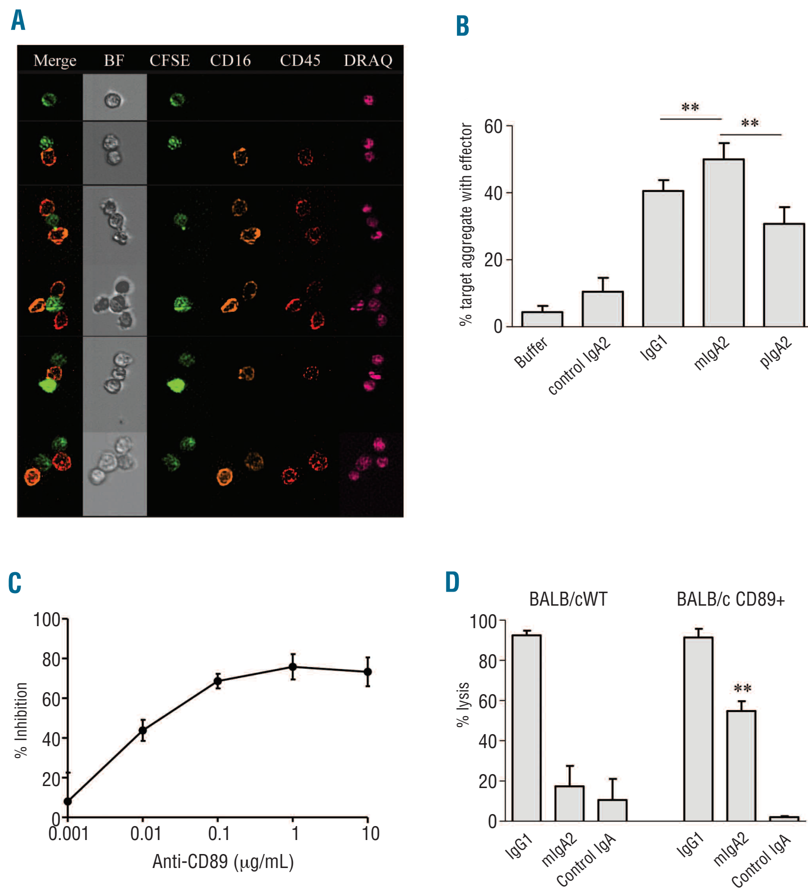

Figure 5. Cytotoxic recruitment of CD89+ effector cells by CD20-IgA. (A-C) The ability of anti-CD20 antibodies to mediate human leukocyte recruitment on lymphoma target cells was assessed using the ImageStream® Imaging Flow Cytometer. CFSE-labeled DHL-4 cells were pre-incubated with 20 μg/mL of each CD20 antibody, mixed with human leukocytes and stained with anti-human CD16 PE, anti-human CD45 ECD and DRAQ5 as a nuclear marker. Analyses were performed using the Amnis IDEAS software to evaluate the percentage of CFSE+ targets associated with CD16+ effector leukocytes. (A) Fluorescence and bright field images of representative CFSE+ cells alone or in aggregates with leukocytes. (BF; bright field) (B) Percentage of DHL-4 cells associated with one or more CD16+ effector leukocytes. Asterisks indicate statistically significant differences with a paired t test ***P*<0.01. (C) Inhibition of mIgA2-mediated target/CD16+ effector aggregation in the presence of anti-human CD89 antibodies. (D) ADCC induced by mIgA2 in human CD89-transgenic or wild-type (WT) mice as evaluated by IVAK; 10^6 mixed labeled target (EL4-CD20) and reference (EL4 WT) cells were injected i.p. before injection of 20 μg anti-CD20 IgG1, mIgA2 or control. Five hours later, hCD20 -specific killing was calculated from ratios of target to reference cells in the peritoneal washings from individual mice. Data are representative of at least six mice for each condition (asterisks indicate statistically significant differences between values observed for transgenic mice compared to WT mice with an unpaired t test ***P*<0.01).

IgA2 expression plasmid resulted in a very low concentration of human IgA2 in blood, it proved sufficient to protect 80% of mice against CD20+ tumor development, demonstrating the potency of this molecule.

Although the IgA2 isotype of anti-CD20 was less effective than IgG1 at extending survival of tumor-bearing mice, the difference between human IgG and IgA in a mouse model should be interpreted with caution particularly because mice do not express FcαRI. Indeed, one of the cytotoxic effects expected from the IgA class in humans would be its strong ability to recruit and activate PMN via the FcαRI receptor, as was highlighted by several previous studies with tumor cells.[18,19,41] We showed that monomeric anti-CD20-IgA was better than IgG1 at recruiting human effector cells on target cells. While it is known that the affinity of FcαRI is identical for both mIgA and pIgA,[42] in our aggregation assays, polymeric anti-CD20-IgA showed a restricted capacity for effector recruitment compared to that observed with the monomeric form (Figure 5). It is likely that the availability or accessibility of IgA bound at the surface of target cells is better for monomeric than for polymeric IgA. We, therefore, chose monomeric IgA2 to test the ADCC of this isotype on a CD20+ tumor target and showed *in vivo* that PMN recruitment via the FcαRI receptor could result in specific lysis of a tumor target. Indeed, using transgenic mice whose neutrophils strongly and constitutively expressed human FcαRI,[15] mIgA2 proved able to specifically kill much higher quantities of CD20+ cells in transgenic mice than in wild-type mice.

Our results show that anti-CD20 IgA has an original "therapeutic" potential against lymphoma targets, highlighting the various therapeutic capacities of the different forms of IgA. Although it is difficult to infer the final *in vivo* effect of a prolonged infusion of anti-CD20 antibodies from short-term *in vitro* experiments, it is interesting to observe that IgA antibodies show significant growth inhibitory or cytotoxic effects in various conditions, including against primary human tumor cells. As for IgG1, these effects were occasionally variable in cell lines such as Raji which does not undergo growth inhibition or BL2 which is resistant to CDC. The strong direct signaling induced by pIgA might be of benefit in situations in which the therapeutic antibody is used as an agonist, for example when targeting the TRAIL receptor[43] or T-cell CD28 for treatment of acute graft-*versus*-host disease.[44] Furthermore, pIgA binds to the pIgR, and is, therefore, actively transported into secretions,[4] thus being a good candidate to treat diseases associated with mucosa. In contrast, monomeric IgA2 appears to be the best candidate for the establishment of indirect cytotoxicity and recruitment of effector cells against a tumor target.

Authorship and Disclosures

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References

1. Ghetie V, Ward ES. Multiple roles for the major histocompatibility complex class I-related receptor FcRn. Annu Rev Immunol. 2000;18:739-66.
2. Bruggemann M, Williams GT, Bindon CI, Clark MR, Walker MR, Jefferis R, et al. Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies. J Exp Med. 1987; 166(5):1351-61.
3. Pakkanen SH, Kantele JM, Moldoveanu Z, Hedges S, Hakkinen M, Mestecky J, et al. Expression of homing receptors on IgA1 and IgA2 plasmablasts in blood reflects differential distribution of IgA1 and IgA2 in various body fluids. Clin Vaccine Immunol. 2010;17(3):393-401.
4. Mostov KE. Transepithelial transport of immunoglobulins. Annu Rev Immunol. 1994;12:63-84.
5. Daha MR, Gorter A, Rits M, van Es LA, Hiemstra PS. Interaction of immunoglobulin A with complement and phagocytic cells. Prog Clin Biol Res. 1989;297:247-60; discussion 60-1.
6. Lohse S, Peipp M, Beyer T, Valerius T, Dechant M. Impact of human IgA antibodies on complement-dependent cytotoxicity mediated by combinations of EGF-R-directed antibodies. Arch Immunol Ther Exp (Warsz). 2010;58(4):303-12.
7. Jarvis GA, Griffiss JM. Human IgA1 initiates complement-mediated killing of Neisseria meningitidis. J Immunol. 1989;143(5):1703-9.
8. Hiemstra PS, Gorter A, Stuurman ME, Van Es LA, Daha MR. Activation of the alternative pathway of complement by human serum IgA. Eur J Immunol. 1987;17(3):321-6.
9. Pfaffenbach G, Lamm ME, Gigli I. Activation of the guinea pig alternative complement pathway by mouse IgA immune complexes. J Exp Med. 1982;155(1):231-47.
10. Roos A, Bouwman LH, van Gijlswijk-Janssen DJ, Faber-Krol MC, Stahl GL, Daha MR. Human IgA activates the complement system via the mannan-binding lectin pathway. J Immunol. 2001;167(5):2861-8.
11. Morton HC, van Egmond M, van de Winkel JG. Structure and function of human IgA Fc receptors (Fc alpha R). Crit Rev Immunol. 1996;16(4):423-40.
12. Monteiro RC, Van De Winkel JG. IgA Fc receptors. Annu Rev Immunol. 2003;21: 177-204.
13. Di Carlo E, Forni G, Lollini P, Colombo MP, Modesti A, Musiani P. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood. 2001;97(2):339-45.
14. Patry C, Herbelin A, Lehuen A, Bach JF, Monteiro RC. Fc alpha receptors mediate release of tumour necrosis factor-alpha and interleukin-6 by human monocytes following receptor aggregation. Immunology. 1995;86(1):1-5.
15. van Egmond M, van Vuuren AJ, Morton HC, van Spriel AB, Shen L, Hofhuis FM, et al. Human immunoglobulin A receptor (FcalphaRI, CD89) function in transgenic mice requires both FcR gamma chain and CR3 (CD11b/CD18). Blood. 1999;93(12): 4387-94.
16. Valerius T, Stockmeyer B, van Spriel AB, Graziano RF, van den Herik-Oudijk IE, Repp R, et al. FcalphaRI (CD89) as a novel trigger molecule for bispecific antibody therapy. Blood. 1997;90(11):4485-92.
17. Huls G, Heijnen IA, Cuomo E, van der Linden J, Boel E, van de Winkel JG, et al. Antitumor immune effector mechanisms recruited by phage display-derived fully human IgG1 and IgA1 monoclonal antibodies. Cancer Res. 1999;59(22):5778-84.
18. Dechant M, Vidarsson G, Stockmeyer B, Repp R, Glennie MJ, Gramatzki M, et al. Chimeric IgA antibodies against HLA class II effectively trigger lymphoma cell killing. Blood. 2002;100(13):4574-80.
19. Dechant M, Beyer T, Schneider-Merck T, Weisner W, Peipp M, van de Winkel JG, et al. Effector mechanisms of recombinant IgA antibodies against epidermal growth factor receptor. J Immunol. 2007;179(5):2936-43.
20. Lohse S, Derer S, Beyer T, Klausz K, Peipp M, Leusen JH, et al. Recombinant dimeric IgA antibodies against the epidermal growth factor receptor mediate effective tumor cell killing. J Immunol. 2011;186(6): 3770-8.
21. Rose AL, Smith BE, Maloney DG. Glucocorticoids and rituximab in vitro: synergistic direct antiproliferative and apoptotic effects. Blood. 2002;100(5):1765-73.
22. Shan D, Ledbetter JA, Press OW. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood. 1998;91(5):1644-52.
23. Golay J, Zaffaroni L, Vaccari T, Lazzari M, Borleri GM, Bernasconi S, et al. Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. Blood. 2000;95(12): 3900-8.
24. Di Gaetano N, Cittera E, Nota R, Vecchi A, Grieco V, Scanziani E, et al. Complement activation determines the therapeutic activity of rituximab in vivo. J Immunol. 2003; 171(3):1581-7.
25. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood. 2002;99(3):754-8.
26. Koene HR, Kleijer M, Algra J, Roos D, von dem Borne AE, de Haas M. Fc gammaRIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gammaRIIIa, independently of the Fc gammaRIIIa-48L/R/H phenotype. Blood. 1997;90(3):1109-14.
27. de Haij S, Jansen JH, Boross P, Beurskens FJ, Bakema JE, Bos DL, et al. In vivo cytotoxicity of type I CD20 antibodies critically depends on Fc receptor ITAM signaling. Cancer Res. 2010;70(8):3209-17.
28. Guyre CA, Gomes D, Smith KA, Kaplan JM, Perricone MA. Development of an in vivo antibody-mediated killing (IVAK) model, a flow cytometric method to rapidly evaluate therapeutic antibodies. J Immunol Methods. 2008;333(1-2):51-60.
29. Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. Gene Ther. 1999;6(7):1258-66.
30. Woof JM, Kerr MA. The function of immunoglobulin A in immunity. J Pathol. 2006;208(2):270-82.
31. Cragg MS, Morgan SM, Chan HT, Morgan BP, Filatov AV, Johnson PW, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. Blood. 2003;101(3):1045-52.
32. Zhang W, Bi B, Oldroyd RG, Lachmann PJ. Neutrophil lactoferrin release induced by IgA immune complexes differed from that induced by cross-linking of fcalpha receptors (FcalphaR) with a monoclonal antibody, MIP8a. Clin Exp Immunol. 2000;121(1):106-11.
33. Tudor D, Derrien M, Diomede L, Drillet AS, Houimel M, Moog C, et al. HIV-1 gp41-specific monoclonal mucosal IgAs derived from highly exposed but IgG-seronegative individuals block HIV-1 epithelial transcytosis and neutralize CD4(+) cell infection: an IgA gene and functional analysis. Mucosal Immunol. 2009;2(5):412-26.
34. Balu S, Reljic R, Lewis MJ, Pleass RJ, McIntosh R, van Kooten C, et al. A novel human IgA monoclonal antibody protects against tuberculosis. J Immunol. 2011;186 (5):3113-9.
35. Shan D, Ledbetter JA, Press OW. Signaling events involved in anti-CD20-induced apoptosis of malignant human B cells. Cancer Immunol Immunother. 2000;48(12): 673-83.
36. Ghetie MA, Bright H, Vitetta ES. Homodimers but not monomers of rituxan (chimeric anti-CD20) induce apoptosis in human B-lymphoma cells and synergize with a chemotherapeutic agent and an immunotoxin. Blood. 2001;97(5):1392-8.
37. Korb LC, Ahearn JM. C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J Immunol. 1997;158(10):4525-8.
38. Nauta AJ, Trouw LA, Daha MR, Tijsma O, Nieuwland R, Schwaeble WJ, et al. Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur J Immunol. 2002;32(6):1726-36.
39. Wang SY, Veeramani S, Racila E, Cagley J, Fritzinger DC, Vogel CW, et al. Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model. Blood. 2009;114(26):5322-30.
40. van der Kolk LE, Grillo-Lopez AJ, Baars JW, Hack CE, van Oers MH. Complement activation plays a key role in the side-effects of rituximab treatment. Br J Haematol. 2001;115(4):807-11.
41. Otten MA, Rudolph E, Dechant M, Tuk CW, Reijmers RM, Beelen RH, et al. Immature neutrophils mediate tumor cell killing via IgA but not IgG Fc receptors. J Immunol. 2005;174(9):5472-80.
42. Oortwijn BD, Roos A, van der Boog PJ, Klar-Mohamad N, van Remoortere A, Deelder AM, et al. Monomeric and polymeric IgA show a similar association with the myeloid FcalphaRI/CD89. Mol Immunol. 2007;44(5): 966-73.
43. Falschlehner C, Ganten TM, Koschny R, Schaefer U, Walczak H. TRAIL and other TRAIL receptor agonists as novel cancer therapeutics. Adv Exp Med Biol. 2009; 647:195-206.
44. Beyersdorf N, Ding X, Hunig T, Kerkau T. Superagonistic CD28 stimulation of allogeneic T cells protects from acute graft versus host disease. Blood. 2009;114(20):4575-82.