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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Online Supplementary Design and Methods

Anti-CD20 chimeric antibodies

Human constant regions for κ-L chain, γ1- or α2m(1)-H chains were cloned and inserted in a pGTRIO vector. Rituximab variable regions were synthesized and inserted in frame upstream of the constant exons of H and L chains. To favor pIgA assembly, an additional J chain gene was included into the construction. In parallel, variable regions of an in-home generated anti-β–galactosidase mouse hybridoma (T9C6) were amplified and inserted into pGTRIO upstream of the Cα2-H chain sequence to produce an anti-βgal IgA2 used as a control. Chinese hamster ovary (CHO) cells were transfected with pGTRIO vectors using the Lyovec system (Invivogen). All IgA preparations were chromatographically purified on SSL7 columns[1] while the chimeric IgG1 was purified using protein G. Concentrations of purified antibodies were determined by 280nm UV adsorption.

The integrity of the purified antibody preparations was determined by sodium dodecylsulphate polyacrylamide gel electrophoresis under reducing or non-reducing conditions using 8% Tris-glycine gels. The assembly state of serum human IgA produced in DNA-injected mice was evaluated by native gel electrophoresis. After transfer, nitrocellulose-membranes (Biorad) were blocked with 0.5% non-fat milk, incubated for 1 h with horseradish peroxidase-conjugated goat anti-human IgA or anti-human IgG antibodies (Southern Biotech). After washing with TBS-0.1%-Tween buffer, blots were revealed with DAB substrate [25 mg DAB in 50 mL phosphate-buffered saline (PBS) + 50 μL H_2O_2].

Antibody polymerization was evaluated by size exclusion chromatography on a Superdex™ 200 10/300 column (GE Healthcare). Human serum (mostly monomeric) and colostrum IgA (exclusively polymeric) (Sigma-Aldrich) were used as standards for elution volumes.

For indirect immunofluorescence, $5x10^5$ non-transfected or hCD20 EL4 cells/sample were incubated with the anti-CD20 antibodies (20 μg/mL). Cells were stained with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human κ-L chain antibodies (Southern biotech) and analyzed by flow cytometry.

Complement-mediated cytotoxicity assays on cell lines and C1q deposition studies

Cells ($2x10^5$) were re-suspended in 50% of pooled normal human serum, heat inactivated normal human serum or depleted human serum (Quidel) with or without anti-CD20- antibodies. For controls, depleted sera were complemented with 60 μg/mL of human C1q (Sigma-Aldrich) or 300 μg/mL of human factor B (Quidel). After incubation for 4 h at 37°C, dead and viable cells were differentially stained by addition of 1 μg/mL propidium iodide (PI) and analyzed by flow cytometry. The percentage of specific cytolysis was calculated from cell counts using the following formula: % of specific lysis = (% $PI^{+}_{experimental}$ - % PI^{+}_{basal}) / (100 - % PI^{+}_{basal}) * 100.

In order to study C1q cell deposition, DHL-4 cells were adjusted to 10^6/mL and incubated with 20 μg/mL of anti-CD20-antibodies in RPMI at 37°C or 4°C. After 20 min, 2 h or 5 h, cells were cooled to 4°C and incubated with 10 μg/mL of C1q for 1 h. Cells were washed and stained with rabbit anti-human C1q-FITC (Dako) and annexin V-phycoerythrin (BD Pharmingen) for flow cytometric analysis.

Complement-dependent cytotoxicity assays on human follicular lymphoma primary cells

Primary follicular lymphoma cells were obtained from four patients recruited after written informed consent, according to the Declaration of Helsinki. They all showed a predominantly follicular growth pattern, CD10 expression, and were classified as grade 1 or 2 according to the World Health Organization diagnostic criteria. Tissues were rapidly dissociated after collection by flushing using a syringe and needle. Cell suspensions were then filtered and washed by centrifugation.

For cytotoxicity assays, primary lymphoma cells were thawed; living cells were purified (using the Dead Cell Removal Kit, Miltenyi Biotec), adjusted to 10^6/mL and incubated with 10 μg/mL of anti-CD20-antibodies in the presence of AB group normal human serum at 37°C for 5 h. Cells were stained with APC-conjugated mouse anti-human CD19 antibodies (BD Biosciences) for 30 min at 4°C, washed and stained with annexin V-V450 (BD Biosciences) and PI in cold PBS-$CaCl_2$-$MgCl_2$. B cells (gated as $CD19^+$ cells) were evaluated for early apoptosis (annexin V^+/PI^-) and late apoptotis/necrosis (PI^+).

Cell proliferation assay

An MTS assay (Promega) was used to analyze the effect of anti-CD20 antibodies on B-cell growth *in vitro*. DHL-4, BL2 and Raji cells were cultured at 37°C in 96-well plates (10^4 cells in 100 μL/well). Proliferation was assessed after 48 h under various concentrations of specific or control monoclonal antibodies. The amount of viable cells was determined (in triplicate) by measuring the absorbance ($A_{490\,nm}$) after addition of MTS for 4 h. Cells incubated in medium without antibodies were used as a control. Growth inhibition is expressed as follows: 100 - (Mean $OD_{experimental\ group}$ – Mean OD_{medium}) / (Mean $OD_{control}$ – Mean OD_{medium}) * 100.

Apoptosis analysis

Briefly, target cells were adjusted to a concentration of 10^6 cells/mL and incubated with the indicated concentration of anti-CD20- antibodies at 37°C. After 5 h, cells were stained with annexin V-FITC (BD Pharmingen) and PI (Sigma) in cold PBS-$CaCl_2$-$MgCl_2$ (Invitrogen). Flow cytometry discriminated living cells (annexin V^-/PI^-), early apoptotic cells (annexin V^+/PI^-), and late apoptotic/necrotic cells (annexin V^+/PI^+). The percentage of apoptosis was calculated using the following formulae:

- % of early apoptotic cells = (%Ann^+ $PI^-_{experimental}$ – % Ann^+ PI^-_{basal}) / %Ann^- PI^-_{basal} * 100;
- % of late apoptotic cells = (%Ann^+ $PI^+_{experimental}$ – %Ann^+ PI^+_{basal})/ %Ann^- PI^-_{basal} * 100.

Cell aggregation in the presence of anti-CD20 antibodies

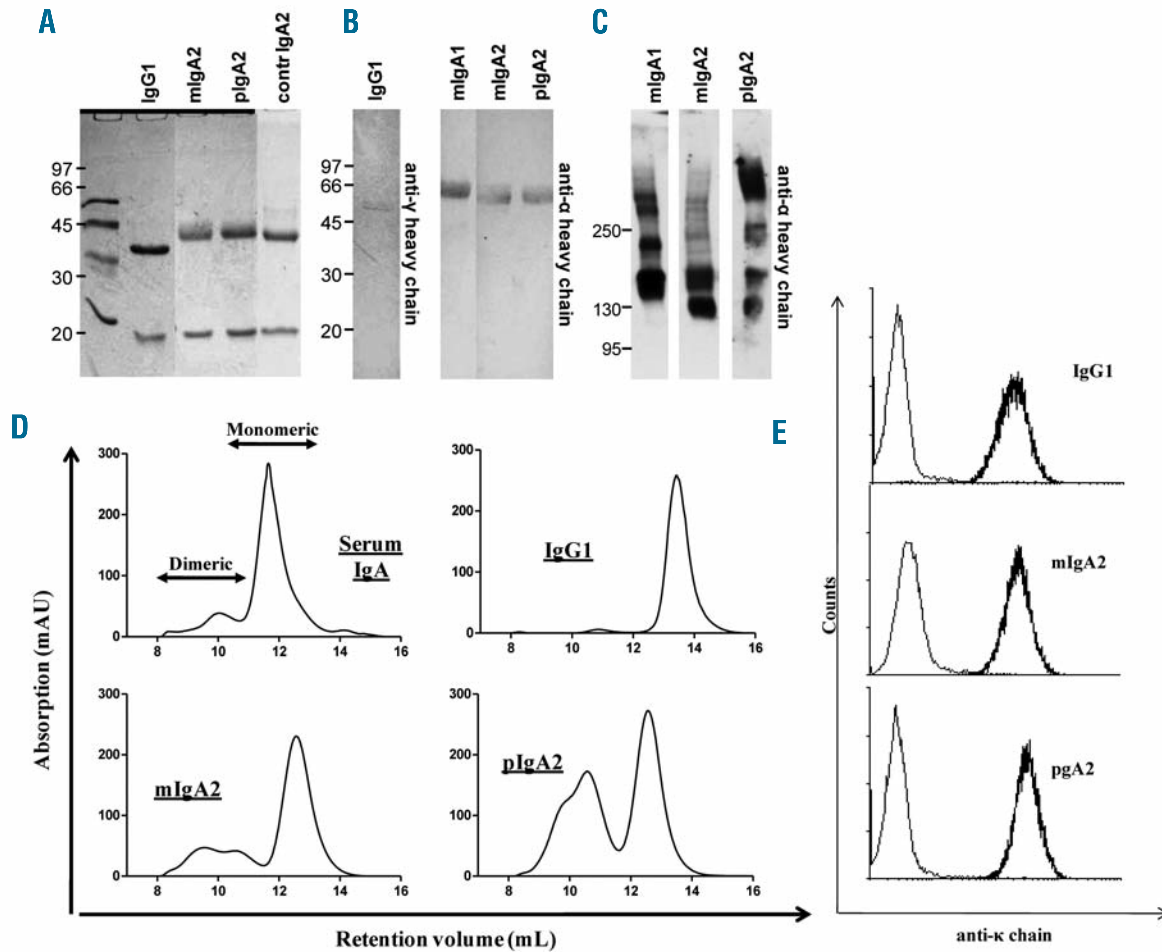
Cells were plated at 2.5x10^5 cells/well in flat-bottomed, 96-well plates in the presence of 10 μg/mL rituximab, mIgA2, pIgA2 or control IgA2. Cell adhesion was assessed 24 h later by examination under an optical microscope.

DNA synthesis by a BrdU pulse-labeling technique

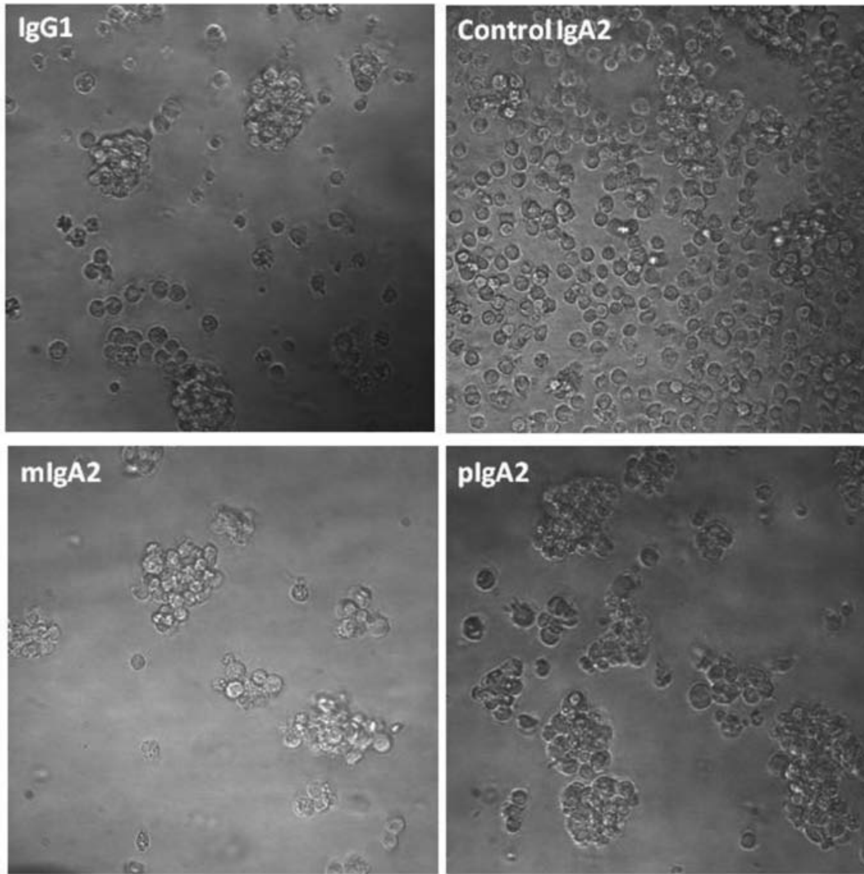
DHL-4 (10^5 cells/mL) were cultured with 1 μg/mL of monoclonal antibodies. After 24 h, exponentially growing cells (10^6) were pulse-labeled with 18 μg/mL bromodeoxyuridine (BrdU; Sigma-Aldrich) for 2 h, washed, and fixed with 50% ice-cold ethanol. Cells were then incubated with 2N HCl to partially denature the DNA, washed and re-suspended in 1% bovine serum albumin/0.5%Tween20/PBS buffer. Incorporated BrdU was stained with 5 μL of FITC-conjugated anti-BrdU mouse monoclonal antibody (BD Pharmingen). Samples were then washed and re-suspended in PBS containing 50 μg/mL PI. Bivariate distributions of amount of BrdU (FITC) *versus* DNA content (PI) were analyzed by flow cytometry, defining regions corresponding to the G_0/G_1, S, and G_2/M phases of the cell cycle.

Online Supplementary Table S1. Recruitment of C1q *versus* incubation time between antibodies and target cells. DHL-4 cells were incubated with anti-CD20 IgG1 or mIgA2 (20 μg/mL) at 37°C for 20 min, 2 h or 5 h. Cells were then washed and incubated successively with C1q and anti-human C1q-FITC. Data are presented as mean percent $C1q^+$ cells ± SEM of four independent experiments (asterisks indicate statistically significant differences between values observed with mIgA2 at 20 min and 2 h or 2 h and 5 h with paired t tests: *P<0.05, **P<0.01).

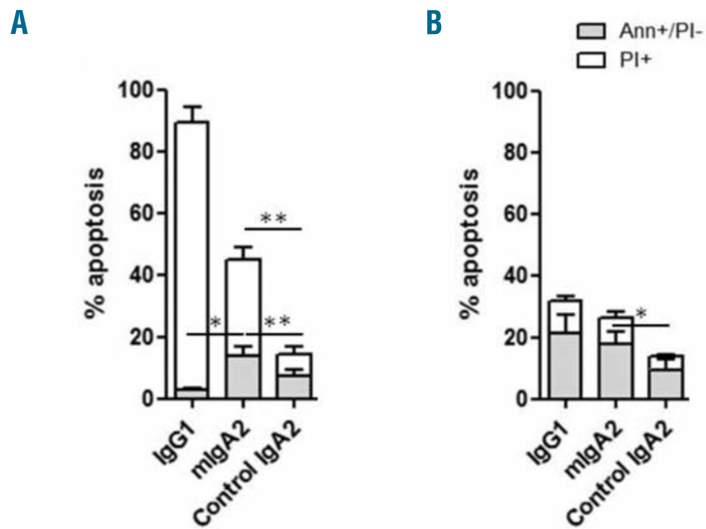
	IgG1	mIgA2
20 minutes	97.06±1.29	23.45±4.31
2 hours	96.34±0.63	29.59±5.07**
5 hours	87.42±5.79	36.49±5.82*



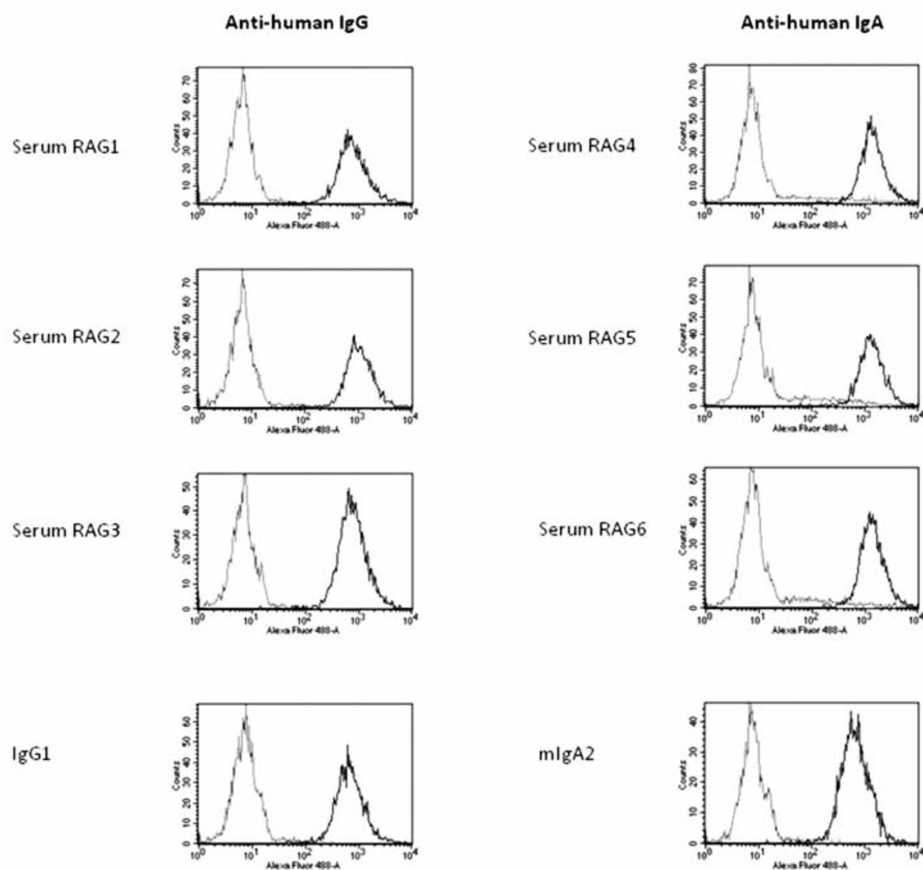
Online Supplementary Figure S1. Molecular structure and binding characteristics of anti-CD20 antibodies. (A) Purified antibodies corresponding to anti-CD20 IgG1, mIgA2, pIgA2 and anti-βgal- mIgA2 were separated by SDS-PAGE under reducing conditions and colored by Coomassie blue. Positions of molecular mass markers (in kDa) are indicated. Purified chimeric antibodies were characterized by western blot with indicated anti-human antibodies under reducing (B) or non-reducing (C) conditions. (D) Analysis of IgA polymerization by size exclusion chromatography. Elution profiles of purified CD20 antibodies and human serum was monitored by UV absorption at 280 nm (mAU, milliabsorbance units). (E) Specificity of anti-CD20 antibodies: WT EL4 (thin line) or CD20-EL4 (bold line) were labeled with anti-human κ light chain after incubation with similar concentrations of chimeric mIgA2, pIgA2, or IgG1.



Online Supplementary Figure S2. Anti-CD20 antibodies induce aggregation of DHL-4 cells. DHL-4 cells were plated in the presence of 10 μg/mL anti-CD20 IgG1, mIgA2, or pIgA2 or unrelated IgA2 as a negative control. Cells were examined by light microscopy after 24 h at 400x magnification.



Online Supplementary Figure S3. Anti-CD20 mediated apoptosis of human primary follicular lymphoma cells. Human primary follicular lymphoma B cells were incubated at 37°C for 5 h with monoclonal antibodies (10 μg/mL) in the presence of complete (A) or heat-inactivated (B) human serum. Early (annexin V^+/propidium iodide$^-$, gray bars) and late (open bars) apoptotic cells were evaluated by cell cytometry after specifically gating $CD19^+$ B cells. Data are presented as mean ± SEM of four independent experiments (asterisks indicate statistically significant differences between values using a one-tailed paired t test: *P<0.05, **P<0.01).



Online Supplementary Figure S4. Specificity control of *in vivo* produced anti-CD20 monoclonal antibodies. Sera from plasmid-injected mice were incubated with either CD20-expressing (black curves) or non-transfected (gray curves) EL4 cells. Specific CD20 staining was revealed with secondary FITC-conjugated anti-human IgG (left panel) or anti-human IgA (right panel) antisera. Sera from three representative hydrodynamic injected mice (three upper graphs) are shown together with *in vitro* synthesized anti-CD20 IgG1 and IgA as positive controls (bottom).