FcRn overexpression in mice results in potent humoral response against weakly immunogenic antigen

Attila Végh,1 Judit Cervenak,1 István Jankovics2 and Imre Kacskovics1,*
1ImmunoGenes Ltd.; Budakeszi; Department of Virology; National Centre for Epidemiology; Budapest, Hungary

Key words: neonatal Fc receptor (FcRn), transgenic mouse, immunogenicity, monoclonal antibody, influenza

The neonatal Fc receptor (FcRn) regulates IgG and albumin homeostasis, mediates maternal IgG transport, is active in phagocytosis and delivers antigen for presentation. We have previously shown that transgenic (tg) mice that have been created to overexpress bovine FcRn (bFcRn) demonstrate increased half-life of mouse IgG, significantly increased antigen-specific IgG in serum and augmented expansion of antigen-specific B cells and plasma cells after immunization. One of the interesting questions surrounding this enhanced immune response is whether these tg mice could effectively induce immune response to weakly immunogenic antigens. To address this question, we immunized these bFcRn tg mice with a conserved hemagglutinin subunit 2 (HA2)-based synthetic peptide that was recently found to be effectively targeted by neutralizing antibodies. Using an ELISA system, we found that, whereas wild-type mice showed a weak immune response and developed only a de minimis amount of antibody against the epitope, FcRn overexpressing animals mounted a robust reaction expressed in specific antibody titers on day 28 that continued to rise through day 50. Consistent with our previous data, the enhanced immune response resulting from the FcRn overexpression was also associated with a substantial increase in the number of spleen derived B cells, dendritic cells, granulocytes and plasma cells. Based on this evidence, we propose that tg mice that overexpress bFcRn offer major advantages in monoclonal antibody production because the tg mice would allow the generation of antibodies (hybridomas) to weakly immunogenic antigens that otherwise would be difficult or even impossible to make.

Introduction

Monoclonal antibodies (mAbs) are essential biotechnology reagents widely used in every phase of the biomedical field from discovery research and diagnosis to therapeutics. More and higher-affinity mAbs are needed for clinical research and newer, improved, faster and more efficient technologies are needed to keep pace with the ever increasing demand for mAbs for use as therapeutic, diagnostic and research agents. In 1975, Kohler and Milstein first reported that B cells harvested from an immunized mouse could be immortalized by fusing them with established myeloma cell lines derived from the Balb/c mouse.1 The Balb/c mouse and its derived cell lines are still the current primary resource used for the generation of mAb producing cells.

The ability of immune complexes to induce potent humoral immune responses has long been known. A series of early experiments demonstrated the activating capacity of these complexes, finding them able to enhance antibody production.2-4 Keler et al. have shown that targeting foreign antigen to human FcγRI (CD64) in transgenic (tg) mice expressing human CD64 can overcome immunological non-responsiveness to a weak immunogen,5 but this approach was intended to facilitate human vaccination6 and not for routine use in hybridoma production. In this case, the approach is not feasible because antigens used in immunization should be combined with a specific targeting molecule.

The neonatal Fc receptor (FcRn) regulates IgG and albumin homeostasis, mediates maternal IgG transport, takes active part in phagocytosis and delivers antigen for presentation.7 We have previously shown that tg mice that have been created to overexpress bovine FcRn (bFcRn) demonstrate increased half-life of mouse IgG as a result of reduced clearance.8 In a more recent study we demonstrated that immunization of these tg mice with T-dependent antigens results in multifold increases of the antigen-specific IgG in serum and that the affinity of these antibodies was at least as good in transgenic mice as in the wild-type (wt) controls.9 We have also shown that FcRn overexpression not only extends the IgG half-life, but also dramatically enhances the expansion of antigen-specific B cells and plasma cells, which indicates a greatly augmented humoral immune response.9 Among the possible explanations for the increased B-cell activity is the much increased antigen specific IgG level in FcRn transgenic...
animals that results in more antigen-IgG immune complexes and thus mimic the natural mechanism to target the antigen to Fc receptors. Furthermore, FcRn overexpression potentially leads to augmented antigen processing in professional antigen presenting cells, which also increases B-cell activation. Current studies in our laboratory attempt to elucidate these mechanisms in greater detail. One of the interesting questions surrounding this augmented immune response is whether these tg mice would effectively induce immune responses to weakly immunogenic antigens.

Recent reports have demonstrated a conserved pocket in the stem region of the influenza hemagglutinin ectodomain that is effectively targeted by neutralizing antibodies to prevent membrane fusion.10,11 Little is known about the immune response to this region13 and this epitope is not particularly exposed in intact virus. We tested the immune competence of the bFcRn tg mice by immunizing them with this hemagglutinin subunit 2 protein (HA2)-based synthetic peptide and report our results here.

Results

Antigen selection. The selected oligopeptide consists of amino acids 41–57 of the α-helix of the influenza hemagglutinin subunit 2 (HA2), Influenza A/California/07/09 (H1N1) (Fig. 1A), which is the core binding site with which recently described neutralizing antibodies interact.10,11,13 The interacting residues of this epitope are highly conserved among the influenza A subtypes (Fig. 1B). We were also interested in predicting antigenicity of this oligopeptide. Although no infallible method to predict antigenic peptides exists, there are several rules that can be followed to determine which peptide fragments are likely to be antigenic. These rules also indicate increased odds of an antibody recognizing the native protein. Antibodies generated in this manner will recognize linear epitopes, and they may or may not recognize the source native protein, but they will be useful for standard laboratory applications such as ELISA assays or western blots. Perhaps the simplest method for the prediction of antigenic determinants is a tool developed by Kolaskar and Tongaonkar that is based on the occurrence of amino acid residues in experimentally determined epitopes.14 This tool showed possible antigenicity in the C-terminal of the oligopeptide (Fig. 1C), while a more comprehensive analysis—the Bepipred Linear Epitope Prediction test15—did not indicate potential B-cell epitopes within this sequence (Fig. 1D).

Potent HA2-specific response was generated only in the bFcRn tg mice. Since peptides alone generally are not able to trigger proper immune response, HA2 peptide was conjugated to KLH via an extra cysteine residue and injected intraperitoneally with Freund’s adjuvant into bFcRn tg and wt mice (Fig. 2). Anti-peptide IgM and IgG titers were determined by ELISA and fundamental differences were detected between tg and wt animals. After the first immunization, all five of the tg, but none of the non-tg littersmates, showed detectable HA2-specific IgG titers. After the first and second booster, constantly rising anti-peptide IgG titer was detected in the sera of all the tg animals, while only one wt animals responded with low level of HA2-specific IgGs (Fig. 2A). In addition, while IgM titers of tg mice increased constantly, wt animals showed only low anti-peptide IgM titers that increased slightly after booster immunizations (Fig. 2B).

HA2-specific antisera did not elicit virus neutralization. We analyzed antibodies derived from tg and wt mice immunized by HA2-KLH and found that they did not elicit virus neutralization activity (data not shown). This was not unexpected given that peptide immunogens are limited in that they present short, linear epitopes that may not be recognized in a whole protein antigen.

Different, but appropriate KLH-specific immune response both in tg and wt mice. To exclude the possibility that the minimal peptide-specific immune response we observed in wt mice was due to improper immunization technique, we next measured the KLH-specific IgM and IgG titers from sera of tg and wt animals. Both tg and wt animals developed high anti-KLH IgM and IgG titers during the experiment, although anti-KLH titers were tripled in tg animals compared to wt mice (p < 0.001) (Fig. 3A and B).

Immunization resulted in substantial differences in cell populations in the spleen between tg and wt mice. We also observed that increased spleen size, along with its cell number, following immunization was more pronounced in tg animals than in wt controls (p < 0.001) (Fig. 4A and B).

To examine whether the elevated immune response and increased spleen size of the tg animals were associated with changes of the cellular composition of the spleen, cell populations from immunized tg and wt mice three days after the booster immunization were characterized by flow cytometry. We observed equal proportions of B (B220+) cells in tg and wt mice, while T (CD3+) lymphocytes were present in significantly lower proportion in the spleen of tg animals compared to their wt controls. Calculation of the total numbers of B and T cells, however, indicated that lymphocytes were present in higher numbers in the spleen of tg animals (Fig. 4C and D).

We also found that immunization significantly increased the number of neutrophil granulocytes (Gr1+/CD11b+) in both groups, and this change was greater in tg animals compared to wt controls (Fig. 5A). The elevated number of neutrophils was reflected also in the proportionate increase of these cell types among splenocytes. These results explained the proportional decrease of cells bearing B220, and those that were CD3+. (Fig. 4C and D). We also detected three times more dendritic cells (CD11b+/CD11c+) (Fig. 5B) and twice as many isotype switched plasma cells (CD138+/IgM) (Fig. 5C) in the spleen of the tg mice than in wt controls.

Discussion

There is great demand for increased hybridoma production efficiency and improvement of the ability to generate antibodies to weakly immunogenic antigens. Increased efficiency would reduce the number of immunizations, and thus the time to find useful hybridomas, and decrease the amount of valuable antigens and the number of animals needed per immunizations, thus lowering the upfront cost of producing mAbs. More importantly, new technologies that would allow the generation of antibodies
of the effects of anti-apoptotic gene expression on B-cell longevity by the prolonged IgG and IgM serum titers to sheep red blood cells in inoculated Bcl-2 transgenic mice; the numbers of splenocytes obtained from β-galactosidase-immunized Bcl-2 transgenic mice were subsequently increased compared to wild-type Balb/c mice.18,19 These results suggest that genes that inhibit apoptosis (endogenous or transgenic) may improve the efficiency of hybridoma production. Nevertheless, these mice are autoimmune-prone and thus they generate a large number of autoreactive B cells that make difficult to find, optimal antigen-specific clones. As a result, the advantage of using these mouse strains for the generation of (hybridomas) to weakly immunogenic antigens would allow the creation of antibodies that otherwise would be difficult or even impossible to make.

Previous publications have described the use of some spontaneous mutant and genetically modified mouse strains that improve the efficiency of hybridoma production in some cases. One such strain is the MRL/MpJ-lpr/lpr mouse, which has a spontaneously formed defect in the apoptosis regulatory gene Fas. Expression of the defected Fas leads to polyclonal B-cell lymphoproliferation and hypergammaglobulinaemia in these autoimmune-prone mice.16,17 Another example is the demonstration of the effects of anti-apoptotic gene expression on B-cell longevity by the prolonged IgG and IgM serum titers to sheep red blood cells in inoculated Bcl-2 transgenic mice; the numbers of splenocytes obtained from β-galactosidase-immunized Bcl-2 transgenic mice were subsequently increased compared to wild-type Balb/c mice.18,19 These results suggest that genes that inhibit apoptosis (endogenous or transgenic) may improve the efficiency of hybridoma production. Nevertheless, these mice are autoimmune-prone and thus they generate a large number of autoreactive B cells that make difficult to find, optimal antigen-specific clones. As a result, the advantage of using these mouse strains for the generation of
haptenated-protein and an influenza vaccine generated a multiple-fold increase of the antigen-specific IgM and IgG levels in serum and displayed two- to three-fold increases in antigen-specific B cells and plasma cells. We also found that bFcRn tg mice produce elevated numbers of antigen-specific hybridomas and that none of these mice displayed symptoms of adverse reactions up to 14 months (data not shown).

Among the possible explanations for the increased B-cell activity in these tg animals is the increased level of antigen-IgG immune complexes that result from the higher levels of antigen-specific IgG. It has been recently demonstrated that antigen-IgG immune complexes, formed in vivo from the antigen and pre-existing antibodies derived from the primary response, activate naïve B cells, inducing them to respond with accelerated kinetics and increased magnitude. Consistent with our previous report that FcRn overexpression boosts humoral immune response along with the increased number of the antigen-specific B cells, such mice, however, exhibit augmented antibody production with increased anaphylactic responses, and they develop spontaneous antinuclear antibodies (ANA) and fatal glomerulonephritis, which excludes use of these FcγRIIB-deficient mice for mAb production.

We recently demonstrated that the humoral immune response of bFcRn-overexpressing tg mice immunized with ovalbumin, antibodies to other antigens, particularly those that are not very immunogenic, awaits further investigation.

Another approach to force more effective immune response against antigens of interest is to take advantage of the ability of immune complexes to induce potent humoral immune responses. Keler et al. have shown that targeting foreign antigen to human FcγRI (CD64) in tg mice expressing human CD64 can overcome immunological non-responsiveness to a weak immunogen. Because antigens should be combined with a specific targeting molecule to accomplish this effect, this technology is better suited to facilitating human vaccination instead of use in hybridoma production. Blocking the negative feedback mechanism that downregulates the immune response in case of increased amount of immune complexes (i.e., elimination of the FcγRIIB), which inhibits B-cell activation, was another promising approach to enhance the humoral immune response along with the increased number of the antigen-specific B cells. Such mice, however, exhibit augmented antibody production with increased anaphylactic responses, and they develop spontaneous antinuclear antibodies (ANA) and fatal glomerulonephritis, which excludes use of these FcγRIIB-deficient mice for mAb production.

We recently demonstrated that the humoral immune response of bFcRn-overexpressing tg mice immunized with ovalbumin, antibodies to other antigens, particularly those that are not very immunogenic, awaits further investigation.

Another approach to force more effective immune response against antigens of interest is to take advantage of the ability of immune complexes to induce potent humoral immune responses. Keler et al. have shown that targeting foreign antigen to human FcγRI (CD64) in tg mice expressing human CD64 can overcome immunological non-responsiveness to a weak immunogen. Because antigens should be combined with a specific targeting molecule to accomplish this effect, this technology is better suited to facilitating human vaccination instead of use in hybridoma production. Blocking the negative feedback mechanism that downregulates the immune response in case of increased amount of immune complexes (i.e., elimination of the FcγRIIB), which inhibits B-cell activation, was another promising approach to enhance the humoral immune response along with the increased number of the antigen-specific B cells. Such mice, however, exhibit augmented antibody production with increased anaphylactic responses, and they develop spontaneous antinuclear antibodies (ANA) and fatal glomerulonephritis, which excludes use of these FcγRIIB-deficient mice for mAb production.

Figure 2. Immunization with HA2-KLH elicits potent anti-peptide immune response in bFcRn tg mice. Tg and wt mice were immunized with HA2 peptide conjugated to KLH in CFA and challenged in IFA on 21st and 42nd day without adjuvant. Sera were analyzed for HA2 and KLH-specific IgG and IgM. (A) HA2-specific IgG titers showed a substantial increase in tg mice compared to the negligible IgG titers of wt mice even before the booster immunization. (B) HA2-specific IgM titers of tg mice were elevated during the secondary immune response compared to wt mice. Each circle represents an individual mouse. Lines represent the mean ± SEM. (*p < 0.05; **p < 0.01; ***p < 0.001). All the experiments were repeated twice with similar results.

We recently demonstrated that the humoral immune response of bFcRn-overexpressing tg mice immunized with ovalbumin, haptenated-protein and an influenza vaccine generated a multiple-fold increase of the antigen-specific IgM and IgG levels in serum and displayed two- to three-fold increases in antigen-specific B cells and plasma cells. We also found that bFcRn tg mice produce elevated numbers of antigen-specific hybridomas and that none of these mice displayed symptoms of adverse reactions up to 14 months (data not shown).

Among the possible explanations for the increased B-cell activity in these tg animals is the increased level of antigen-IgG immune complexes that result from the higher levels of antigen-specific IgG. It has been recently demonstrated that antigen-IgG immune complexes, formed in vivo from the antigen and pre-existing antibodies derived from the primary response, activate naïve B cells, inducing them to respond with accelerated kinetics and increased magnitude. Consistent with our previous report that FcRn overexpression boosts humoral immune response in transgenic mice, we propose that the elevated antigen-specific IgM and IgG levels observed during the secondary immune response were the result of the more potent activation of naïve and memory B cells in tg mice (Figs. 2 and 3). In addition, it is possible that an increased number of dendritic cells in these tg animals contributed to the augmented immune response. FcRn expression in professional antigen presenting cells has been
recently described in reference 26–29. Although we have not
determined whether bFcRn is expressed by professional antigen
presenting cells, or if these cells differ in antigen presentation
compared to wt controls, the higher number of dendritic cells in tg
animals compared to the wt controls after immunization (Fig. 5)
suggests that these cells contribute to the augmented immune
response we observed. Our data showing robust neutrophil influx
in the spleen is also consistent with our earlier observations9 and
with those of others who showed that in the presence of antigen-
IgG immune complexes the main antigen-specific cells recruited
in draining lymph nodes were neutrophils.24 Since these tg mice
produced much more antigen-specific IgGs than the controls, we
concluded that the difference in the number of granulocytes we
observed (Fig. 5) could be explained at least partly by the greater
amount of immune complexes formed in tg animals. We believe
that these factors collectively contribute to the augmented anti-
gen specific B-cell production in bFcRn tg mice compared with
controls.

One of the interesting questions surrounding this augmented
immune response is whether the bFcRn tg mice would effectively
induce immune response to weakly immunogenic antigens.
To address this point, we selected a highly conserved heli-
cal region in the membrane-proximal stem of the influenza
hemagglutinin HA1 and HA2 that is effectively targeted by
neutralizing antibodies to prevent membrane fusion.10,11,13
Crystal structure analyses revealed that the neutralizing antibody
recognizes a conformational epitope between HA1 and HA2,
and that the epitope consists of two components: (1) the A helix, which accounts for most of the interacting surface and most of the polar contacts and (2) the HA1 region adjacent to the A helix, which makes primarily hydrophobic contacts with this antibody. The authors hypothesized that it may only be necessary to mimic the A helix as a linear peptide in any rationally designed antigen.10

A range of immunization techniques can be used for the successful production of antibodies. Generation of antibodies that recognize native three-dimensional epitopes require the use of whole native protein as immunogens. Peptides provide quick and cost-effective ways to generate an antigen necessary to begin an immunization protocol, particularly if native or recombinant protein may not be available as a source of antigen. Peptide immunogens are limited in that they present short, linear epitopes that may not be recognized in a whole protein antigen. On the other hand, they generally work well in ELISA, western blots and other applications in which antibodies recognize denatured proteins. Nevertheless, in many cases, it has been demonstrated that synthetic peptides are able to elicit appropriate immune responses and generate antibodies that react with the native protein. An excellent example is a recent report about vaccination with a synthetic peptide from the influenza virus hemagglutinin (another conserved segment of HA2) that provides protection against distinct viral subtypes.30 The segment of the A helix we selected (Fig. 1A and B) was predicted to be slightly antigenic by a commonly used analysis (Fig. 1C), while another more complex prediction did not find potential B-cell epitope within this sequence (Fig. 1D). Thus the synthetic version of the conserved HA2 epitope conjugated to KLH nicely served our intention to test the immune competence of the bFcRn tg mice.

Figure 5. Differences were observed between the spleen cell populations of tg and wt mice after immunization as revealed by FACS analysis. Granulocytes, dendritic cells and IgM/CD138+ cells (A–C, respectively) were present in significantly higher numbers in the spleen of tg mice. Absolute numbers of these cell types calculated based on the total spleen cell number showed multiple fold increase in tg animals. Values shown are the mean ± SEM.
Whereas wild-type mice showed a weak immune response and developed only a de minimis amount of antibody against the epitope, FcRn overexpressing animals mounted a robust reaction expressed in specific antibody titers on day 28 which continued to rise through day 50; titers were more than 13-fold higher in FcRn transgenic mice than in their wild-type counterparts (Fig. 2). To exclude the possibility that the minimal peptide-specific immune response we observed in wt mice was due to improper immunization technique or other technical errors, we next measured the KLH-specific IgM and IgG titers and found that both tg and wt animals developed high anti-KLH IgM and IgG titers during the experiment, where anti-KLH titers were tripled in tg animals compared to wt mice (Fig. 3). Consistent with our previous data, the enhanced immune response resulting from the FcRn overexpression was also associated with a substantial increase in the number of spleen derived B cells, dendritic cells, granulocytes and plasma cells (Figs. 4 and 5).

We demonstrated here that an influenza derived conserved peptide resulted in a significant antigen-specific IgG titer in the FcRn transgenic mice while wt Balb/c controls produced only minimal immune response. Based on this evidence, and the fact that these bFcRn tg mice generate high titer of antigen-specific IgGs against weakly immunogenic proteins and peptides, resulting in increased quantity and quality of hybridomas.

Materials and Methods

Mice. Female 10- to 12-week old wt and bFcRn transgenic mice on Balb/c background (Balb/c_tg5) were used. bFcRn transgenic Balb/c line was created by back-crossing the line#19 of bFcRn transgenic FVB/N mice to a Balb/c background. We used off-springs of tenth generation carrying five transgene copies. The generation and basic phenotype of the tg mice have been recently published. All animals were kept in the specified pathogen free (SPF) animal house of the University Eotvos Lorand, Budapest, in compliance with Institutional Animal Care and Ethics Committee-approved protocols.

Antigen and immunization. The selected oligopeptide sequence is N'-TQN AIN GIT NKV NSV IE-C' (HA2) and consists of the highly conserved amino acids 41–57 of the α-helix of the influenza hemagglutinin subunit 2, Influenza A/California/07/09 (H1N1). Epitope prediction was performed by B-cell Epitope Prediction Tools at IEDB Analysis Resource using Kolaskar & Tongaonkar Antigenicity and Bepipred Linear Epitope prediction tests. The oligopeptide was synthesized with a cysteine at the N-terminal end of HA2 sequence is N'-TQN AIN GIT NKV NSV IE-C' (HA2) and was covalently linked to the carrier protein keyhole limpet hemocyanin (KLH) using Imject Maleimide Activated Immunogen Conjugation kit according to manufacturer’s instructions. KLH, complete Freund’s adjuvant (CFA) and incomplete Freund’s adjuvant (IFA) were purchased from Sigma-Aldrich Company, Budapest, Hungary.

Tg and wt mice (age- and sex-matched; five in each group) were intraperitoneally immunized with 100 μg of the HA2 peptide-KLH conjugate (HA2-KLH) in CFA and challenged 21 days later with 100 μg of the conjugate in IFA. A second booster immunization was performed with 100 μg of the conjugate without adjuvant on the 42nd day. Blood samples were taken on days 0, 21, 28, 42, 50. Mice were sacrificed on the 50th day.

ELISA measurements of the antigen-specific immunoglobulin levels. High-binding ELISA plates (Costar 9018, Corning Inc., NY) were coated with HA2-peptide (5 μg/ml) or KLH (5 μg/ml), respectively in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) for 2 h at room temperature and then were washed with 0.1 M phosphate-buffered saline (PBS, pH 7.2) containing 0.05%Tween 20 (PBS-Tween) and blocked with PBS containing 1% BSA for 1 h at room temperature. After washing with PBS-Tween serially diluted serum samples were added to the wells and incubated for 1 h at room temperature. Each plate included standard controls of serially diluted antigen-specific immune sera. After washing, bound serum antibody was revealed by horse-radish peroxidase (HRP)-labeled goat anti-mouse IgM or IgG (1:4,000-fold dilution, Southern Biotechnology Associates Inc., Birmingham, AL). The peroxidase-conjugated antibodies were detected using tetramethyl-benzidine (TMB) (Sigma-Aldrich, Budapest, Hungary) as the substrate and optical density at 450 nm was measured with Multiscan ELISA Plate Reader (Thermo EC). Serial dilutions of each serum samples were applied and antigen-specific IgM or IgG titers as end-point titers or as half-maximal values respectively were determined by GraphPad Prism5 non-linear regression to the hyperbolic saturation function. Samples were assayed in duplicates.

Virus neutralization assay. Serum antibody titers against the HA2-KLH were measured by microneutralization assay detected with chicken red blood cell hemagglutination following standard procedures. Briefly, sera were heat inactivated at 56°C and serially diluted in 0.05 ml protein-free RPMI 1640. Virus [100 tissue culture infective doses (TCID) in 0.05 ml/well] was then added to the plates containing test sera, and they were incubated at 37°C for 1 h. After incubation, the virus-serum mixtures were transferred to MDCK monolayer plates, and virus was allowed to adsorb for 18–24 h. The neutralization mixture was then aspirated; the plates were re-fed with 0.1 ml of protein-free RPMI 1640 per well, containing 2 μg of trypsin per ml and incubated in 5% CO2 at 34°C for five days. Then, 0.025 ml of 1% chicken erythrocytes in PBS was added per well. After 1–4 h at room temperature, absence of macroscopically visible hemagglutination in a well was interpreted as neutralization. At least two microtiter rows were run for each serum sample. Neutralization titers were calculated, using the Karber method, as the dilution (in log2) giving 50% neutralization and expressed arithmetically as the reciprocal of the dilution.

Flow cytometry. Single-cell suspensions from the spleens were isolated and first incubated with anti-CD32/CD16 (clone 2.4G2) for 30 min. Then the cells were incubated with fluorochrome conjugated specific antibodies at 4°C for 50 min in staining buffer (PBS with 0.1% BSA and 0.1% sodium azide), washed twice, and then analyzed using a FACSCalibur equipped with...
were considered to differ significantly if p < 0.05. Values obtained from BD Pharmingen or eBioscience. Isotype controls were obtained from eBioscience (San Diego, CA). Anti-mouse CD45R/B220-PECy5, CD3-PE, IgM-FITC and CD11b-A647 were purchased from BD Pharmingen (San Diego, CA). Isotype controls were obtained from BD Pharmingen or eBioscience.

**Statistics.** Student's two-tailed t-test was used to evaluate the statistical significance of mean values of treatment groups. Values were considered to differ significantly if p < 0.05.

**References**

1. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975; 256:495-7.
2. Kunkl A, Klaus GG. The generation of memory cells. IV. Immunization with antigen-antibody complexes accelerates the development of B-memory cells, the formation of germinal centres and the maturation of antibody affinity in the secondary response. Immunology 1981; 43:371-8.
3. Lassie J, Courtier H, Hess MW, Stoner RD. Early and enhanced germinal center formation and antibody responses in mice after primary stimulation with antigen-isoelogous antibody complexes as compared with antigen alone. J Immunol 1971; 107:822-31.
4. Couleif PG, Van Snick J. Enhancement of IgG anti-carrier responses by IgG2 anti-hapten antibodies in mice. Eur J Immunol 1985; 15:793-8.
5. Keler T, Guyre PM, Vila F, Srinivasan K, van De Winkel JG, Deo YM, et al. Targeting weak antigens to CD64 elicits potent humoral responses in human CD64 transgenic mice. J Immunol 2000; 165:6738-42.
6. Keler T, He L, Graziano RF. Development of antibody-targeted vaccines. Curr Opin Mol Ther 2005; 7:157-63.
7. Ward ES, Ober RJ. Chapter 4: Multitasking by exploitation of intracellular transport functions the many faces of FcRNs. Adv Immunol 2009; 103:77-115.
8. Bender B, Bodrog L, Mayer B, Schneider Z, Zhao Y, Hammarstrom L, et al. Position independent and copy-number-related expression of the bovine neonatal Fc receptor alpha-chain in transgenic mice carrying a 102 kb BAC genomic fragment. Transgenic Res 2007; 16:613-27.
9. Cervenak J, Bender B, Schneider Z, Magna M, Carenea BV, Lilom K, et al. Neonatal FcR overexpression boosts humoral immune response in transgenic mice. J Immunol 2011; 186:959-68.
10. Eikert DC, Bhalla G, Ehliger MA, Friesen RH, Jongmeelen M, Throsby M, et al. Antibody recognition of a highly conserved influenza virus epitope. Science 2009; 324:246-51.
11. Su J, Hwang WC, Perez S, Wei G, Aird D, Chen LM, et al. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 2009; 16:265-73.
12. Yang H, Carney P, Stevens J. Structure and receptor binding properties of a pandemic H1N1 virus hemagglutinin. PLoS Curr 2010; 2:1152.
13. Throsby M, van den Brink E, Jongmeelen M, Poon LL, Alard P, Cornelissen L, et al. Heterosubtypic neutralizing monoclonal antibodies against H5N1 and H1N1 recovered from human IgM memory B cells. PLoS One 2008; 3:3942.
14. Kolaskar AS, Tongaonkar PC. A semi-empirical method for prediction of antigenic determinants on protein antigens. FEBS Lett 1990; 276:172-4.
15. Larsen JE, Lund O, Nielsen M. Improved method for predicting linear B-cell epitopes. Immuneun Res 2006; 2:2. DOI: 10.1186/1745-7580-2-2.
16. Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. Nature 1992; 356:314-7.
17. Elkob KB, Marshak-Rothstein A. B cells in systemic autoimmune disease: recent insights from Fas-deficient mice and men. Curr Opin Immunol 1996; 8:852-9.
18. Strasser A, Whitington S, Vaux DL, Bath ML, Adams MJ, Gery S, et al. Enforced BCL2 expression in Blymphoid cells prolongs antibody responses and elicits autoimmune disease. Proc Natl Acad Sci USA 1991; 88:8661-5.
19. Knott CL, Reed JC, Bodrug S, Saedi MS, Kumar AJ, Klaus-Reichel K. Evaluation of Bcl-2/B cell transgenic mice (B6) for hybridoma production. Hybridoma 1996; 15:365-71.
20. Peterson NC. Advances in monoclonal antibody technology: genetic engineering of mice, cells and immunoglobulins. ILAR J 2005; 46:314-9.
21. Takai T, Ono M, Hikida M, Ohmori H, Ravetch JV. Augmented humoral and anaphylactic responses in Fc gamma RI-deficient mice. Nature 1996; 379:346-9.
22. Bolland S, Yim YS, Tius K, Wakeland EK, Ravetch JV. Genetic modifiers of systemic lupus erythematosus in FcgammaRIIB(-/-) mice. J Exp Med 2002; 195:1167-74.
23. Tillier T, Kofe J, Kreschel C, Bause CE, Riebel S, Wickert S, et al. Development of self-reactive germinal center B cells and plasma cells in autoimmune Fc(gamma)RIIB-deficient mice. J Exp Med 2010; 207:267-78.
24. Maletto BA, Ropolo AS, Alignani DO, Liscovsky MV, Ranocchia RP, Moron YG, et al. Presence of neutrophil-bearing antigen in lymphoid organs of immune mice. Blood 2006; 108:3094-102.
25. Goins CL, Chappell CP, Shashidharamurthy R, Selvaraj P, Jacob J. Immune complex-mediated enhancement of secondary antibody responses. J Immunol 2010; 184:6293-8.
26. Qiao SW, Kohayashi K, Johansen FE, Solid LM, Andersen JT, Milford E, et al. Dependence of antibody-mediated presentation of antigen on FcRn. Proc Natl Acad Sci USA 2008; 105:9337-42.
27. Zhu X, Meng G, Dickinson BL, Li X, Mizoguchi E, Miao L, et al. MHC class I-related neonatal Fc receptor for IgG is functionally expressed in monocytes, intestinal macrophages and dendritic cells. J Immunol 2001; 166:3266-76.
28. Liu X, Ye L, Christianson GJ, Yang JQ, Roopenian DC, Zhu X. NF{kappa}B signaling regulates functional expression of the MHC class I-related neonatal Fc receptor for IgG via intronic binding sequences. J Immunol 2007; 179:2999-3011.
29. Mi W, Wanjie S, Lo ST, Gan Z, Pick-erk H, Ober RJ, et al. Targeting the neonatal Fc receptor for antigen delivery using engineered Fc fragments. J Immunol 2008; 181:7550-61.
30. Wang TT, Tan GS, Hai R, Pica N, Ngi L, Ekiert DC, et al. Vaccination with a synthetic peptide from the influenza virus hemagglutinin provides protection against distinct viral subtypes. Proc Natl Acad Sci USA 2010; 107:18979-84.
31. Frank AL, Puck J, Hughes BJ, Cate TR. Micronutralization test for influenza A and B and parainfluenza 1 and 2 viruses that uses continuous cell lines and fresh serum enhancement. J Clin Microbiol 1980; 12:426-32.
32. Wang Y, Addes KJ, Chen J, Geer LY, He J, He S, et al. MDMB: annotating protein sequences with Entrez's 3D-structure database. Nucleic Acids Res 2007; 35:298-300.
33. Chen J, Anderson JB, DeWeese-Scott C, Fedorova ND, Geer LY, He S, et al. MDMB: Entrez's 3D-structure database. Nucleic Acids Res 2003; 31:474-7.