American Journal of Transplantation xxx (xxxx) xxx

Contents lists available at ScienceDirect

American Journal of Transplantation

journal homepage: www.amjtransplant.org

Original Article

ABO-A antibody induction in mice is T cell-dependent, estrogen-independent, and modulated by CD22

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ARTICLE INFO

Keywords:
ABO antibodies in mice
natural antibodies in mice
ABO-incompatible transplantation
CD22
T cell-dependent vs T cell-independent antibodies

ABSTRACT

ABO antibodies pose barriers in transplantation but remain poorly studied. We investigated anti-A natural antibodies (nAbs) and induced antibodies (iAbs) in wild-type (WT), CD19KO, and CD22KO mice in the context of major histocompatibility complex-syngeneic or major histocompatibility complex-allogeneic stimulation by ABO-A blood cell membranes (BCM) from A-transgenic mice, or xenogeneic human (Hu-A) BCM. CD19KO mice failed to produce anti-A nAbs and iAbs. Syngeneic A-transgenic-BCM failed to stimulate anti-A iAbs in WT mice, in contrast to allogeneic A-transgenic-BCM and xenogeneic Hu-A-BCM. Hu-A-BCM failed to stimulate anti-A iAbs in CD4-T cell-depleted or CD4KO mice, reversed with CD4-T cell reconstitution. Although anti-A nAbs were absent in estrogen-receptor-α-deficient mice, anti-A iAbs were easily stimulated. Anti-A nAbs were higher in CD22KO than in WT mice, with pubertal females producing higher levels than males. Anti-A iAbs were stimulated in CD22KO mice by syngeneic A-transgenic-BCM or by Hu-A-BCM after CD4T cell depletion. We conclude that anti-A nAbs and iAbs are produced by B1a-cells. In WT mice, stimulation of anti-A iAbs requires exposure to nonself A-antigen together with foreign proteins and is T cell dependent. Without CD22-mediated inhibition, anti-A iAb stimulation does not require foreign protein and is T cell-independent. Anti-A iAbs are estrogen-independent, whereas anti-A nAbs are estrogen-dependent and could be elicited by estrogen in males.

Abbreviations: α-gal, Galα1,3Galβ1,4GlcNAc-R (α-gal); B6, C57BL/6; BALB, BALB/c; BCM, blood cell membranes; C3H, C3H/He; Hu-BCM, human blood cell membranes; iAbs, induced antibodies; KO, knockout; MHC, major histocompatibility complex; nAbs, natural antibodies; PBMC, peripheral blood mononuclear cells; Siglecs, sialic acid-binding immunoglobulin-like lectins; WT, wild-type.

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https://doi.org/10.1016/j.ajt.2025.03.001
Received 18 December 2024; Received in revised form 7 February 2025; Accepted 1 March 2025
Available online xxxx

1. Introduction

The ABO histo-blood groups are defined by ABH glycans that decorate many tissues including erythrocytes and vascular endothelium.[1-5] A- and B-antigen synthesis is mediated by glycosyltransferases that sequentially add fucose and either N-acetylgalactosamine or D-galactose residues, respectively, to glycolipid and glycoprotein core chains during embryonic life.[1,5-7] Natural antibodies (nAbs) with specificities to nonself ABH glycans are produced without known antigen exposure. ABO nAbs as well as induced ABO antibodies (iAbs) pose clinical risks in transplantation. ABO-incompatible organ transplants carry a high risk of rejection except during infancy when ABO nAbs are notably absent, and during which ABO-incompatible heart transplantation leads to donor-specific B cell tolerance.[8,9] ABO-incompatible transplantation can be undertaken safely, but a precise understanding of ABO nAbs and iAbs is necessary to optimize this strategy.

In contrast to proteins, exposure to glycans induces B cell responses described as occurring without $CD4^+$ T cell participation.[10] In T-independent-type-1 responses, complex glycans such as bacterial lipopolysaccharides, engage the B cell receptor and toll-like receptors to stimulate antibody production.[10-13] In T-independent-type-2 responses, extensive B cell receptor cross-linking by glycans containing repetitive epitopes is thought to provide strong B cell receptor signaling sufficient to stimulate glycan-specific B cell responses.[11-13] In mice, antibodies to repetitive A/B-antigens are thought to be produced by B1-cells residing in peritoneal and pleural cavities, among other locations[14,15] and have been reported to occur without $CD4^+$ T cell help.[10,16-19] However, ABO nAbs were not distinguished from iAbs. Moreover, this conclusion was based on studies in mice with inherent limitations, such as nu/nu mice[18] that produce extrathymic T cells[20-22] or CBA/xid mice that have impaired B cell maturation and antibody production.[23,24] Additionally, previous studies relied on chemically synthesized glycans such as trinitrophenyl-*Ficoll*,[25-29] which may not be appropriate surrogates for naturally occurring glycans.

We showed that anti-A nAbs are T cell independent and sex- and age-dependent, suggesting that sex hormones and/or chromosomes may regulate anti-A nAbs.[30] We further showed, in contrast, that anti-A iAbs are sex-independent. Here, we studied anti-A nAbs and iAbs in CD19-deficient mice to confirm their B1a-cell origin, and anti-A iAb production in response to stimulation with naturally occurring A-glycans. To study immunity to nonself A-antigens in a transplant-relevant setting, we previously generated A-antigen transgenic mice on C57BL/6 (B6) and BALB/c (BALB) backgrounds in which tissues and cells, including vascular endothelium, erythrocytes, and leukocytes, are decorated with A-antigens.[31] Here, we used A-transgenic mice to investigate anti-A production in response to stimulation by A-antigen in the context of syngeneic (A-transgenic-B6 into wild-type (WT) B6; A-transgenic-BALB into WT BALB), allogeneic (A-transgenic-B6 into WT BALB; A-transgenic-BALB into WT B6), and xenogeneic (human into mouse) cells.

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are cell surface proteins involved in the regulation of B cell signaling.[32,33] In mice, Siglec-g and CD22 are highly expressed by B1 and marginal-zone B cells,[34-36] B cell subsets reported to produce antibodies against T-independent antigens.[37-39] In humans, we found that anti-A/B antibodies are produced mainly by $CD27^+IgM^+$ B cells and that CD22 is more highly expressed in this splenic B cell subset in infants than adults, diminishing with age,[40,41] suggesting CD22 involvement in ABO-tolerance after ABO-incompatible infant transplantation. Despite CD22 being identified as an important B cell inhibitory molecule,[42,43] its potential role in anti-glycan antibodies remains unclear,[34,36,44-50] and its impact in ABO antibody responses has not been studied. Here, we studied CD22 and sex in anti-A antibody responses in mice.

2. Materials and methods

2.1. Mice

WT B6 (H-2^b), BALB (H-2^d), and C3H/He (C3H, H-2^k) mice were purchased from Charles River Laboratories (Quebec City, QC). Mice homozygous for B6.129S2-$Cd4^{tm1Mak}$ targeted mutation (CD4KO) and B6.129P2(C)-$Cd19^{tm1(cre)Cgn}$/J (CD19KO) and mice homozygous for B6N(Cg)-$Esr1^{tm4.2Ksk}$/J targeted mutation (estrogen receptor-α-deficient) on the B6 background were purchased from Jackson Laboratory. C57BL/6-$CD22^{tm1Lam}$/J mice (CD22KO) were kindly provided by Dr L. Nitschke, Erlangen, Germany. A-transgenic mice[31] were bred onto both B6 and BALB backgrounds; A-antigen expression was confirmed on various tissues, including erythrocytes and leukocytes.[31] Mice were used at 6 to 12 weeks of age unless otherwise noted.

2.2. Blood cell membrane (BCM) preparation

BCM were prepared from sex-mixed pooled human erythrocytes (Immucor Inc) of blood group ABO-A_1 (Hu-A-BCM), or sex-mixed whole blood from A-transgenic mice (A-transgenic-BCM).[51] Briefly, washed cells were lysed in hypotonic buffer. Following multiple centrifugations at 20 000 × *g*, the membranes were suspended in phosphate-buffered saline at 10% (v/v) and stored at −30 °C until thawed for injection.

2.3. Immunization

Mice were injected intraperitoneally with 100 to 150 μL of 10% v/v human ABO-A_1 (Hu-A-BCM), ABO-O (Hu-O-BCM), or mouse A-transgenic blood (A-transgenic-BCM), with incomplete Freund's adjuvant (1:1 mixture, Sigma-Aldrich). Mice received 3 weekly injections beginning at 6-7 weeks. In some experiments, the mice were first injected with syngeneic A-transgenic-BCM and 5 weeks later with Hu-A-BCM without adjuvant. Some B6 male mice were injected (twice per week on weeks 6 and 7) with 40-50 μg of β-estradiol in adjuvant (E2758, Sigma-Aldrich).

2.4. Heart transplantation

Seven-week-old B6, BALB, or C3H WT mice were transplanted with age- and sex-matched major histocompatibility complex

(MHC)-syngeneic or -allogeneic A-transgenic B6 or BALB hearts, as described previously;[52,53] graft rejection was indicated as cessation of graft pulsation on abdominal palpation, as described previously.[54]

2.5. T cell depletion

WT B6 mice were intraperitoneally injected (7-9 injections, on days −2, −1, 0, and thereafter twice per week) with 150 to 200 μg of purified antimouse CD4 (clone GK1.5) or CD8 antibody (clone 2.43, Bio-X-cell). T cell depletion was confirmed and monitored by flow cytometry staining of peripheral blood mononuclear cells at weeks 7, 8, 9, and 10.

2.6. $CD4^+$ T cell isolation and adoptive transfer

$CD4^+$ T cells were isolated and purified from spleens of A-transgenic mice using a $CD4^+$ T cell isolation kit (Easy-Sep, STEMCELL Technologies). After confirmation of purity by flow cytometry, $CD4^+$ T cells were injected via tail vein into 4-week-old male CD4KO mice at 8 to 12 $\times$ 10^6 T cells per mouse in 150 μL of 0.9% phosphate-buffered saline. Peripheral $CD4^+$ T cell reconstitution was assessed 2 weeks later by flow cytometry. These experiments were performed in male mice only due to previously reported high levels of anti-A nAbs in female CD4KO mice.[30]

2.7. Flow cytometry

Mouse splenocytes and peripheral blood mononuclear cells were labeled with rat monoclonal antibodies (fluorescein isothiocyanate (FITC)-anti-CD3, AlexaFluor647-anti-CD4, and PE-anti-CD19, eBioscience) and incubated for 30 minutes at 4 °C. Cells were acquired using BD-LSR-Fortessa Cell Analyzer and analyzed using FlowJo-7.6.4 software (Tree-Star Inc).

2.8. Hemagglutination assay

Before each A-transgenic-BCM or Hu-A-BCM injection, anti-A antibody titers were measured by incubating serially diluted serum with 1% to 2% (v/v) A-transgenic reagent erythrocytes in 96-well plates. After room temperature incubation for 1 hour (ImmunoSpot), the highest dilution with visualized agglutination was designated as the anti-A titer.[51]

2.9. Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software Inc). Student's *t* test compared groups for anti-A antibody production. We used the following to indicate the significance and nonsignificance of the test results: ns, not significant; *: $P \leq .05$; **: $P \leq .01$; ***: $P \leq .001$; ****: $P \leq .0001$.

2.10. Study approval

The University of Alberta Health Sciences Laboratory Animal Services approved animal protocols according to the guidelines of the Canadian Council on Animal Care.

3. Results

3.1. The role of B-1 cells: CD19KO mice lacking B1a-cells failed to produce anti-A nAbs and iAbs

Young adult B6 mice produce minimal anti-A nAbs, widely variable in later life (Fig. 1A); thus, we studied 7- to 8-week-old mice. We observed low levels of anti-A nAbs in nonimmunized WT B6, BALB, and C3H mice (Fig. 1B) and abundant anti-A iAbs after immunization with xenogeneic Hu-A-BCM (mean titer: 1/512, Fig. 1B, Supplementary Fig. S1) with no sex differences (Supplementary Table S1).

Anti-A antibodies are reported to be produced by B1a cells[15]; however, whether both anti-A nAbs and iAbs are produced by B1a cells has not been investigated. Using CD19KO mice lacking B1a cells,[55] we found that anti-A nAbs neither produced (up to 24 weeks) nor could anti-A iAbs be stimulated by xenogeneic Hu-A-BCM, whereas abundant anti-erythrocyte antibodies were induced by Hu-A-BCM (mean titer: 1/512, Fig. 1C).

3.2. The role of foreign protein: exposure to nonself A-antigen in syngeneic A-transgenic-BCM failed to stimulate anti-A iAbs

Galα1,3Galβ1,4GlcNAc-R (α-gal) is an important glycan in xenotransplantation.[56] In the absence of α-gal expression, anti-α-gal nAbs are produced, and stimulation of anti-α-gal iAbs requires the presentation of the nonself-glycan antigen together with xenogeneic protein.[57] Due to the similarity of α-gal and A/B-glycans,[58,59] we hypothesized that foreign proteins are likewise required for ABO iAbs. We tested if A-antigen exposure in the context of syngeneic and allogeneic proteins stimulated anti-A iAbs. WT B6 and BALB mice injected with syngeneic A-transgenic-BCM failed to produce anti-A iAbs (mean titer: 1/4), whereas injection of WT B6, BALB, and C3H mice with allogeneic A-transgenic-BCM stimulated abundant anti-A iAbs (mean titer: 1/128, Fig. 2A). We further examined whether allogeneic A-transgenic heart transplants would also induce anti-A iAbs. A-transgenic-B6 allografts left in situ stimulated abundant anti-A iAbs in both WT BALB and C3H recipients (mean titer 1/256) compared with recipients of syngeneic A-transgenic-B6 grafts and nontransplanted mice (mean titer: 1/4, Fig. 2B), confirming that A-antigen in the context of allogeneic protein was needed to stimulate anti-A iAbs. A-transgenic-B6 allografts transplanted into WT BALB or C3H recipients without preformed anti-A nAbs were rejected by 8 to 11 days after transplant, as expected, whereas A-transgenic-B6 grafts in syngeneic recipients survived for >100 days (Supplementary Fig. S2).

Carbohydrates that induce T-independent-2 immune responses have been shown to generate memory B cell responses,[60,61] suggesting that mice with pre-existing anti-A nAbs could have A-specific memory B-cells or "primed" B cells requiring less stimulation for antibody production than naïve B cells.[62] We tested whether syngeneic A-transgenic-BCM would stimulate anti-A iAbs in the setting of pre-existing anti-A nAbs, selecting mice with the highest anti-A nAb titers at age 12 weeks.

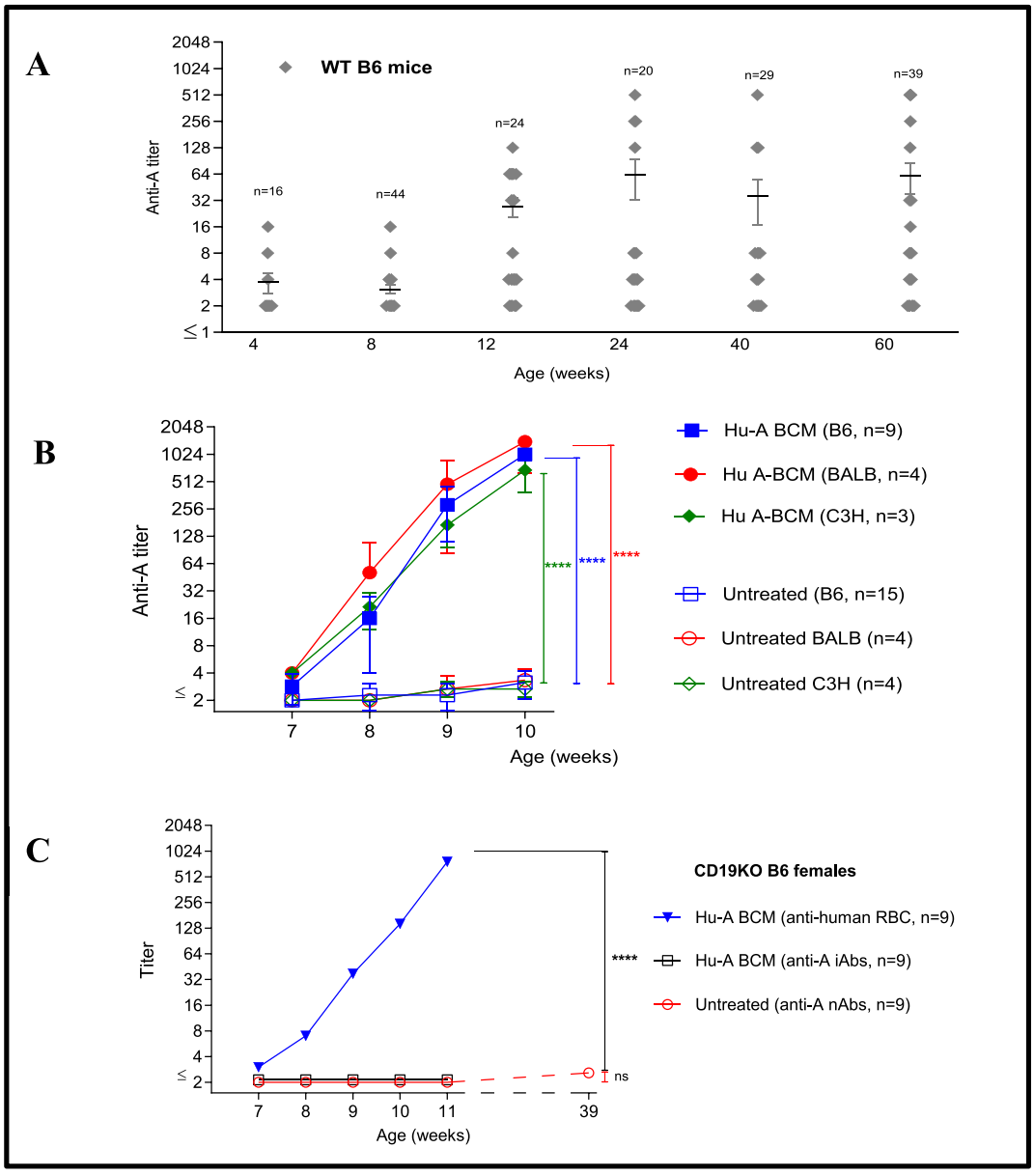

Figure 1. Several WT strains produced anti-A nAbs and iAbs; B1-deficient mice failed to produce both anti-A nAbs and iAbs (A) WT B6 mice (both sexes) produced low levels of anti-A nAbs in early life and widely variable in later life. (B) WT B6 (13 males, 15 females), BALB (4 males, 4 females), and C3H mice (5 males, 2 females) were untreated or injected with xenogeneic Hu-A-BCM [weekly ×3 injections at 7-10 weeks old (B6: 6 males, 3 females; BALB: 2 males, 2 females; C3H: 12 males, 1 female)]. Tail blood was collected at week 7, 8, 9, and 10 to measure anti-A nAbs and iAbs by hemagglutination assay using A-transgenic reagent erythrocytes. (C) CD19KO (B1a-deficient) mice were untreated (4 males, 5 females) or injected with xenogeneic Hu-A-BCM (5 males, 4 females) and assessed for anti-A antibodies by hemagglutination assay using A-transgenic reagent erythrocytes, and for anti-erythrocyte antibodies with human ABO-O reagent erythrocytes. Data are presented using standard error of mean (mean + SEM) in *t* test. ****$P \leq .0001$.

Similar to younger B6 mice without anti-A nAbs, syngeneic A-transgenic-BCM did not further increase anti-A iAbs (Fig. 2C), whereas Hu-A-BCM dramatically stimulated anti-A iAbs (mean titer 1/2048), suggesting that memory B cells do not respond to A-antigen in the absence of foreign proteins.

Vaccine studies showed that proteins are required for optimal anti-glycan antibody production.[63] Accordingly, we tested whether foreign proteins in xenogeneic Hu-O-BCM mixed with syngeneic A-transgenic-BCM could stimulate anti-A iAbs. Compared with xenogeneic Hu-A-BCM that induced abundant anti-A iAbs (mean titer: 1/1024), xenogeneic Hu-O-BCM/syngeneic A-transgenic-BCM (1:1) mixture did not induce anti-A iAbs (mean titer: 1/16, Fig. 2D), suggesting that the foreign protein mixture was not sufficient to render syngeneic A-transgenic-BCM immunogenic, consistent with a requirement for linkage of foreign protein with the glycan antigen.[64,65]

Failure of syngeneic A-transgenic-BCM to stimulate anti-A iAbs prompted us to examine whether exposure to A-antigen in syngeneic A-transgenic-BCM had induced unresponsiveness or tolerance to subsequent stimulation, as previously reported in

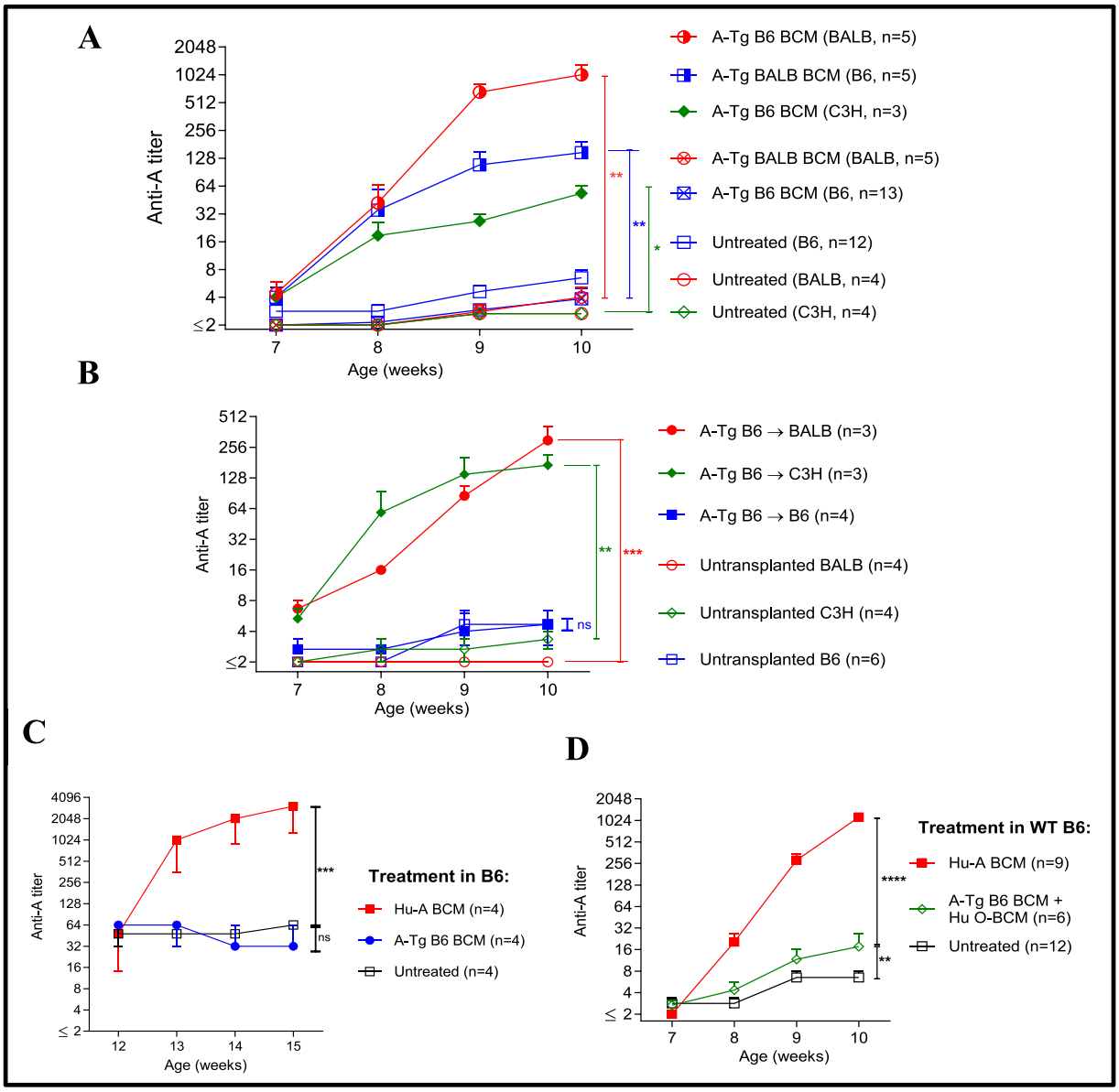

Figure 2. A-antigen in the context of syngeneic stimulation failed to induce anti-A iAb production in WT mice in contrast to A-antigen-expressing xenogeneic or allogeneic stimulation. (A) WT B6 (10 males, 7 females), BALB (4 males, 5 females), and C3H mice (5 males, 2 female) received 3 weekly injections of allogeneic (B6: 3 males, 2 females; BALB: 2 males, 3 females; C3H: 2 males, 1 female), or syngeneic A-transgenic (A-Tg) BCM (B6: 6 males, 7 females; BALB: 2 males, 3 females) and sera were assessed for anti-A antibodies. (B) Some B6, BALB, and C3H mice (all males) were transplanted with age-and sex-matched syngeneic (B6 and BALB) or allogeneic A-Tg hearts (B6, BALB, and C3H); A-Tg B6 heart grafts were left *in situ* and tail blood was collected to measure anti-A antibodies in B6, BALB, and C3H recipients. (C) Aged B6 mice (all males) with pre-existing anti-A nAbs received 3 weekly injections of syngeneic A-Tg BCM or Hu-A-BCM and tail blood was collected to assess anti-A antibodies. (D) B6 mice received 3 weekly injections of Hu-A-BCM (4 males, 5 females), or xenogeneic Hu-O-BCM mixed 1:1 with syngeneic A-Tg BCM (2 males, 4 females). Tail blood was assessed for anti-A antibody. Anti-A antibodies were measured and presented as described in the legend to Figure 1. Data are presented using standard error of mean (mean + SEM) in *t* test. ns, nonsignificant; $*P \leq 0.05$; $**P \leq .01$; $***P \leq .001$; $****P \leq .0001$.

α-gal-KO mice.[66] WT B6 mice failing to produce anti-A iAbs in response to syngeneic A-transgenic-BCM challenged 5 weeks later with Hu-A-BCM produced abundant anti-A iAbs (mean titer: 1/512, Supplementary Fig. 3A), indicating that the lack of response to A-antigen in the context of syngeneic cells could not be explained by this exposure having induced tolerance. Similarly, we examined whether early life introduction of A-antigen induced tolerance to subsequent challenges with Hu-A-BCM. We injected juvenile B6 mice with syngeneic A-transgenic-BCM or Hu-A-BCM. Five weeks after injection with A-transgenic-BCM, which did not stimulate anti-A iAbs, Hu-A-BCM were injected, which stimulated abundant anti-A iAbs (mean titer 1/512, Supplementary Fig. 3B), indicating that the lack of response to syngeneic A-transgenic-BCM could not be explained by tolerance induction, but rather, that A-transgenic-BCM provided insufficient stimulus for anti-A iAbs in syngeneic mice.

These findings indicate that stimulation of anti-A iAbs requires exposure to A-antigen in the context of foreign proteins (ie, the

requirement of allogeneic or xenogeneic antigens, implying the need for nonself-proteins together with nonself A-antigen).

3.3. The role of T cells: stimulation of anti-A iAbs required the participation of CD4$^+$ T cells

Our preliminary data showed that CD40-CD40 ligand was required to stimulate anti-A iAbs in WT mice.[67] Here, we examined the role of T cells in anti-A iAbs by depleting CD4$^+$ or CD8$^+$ T cells in WT mice. CD4-depleted B6 mice were unable to produce anti-A iAbs in response to Hu-A-BCM, in contrast to CD8-depleted or undepleted mice (mean titer 1/1024, Fig. 3A).

B cells in A-transgenic mice do not produce antibodies against self A-antigen;[31] however, it is less clear whether T cells are involved in tolerance to self-glycans.[68] Our previous data showed that CD4$^+$ T cells were required to stimulate anti-A iAb production in WT mice; thus, we tested whether adoptively transferred CD4$^+$ T cells from A-transgenic mice would provide help stimulating iAbs against self A-antigen. We used only male mice as we previously showed that CD4KO females produced high anti-A nAbs.[30] CD4KO males reconstituted with syngeneic sex-matched A-transgenic CD4$^+$ T cells (Fig. 3B), as with WT CD4$^+$ T cells,[30] produced anti-A iAbs in response to Hu-A-BCM stimulation (mean titer: 1/512, Fig. 3C). These data confirmed our previous observations and demonstrated that CD4$^+$ cells from

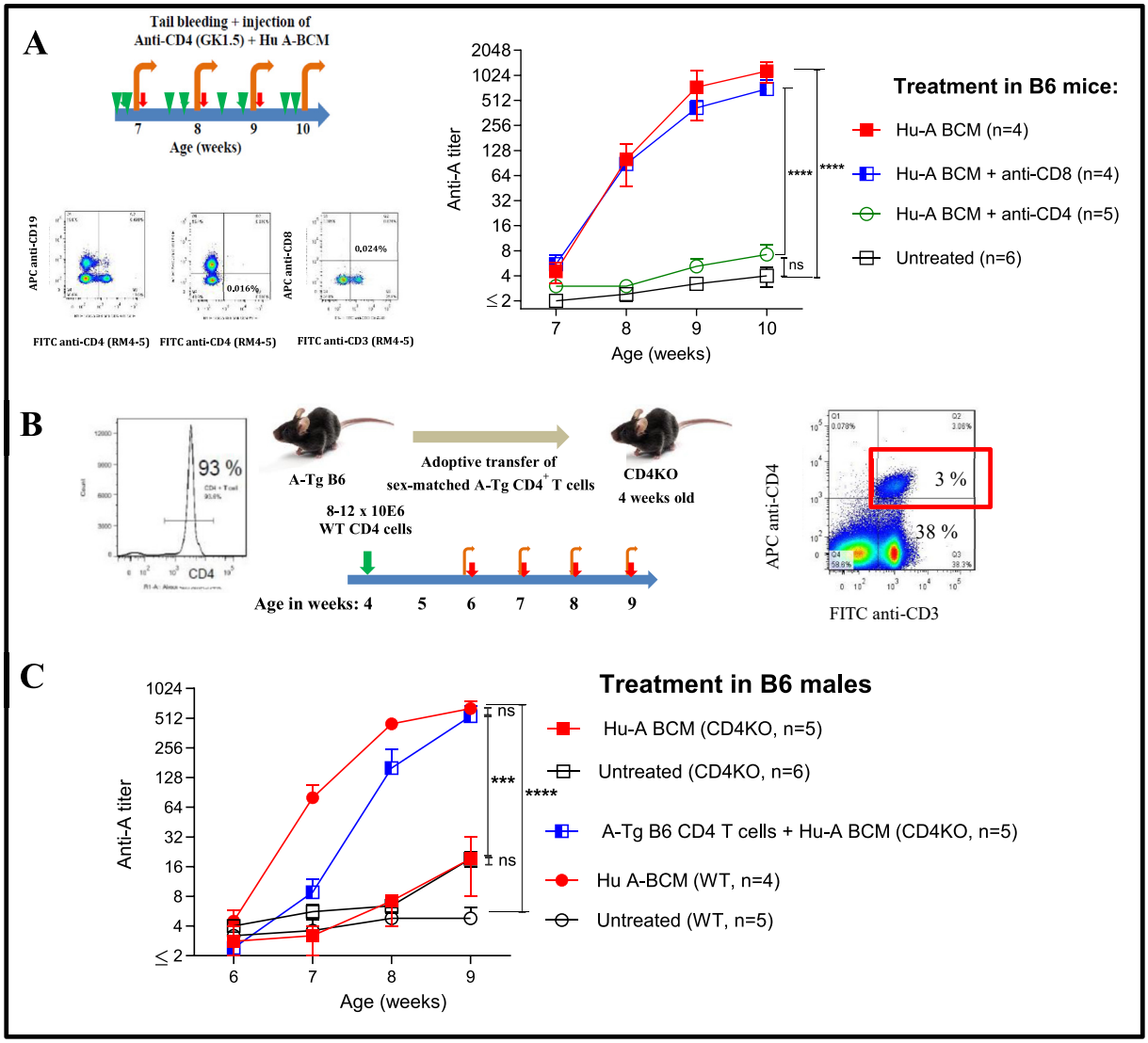

Figure 3. CD4$^+$ T cell depletion abolished the ability to induce anti-A antibodies in WT mice; adoptive transfer of CD4$^+$ T cells to CD4KO mice allowed stimulation of anti-A iAbs. (A) WT B6 mice (10 males, 9 females) received injection of GK1.5 anti-CD4 (250 μg, intraperitoneally twice weekly for 4 weeks, green triangles) and Hu-A-BCM (red arrows). Effective depletion of CD4$^+$ T cells was demonstrated by flow cytometry of peripheral blood mononuclear cells on week 10. Impact of CD4$^+$ (3 males, 2 females) and CD8$^+$ T cell depletion (2 males and 2 females) on stimulation of anti-A iAbs is shown. (B) CD4$^+$ T cells isolated from splenocytes obtained from A-transgenic (A-Tg) B6 male mice were examined by flow cytometry and injected into 4-week-old CD4KO male mice (8 to 12 $\times$ 10^6 cells, green arrow). Two weeks later, CD4$^+$ T cell reconstitution was assessed by flow cytometry. Mice were then injected with Hu-A-BCM ($\times$3, red arrows). (C) Shown is the impact of CD4$^+$ T cells isolated from A-transgenic mice on anti-A iAb production in sex-matched syngeneic CD4KO male mice. Anti-A antibodies were measured and presented as described in the legend to Figure 1. Data are presented using standard error of mean (mean + SEM) in *t* test. ns, nonsignificant; ***$P \leq .001$; ****$P \leq .0001$.

A-transgenic mice, which are tolerant to self A-antigen, provided help for induction of anti-A iAb, presumably engaged by foreign proteins expressed in allogeneic or xenogeneic BCM.

3.4. The role of estrogen: production of anti-A nAbs in mice was enhanced with estrogen

Our recent work with CD4KO females demonstrated the influence of sex on anti-A nAbs but not iAbs.[30] NAbs, mostly IgM isotype, are thought to be produced spontaneously by B1 cells[69,70] and have specificity for epitopes expressed on microorganisms[71,72] or host cells.[73,74] Estrogen was found to be responsible for the production of anti-*Escherichia coli* nAbs in female mice at puberty, and estrogen injection elicited anti-*E. coli* nAbs.[75] Analyzing anti-A production in female mice deficient for estrogen receptor-α, we similarly found complete absence of anti-A nAbs; however, high anti-A iAb production was induced by Hu-A-BCM (mean titer: 1/2048, Fig. 4A). Injecting prepubertal WT B6 males with estrogen, we found that anti-A nAb levels after puberty were 1-fold higher than untreated mice (Fig. 4B), similar to anti-*E. coli* nAbs.[75] These data indicate that estrogen enhances anti-A nAb production, presumably via estrogen receptor-α.

3.5. The role of CD22: in the absence of CD22-mediated inhibition, $CD4^+$ T cells were not required to stimulate anti-A iAbs and iAb production was not influenced by sex

There is inconsistency in the literature regarding the role of CD22 and Siglec-g in antibody production. CD22-deficient mice have more peritoneal cavity B1 cells than WT mice and higher levels of IgM nAbs,[34,36] with expansion of B1 cells and hyper-responsiveness of B cells.[36,44,45,76] CD22/Siglec-g-deficiency has been variably reported to enhance,[36,44,45,76] impair,[35,45-48] or have no impact on stimulation by chemically synthesized glycans,[34,35,49,50,76-78] which may not represent in vivo antibody production to biologic antigens. A further confounding factor may be the lack of differentiation between nAbs and iAbs.

We explored the absence of CD22-mediated inhibition on anti-A nAbs and iAbs. We first analyzed anti-A nAbs in CD22KO mice, finding that anti-A nAbs were higher than WT mice at all ages (Supplementary Fig. 4). In contrast to WT mice at 6-10 weeks old, a sex difference emerged in CD22KO mice at pubertal ages, with females producing 2-fold to 4-fold higher anti-A nAb levels than males (Fig. 5A); this difference diminished after puberty. In contrast, anti-A nAbs continued to increase in older WT females while plateauing in males after 5 months (Fig. 5B).

Examining anti-A iAbs in CD22KO mice, we found that Hu-A-BCM stimulated 3-fold to 4-fold higher anti-A production in both females and males than in WT mice (Fig. 5C, D), reflecting the hyper-responsiveness of CD22KO mice. We examined if A-antigen alone would induce anti-A production in the absence of CD22 by stimulating with syngeneic A-transgenic-BCM. Unlike WT mice, syngeneic A-transgenic-BCM induced anti-A iAb production (mean titer: 1/512-1/2048, Fig. 5C, D). Thus, in the absence of CD22-mediated inhibition, a foreign protein known to engage $CD4^+$ T cell help was not necessary for anti-A iAb stimulation by nonself A-antigen alone. To confirm our results, we depleted $CD4^+$ T cells in CD22KO mice. We found that Hu-A-

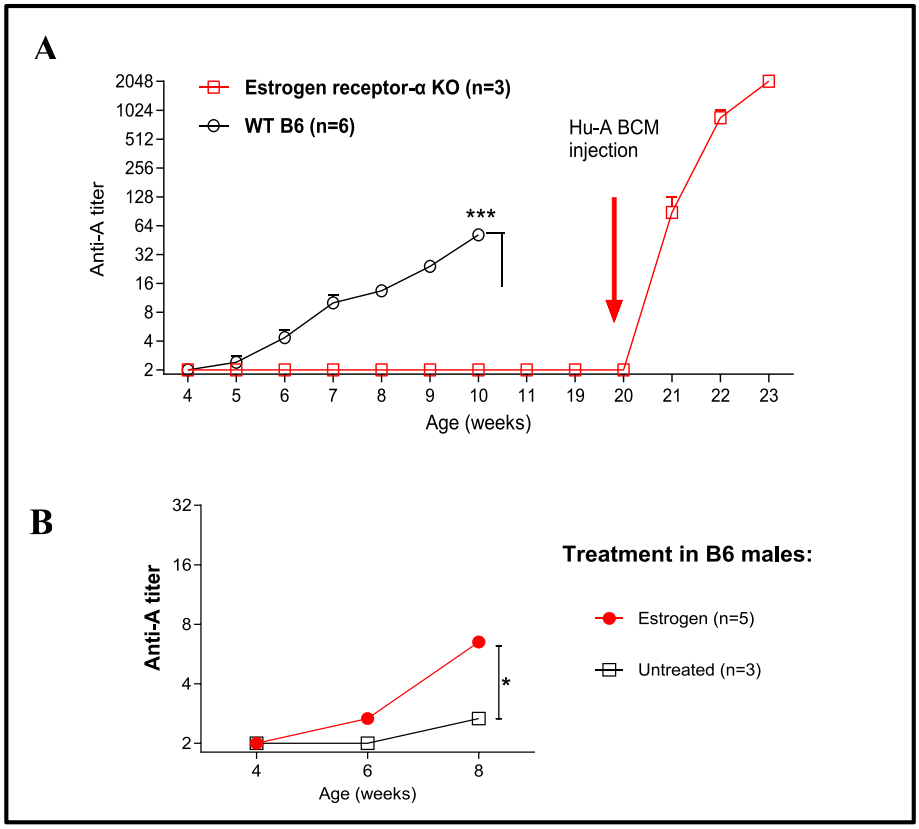

Figure 4. Estrogen receptor-α deficient females failed to produce anti-A nAbs, but not anti-A iAbs, and estrogen injection in males enhanced anti-A nAbs. (A) Anti-A nAbs were monitored in WT and estrogen receptor-α deficient females. Hu-A-BCM were injected in 20-week-old estrogen receptor-α deficient females. (B) WT B6 males were intraperitoneally injected with estrogen mixed with adjuvant [twice per weeks on weeks 6 and 7 (4 injections) of 40-50 μg of estrogen]. Anti-A antibodies were measured and presented as described in the legend to Figure 1. Data are presented using standard error mean (mean + SEM) in *t* test. ***$P \leq .001$.

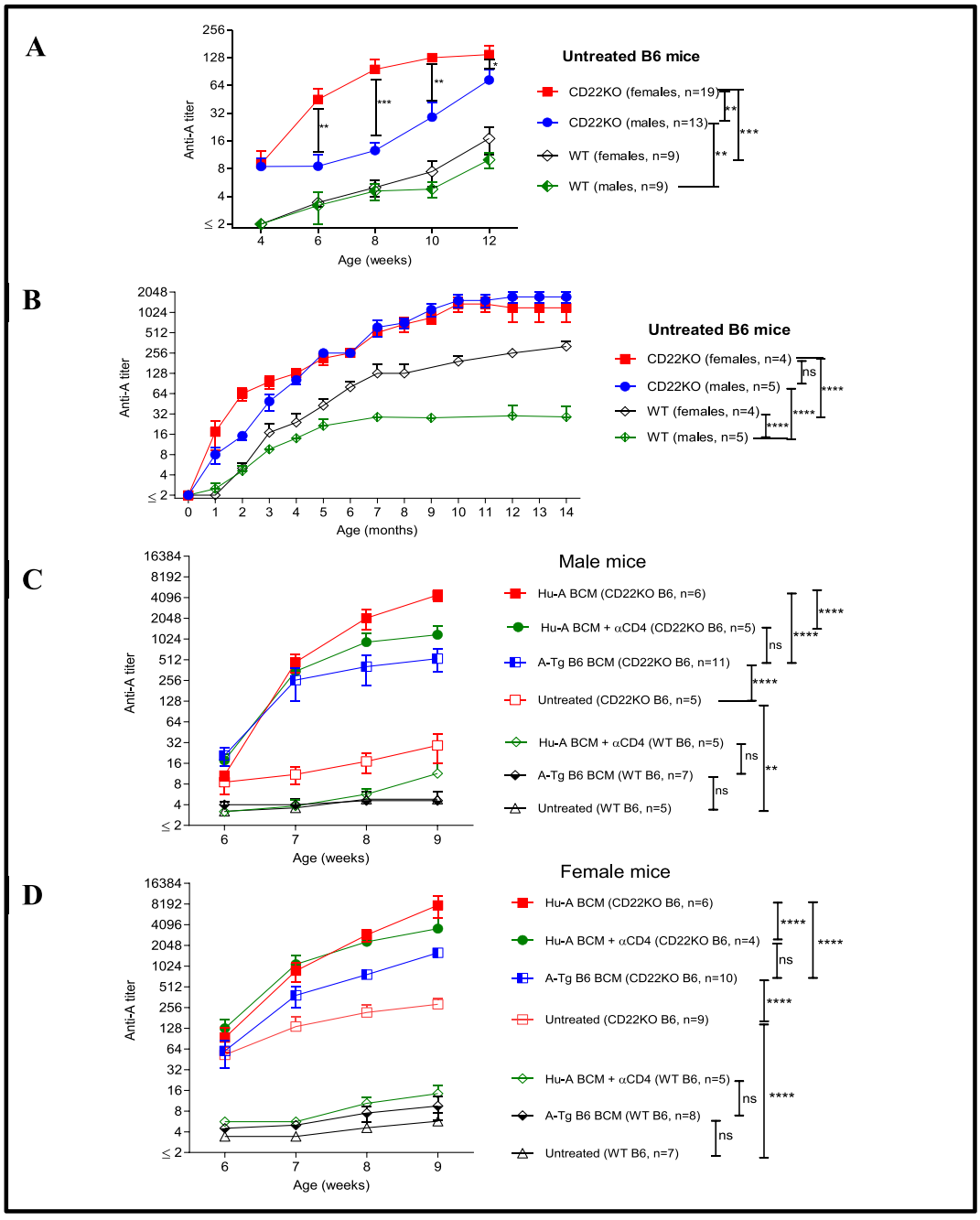

Figure 5. CD22KO and WT female mice developed higher anti-A nAbs than CD22KO and WT males, respectively. Stimulation of anti-A iAbs in CD22KO mice is $CD4^+$ T independent. (A) Measured longitudinally at 4-12 weeks of age, WT and CD22KO females developed significantly higher anti-A nAb titers than WT and CD22KO males, respectively. (B) Using some mice shown in A) for longer follow-up, there was no difference in anti-A nAb production between CD22KO males and females. However, higher anti-A nAbs were produced by CD22KO females than CD22KO males and WT mice during puberty, and by WT females than WT males. In males (C) and females (D), Hu-A-BCM injection stimulated massive anti-A iAb production in CD22KO mice. In addition, *syngeneic* A-Tg BCM injection stimulated anti-A iAb production in CD22KO mice. $CD4^+$ T cells were depleted by injection of GK1.5 anti-CD4 mAb (αCD4). After $CD4^+$ T cell depletion, Hu-A-BCM and A-Tg BCM injection stimulated anti-A iAbs in CD22KO mice but not in WT mice. Anti-A antibodies were measured and presented as described in the legend to Figure 1. Data are presented using standard error mean (mean + SEM) in *t* test. ns, nonsignificant; $*P \leq .05$; $**P \leq .01$; $***P \leq .001$; $****P \leq .0001$.

BCM injection stimulated anti-A iAbs independent of $CD4^+$ T cells, demonstrating that in the absence of CD22, $CD4^+$ T cells were not required for stimulation of anti-A iAbs (mean titer: 1/1024-1/2048, Fig. 5C, D).

Altogether, these data show that in the absence of the inhibitory CD22 co-receptor, high levels of anti-A nAbs are produced, especially prominent in females at puberty, and stimulation of anti-A iAbs is $CD4^+$ T cell-independent and sex-independent.

4. Discussion

Understanding the production of natural and induced antibodies to ABH glycans, an important goal in ABO-incompatible transplantation, is hampered by inherent limitations in available mouse models. In this study, we used A-antigen transgenic mice to explore the roles of B-1a cells, allogeneic proteins, sex, estrogen, and the B cell inhibitory co-receptor CD22 in the generation of anti-A nAbs and the stimulation of iAbs. We showed that WT mice produced anti-A iAbs in abundance in response to stimulation by Hu-A-BCM and that these antibodies were derived from B1a-cells. Stimulation of WT mice by allogeneic A-transgenic-BCM or heart grafts also induced abundant anti-A iAbs. In contrast, there was no response to syngeneic A-transgenic-BCM either alone or coinjected with Hu-O-BCM. $CD4^+$ T cell depletion in WT mice abolished the ability of Hu-A-BCM to induce anti-A iAbs, which was restored in CD4KO mice after reconstitution with $CD4^+$ T cells. In the absence of CD22 signaling, sex emerged as an important variable in anti-A nAb production, where higher levels of nAbs were produced in CD22KO females than in males, WT mice, or estrogen receptor-α-deficient females. Moreover, in the absence of CD22, stimulation of anti-A iAb production no longer required $CD4^+$ T cells, but T cell help resulted in higher anti-A iAb production.

We previously showed that CD4KO females spontaneously produced very high titer anti-A nAbs,[30] rendering females unsuitable for T cell transfer, as we are unable to distinguish anti-A nAbs vs iAbs. Nevertheless, anti-A Abs induction in female mice is also $CD4^+$ T cell dependent as injection of syngeneic A-transgenic-BCM failed to induce anti-A, in contrast to allogeneic A-transgenic or xenogeneic Hu-A BCM, indicating that foreign protein known to engage $CD4^+$ T cells is required. Additionally, anti-A iAb stimulation by xenogeneic Hu-A BCM was abolished after $CD4^+$ T cell depletion, confirming that $CD4^+$ T cells are required for anti-A iAb production.

In contrast to previous studies,[25–29] we used biologically relevant ABH glycans. Moreover, we differentiated between nAbs and those induced with intentional stimulation by A-antigens.[17,18,67,79,80] We showed that mice not only developed anti-A nAbs to nonself A-antigens as in other mammals,[31,81,82] but they also produced anti-A iAbs in response to A-antigen in the context of allogeneic and xenogeneic stimulation. Syngeneic A-transgenic-BCM not inducing anti-A iAbs is consistent with our observation that syngeneic A-transgenic heart grafts are not immunogenic in WT mice. Furthermore, the lack of anti-A iAb response in WT mice with pre-existing anti-A nAbs suggests that memory B cells also do not respond to A-antigen in the context of self-protein. Our finding that syngeneic A-transgenic-BCM mixed with xenogeneic Hu-O-BCM did not induce abundant anti-A iAbs, compared with xenogeneic Hu-A-BCM, is consistent with the requirement for conjugation of protein/carbohydrates for optimal stimulation of antibodies to glycoconjugate vaccines.[63,83]

Others showed that stimulation of α-gal-KO mice with α-gal-antigen induces α-gal-specific B cell tolerance.[57,66,84] However, the lack of response to syngeneic A-transgenic-BCM in our studies did not reflect tolerance to A-antigen because subsequent injection with xenogeneic Hu-A-BCM induced anti-A iAbs. Rather, anti-A iAbs to Hu-A-BCM stimulation after the initial failure to respond to A-transgenic-BCM indicates that the initial challenge with syngeneic A-transgenic-BCM was not sufficiently immunogenic to stimulate a B cell response.

Induction of a B cell response to A-antigen in WT mice by xenogeneic Hu-A-BCM, allogeneic A-transgenic-BCM and heart grafts, or after reconstitution of CD4KO mice with $CD4^+$ T cells, in addition to abrogation of these responses by depleting $CD4^+$ T cells, suggests a requirement for $CD4^+$ T cells to stimulate anti-A iAbs. Thus, in contrast to the commonly held paradigm that T cells are not required for antibody responses to carbohydrate antigens,[17,85] we found that anti-A antibody stimulation requires protein and $CD4^+$ T cell participation, similar to glycoconjugate vaccines.[86-88] Furthermore, glycoconjugate vaccines with a specific carrier protein (such as tetanus toxoid) prime the recipient, who responds strongly to subsequent vaccination with new carbohydrate vaccines conjugated to the same carrier protein.[89,90] Since these responses to glycoconjugate vaccines are $CD4^+$ T cell-dependent,[91] responding $CD4^+$ T cells are carrier protein-specific. Accordingly, we expect that some adoptively transferred T cells in our experiments are specific to xenogeneic proteins expressed in BCM.

In contrast to transferred $CD4^+CD25^+$ T cells,[30] reconstitution of CD4KO mice with total $CD4^+$ T cells rendered them responsive to stimulation by Hu-A-BCM, indicating that the normal concentration of $CD4^+CD25^+$ T cells within the total $CD4^+$ T cell population is not sufficient to suppress induction of anti-A antibodies. How many peptide-specific $CD4^+$ T cells are required to respond to xenogeneic peptides is unknown. Studies showed that 0.025% to 0.03% of total $CD4^+$ T cells are antigen-specific to glycoconjugate vaccines;[68] we speculate that the proportion of $CD4^+$ T cells responding to Hu-A-BCM would be similar. Without CD22-mediated inhibition, anti-A nAbs were higher than in WT mice at all ages; thus, CD22 suppresses anti-A nAb production. Increased anti-A nAbs may additionally be due to B cell hyper-responsiveness and/or B1-cell expansion.[36,44,45,76] As stimulation of anti-A antibodies in WT mice required $CD4^+$ T cells, the massive anti-A iAb production following xenogeneic Hu-A-BCM injection in CD22KO mice may be a result of the combination of $CD4^+$ T cell help, B cell "hyper-responsiveness," and/or B1-cell expansion.

In addition to the induction of anti-A iAbs with Hu-A-BCM, injection of CD22KO mice with syngeneic A-transgenic-BCM (with no foreign protein to engage T cells) induced anti-A iAb production, suggesting that the "hyper-responsive" B cells in CD22KO mice respond to A-antigen without $CD4^+$ T cell participation. Hu-A-BCM injection in T cell-depleted CD22KO mice induced comparable anti-A iAbs as induced by syngeneic A-transgenic-BCM. How B cells without CD22 respond to A-antigen in the absence of $CD4^+$ T cells is unknown but is consistent with previous reports that B cell FcγR coinhibitory receptor blockade reduced the requirement for T cell help.[92] We suggest that CD22 provides inhibitory signaling preventing anti-A iAb induction in WT mice, but this signaling could be overcome with T cell help engaged with foreign proteins in Hu-A-BCM and allogeneic A-transgenic-BCM (depicted in Fig. 6).

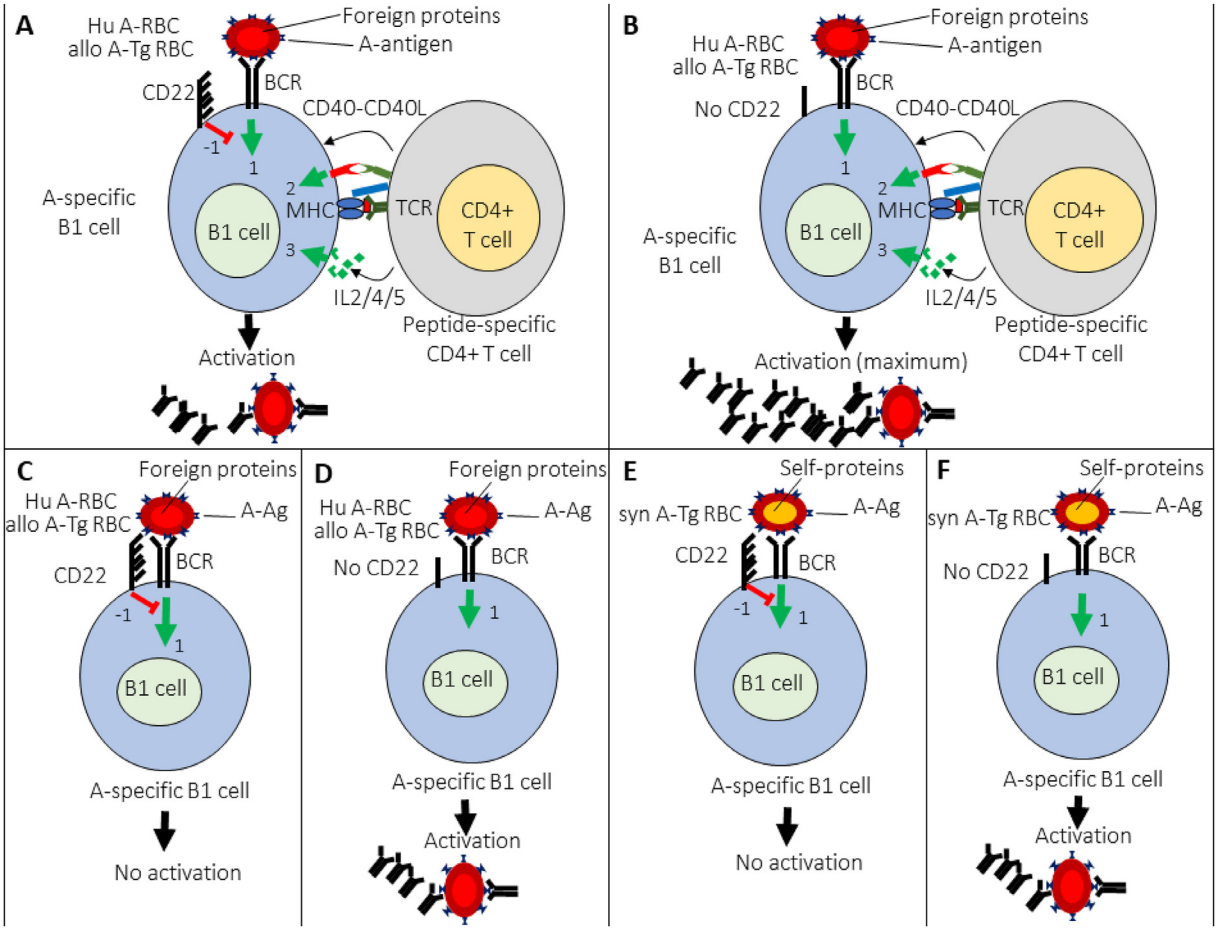

Figure 6. Illustration for impact of CD22 and $CD4^+$ T cells during anti-A antibody production in mice. (A) In a WT mouse, B cell stimulation by xenogeneic Hu-A BCM or allogeneic A-transgenic BCM induces anti-A antibody production due to engagement of $CD4^+$ T cell help presumably by foreign proteins expressed in xenogeneic Hu-A BCM or allogeneic A-transgenic BCM. $CD4^+$ T cell help can overcome the inhibitory signals provided by CD22 molecules expressed on B cells in WT mice. (B) In CD22-deficient mice, B cells are "hyper-responsive" to stimulation; injection of xenogeneic Hu-A BCM induces "hyper" anti-A production due to (1) induction of B cell receptor signaling by A-antigen, (2) lack of CD22-inhibitory signaling, and 3) $CD4^+$ T cell participation presumably induced by foreign proteins in xenogeneic Hu-A BCM or allogeneic A-transgenic BCM. (C) In the absence of $CD4^+$ T cell help (ie, $CD4^+$ T cell depletion), injection of xenogeneic Hu-A BCM (and allogeneic A-transgenic BCM) in WT mice does not induce anti-A production, possibly because of CD22 inhibitory signals that negate B cell-receptor stimulation by A-Ag. (D) In the absence of $CD4^+$ T cell help, injection of xenogeneic Hu-A BCM in CD22KO mice induces anti-A because A-antigen in xenogeneic Hu-A-BCM would stimulate B cell receptor in the absence of CD22-mediated inhibition. (E) Syngeneic A-transgenic BCM express A-antigen known to engage B cell receptor, but not $CD4^+$ T cell participation, due to absence of foreign protein. Therefore, injection of syngeneic A-transgenic BCM does not induce anti-A antibody production because CD22 expressed on B cells in WT mice provides an inhibitory signaling that prevents B cell activation. (F) Injection of syngeneic A transgenic-BCM (no foreign protein that could recruit $CD4^+$ T cells participation) in CD22KO mice induces anti-A antibody production because of "hyper-responsive" B cells in the absence of CD22 inhibitory signals. Therefore, anti-A antibody is stimulated by syngeneic A-transgenic-BCM to produce antibodies at comparable level with anti-A stimulated by Hu-A-BCM in CD4-depleted mice in (D). A-Ag, A-antigen; A-Tg, A-transgenic; BCR, B cell receptor; CD40L, CD40 ligand; RBC, Red Blood Cell; TCR, T cell receptor.

We previously showed that female mice produced higher anti-A nAbs than males,[30] suggesting that female sex hormones and/or chromosomes are important for nAb production. It was recently shown that anti-*E. coli* nAbs were produced in germ-free females during puberty in response to estrogen.[75] Similarly, estrogen also plays an important role in anti-A nAbs in WT females. Our findings that peripubertal CD22KO females produced higher anti-A nAbs than males, estrogen receptor-α-deficient females failed to produce anti-A nAbs, and estrogen injection elicited anti-A nAbs all suggest an influence of estrogen and/or sex chromosomes. Nonetheless, defining the precise interactions between estrogen, sex chromosomes, CD22, and ABO antibodies requires further investigation.

These data provide new insights into mechanisms of immunity to ABH antigens. A-transgenic mice on B6 and BALB backgrounds[31] allowed us to study immunity to naturally occurring A-antigens in a setting not possible in humans, and we showed that stimulation of anti-A iAbs is T-dependent, in contrast to anti-A nAbs. Additionally, we showed that when CD22-related inhibition is absent, $CD4^+$ T cells are not required to stimulate anti-A iAbs. Since ABO antibodies are reported to be produced by human B1 cells,[93] it may be possible to target these populations in patients

awaiting transplantation, expanding the potential donor pool to include ABO-incompatible organs.

Acknowledgments

This work was supported in part by a grant from Heart and Stroke Foundation of Canada, by contributions from the Gordon English family through the Stollery Children's Hospital Foundation and the Women and Children's Health Research Institute at the University of Alberta, and with in-kind support from the Canadian Donation and Transplantation Research Program. The authors thank Drs. Mylvaganam Jeyakanthan, Colin Anderson, and Robert Ingham for their insightful discussion and constructive feedback on this work.

Declaration of competing interest

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ajt.2025.03.001.

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