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Critical role of NKT Cells in Posttransplant Alloantibody Production

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Abstract

We previously reported that posttransplant alloantibody production in CD8-deficient hosts is IL-4$^+$CD4$^+$ T cell-dependent and IgG1 isotype-dominant. The current studies investigated the hypothesis that IL-4-producing NKT cells contribute to maximal alloantibody production. To investigate this, alloantibody levels were examined in CD8-deficient wild-type, CD1d KO and Jα18 KO transplant recipients. We found that the magnitude of IgG1 alloantibody production was critically dependent on the presence of type I NKT cells, which are activated by day 1 posttransplant. Unexpectedly, type I NKT cell contribution to enhanced IgG1 alloantibody levels was IFN-γ-dependent and IL-4-*in*dependent. Cognate interactions between Type I NKT and B cells alone do not stimulate alloantibody production. Instead, NKT cells appear to enhance maturation of IL-4$^+$CD4$^+$ T cells. To our knowledge, this is the first report to substantiate a critical role for type I NKT cells in enhancing *in vivo* antibody production in response to endogenous antigenic stimuli.

Introduction

Alloantibodies play a critical role in acute and chronic rejection after transplantation (1, 2). Acute antibody-mediated rejection is associated with worse graft outcome than T cell-mediated rejection (3). This suggests that conventional agents, targeting T cells (4, 5), suboptimally prevent the development or pathogenicity of alloantibody on allograft function and survival. Many factors have the potential to impact humoral alloimmunity after transplantation. Recipient and donor genetics impact the degree and specificity of alloantigen disparity (6-8), and influence the repertoire of cellular, cytokine and other

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factors which contribute to the resulting immune response (9, 10). The cells or organ to be transplanted determine the antigen load and expression of MHC and other molecules impacting the humoral immune responses evoked. Additionally, the site where the cells or organs are transplanted determines local microenvironmental factors such as resident cell populations, lymph nodes, and vasculature (11). Despite the importance of humoral alloimmunity in clinical transplantation, mechanisms mediating posttransplant alloantibody production and regulation are complex and not well understood.

A conceptual barrier to progress in understanding mechanisms regulating posttransplant humoral alloimmunity is the conventional focus on $CD4^+$ T cells as the dominant cell population influencing B cell antibody responses (12, 13). Using a well characterized *in vivo* model of posttransplant alloantibody production, we provided first evidence supporting a pivotal role for IFN-γ^+CD8^+ T cells in the inhibition of posttransplant IgG1 (IL-4-dependent) alloantibody production, in part, by downregulating the development of IL-4^+CD4^+ T cells (14) and, in part, by killing $IgG1^+$ B cells (15). Our data raise the possibility that current agents suppress T cell immune pathways which both promote ($CD4^+$ T cells) and downregulate ($CD8^+$ T cells) alloantibody production. In addition, other cells may be important mediators of alloantibody formation that are not currently targeted by current immunosuppressive therapies.

In our model, the IgG1 alloantibody generated is non-complement fixing and mediates antibody-dependent cell-mediated hepatocellular toxicity (ADCC). Our *in vitro* studies found that ADCC was mediated by macrophages, which was confirmed through *in vivo* studies where we found that survival of hepatocellular allografts was significantly prolonged in macrophage-deficient recipients, even in the presence of significant amounts of serum alloantibody (16). Studies by others also demonstrate a role for IgG1 in the induction of ADCC cytotoxicity and macrophage-mediated phagocytosis through FcγRIII (17-19).

Preliminary observations in our lab showing reduced alloantibody levels in CD8-depleted CD1d KO recipients suggested a novel role for NKT cells in promoting posttransplant alloantibody production. NKT cells, consisting of type I and type II NKT cell subsets, have a T cell receptor (TCR) that is activated by (glycol)lipid antigens presented through CD1d (20). CD1d, a MHC-like complex, is expressed on antigen presenting cells including dendritic cells, B cells and macrophages (21). Following type I NKT TCR binding to glycolipid antigen and CD1d, activated type I NKT cells can play an important role in the activation and regulation of multiple immune cells subsets including NK, T, and B cells (22-26). NKT cells have pleiotropic functions heavily influenced by microenvironmental factors (27). Type I NKT cells tend to be proinflammatory while type II NKT cells are anti-inflammatory and can downregulate type I NKT cells, as can T regulatory cells (28). While CD1d is identified as the dominant trigger for NKT cell activation, in some circumstances NKG2D may activate NKT cell function through interaction with RAE1, a MHC I like molecule (29). Of particular interest, it has been shown that type I NKT cells can induce antibody production in response to exogenous protein antigens in conjunction with α-Galactosylceramide (α-GalCer; the canonical CD1d ligand that stimulates type I NKT cells) (25, 26, 30-33). Type I NKT cells produce a variety of pro- and anti-inflammatory cytokines (IFN-γ, IL-4, IL-6, IL-13, etc.) and chemokines (RANTES, CCL22, CCL3, CCL4) (34). We

therefore hypothesized that type I NKT cells, without the requirement for exogenous NKT cell antigens or ligands, contribute to enhanced posttransplant IgG1 alloantibody levels through the production of IL-4 and perhaps other Th2 like cytokines which promote $CD4^+$ T cell maturation. However, our hypothesis proved to be incorrect since we unexpectedly found that IFN-γ^+NKT (and not IL-4^+NKT) cells are necessary to enhance the magnitude of alloantibody production in our model.

Materials and Methods

Experimental animals

FVB/N (H-2^q MHC haplotype, Taconic), C57BL/6 (wild-type; WT), and CD8 KO (both H-2^b, Jackson Labs) mouse strains (all 6-10 weeks of age) were used in this study. Jα18 KO mice (35) and CD1d KO mice (36) (H-2^b, both backcrossed >8 times onto a C57BL/6 background) were provided to Dr. Randy Brutkiewicz by Dr. Luc van Kaer (Vanderbilt University, Nashville, TN) with permission (for the Jα18 KO mice) from Dr. Masaru Taniguchi (Chiba University, Chiba, Japan). Transgenic FVB/N mice expressing human α-1 antitrypsin (hA1AT) were the source of “donor” hepatocytes, as previously described (37). All experiments were performed in compliance with the guidelines of the IACUC of The Ohio State University (Protocol 2008A0068-R1).

Hepatocyte isolation, purification, and transplantation

. Hepatocyte isolation and purification was completed, as previously described (37), by perfusing the liver with a 0.09% EGTA-containing calcium-free solution. The liver was then perfused with a 0.05% collagenase (type IV; Sigma Aldrich, St. Louis, MO) in 1% albumin. Liver tissue was minced, filtered, and washed with RPMI 1640 containing 10% FBS. Hepatocytes were purified on a 50% Percoll gradient (Sigma Aldrich). Hepatocyte viability and purity was consistently >95%. Donor FVB/N hepatocytes (2×10^6) were transplanted by intrasplenic injection with circulation of donor hepatocytes to the host liver, as previously described (37). Graft survival was determined by detection of secreted hA1AT in serial recipient serum samples by ELISA (37, 38). The reporter protein hA1AT does not elicit an immune response since syngeneic, hA1AT-expressing hepatocytes survive long term (37).

$CD8^+$ T cell depletion

Recipients were depleted of circulating $CD8^+$ T cells, by intraperitoneal (i.p.) injection of 100 μg of mAb (clone 53.6.72; day -2,-1), as described (14). Depletion was confirmed through flow cytometric analysis of recipient peripheral blood lymphocytes.

Type I NKT cells isolation and purification

Type I NKTs were isolated from liver mononuclear cells (LMNCs) obtained from syngeneic C57BL/6 mice. LMNC isolation was performed as described (39). In brief, the liver was perfused with PBS. Liver tissue was minced, filtered, and washed with PBS (2% FBS, 0.02% sodium azide). LMNCs were purified by a 33.75% Percoll gradient. Type I NKT cell staining and sorting was performed as described (40). Briefly, isolated LMNCs were Fc receptor blocked (2.42G hybridoma supernatant), washed, and with PBS-57-loaded APC-conjugated CD1d tetramers (1:2000; NIH NIAID Tetramer Facility, Emory University

Vaccine Center, Atlanta, GA). Additional samples of LMNCs were unstained or stained with unloaded APC-conjugated CD1d tetramers (1:2000) for flow cytometric gating purposes. Cells were sorted at The Ohio State University Comprehensive Cancer Center's Flow Cytometry Core Laboratory using FACSAria (Becton Dickinson, Franklin Lake, NJ). PBS-57-loaded CD1d positive cells represented the type I NKT cell population. In general, this method yielded approximately 3 million LMNC's per liver. LMNC's ranged from 10%-30% tetramer positive type I NKTs. Type I NKT cells (>98% pure) were pooled from multiple mice for AT.

ELISPOT

Analysis of B cells for IgG1 production by ELISPOT was performed as previously described (41). Plates were analyzed by computer-assisted image analysis using a KS ELISPOT Automated Reader with KS ELISPOT software 4.2 (Carl Zeiss Inc, Thornwood, NY). The data are reported as the number of relative Spot Forming Cells (SFC) per $1x10^6$ splenocytes. A side-by-side ELISA was run in a similar fashion and performed as previously described (42). Colormetric analysis was utilized to quantitate *in vitro* antibody production by a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA).

Assay of allospecific antibody

Recipient serum was tested for allospecificity first by incubation with allogeneic FVB/N target splenocytes, as previously described (14). The percent binding of total IgG to splenocyte targets was determined by a second incubation with FITC-conjugated goat anti-mouse IgG Fc (Organon Teknika, Durham, NC) and analysis by flow cytometry. Alloantibody level is represented as the percentage of target cells labeled by secondary fluorescent antibody as described previously (12). *Total alloantibody and isotype titering*- To quantitate alloantibody titer, we reanalyzed the recipient serum, using published methods (43). Briefly, serum was serially diluted and incubated with allogeneic FVB/N target splenocytes. Splenocytes were then stained with FITC-conjugated goat anti-mouse IgG Fc or IgG isotypes (IgG1, IgG2b, IgG2c, IgG3; Bio-Rad Laboratories, Hercules, CA). The mean channel fluorescence was measured for each sample and the dilution that returned the mean channel fluorescence observed when the splenocytes were stained with the 1:4 dilution of naïve C57BL/6 serum was divided by two and recorded as the titer.

Intracellular cytokine staining

Splenocytes were isolated from transplant recipients (day 7) and incubated with Leukocyte Activation Cocktail (Becton Dickinson). Splenocytes were treated with anti-FcγR mAb and subsequently stained with anti-CD4 mAb (clone GK1.5; Becton Dickinson) and PBS-57-loaded APC-conjugated CD1d tetramers. Intracellular staining was performed following eBiosciences recommendations for IFN-γ (XMG1.2) and IL-4 (11B11; both Becton Dickinson). $CD4^+NKT^-$ T cells were utilized for gating.

Statistical analysis

General linear models were fit for each outcome and contrasts were used to compare relevant groups to test the primary hypothesis/hypotheses in each experiment. Model

assumptions were assessed and violations to the normality assumption were addressed by transforming the data to the natural log scale. Where log transformations did not resolve the violation of the normally distributed residuals assumption, non-parametric hypothesis testing using the Wilcoxon rank-sum test were conducted (**Figure 1B** and **3B**). In cases where multiple group testing is conducted for an outcome, the overall type 1 error rate was maintained at 5% using Holm's step-down procedure to adjust for multiple comparisons. All analyses were conducted using SAS Statistical Software Version 9.3 (SAS Institute, Inc., Cary, NC). Summary statistics are listed as the mean plus or minus the standard error.

Results

Type I NKT cells significantly contribute to the magnitude of alloantibody production posttransplant

We previously reported that CD8-deficient transplant recipients are high IgG1 alloantibody producers which is IL-4-dependent (no antibody was detected in CD8-depleted IL-4 KO recipients). AT of WT (IL-4$^+$) CD4$^+$ T cells into CD8-depleted IL-4 KO mice restored alloantibody production but not to levels expected in CD8-depleted WT recipients (14). This suggests that other IL-4$^+$ cells are required for high alloantibody production. To determine if NKT cells, known strong producers of IL-4 (20), contribute to maximal alloantibody in CD8-deficient high alloantibody producers, CD1d KO recipients [NKT-deficient; (36)] were CD8-depleted (day -2,-1 with respect to transplant) and transplanted with allogeneic hepatocytes. Serum samples were collected to measure alloantibody (by two methods-percent allogeneic target splenocytes bound and by serum dilution to calculate alloantibody titer). We found day 14 alloantibody levels were significantly reduced in CD8-depleted CD1d KO (34.8±4.0%; titer=333±39) compared to CD8-depleted WT recipients (76.9±3.4%; titer=1,800±89; **Figure 1A, 1B**) on day 14 posttransplant. Despite alloantibody reduction in CD8-depleted CD1d KO recipients, rejection was not delayed (MST= day 14) compared to CD8-deficient WT (MST= day 14) or CD8-sufficient CD1d KO recipients (MST= day 10; $p>0.05$ ns for both). To determine if a deficiency in type I NKT cells was responsible for reduced alloantibody levels, we performed similar experiments utilizing Jα18 KO (type I NKT cell-deficient) recipients. Serum alloantibody levels in CD8-depleted Jα18 KO recipients (6.6±2.4%; titer=22±2) were markedly reduced compared to CD8-depleted WT recipients (76.9±3.4%; titer=1,800±89) and CD8-depleted CD1d KO recipients (34.8±4.0%; titer=333±39). This reduction in alloantibody was accompanied by delayed allograft rejection in CD8-deficient Jα18 KO recipients (MST= day 17) compared to CD8-sufficient Jα18 KO recipients (MST= day 10; $p<0.001$). Serial analysis of alloantibody levels in CD8-depleted WT and Jα18 KO recipients showed both exhibited peak circulating alloantibody levels on day 14-21 posttransplant (data not shown), consistent with our previous studies (14). Therefore, the difference in quantity of alloantibody between the two groups is not due to a difference in the kinetics of alloantibody production. Additionally, as previously reported (14), alloantibody isotype is IgG1-dominant with minimal contribution of IgG2b, IgG2c, and IgG3 (**Figure 1C**).

To determine if the higher magnitude of alloantibody produced in type I NKT cell-sufficient recipients was due to higher numbers of antibody-producing B cells (versus higher

production of antibody per B cell), we analyzed the number of B cells that produced IgG1 antibody within WT and Jα18 KO recipient splenocytes by ELISPOT on days 4, 8, 11, and 15 posttransplant. CD8-depleted WT recipient splenocytes exhibited significantly more IgG1-producing cells (spot forming cells, SFC) per 10^6 cells analyzed on days 8 (288±42) and 11 (266±53) posttransplant compared to naïve WT mice (45±15; **Figure 2**). CD8-depleted Jα18 KO recipient splenocytes exhibited significantly reduced numbers of IgG1-producing cells per 10^6 cells on day 8 (169±28) and 11 (94±30) posttransplant compared to CD8-depleted WT recipient splenocytes. Notably, splenocytes from Jα18 KO and WT recipients have similar numbers of B cells ($B220^+$; 41.1±3.5% versus 39.1±7.5%, respectively; $p>0.05$). Although not quantitative, the magnitude of IgG1 produced per B cell, determined by spot size, was similar between WT and Jα18 KO recipients, suggesting that B cells from both groups secreted similar amounts of IgG1 (data not shown). The significant difference in the number of IgG1-producing cells between CD8-deficient WT and Jα18 KO recipients strongly suggests NKTs play a critical role in enhancing alloprimed B cell activation and/or maturation.

Type I NKT cell-enhancement of IgG1 isotype-dominant alloantibody production is IFN-γ- (but not IL-4-)dependent

AT studies were used to definitively establish an *in vivo* role for type I NKT cells in enhancing alloantibody production. Type I NKTs were transferred (1×10^6 cells i.v.) into CD8-depleted Jα18 KO mice immediately following allogeneic hepatocyte transplant, and serum samples collected for the measurement of alloantibody levels. We found AT of WT NKTs into CD8-depleted Jα18 KO recipients (33.3±1.7%; titer=142±11) significantly enhanced alloantibody production compared to control CD8-depleted Jα18 KO recipients (6.6±2.4%; titer=22±2; **Figure 3A, 3B**). AT of NKTs resulted in increased alloantibody production and faster allograft rejection in CD8-depleted Jα18 KO recipients (MST= day 10) compared to CD8-depleted Jα18 KO recipients without AT (MST= day 17; $p<0.05$). To determine whether type I NKTs required IL-4 to promote alloantibody production, Jα18 KO recipients received AT of IL-4-deficient (IL-4 KO) or WT NKTs. Surprisingly, IL-4-deficient NKTs (27.0±2.1%; titer=113±16) were equally capable of enhancing posttransplant alloantibody levels as WT NKTs. Contrastingly, IFN-γ-deficient (IFN-γ KO) NKTs did not enhance alloantibody production in Jα18 KO recipients (4.8±2.3%; titer=18±1). Alloantibody isotype is IgG1-dominant in Jα18 KO recipients AT'ed with WT or IL-4 KO type I NKTs with minimal contribution of IgG2b, IgG2c, and IgG3 (**Figure 3C**). Type I NKTs upregulated intracellular IL-4 and IFN-γ by flow cytometry as early as day 1 posttransplant (data not shown). These results support the conclusion that type I NKTs enhance posttransplant IgG1 alloantibody production in an IFN-γ-dependent (but IL-4-*in*dependent) manner.

Enhanced number of IL-$4^+$$CD4^+$ T cells in NKT-sufficient high alloantibody producers

To determine if NKT cell cognate interactions with B cells are sufficient to induce alloantibody production, we utilized MHC II KO recipients which are $CD4^+$ T cell-deficient. While others have reported that NKTs facilitate antigen-specific antibody production in MHC II KO mice in response to viral immunization (33), neither MHC II KO nor CD8-depleted MHC II KO recipients developed alloantibody posttransplant (**Figure S1**). Because

NKTs alone are insufficient to drive alloantibody production by B cells in our model, we hypothesized that NKTs drive the maturation of IL-4$^+$CD4$^+$ T cells. To address this hypothesis, CD4$^+$ T cells from CD8-depleted WT and CD8-depleted CD1d KO recipient mice were evaluated on day 7 for intracellular IFN-γ or IL-4 expression (no significant difference in splenocyte numbers between CD1d KO and WT recipients). Of note, CD4$^+$ type I NKTs constitute approximately 0.5% of all CD4$^+$ T cells within the spleen and less than 0.1% of all IL-4$^+$ and IFN-γ$^+$ CD4$^+$ T cells (data not shown). WT recipients exhibited a similar percentage of IFN-γ$^+$CD4$^+$ T cells (5.0%±0.4%) and IL-4$^+$CD4$^+$ T cells (1.1% ±0.3%) compared to CD1d KO recipients (IFN-γ$^+$CD4$^+$ T cells= 5.6%±0.5%, IL-4$^+$CD4$^+$ T cells= 1.5%±0.3%; **Figure 4**; representative data shown in **Figure S2**). In contrast, CD8-depleted CD1d KO recipients exhibited significantly fewer IL-4$^+$CD4$^+$ T cells (1.5%±0.2%) (and similar numbers of IFN-γ$^+$CD4$^+$ T cells, 4.3%±0.2%) compared to CD8-depleted WT recipients (IL-4$^+$CD4$^+$ T cells= 2.6%±0.2% and IFN-γ$^+$CD4$^+$ T cells= 5.2%±0.4%). This suggests that NKTs drive the maturation and/or proliferation of Th2 IL-4$^+$CD4$^+$ T cells.

Discussion

Preliminary studies in our well characterized transplant model suggested that cells other than CD4$^+$ T cells and B cells might be critical for maximal IgG1 alloantibody production. We and others have shown that depletion of CD8$^+$ T cells significantly increased antigen-specific antibody production in allergy, bacterial infection, viral infection, and platelet transfusion (15, 44-52). We reported a dominance of IgG1 alloantibody produced in CD8-deficient recipients (14) (confirmed in the current studies) but which differ from a report by Sayeh *et al.* which found an increase in IgG2a following CD8-depletion (53). This may be attributable to differences in the antigenic stimuli and other experimental conditions (repeated antigenic stimuli of five platelet infusions over five weeks). Interestingly, they reported increased IL-4 levels (expected to drive IgG1 production) in these CD8-depleted recipients.

In the current study, we found that IgG1 alloantibody levels in CD8-deficient recipients were reduced in the absence of CD1d or type I NKT cells. Furthermore, AT of type I NKT cells enhanced IgG1 alloantibody levels (and allograft rejection) in NKT cell-deficient recipients in an IFN-γ-dependent, IL-4-*in*dependent manner. This study provides the first evidence that type I NKT cells, responding to endogenous signals/stimuli, significantly contribute to IgG1 alloantibody production posttransplant. Reports by others also show a role for NKT cells in antibody production. However, the majority of these reports have occurred in the context of NKT cell activation by exogenous administration of α-GalCer (25, 26, 30-33, 54, 55) or other exogenous glycolipids including OCH (56), which is a glycolipid known to skew conditions toward Th2 conditions (57). Thus, another novel aspect of the current study is NKT-mediated enhancement of *in vivo* antibody production without concurrent exogenous glycolipid administration to activate NKT cells.

There are some aspects of the animal models used in this study which require consideration for interpretation of our data. **Figure 1** shows that Jα18 KO recipients produce less antibody than the CD1d KO recipients which might be due to altered TCR repertoire diversity in Jα18 KO mice (58). In addition, Jα18 KO recipients have inhibitory type II NKT cells (59) which

may downregulate antibody production, in contrast to CD1d KO recipients that lack all NKT cells. Nevertheless, AT studies clearly support that type I NKT cells significantly enhance alloantibody production posttransplant (though not to the levels observed in WT recipients). The lower levels may be due to the limited number of NKT cells transferred. However, increasing the number of type I NKT cells for AT (2×10^6) did not result in further enhancement of posttransplant alloantibody levels (data not shown). While impairment of *in vivo* trafficking may limit the availability of AT'ed type I NKT cells, previous reports (60), as well as our unpublished data, suggest that the majority of transferred immune cells do indeed traffic to the liver, spleen, and lymphoid tissue.

NKT cells may enhance antibody production through direct interaction with B cells or indirectly, by stimulating non-B cell APCs (e.g., DCs) and $CD4^+$ T cell activation (25, 26, 30-33). We know from previous studies that alloantibody production in our model is IL-4-dependent and IL-4^+CD4^+ T cells drive IgG1 alloantibody production (14). In AT studies we have demonstrated that alloantibody in this model is detrimental to graft survival, since AT of alloantibody into immunodeficient SCID hepatocyte recipients prompts rejection within 7 days (44). Furthermore our published *in vitro* and *in vivo* studies demonstrate that alloantibody-mediated parenchymal cell rejection is mediated by macrophages (16).

The role of specific alloantibody isotypes in graft rejection or tolerance is complex and likely influenced by many recipient and donor factors. For example, in alignment with our results showing a detrimental role for IgG1 alloantibody are results from Hirohashi *et al.* who reported that IgG1 alloantibody triggers chronic arterial lesions within murine cardiac allografts in the absence of C4d deposition (61). Additionally, Yin *et al.* reported IgG1-mediated rejection of cardiac xenografts in mice (62). Interestingly, Rahimi *et al.* reported that cardiac rejection is not induced following the transfer of 25 μg IgG2b nor 100 μg IgG1. However, if 25 μg IgG2b and 100 μg IgG1 are co-transferred, rejection occurs (63). These results suggest that IgG1 is deleterious to allografts in this model under certain conditions and highlight the complexity of humoral alloimmune responses on transplant outcomes. In contrast, other reports using a model of renal allograft rejection found that production of IgG2a was associated with rejection, whereas IgG1 was associated with tolerance (64). In these studies tolerance was transferable by AT of $CD4^+$ T cells, presumably T regulatory cells, but it is not clear whether or not AT of IgG1 or IgG2a alloantibodies was protective or deleterious.

Our data with CD8-depleted MHC class II KO recipient mice indicate that NKT cells and B cells alone are not sufficient for alloantibody production under physiological conditions posttransplant. Experiments using mixed bone marrow chimeras are currently in progress to directly examine whether cognate interactions between NKT cells and B cells facilitate alloantibody production. It is somewhat surprising that IFN-γ, not IL-4 (both dominant NKT-produced cytokines), is required by NKT cells to enhance IgG1 alloantibody production *in vivo*. However, it is possible that these results could be explained by previous reports which indicate that IFN-γ can induce IL-18 (65), a pleiotropic cytokine, capable of inducing Th2 cytokines, including IL-4 (66, 67). Additionally, IL-4 and IL-4Rα have been shown to be unnecessary for the development of NKT effector functions (68).

We found that the number of IL-4$^+$CD4$^+$ T cells is reduced in CD8-depleted NKT deficient recipients compared to CD8-depleted WT recipients which correlates with reduced levels of serum alloantibody. One hypothesis to explain these results is that NKT cells might "license" DCs to stimulate the maturation of IL-4$^+$CD4$^+$ T cells necessary to stimulate B cell production of IgG1 alloantibody. Studies are underway to investigate if NKT cells license APCs to stimulate Th2 CD4$^+$ T cell responses, indirectly enhance CD4$^+$ T cell activation and/or trafficking, and/or stimulate B cells directly in concert with CD4$^+$ T cell/B cell interactions (**Figure S3**). Many other interesting questions require further investigation such as the role of CD1d-dependent cognate interactions and the nature of the ligand in this response since glycolipid moieties are known to differ in their APC preference (69) and their Th1/Th2 bias (70). Hepatocytes are known to express CD1d and may directly interact with type I NKT cells. We are also interested in determining the immune depots where NKT cell interactions occur.

In conclusion, the present study highlights a novel and important role for NKT cells in posttransplant IgG1 alloantibody production. Human IgG1 antibodies directly correlate with human kidney allograft rejection and function (71) and this human isotype is, in part, driven by IL-4 (72-76). This would suggest that the mechanisms promoting IgG1 antibody production, in mice (IL-4-dependent) and human, may be similar. Studies are ongoing to further clarify the mechanisms by which NKT cells promote humoral alloimmunity. The results of our current studies predict that interference with NKT mechanisms promoting alloantibody production or with type I NKT cells directly is expected to suppress the magnitude of posttransplant alloantibody production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations

AT	Adoptive transfer
hA1AT	human alpha-1 antitrypsin

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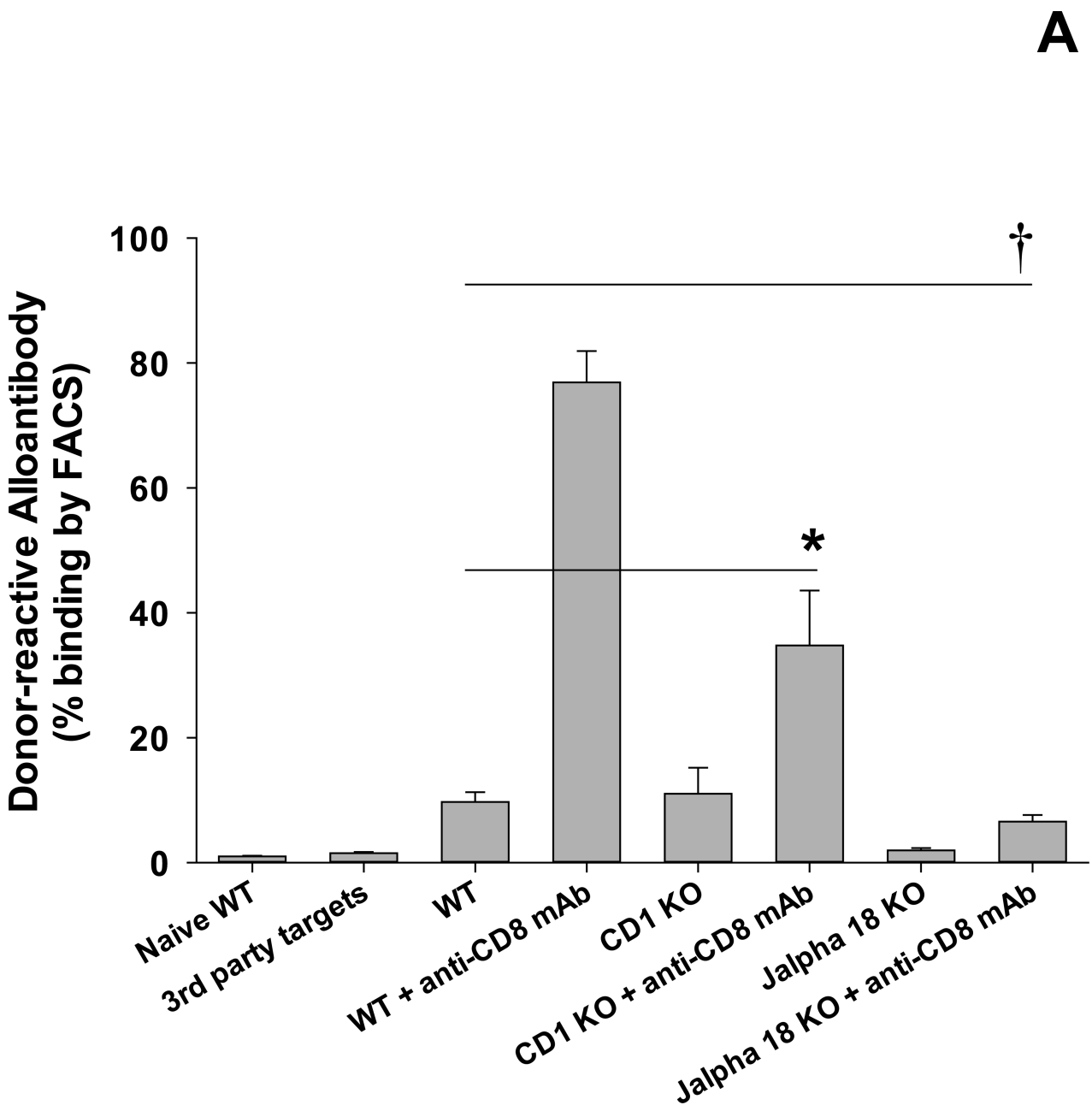

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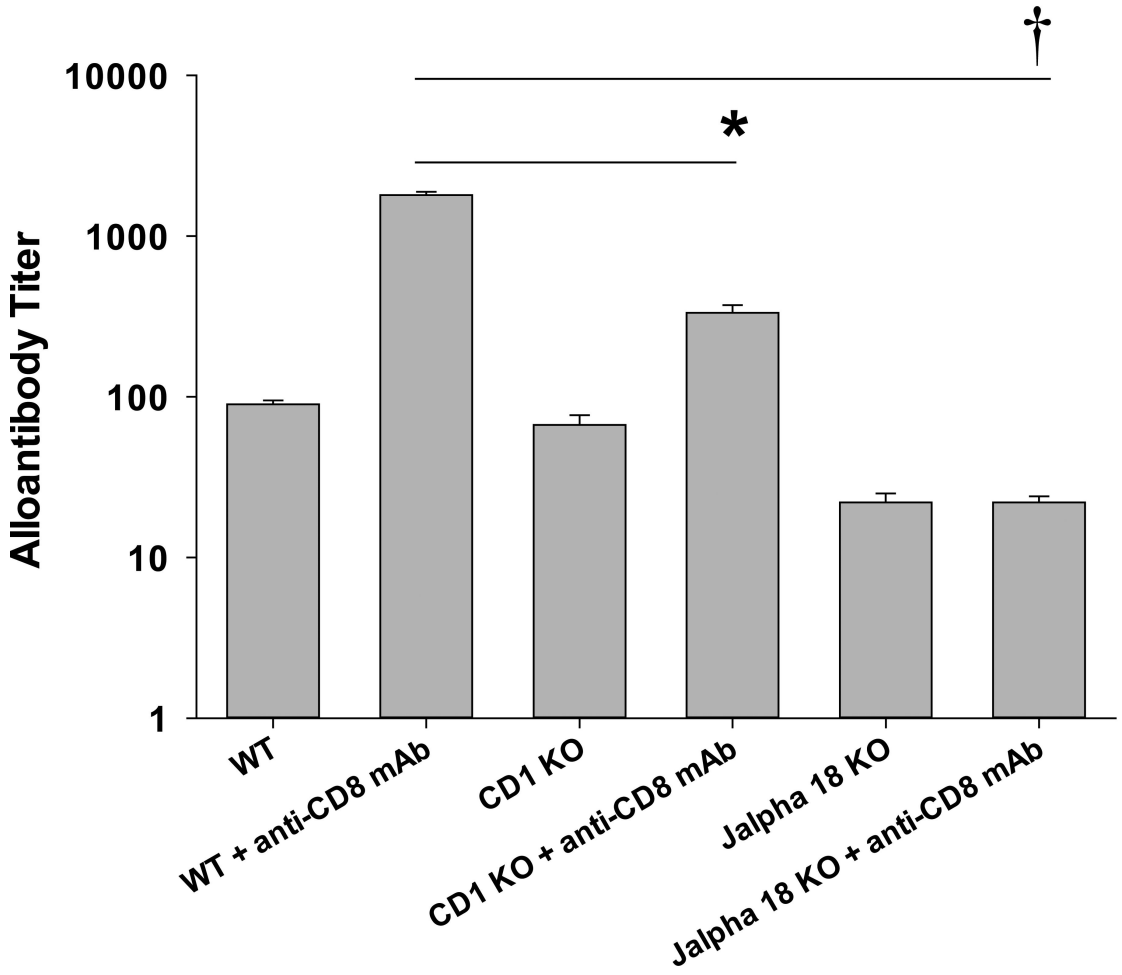

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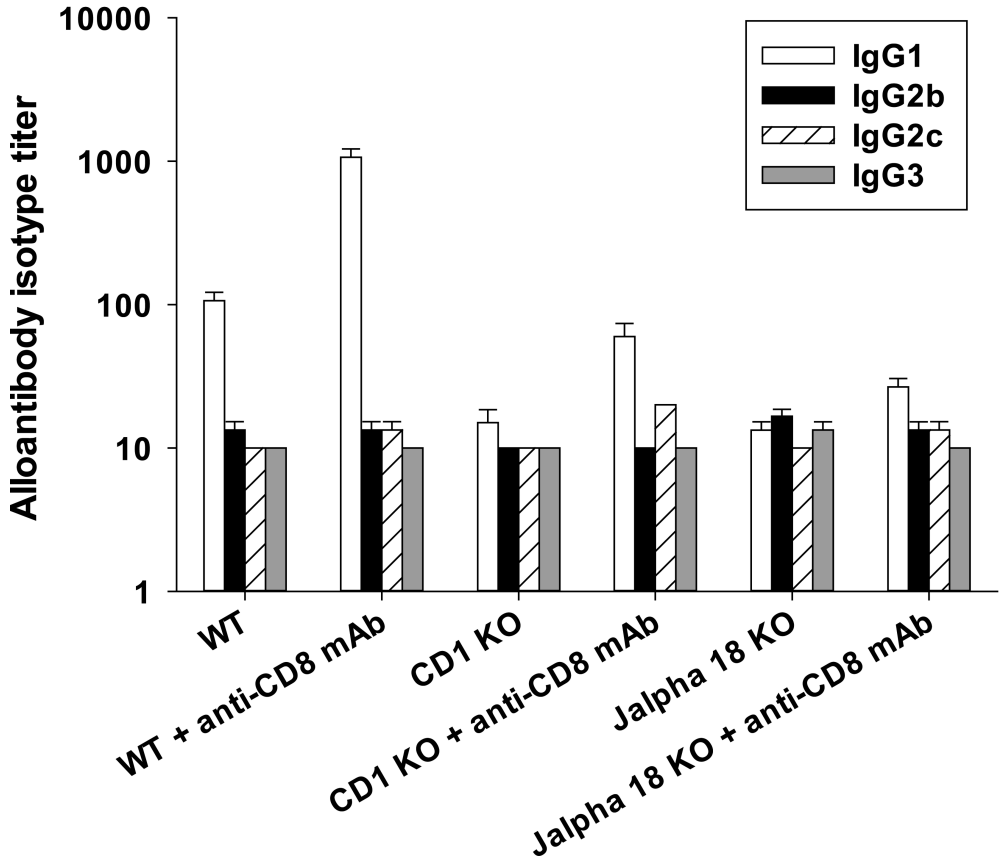

Figure 1. Type I NKT cells are critical for maximal alloantibody production following allogeneic hepatocyte transplant

C57BL/6 (wild-type; WT), CD1d KO, and Jα18 KO mice (H-2^b) were transplanted with allogeneic FVB/N (H-2^q) hepatocytes. Recipients were left untreated or CD8-depleted (day −2,−1). Serum was tested for alloantibody on day 14 posttransplant by two methods- **A)** percent allogeneic target splenocytes bound and **B)** by serum dilution to calculate alloantibody titer. Naïve serum and third party B10.BR targets were used as negative controls. Both WT (9.7±2.5%; n=13; titer=90±5 n=5) and CD1d KO (11.0±4.5%; n=4; titer=66.7±10 n=3) recipients produced similar low, but significant alloantibody levels

compared to naïve controls ($p<0.05$). CD8-depleted WT mice (76.9±3.4%; n=7; titer=1800±89 n=5) produced increased alloantibody compared to WT recipients (9.7±2.5%; titer=90±5; $p<0.0001$). CD8-depleted CD1d KO recipients (34.8±4.0%, n=5; titer=333±39 n=3) produced significantly less alloantibody than CD8-depleted WT recipients (76.9±3.4%, $p<0.0001$; titer=1800±89, $p=0.016$; as signified by "*" for both). Jα18 KO recipients exhibited low alloantibody production (2.0±0.4%, n=5; titer=22±3 n=6). CD8-depleted Jα18 KO recipients (6.6±2.4%, n=14; titer=22±2 n=6) produced significantly less alloantibody than CD8-depleted WT recipients (76.9±5.0%, $p<0.001$; titer=1800±89, $p=0.0041$; as signified by "†" for both). **C)** Analysis of alloantibody isotype by titer analysis shows that IgG1 is the dominant posttransplant alloantibody isotype with relatively low contributions from IgG2b, IgG2c, and IgG3.

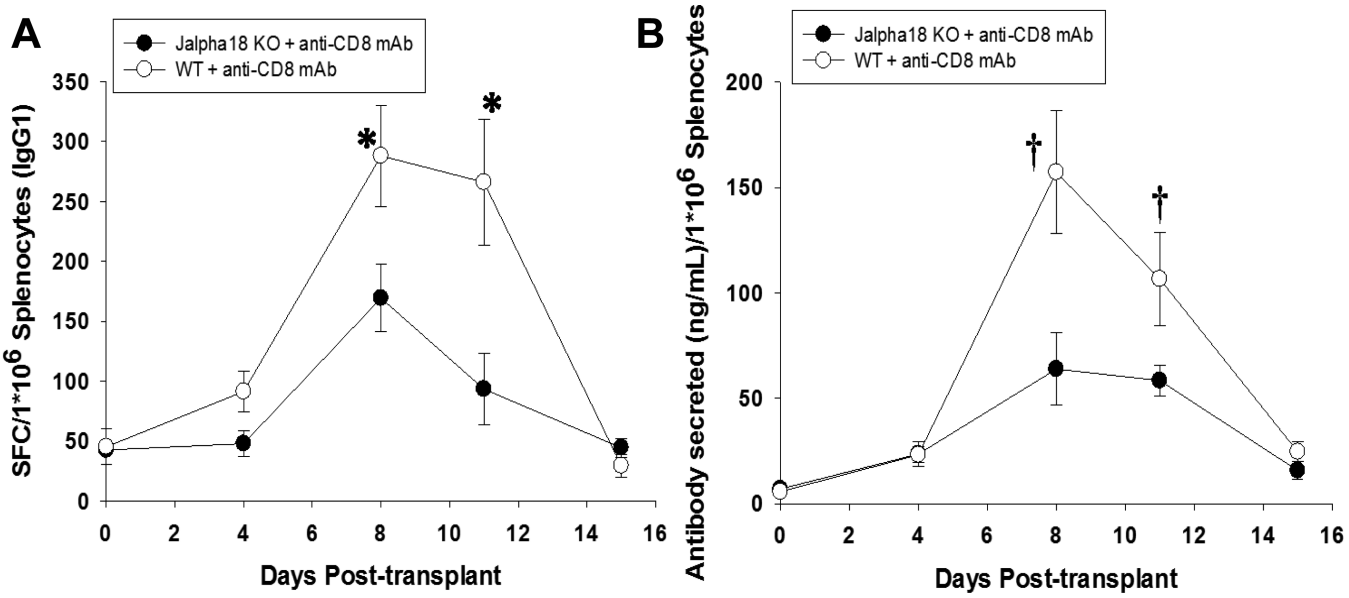

Figure 2. Type I NKT cell-deficient recipients have fewer antibody producing cells

CD8-depleted wild-type (WT) and Jα18 KO mice were transplanted with FVB/N hepatocytes. ELISPOT was used to determine the number of antibody producing cells on days 4, 8, 11, and 15 posttransplant. **A)** WT transplant recipients exhibited a significant induction of IgG1-producing cells (spot forming cells, SFC) per 10^6 cells analyzed on days 8 (288±42) and 11 (266±53) posttransplant, compared to naïve (day 0) WT mice (45±15; $p<0.0001$ for both comparisons). Jα18 KO recipients exhibited significantly fewer IgG1-producing cells per 10^6 cells on days 8 (169±28) and 11 (94±30) posttransplant, compared to WT recipients ($p<0.006$ for both comparisons, as signified by "*"). **B)** *In vitro* antibody production, as demonstrated by a side-by-side ELISA, was lower in Jα18 KO splenocytes (day 8= 63.9±17.1, day 11= 58.4±7.2 ng/mL per 10^6 splenocytes) versus WT splenocytes (day 8= 157.2±29.2, day 11= 106.6±22.0 ng/mL per 10^6 splenocytes; $p<0.02$ for both days, as signified by "†").The experimental results are representative of duplicate experiments. Error bars designate the standard error (based on 4 to 10 wells per data point from 2 mouse spleens).

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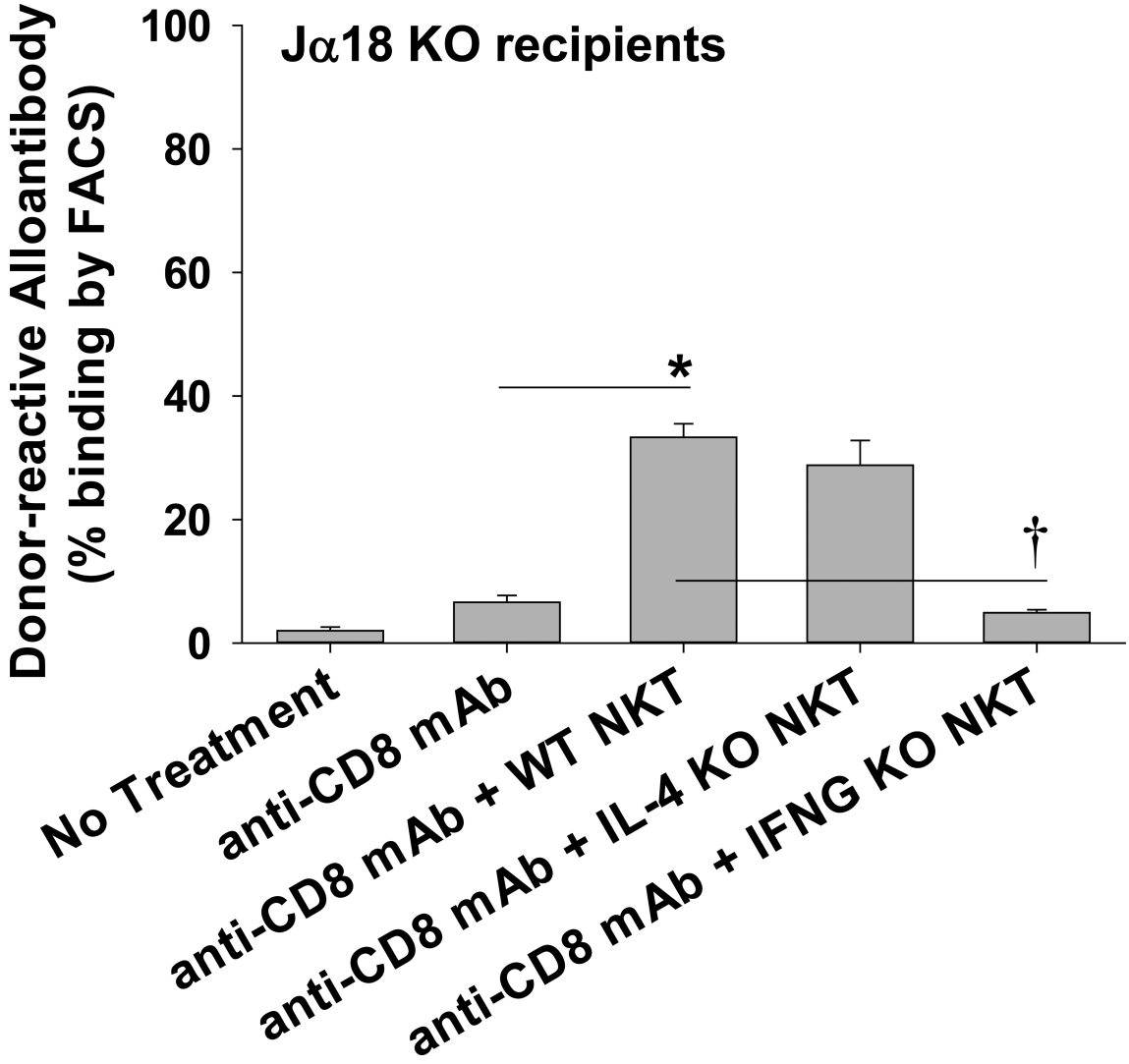

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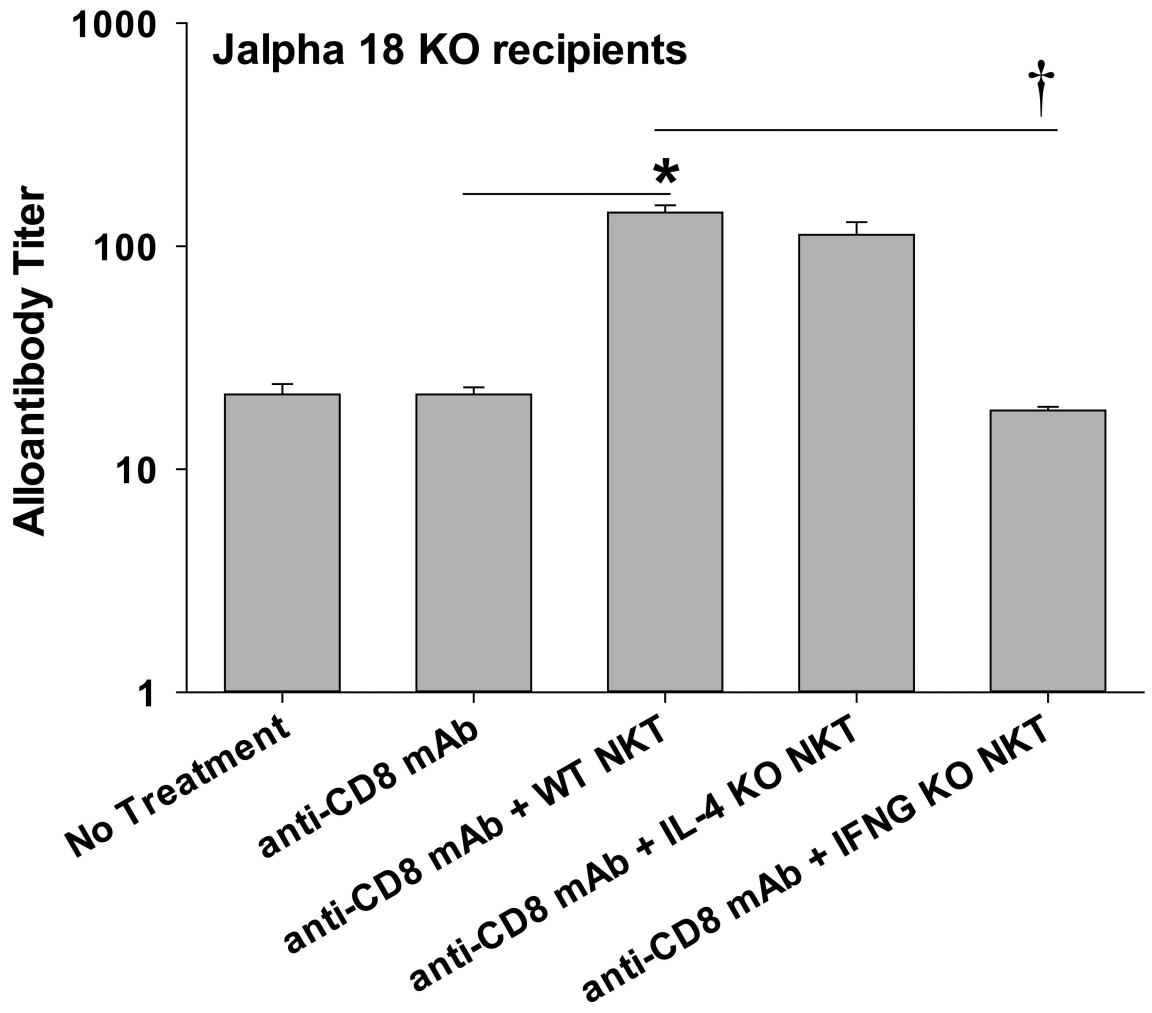

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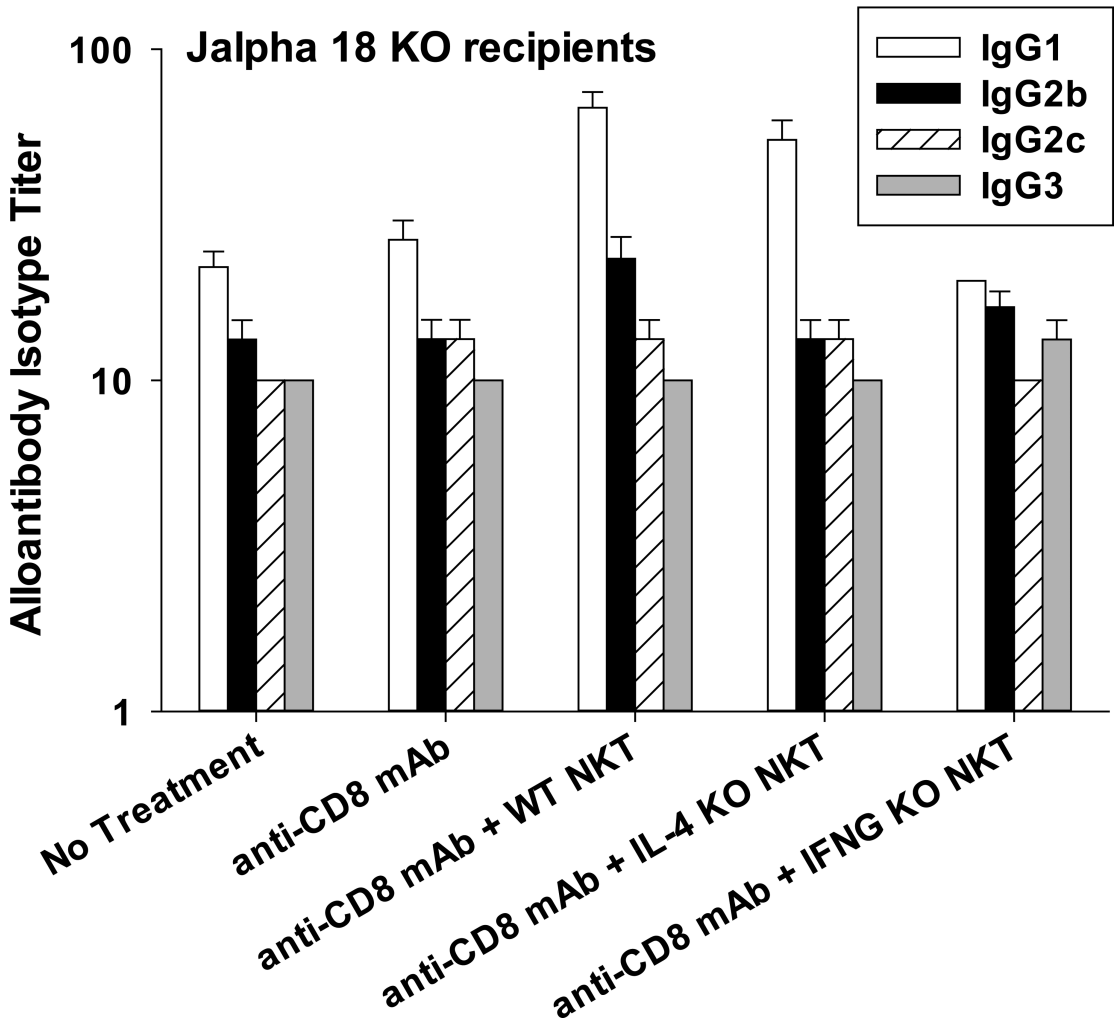

Figure 3. Type I NKT cell enhanced alloantibody production is IFN-γ-dependent, but IL-4-independent

Jα18 KO mice (H-2^b) were transplanted with allogeneic FVB/N (H-2^q) hepatocytes. Recipients were left untreated or CD8-depleted (day -2,-1). Type I NKT cells were adoptively transferred (AT; $1x10^6$ cells i.v.) into CD8-depleted Jα18 KO mice immediately following allogeneic hepatocyte transplant. Serum alloantibody was tested on day 14 by two methods- **A)** percent allogeneic target splenocytes bound and **B)** by serum dilution to calculate alloantibody titer. Naïve serum was used as a negative control. AT of wild-type (WT) NKT cells into CD8-depleted Jα18 KO recipients induced significant enhancement of

alloantibody production (33.3±1.7%; n=7; titer=142±11 n=6) compared to CD8-depleted Jα18 KO recipients (6.6±1.2%, n=14, $p<0.0001$; titer=22±2, n=6; $p<0.0031$; as signified by "*" for both). Jα18 KO recipients adoptively transferred with IL-4 KO NKT cells (27.0±2.1%; n=5; titer=113±16 n=4) produced similar alloantibody levels compared to recipients which received AT of WT NKT cells (p=ns). CD8-depleted Jα18 KO recipients adoptively transferred with IFN-γ KO NKT cells (4.8±2.3%; n=4; titer=18±1 n=6) produced significantly less alloantibody compared to recipients which received AT of WT NKT cells (†, $p<0.003$ for both methods). **C)** Analysis of alloantibody isotype by titer analysis shows that IgG1 is the dominant posttransplant alloantibody isotype with relatively low contributions from IgG2b, IgG2c, and IgG3.

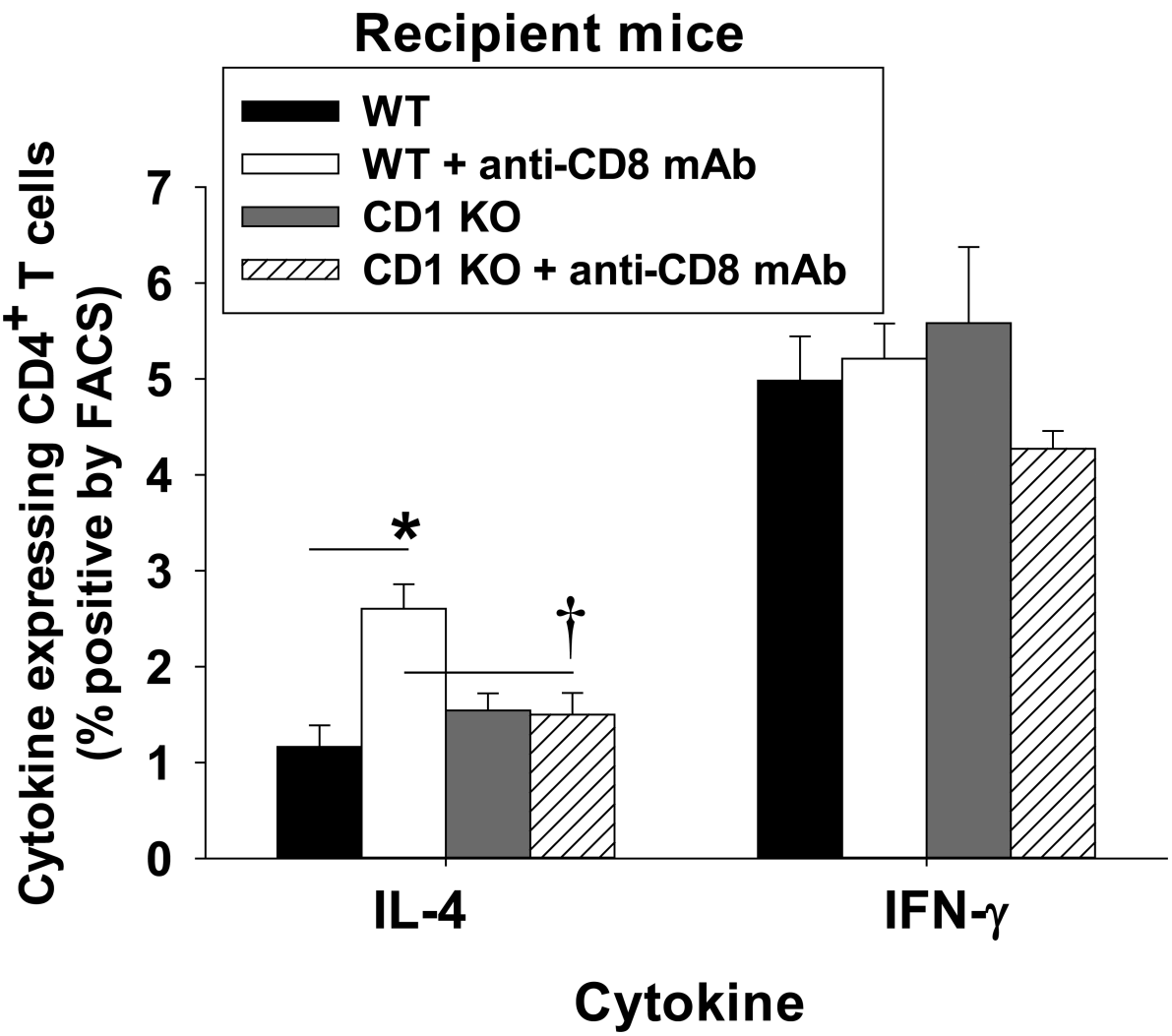

Figure 4. NKT cells enhance maturation of IL-4-producing CD4$^+$ T cells following allogeneic hepatocyte transplant

Wild-type (WT) and CD1d KO mice (H-2^b) were transplanted with allogeneic FVB/N (H-2^q) hepatocytes. Recipients were left untreated or CD8-depleted (day -2,-1). On day 7, splenocytes were harvested and analyzed for intracellular IFN-γ and IL-4 within CD4$^+$ T cells. WT and CD1d KO recipients both exhibited increased percentages of IL-4$^+$CD4$^+$ T cells (1.1%±0.3%, n=3 and 1.5%±0.3%, n=3, respectively) compared to naïve controls (0.2%±0.0%, n=3; $p<0.02$ for both). CD8-depleted WT recipients exhibited a greater percentage of IL-4$^+$CD4$^+$ T cells (2.6%±0.2%, n=5; $p=0.0005$, as signified by "*") compared to CD8-sufficient WT recipients. In contrast, IL-4$^+$CD4$^+$ T cells were significantly lower in CD8-depleted CD1d KO recipients (1.5%±0.2%, n=5; $p=0.0028$, as signified by "†") compared to CD8-depleted WT recipients. WT and CD1d KO recipients exhibited a significantly greater percentage of IFN-γ$^+$CD4$^+$ T cells (5.0%±0.4%, n=3 and 5.6%±0.5%, n=3, respectively) compared to naïve controls (0.3%±0.1%, n=3; $p<0.01$ for both). IFN-γ$^+$CD4$^+$ T cells from CD8-deficient WT and CD1d KO recipients were

comparable to each other (5.2%±0.4%, n=5 and 4.3%±0.4%, n=5) and their CD8-sufficient counterparts.