Published in final edited form as:

Transplantation. 2009 October 15; 88(7): 874–878. doi:10.1097/TP.0b013e3181b6eca2.

B cells are dispensable for neonatal transplant tolerance induction¹

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Abstract

Background—Prior studies have demonstrated that neonatal B cells possess unique immunoregulatory properties. Specifically, neonatal B-1 cells produce IL-10 in response to TLR stimulation; this modulates innate and adaptive alloimmune responses. Here, we examine whether this process plays a critical role in neonatal transplant tolerance induction.

Methods—We employed a murine model of neonatal transplant tolerance induction whereby female C57BL/6 mice injected with male spleen cells 3 days after birth acquire the ability to accept a male skin transplant in adulthood.

Results—We investigated the role of B cells in this model by employing mice with targeted genetic B cell deficiencies (including muMT, JH^{−/−} and XID mice). Additionally, we examined the role of IL-10 in this model using IL-10^{−/−} mice. Transplant tolerance induction was neither dependent on B cells nor on IL-10.

Conclusions—Despite the role of neonatal B cells in suppressing innate and adaptive alloimmune responses, B cells are dispensable for neonatal transplant tolerance induction in this experimental model.

Keywords

neonate; transplantation; tolerance; B cell

Introduction

Neonates have a unique susceptibility to transplant tolerance induction (1–3), although the mechanisms behind this susceptibility remain to be fully elucidated. Under the right experimental conditions, neonates are able to mount adult-like innate and adaptive immune responses (4–7). However, neonates are more susceptible to certain infections and do not respond to vaccines as readily as adults (8–11). This impaired immunity is associated with a bias towards Th2 vs. Th1 responses (5,6,11–13). It is likely that the mechanisms behind impaired neonatal immunity also contribute to their susceptibility to tolerance induction. Understanding these mechanisms will aid the development of protocols to combat infection in neonates and protocols to induce transplant tolerance in adults.

¹This work was supported by grants from the Roche Organ Transplant Research Foundation (29991650) and National Institute of Health Grant (AI064666) awarded to DRG. Wendy E Walker was supported in part by a National Institutes of Health Grant T32 (HL007778-12).

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The authors have no conflict of interest to declare.

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Prior work has demonstrated that neonatal B cells (specifically, B-1 cells) produce IL-10 in response to Toll-like Receptor (TLR) stimulation (14–16). This in turn suppresses interleukin (IL)-12 production and costimulatory molecule upregulation by TLR-stimulated dendritic cells (DCs) (14–16). IL-12 is a key cytokine, which promotes Th1 adaptive immunity. Furthermore, neonatal B cells suppress Th1 but not Th2 adaptive immune responses (14).

Regarding transplantation, TLRs have been implicated in modulating the balance between rejection and allograft acceptance. Mice lacking Myeloid Differentiation Factor 88 are more susceptible to transplant tolerance induction (17–19). Furthermore, exogenous TLR agonists can break transplant tolerance induction in mice (20–22). Hence, it seems plausible that, by modulating TLR responses, neonatal B cells could impact allograft survival. In our prior report, we demonstrated that injecting neonatal B cells into adult transplant recipients suppresses Th1, but not Th2, alloimmune responses during transplant rejection (14). In support of our hypothesis, neonatal transplant tolerance is associated with increased production of IL-10 and Th2 cytokines; furthermore, IL-12 administration prevents neonatal tolerance induction (7, 23–30). In this report, we directly examined whether B cells play an essential role in a murine model of neonatal transplant tolerance, wherein female C57BL/6 neonatal mice injected with male spleen cells acquire the ability to accept a male skin graft in adulthood (30).

Materials and Methods

Mice

C57BL/6 mice and muMT and IL-10^{−/−} mice on the C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). XID mice on the C57BL/6 background were a gift from Dr. Woodland (University of Massachusetts, MA, USA). JH^{−/−} mice were purchased from Taconic (Hudson, NY, USA) and backcrossed to the C57BL/6 background. ACT m.OVA mice on the C57BL/6 background were purchased from The Jackson Laboratory; these mice express the chicken ovalbumin transgene under the control of a chicken beta actin promoter, coupled with a cytomegalovirus immediate-early enhancer (31). All strains were bred in our animal facility to produce the neonatal and adult mice used in these studies.

Tolerance induction and skin transplantation

Spleen cells were prepared by masticating the spleens between frosted glass slides. The cells were spun down and re-suspended in red blood cell lysis buffer (0.8% NH₄Cl, 0.01% KHCO₃ by volume in water plus 100 mM EDTA), then incubated on ice for five minutes to lyse red blood cells. The cells were then washed with PBS and filtered through a nylon mesh. To induce tolerance of skin transplants, 5×10⁷ male C57BL/6 spleen cells were injected into the peritoneum (ip) of neonatal mice 3 days after birth. Once the mice reached adulthood (at approximately 6 weeks of age and ≥ 17g weight), they were transplanted with a male skin trunk graft, according to our previously published protocol (17). The recipient mouse was anesthetized with ketamine/xylazine then the thorax was shaved and cleaned with betadine and ethanol. A small piece of skin (~2cm square) was cut away from the thorax and a donor graft of the same size was placed in the graft bed and stapled into place. The graft was impregnated with antibiotic ointment and bandages were applied for 6 days. The staples were removed after 14 days. Rejection was defined as hardness and scabbiness of ≥90% of the graft surface. Surviving grafts remained supple and grew fur.

Flow cytometric staining

Spleen cells were prepared as described above and re-suspended at a concentration of 2 × 10⁷/ml in 2% fetal bovine serum (FBS) in PBS. Antibodies to cell surface markers (eBioscience, San Diego, CA, USA) were added and the cells were incubated on ice for 30 minutes. The cells were washed with 2% FBS in PBS then fixed with Intracellular Fixation

Buffer (eBioscience) on ice 5 minutes. The cells were washed and re-suspended in PBS. Fluorescence data was acquired with a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) then analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Results

We used a model of transplant tolerance whereby female C57BL/6 neonatal mice injected with male spleen cells ip three days after birth accept a male skin graft in adulthood. To confirm that this transplant tolerance induction model was neonate-specific, we injected 5×10^7 male C57BL/6 spleen cells ip into female C57BL/6 neonatal mice (3 days old) or adult mice (6–8 weeks old). 5–6 weeks later, the mice received a male trunk skin transplant. Female mice that were injected with male spleen cells as neonates accepted their grafts for >150 days (Fig. 1a). In contrast, female mice that were injected with male spleen cells as adults rejected their grafts at a similar tempo to naïve female recipients. Hence, only neonatal mice were susceptible to this tolerance induction protocol.

To demonstrate that the neonatal tolerance model was antigen specific, we injected female C57BL/6 neonates with female C57BL/6 spleen cells (Fig 1a). These mice did not accept a male skin transplant in adulthood. Hence, tolerance induction was specific to the antigens present on the donor spleen cells: infusion of splenocytes per se did not induce tolerance. Furthermore, female C57BL/6 mice injected with male spleen cells did not accept a female C57BL/6 ACT m.OVA skin transplant in adulthood (Fig. 1b; C57BL/6 ACT m.OVA mice express the ovalbumin minor histocompatibility antigen, see methods). The tempo of rejection was similar to naïve female C57BL/6 mice that received a female C57BL/6 ACT m.OVA skin transplant. Hence, injection of the male spleen cells did not induce nonspecific tolerance to an unrelated antigen.

In order to test the role of neonatal B cells in this tolerance model, we used three genetic mutants. muMT mice have a targeted mutation of the mu chain constant region, consequently B cell development is arrested at the pro-B cell stage (32). We subjected muMT neonates to the tolerance induction protocol. In contrast to our hypothesis, female muMT neonates injected with male wild-type cells accepted a male wild-type skin graft in adulthood for >150 days (Fig. 2a), while naïve muMT females rapidly rejected the male skin graft. However, when we examined the B cell populations in the spleens of muMT mice (by antibody staining and FACS analysis (14)), we found that these mice did possess some cells that fell into the CD19+CD5+ B-1 gate (Fig. 2b) (prior work indicates that it is the B-1 neonatal subset that possesses immunoregulatory properties (14,15)). However, the intensity of CD5 staining was notably lower in this gated population with the muMT mice vs. the wild-type mice. Our data indicate that the B cell abnormalities in muMT mice did not prevent tolerance induction in this model.

JH^{-/-} mice have a targeted deletion of all JH gene segments and consequently do not produce mature B cells (33); these mice were used to more fully test the role of B cells in tolerance induction. Male JH^{-/-} mice accepted a male wild-type skin graft for >50 days, confirming that the mice had been sufficiently backcrossed (Fig. 3a), whereas naïve female JH^{-/-} mice rapidly rejected a male wild-type skin graft. In contrast, female JH^{-/-} neonates that were injected with male wild-type spleen cells accepted a male wild-type skin graft in adulthood for >50 days (Fig. 3a). Our data indicate that the B cell abnormalities in JH^{-/-} mice did not prevent tolerance induction in this model.

In order to test the role of the B-1 subset in tolerance induction, we used XID mutant mice: these mice have a spontaneous mutation in the Burton's tyrosine kinase gene and consequently lack functional B-1 cells (34,35). In agreement with our data in the JH^{-/-} and muMT models, female XID neonates that were injected with male wild-type spleen cells accepted a male wild-

type skin graft in adulthood for >150 days, whereas naïve XID females rapidly rejected a male skin graft (Fig. 3b). Our data indicates that a functional B-1 subset was not required for tolerance induction in this model.

We reasoned that IL-10 production might not be limited to neonatal B cells; thus IL-10 might still be important to tolerance induction in this model, despite the independence of B cells. Female IL-10^{-/-} neonates were injected with male wild-type spleen cells three days after birth. These mice were subsequently able to accept a male wild-type skin graft in adulthood for >150 days, whereas naïve female IL-10^{-/-} mice rapidly rejected male wild-type skin grafts. Thus, our data indicate that IL-10 was dispensable for tolerance induction in this model (Fig. 4).

Discussion

To conclude, it has been known for many years that neonates have altered immune function and are susceptible to transplant tolerance induction. Prior work demonstrated that neonatal B cells possess unique immunomodulatory functions, which impact innate and adaptive immune responses (14–16). In addition, neonatal B cells can reduce Th1 alloimmune responses, following their injection into adult transplant recipients (14).

The above findings led us to examine whether neonatal B cells are critical for a murine model of transplant tolerance induction, whereby female C57BL/6 neonates injected with male spleen cells accept a male skin graft in adulthood. Through the use of genetic approaches, we demonstrated that B cells (including B-1 cells) were dispensable to tolerance induction in this model. muMT mice (with arrested B cell development) and JH^{-/-} mice (lacking mature B cells) were susceptible to tolerance induction. XID mice (lacking functional B-1 cells) were also susceptible to tolerance induction in this model. Finally, IL-10 was also dispensable to this process as IL-10^{-/-} mice were susceptible to tolerance induction.

Despite our findings in this transplant model, B cells and IL-10 are still likely to modify immune function in neonates. In future studies, it would be interesting to investigate the role of neonatal B cells in alternative transplant models, including models with major histocompatibility antigens. In addition, it will be important to investigate if neonatal B cells play a role in the impaired neonatal immune response to certain infections, such as pneumonia and influenza. It will also be important to determine if B cells contribute to neonatal resistance to vaccination. Multiple mechanisms may contribute to the altered immune phenotype in neonates and there may be some redundancy between them. These mechanisms may have evolved to maintain immune tolerance at the maternal-fetal interface and to facilitate education to self-antigens during development to prevent deleterious immune responses and the development of autoimmunity.

Abbreviations

DC, dendritic cells; FBS, fetal bovine serum; IL, interleukin; ip, into the peritoneum; TLR, Toll-Like Receptor.

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Figure 1. Injection of male spleen cells during the neonatal period induces antigen specific acceptance of male skin transplants in adulthood

C57BL/6 strain female mice were injected with 5×10^7 male spleen cells at 3 days of age (unless otherwise noted). Five weeks later the mice received a male skin graft. **(a)** Wild type (WT) female mice injected with male spleen cells as neonates accepted male grafts in adulthood for >150 days, whereas male grafts were rapidly rejected by WT female mice injected with female spleen cells as neonates. WT female mice injected with male spleen cells as adults (5–6 weeks of age) and WT naïve female controls also rejected male skin grafts. **(b)** WT naïve adult females and wild type females injected with male spleen cells as neonates rapidly rejected C57BL/6 ACT m.OVA skin transplants.

(a) tolerance induction in muMT neonates

- ▲ muMT naive females (n=3)
 -○- muMT females + male spleen cells as neonates (n=7)

Figure 2. Mature B cells are not required for neonatal transplant tolerance induction

(A) muMT females (with arrested B cell development) that were injected with WT male spleen cells as neonates accepted WT male grafts >150 days, whereas muMT naïve adult females rapidly rejected WT male grafts. **(B)** Spleen cells from adult wild type and muMT mice were stained with CD19-APC and CD5-FITC to identify the B-1 subset (CD19⁺CD5⁺) and the B-2 subset (CD19⁺CD5⁻).

Figure 3. Alternate genetic mutants demonstrate that B cells are not required for neonatal transplant tolerance induction

(A). JH^{-/-} females (lacking B cells) that were injected with WT male spleen cells as neonates accepted WT male grafts >50 days, whereas JH^{-/-} naïve adult females rapidly rejected WT male grafts. JH^{-/-} adult males also accepted WT male grafts >50 days, demonstrating that these mice were sufficiently backcrossed to the C57BL/6 background. **(B)** XID females (lacking B-1 cells) that were injected with WT male spleen cells as neonates accepted WT male grafts >150 days, whereas XID naïve adult females rapidly rejected WT male grafts.

Figure 4. IL-10 is dispensable for neonatal transplant tolerance induction

IL-10^{-/-} females that were injected with WT male spleen cells as neonates accepted WT male grafts >150 days, whereas IL-10^{-/-} naïve females rapidly rejected WT male grafts.