Published in final edited form as:

Am J Transplant. 2008 January ; 8(1): 21–31. doi:10.1111/j.1600-6143.2007.02038.x.

Epidermal Langerhans cells promote skin allograft rejection in mice with NF- κ B-impaired T cells¹

LL Molinero^{*}, P Zhou^{*,2}, Y Wang^{*}, H Harlin[†], B Kee[§], C Abraham^{*}, and ML Alegre^{*}

^{*}Department of Medicine, and Committee on Immunology, University of Chicago, Chicago, IL 60637

[§]Department of Pathology, and Committee on Immunology, University of Chicago, Chicago, IL 60637

[†] Medical College of Wisconsin, WI 53226

Abstract

T cells play a major role in the acute rejection of transplanted organs. Using mice transgenic for a T cell-restricted NF- κ B super-repressor (IkB α Δ N-Tg mice), we have previously shown that T cell-NF- κ B is essential for the acute rejection of cardiac but not skin allografts. In this study, we investigated the mechanism by which skin grafts activate IkB α Δ N-Tg T cells. Rejection was not due to residual T cell-NF- κ B activity as mice with p50/p52^{-/-} T cells successfully rejected skin grafts. Rather, skin but not cardiac allografts effectively induced proliferation of graft-specific IkB α Δ N-Tg T cells. Rejection of skin grafts by IkB α Δ N-Tg mice was in part dependent on the presence of donor Langerhans cells (LC), a type of epidermal dendritic cell (DC), as lack of LC in donor skin grafts resulted in prolongation of skin allograft survival and injection of LC at the time of cardiac transplantation was sufficient to promote cardiac allograft rejection by IkB α Δ N-Tg mice. Our results suggest that LC allow NF- κ B-impaired T cells to reach an activation threshold sufficient for transplant rejection. The combined blockade of T cell-NF- κ B with that of alternative pathways allowing activation of NF- κ B-impaired T cells may be an effective strategy for tolerance induction to highly immunogenic organs.

Introduction

T cell-dependent immune responses depend on concomitant TCR and costimulatory receptor engagement. These interactions promote a cascade of intracellular events that ultimately activate the transcription factors NF-AT, AP-1 and NF- κ B. In unstimulated T cells, the NF- κ B dimers p65/p50 are sequestered in the cytoplasm by an inhibitory subunit, IkB α . Upon TCR stimulation, the CARMA1-Bcl10-Malt1-ADAP signalosome activates the IKK complex that phosphorylates and promotes the degradation of IkB α , therefore liberating NF- κ B to translocate into the nucleus (1). The generation of mice that are deficient in NF- κ B signaling in T cells via the overexpression of a super-repressor form of IkB α [IkB α Δ N-Tg, (2)] helped to elucidate the role of this transcription factor in several types of T cell responses. Secretion of the pro-inflammatory cytokines IL-2 and IFN- γ is dependent on NF-

¹This work was supported by NIH/NIAID RO1 #AI052352-01 (MLA)

Corresponding Author: LL Molinero, 5841 S. Maryland Ave., Room AMB-N011, MC0930, Chicago, IL 60637, USA; lmoliner@medicine.bsd.uchicago.edu; tel: (773) 834 4510; fax: (773) 702 8702.

²Current Address: Tongji Hospital, Transplant Research Institute, 1059 Jiefang Road, Wuhan, Hubei 430030, P.R. China

Disclosures

The authors have no financial conflict of interest.

κ B signaling (3,4), and lack of T cell-NF- κ B prevents onset of collagen-induced arthritis (5) and abrogates resistance to *Toxoplasma gondii* infection (6). The selective role of TCR-driven NF- κ B has been investigated using CARMA1-deficient mice in which T cell-NF- κ B is only impaired downstream of the TCR but not of other surface receptors such as TNFR family members. CARMA1-deficient T cells display reduced IL-2 production and proliferation (7).

T-cell activation is essential for acute allograft rejection. Using IkB α Δ N-Tg mice, we and others have previously shown that T cell-specific NF- κ B activity is required for allograft rejection of cardiac transplants (8,9). In contrast, IkB α Δ N-Tg mice effectively rejected skin allografts, with similar kinetics as wild type (WT) mice (9). Cardiac allograft rejection was restored in IkB α Δ N-Tg mice overexpressing the anti-apoptotic factor Bcl-x_L selectively in T cells (10), suggesting that cardiac allograft antigens induced cell death of NF- κ B-impaired alloreactive T cells. However, whether selective impairment of TCR-driven NF- κ B is sufficient to permit acceptance of cardiac allografts, as well as the mechanism by which IkB α Δ N-Tg mice retain the ability to reject skin grafts remained to be established.

Skin allografts have long been thought to be more “immunogenic” than other tissues, as interventions that can successfully prevent rejection of heart or pancreatic islets fail to elicit acceptance of skin transplants (11). After testing and disproving the hypotheses that vascularization, transplant location or graft size determined the differences in the graft fate between skin and heart transplants (9), we postulated that an allograft-intrinsic factor, such as the type of donor resident APC, may dictate the intensity of the alloresponse and be responsible for skin allograft rejection despite impaired NF- κ B-dependent T cell activation in IkB α Δ N-Tg mice.

Mature DC are the most potent APC for naïve WT T cells (12), and influence their Th1/Th2 differentiation and susceptibility to suppression by regulatory T cells and to peripheral T cell deletion (13). At least six types of DC have been described, all of them bearing varying levels of CD11c expression. Blood-derived DC, also called conventional DC, are present in spleen and lymph nodes (12) and comprise the “myeloid” (MDC) populations CD11c⁺CD11b⁺ and the “lymphoid” (LDC) CD11c⁺CD11b⁻CD8 α ⁺ subset. LDC are different from MDC in that they secrete much higher levels of IL-12, they can cross-present antigen, and they are located in the T cell areas of the spleen whereas MDC are found in the marginal zone (14). The plasmacytoid DC (pDC, CD11c⁺B220⁺Gr1⁺mPDCA1⁺), found in tissues as well as in blood, are specially important for the production of type 1 interferon upon viral infection (15) and have also been reported to play an essential role in the onset of tolerance to cardiac allografts induced by anti-CD154 (16). In peripheral lymph nodes, two additional populations of CD11c⁺ cells are found, with one expressing Langerin and not the other (13). The latter are dermal DC and epidermal LC that emigrated from the skin or the oral and vaginal mucosa (17), while the former are the tissue resident DC. LC depend on the expression of the transcription factor Id2 for their differentiation (18). Although the role of LC is somewhat controversial, LC are important in the onset of graft versus host disease (19), and become very potent immunostimulators upon maturation either following *in vivo* migration to draining lymph nodes or *in vitro* (20,21). LC are also known to be potent stimulators of IFN- γ secretion by T cells (22,23), hence promoting a type 1 T cell phenotype that correlates with allograft rejection (24).

Our results indicate that LC contained in donor skin enable proliferation and IFN- γ secretion by NF- κ B-impaired T cells and play an important role in promoting effective skin allograft rejection in mice with a T cell intrinsic defect in NF- κ B activity.

Materials and Methods

Mice

6-8 week-old C57BL/6 (B6, H-2^b), BALB/c (H-2^d), MHC class II^{-/-} (C2ta^{tm1Ccum}, H-2^b) and RAG-2^{-/-} (H-2^b) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). CARMA1-deficient mice (H-2^b) were generously provided by Daniel Littman (New-York University, NY)(7). Mice transgenic for a T cell-restricted IκBα super-repressor, IκBαΔN-Tg mice, were obtained from Mark Boothby (Vanderbilt University, TE) (2) on B6 and BALB/c backgrounds (following backcrosses for over 10 generations), or were transgenic for an ovalbumin (OVA)-specific TCR (DO11.10, BALB/c background). Id2-deficient mice were backcrossed onto the FvB background [H-2^d, (18)]. OVA-transgenic (OVA-Tg) mice on a BALB/c background, that express membrane OVA under the control of the β-actin promoter, were a generous gift by Elizabeth Ingulli (University of Minnesota, MN). *Nfkb1*^{-/-} (p50^{-/-}, H-2^b) and *Nfkb2*^{-/-} (p52^{-/-}, H-2^b) mice were a gift by Ulrich Siebenlist (NIH/NIAID) and Guido Franzoso (University of Chicago) and were intercrossed as previously described (25). CD80/C86 double deficient mice were a generous gift by Thomas Gajewski (University of Chicago). Animals were housed in individually ventilated cages in a specific pathogen-free animal facility. Experiments were performed in agreement with our Institutional Animal Care and Use Committee and according to the NIH guidelines for animal use.

Heart and skin transplantation

Abdominal heterotopic cardiac transplantation was performed using a technique adapted from that originally described by Corry et al. (26). Cardiac allografts were transplanted in the abdominal cavity by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava, respectively. The day of rejection was defined as the last day of a detectable heartbeat. Graft rejection was verified in selected cases by necropsy and pathological examination of hematoxylin/eosin-stained graft sections. Skin transplantation was performed as previously described (27). Briefly, full-thickness donor tail-skin pieces (0.5-1 cm²) were positioned on a graft bed prepared on the flank of the recipient. The time point of rejection was defined as the complete necrosis of the graft.

Antibodies

Antibodies to CD4-APC and I-A^d-FITC were purchased from BD Pharmingen (San Jose, CA). Anti-Langerin-Alexa Fluor 647, anti-CD11c-biotin (N418), and streptavidin-APC were purchased from eBioscience (San Diego, CA). The clonotypic antibody KJ1-26 that detects the DO11.10 TCR was purchased from Caltag (Burlingame, CA). Anti-CD3 mAb (clone 145-2C11) and anti-CD28 mAb (clone PV1) were purified from hybridoma cell culture using binding to protein G.

p50^{-/-}/p52^{-/-} bone marrow reconstitution

Bone marrow cells (2-3 × 10⁶), isolated from the femurs of 22 days-old p50^{-/-}/p52^{-/-} or control p50^{+/+}/p52^{-/-} donor mice were injected intravenously into syngeneic RAG-2-deficient mice that had been lightly irradiated (350 rads) 24h earlier. T and B cell development were verified in all recipients by flow cytometry on peripheral blood 8 weeks following bone marrow transplantation. Four weeks later, mice were used as recipients of BALB/c allogeneic skin transplant, or sacrificed for purification of T cells.

Electrophoretic Mobility Shift Assays (EMSA)

T cells were purified by negative selection using a magnetic bead separation system, according to the manufacturer's instructions (Stem Cell Technologies). In all cases, T-cell

purity was greater than 95%, as analyzed by flow cytometry. Nuclear proteins were extracted from T cells that had been stimulated with immobilized anti-CD3 mAb and anti-CD28 mAb (1 µg/ml each) for 12 h. Nuclear extracts were quantified as described previously (28). An NF-κB consensus oligonucleotide, [CAA CGG CAG GGG AAT TCC CCT CTC CTT, (2)] or a commercially available AP-1 consensus oligonucleotide (Promega, Madison, WI) were labeled with [γ -³²P] ATP (MP Biomedicals, Aurora, OH). Equal nuclear protein input from the different samples was analyzed.

CD4⁺ T cell purification, CFSE labeling and adoptive transfer of DO11.10 T cells

Spleens and lymph nodes were harvested from DO11.10 or DO11.10/IκBαΔN-Tg mice and prepared into single-cell suspensions. CD4⁺ T cells were purified by negative selection using a magnetic bead separation (Stem Cell Technologies). Purified CD4⁺ T cells were stained with CFSE (2.5 µM, Molecular Probes, Eugene, OR) and adoptively transferred ($2-2.5 \times 10^6$ in 100 µl of PBS/mouse) intravenously into syngeneic BALB/c mice. One day later, mice also received OVA-Tg skin or cardiac allografts. Animals were sacrificed 5 days after transplantation and lymphoid organs harvested for analysis by flow cytometry. Cells falling in the live gate were further gated based on expression of CD4 and KJ1-26, and CFSE dilution was analyzed.

IFN-γ ELISPOTs and alloantibodies

Axillary and inguinal peripheral lymph node cells from untransplanted mice or from mice transplanted 18 days prior with BALB/c skin were adjusted for the numbers of T cells such that 0.5×10^6 T cells were plated in each well. Cells were stimulated for 18h with irradiated B6 or BALB/c splenocytes (4×10^5 /well). The ELISPOT assay was conducted according to the instructions of the manufacturer (BD Biosciences), and the numbers of IFN-γ-producing spots per well were calculated using the ImmunoSpot Analyzer (CTL Analyzers LLC). Serum alloantibodies were detected as previously described (29).

Immunohistochemistry

Transverse tissue sections were obtained from tail skin of Id2^{+/-} or Id2^{-/-} mice and processed as previously described (30). Sections were stained with biotinylated anti-I-A/I-E followed by streptavidin-alkaline-phosphatase and developed with vecton red (Invitrogen). Images were acquired using an upright Zeiss Axioscop microscope (Carl Zeiss MicroImaging, Inc. Thornwood, NY) with a color Zeiss Axiocam camera and a 40x oil objective. The images were analyzed with the AxioVision 3.0 software (Carl Zeiss Microimaging, Thornwood, NY).

DC purification

Splenic DC were obtained after digestion of spleens for 30 min with type IV collagenase (400 U/ml, Sigma-Aldrich, St. Louis, MO), and EDTA treatment to disrupt DC-T cell complexes. Cells were resuspended in isoosmotic Histodenz medium (1.080 g/cm³, Sigma-Aldrich, St. Louis, MO), and centrifuged (3,000 rpm for 15 min at 4°C). The low-density fraction at the interface was collected and washed twice. Subsequently, DC were FACS-sorted based on CD11c (clone N418) and I-A^d expression in a high-speed cell sorter (Dakocytomation MoFlo HTS, Glostrup, Denmark).

For LC purification, ears from BALB/c mice were washed in 70% ethanol, split and placed dermal side down on a 0.25% trypsin (CellGro, Herndon, VA)-HBSS solution and incubated for 45 min at 37°C. The cell suspension was filtered through a Nylon mesh and then applied to density gradient to separate dead cells. Finally, cells were sorted as above mentioned. Purity was routinely over 95%. In the T cell stimulation assays, the different DC were

pulsed for 2h with OVA protein or OVA peptide 323-339 (1 µg/ml), extensively washed and resuspended in media (*in vitro* stimulation) or PBS (*in vivo* stimulation). In the case of LC and SpDC used during cardiac allograft rejection, cell preparations were cultured one day prior to FACS-based sorting.

Flow cytometry

Multiparameter analyses were performed on an LSR II (Becton Dickinson, Franklin Lakes, NJ) and were analyzed using FlowJo software (Tree Star, Ashland, OR). Except for the staining of intracellular Langerin, all other analyses were conventional cell surface stainings. To detect expression of Langerin, cells were fixed with 4% paraformaldehyde for 5 min at room temperature, permeabilized with 0.1 % saponin and stained with 1 µg of anti-Langerin antibody.

Statistical analysis

Skin graft mean survival time (MST), standard deviation, and p-values were calculated using Kaplan-Meier/log rank test methods. Comparisons of means were performed using the Student's t test or the Tukey test for multiple comparisons.

Results

Lack of TCR-mediated NF-κB in recipient mice is sufficient to ensure different fates of heart versus skin allografts

We have previously shown that IκBαDN-Tg mice accept cardiac allografts permanently but promptly reject skin allografts (9). The IκBαDN transgene can inhibit NF-κB activation downstream of several receptors expressed in T cells, such as TCR, TNFR family members and TLR family members. To determine if inhibition of TCR-mediated NF-κB alone could recapitulate the phenotype observed in IκBαDN-Tg mice, we used CARMA1-deficient mice that lack an adaptor essential for linking the TCR with the IKK complex. Whereas IκBαDN-Tg mice have reduced numbers of peripheral T cells, especially CD8⁺ T cells (2), CARMA1-deficient mice display normal numbers and proportions of conventional CD4⁺ and CD8⁺ T cells (7). T cells from both strains of mice have reduced IL-2 and IFNγ production upon TCR stimulation. Similarly to IκBαDN-Tg mice, CARMA1-deficient mice accepted BALB/c cardiac allografts permanently but retained the ability to reject skin allografts (Figure 1). Therefore, lack of TCR-mediated NF-κB is sufficient to enable cardiac allograft acceptance but is not essential for skin allografts to successfully induce T cell priming.

Skin allograft rejection in CARMA1-KO and IκBαDN-Tg mice is not due to residual NF-κB activity

It was possible that skin graft rejection by IκBαDN-Tg and CARMA1-deficient mice was due to incomplete inhibition of NF-κB, as lack of CARMA1 only prevents NF-κB driven by stimulation of the TCR but not of other receptors (7), and the super-repressor in IκBαDN-Tg T cells reduces, but does not abolish T cell-NF-κB activity (Figure 2A). Therefore, it was conceivable that residual NF-κB activity in T cells afforded partial T cell activation sufficient to promote rejection of a small skin allograft. To investigate this possibility, we generated mice with profound inhibition of T cell-NF-κB activity. p50^{-/-}/p52^{-/-} mice are devoid of T cells because NF-κB activity in thymic epithelium is necessary for T cell positive selection (25). However, p50^{-/-}/p52^{-/-} T cells can develop following generation of bone marrow chimeras (25). Therefore, p50^{-/-}/p52^{-/-} and control p50^{+/+}/p52^{-/-} bone marrow was utilized to reconstitute the immune system of lightly irradiated syngeneic RAG-2-deficient mice. In contrast to T cells from p50^{+/+}/p52^{-/-} control littermates, p50^{-/-}/

p52^{-/-} T cells obtained 12 weeks after bone marrow transfer displayed no detectable NF-κB activity when stimulated with anti-CD3 and anti-CD28 mAb, but retained AP-1 activity (Figure 2B). Despite this profound deficiency in T cell-NF-κB activity, RAG-2-deficient mice reconstituted with p50^{-/-}/p52^{-/-} bone marrow successfully rejected BALB/c allogeneic skin grafts similarly to those reconstituted with p50^{+/+}/p52^{-/-} control bone marrow (Figure 2C). These data suggest that skin allograft rejection can occur even in the context of a more complete deficiency in T cell-NF-κB activity.

Skin allografts promote T cell proliferation and Th1 differentiation in IκBαΔN-Tg mice

To determine if skin allografts were indeed promoting activation of NF-κB-impaired allogeneic T cells, CFSE-labeled DO11.10 WT or IκBαΔN-Tg CD4⁺ T cells were adoptively transferred into syngeneic BALB/c recipients one day prior to transplantation with OVA-expressing BALB/c heart or skin. Five days later, cells were isolated from the spleen and lymph nodes and CFSE-dilution in DO11.10 T cells was assessed by flow cytometry. It has previously been shown that initial T cell activation occurs in draining lymph nodes following skin transplantation and in spleen following heart transplantation (31). As shown in Figure 3A, higher proliferation of IκBαΔN-Tg DO11.10 T cells was observed in draining lymph nodes from mice transplanted with OVA-Tg skin when compared to the spleen of mice that received OVA-Tg hearts. In addition, although cell recovery was lower than that of WT T cells, higher numbers of IκBαΔN-Tg DO11.10 T cells were recovered after transplantation with OVA-Tg skin than heart (data not shown), consistent with greater T cell proliferation/survival induced by skin than heart allografts. No proliferation was observed in animals transplanted with non-OVA-expressing syngeneic BALB/c heart (Figure 3A) or skin (data not shown).

The transcription factor NF-κB plays an important role in production of type 1 cytokines (2). To determine if skin grafts could induce type 1 T cell differentiation in IκBαΔN-Tg T cells, we analyzed IFN-γ production following skin allograft rejection. B6 IκBαΔN-Tg and WT mice received a BALB/c skin allograft. Following rejection, cells from the draining LN were adjusted for the number of T cells and restimulated with irradiated syngeneic or donor APC. Transplanted IκBαΔN-Tg mice displayed an increased frequency of donor-reactive IFN-γ-producing cells when compared to naïve mice (Figure 3B). This was in contrast to the markedly reduced IFN-γ-production that we had previously observed in IκBαΔN-Tg cardiac allograft recipients (10). Together, these results indicate that skin grafts can efficiently prime and induce differentiation of IκBαΔN-Tg T cells, despite their impairment in NF-κB activity.

Donor LC promote skin allograft rejection by IκBαΔN-Tg mice

Skin but not heart contains LC (32), which are potent stimulators of WT T cells, both *in vitro* and *in vivo* following their migration into the local LN (33). We hypothesized that skin allograft rejection by IκBαΔN-Tg mice may be dependent on the presence of donor LC. To test this possibility, skin from Id2^{-/-} mice (H2^q), which lacks LC but not dermal DC or skin macrophages [(18), Figure 4A], was transplanted into B6 IκBαΔN-Tg or WT mice (H-2^b). Significantly delayed rejection of Id2^{-/-} skin was observed in IκBαΔN-Tg (p=0.0017) but not control littermates (Figure 4B). Lack of donor LC resulted in a reduced precursor frequency of IFN-γ-producing alloreactive splenocytes in IκBαΔN-Tg but not WT mice (Figure 4C), suggesting that donor LC may contribute to Th1 differentiation in NF-κB-impaired T cells. Eventual rejection of Id2^{-/-} skin allografts by IκBαΔN-Tg mice was probably not dependent on alloantibody production, as IκBαΔN-Tg mice failed to generate allospecific IgG antibodies following skin transplantation (Figure 4D). Together, these results imply that donor LC are important for the onset of skin allograft rejection in mice with impaired T cell-NF-κB.

Donor LC activate NF- κ B impaired T cells *in vitro*

To test if LC can stimulate I κ B α Δ N-Tg T cells despite impaired NF- κ B activity, the activating capacity of sorted epidermal LC, splenic LDC/MDC and splenic pDC were compared (Figure 5A). Sorted APC were cultured with OVA protein in the presence or absence of the inflammatory cytokine IL-1 β to induce maximal DC maturation. Two days later, these cells were used as stimulators of OVA-specific DO11.10 CD4⁺ WT or I κ B α Δ N-Tg T cells. IL-1 β -stimulated-LC promoted a stronger proliferation of both WT and I κ B α Δ N-Tg T cells when compared to splenic DC, whether pDC or MDC/LDC (Figure 5B). This was not due to greater antigen processing by LC, as the same results were observed when LC and splenic DC were pulsed with OVA peptide rather than protein (data not shown). These results underscore the greater capacity of LC to stimulate T cells, even if they are impaired in NF- κ B activity.

B6/CD80/CD86-double deficient skin grafts were acutely rejected by BALB/c I κ B α Δ N-Tg mice with little delay when compared with control skin grafts (Figure 6A), suggesting that B7 family members in donor LC play a minimal role in promoting activation of NF- κ B-impaired T cells. In contrast, different levels of MHC Class II between the different types of donor DC subsets (Figure 5A) may play a role in the enhanced capacity of LC to activate NF- κ B-impaired T cells, as acute rejection of B6/MHC class II-deficient B6 skin was delayed in BALB/c I κ B α Δ N-Tg ($p=0.0021$) but not in WT mice (Figure 6B). Alternatively, lack of rejection of MHC class II-deficient skin by I κ B α Δ N-Tg mice may be due to the fact that unlike direct presentation of alloantigen, indirect presentation cannot overcome the T cell-NF- κ B deficiency. Analysis of splenic DC subsets in I κ B α Δ N-Tg mice showed similar phenotype and function as WT DC (data not shown).

LC are sufficient to promote cardiac allograft rejection

Our previous results indicate that donor LC play an important role in the activation of NF- κ B-impaired T cells. We next tested whether they are sufficient to promote rejection of cardiac allografts, which are otherwise permanently accepted by I κ B α Δ N-Tg mice (9). B6/I κ B α Δ N-Tg mice were transplanted with BALB/c hearts and received an injection of donor LC or splenic CD11c^{hi} DC. Administration of donor LC was more efficient than that of splenic DC at inducing rejection of cardiac allografts by I κ B α Δ N-Tg mice (Figure 7). This result suggests that LC are sufficient to activate NF- κ B-impaired T cells, thus resulting in subsequent rejection of allogeneic hearts.

Discussion

This study demonstrates that donor LC play an important role in promoting acute rejection of skin allografts in mice with deficient T cell-NF- κ B activity. Our data suggest that LC can bypass T cell-NF- κ B impairment allowing T cells to reach an activation threshold sufficient for allograft rejection.

We and others have previously shown that mice over-expressing the super-repressor form of I κ B α in T cells permanently accept cardiac allografts (8,9), but reject skin allografts. In this study, we show similar acceptance of cardiac allografts and rejection of skin allografts by CARMA1-deficient mice, in which TCR-driven NF- κ B activation is selectively impaired. Together, these results suggest that some tissue allografts may promote NF- κ B-dependent responses while others may be capable of triggering T cell-NF- κ B-independent responses that can in turn lead to allograft rejection. The presence or absence of regulatory T cells does also not appear to affect graft fate in mice with T cell-impaired NF- κ B activity. Indeed similar acceptance of heart allografts but rejection of skin allografts was observed in

I κ B α Δ N-Tg mice that have normal percentages of regulatory T cells (10) and in CARMA1KO mice that lack regulatory T cells (Molinero et al, unpublished results).

OVA-Tg skin promoted a higher proliferation of antigen-specific DO11.10/I κ B α Δ N-Tg T cells when compared to OVA-Tg heart. It is possible that I κ B α Δ N-Tg T cells, when weakly activated by cardiac donor and splenic recipient DC, do not reach an activation threshold sufficient to maintain their oligoclonal proliferation. We have recently shown that overexpression of Bcl-x_L on I κ B α Δ N-Tg T cells restored cardiac allograft rejection in these mice (10), suggesting that donor cardiac DC and recipient splenic DC may not support survival of I κ B α Δ N-Tg T cells.

Our results show that rejection of skin devoid of LC is markedly delayed in mice with impaired NF- κ B signaling in T cells but not in WT mice. In keeping with the lack of effect of LC deficiency in WT recipients, LC have been shown to be dispensable for contact hypersensitivity reactions in WT mice where they may even serve a regulatory function (34). Our data suggest that presentation of alloantigen to WT T cells by non-LC donor APC or by recipient APC is sufficient for maintaining normal rejection kinetics when T cell function is normal. In contrast, when T cell function is impaired such as in I κ B α Δ N-Tg T cells, lack of donor LC has significant consequences. Other mouse models of T cell-impaired function have similarly revealed the importance of discrete pathways of alloreactivity. Use of CD28^{-/-} mice, for instance, which contain T cells with markedly impaired function but retain the ability to reject allografts, revealed the requirement for CD8⁺ T cells and NK cells (9,35,36) in the acute rejection of cardiac allografts, whereas those cell subsets participate in, but are not essential for, rejection in WT mice. Nevertheless, rejection of Id2-deficient skin allograft does eventually occur in I κ B α Δ N-Tg mice, suggesting that non-LC donor APC or recipient APC are sufficient to activate NF- κ B-impaired T cells. In fact the potency of donor dermal DC has not been compared to that of LC, such that we cannot exclude that delayed rejection of Id2-deficient skin allografts in I κ B α Δ N-Tg mice is due to reduced total number of donor DC. Nevertheless, our results indicate an important role of donor LC in promoting alloresponses of NF- κ B-impaired T cells *in vivo*.

The superior immunostimulatory capacity of LC for WT T cells when compared with splenic DC has long been known (21), (37) making LC interesting candidates for anti-tumor vaccines (38,39). In this report we observed that LC can also successfully activate NF- κ B-impaired T cells, both *in vitro* and *in vivo*. Most remarkable is the capacity of LC to induce the production of IFN- γ by I κ B α Δ N-Tg T cells, as the IFN- γ gene is strongly regulated by NF- κ B (4). Our results suggest that the enhanced stimulatory capacity of LC is not due to higher expression of CD80 or CD86. It is conceivable that stimulatory capacity by LC is due to greater expression of other costimulatory ligands. Recently, it has been shown that OX40L plays a role in murine skin allograft rejection (40), as combined targeting of OX40/OX40L, CD28/B7 and CD40/CD40L costimulation enabled long-term skin allograft survival in WT recipients (40). However, we could not detect expression of OX40L mRNA or protein on either freshly isolated or cultured LC, whereas cultured splenic DC expressed low but detectable levels of this molecule (data not shown). Furthermore, treatment of I κ B α Δ N-Tg skin allograft recipients with high doses of anti-OX40L did not result in prolongation of skin allograft survival (data not shown). Expression of other members of the TNF family (4.1BBL, CD70) or of the B7 family (ICOSL, B7h3, B7h4) (41) of costimulatory molecules have not been thoroughly studied in LC, raising the possibility that any or all of them may play a role in the enhanced costimulatory capacity of LC.

Our results support the hypothesis that donor LC induce sufficient function in I κ B α Δ N-Tg mice, allowing NF- κ B-impaired T cells to reach an activation threshold necessary for T cell

differentiation and allograft rejection. They also suggest that immunosuppression may have to be tailored to the type of tissue that is being transplanted. Development of new reagents that selectively block NF- κ B activation in T cells may be useful to prevent rejection of some, but not all, organ allografts in the clinic. A combination of NF- κ B inhibitors with therapies that suppress specific factors expressed by donor APC infiltrating the transplanted organ may prove effective to prevent rejection of very immunogenic organs.

Acknowledgments

We thank the technical help of David Leclerc and Ryan Duggan (Flow Cytometry Facility), Terry Li (Immunohistochemistry Facility) and Shirley Bond (Light Microscopy Facility).

REFERENCES

1. Medeiros RB, Burbach BJ, Mueller KL, Srivastava R, Moon JJ, Highfill S, et al. Regulation of NF- κ B activation in T cells via association of the adapter proteins ADAP and CARMA1. *Science* 2007;316(5825):754–758. [PubMed: 17478723]
2. Boothby MR, Mora AL, Scherer DC, Brockman JA, Ballard DW. Perturbation of the T lymphocyte lineage in transgenic mice expressing a constitutive repressor of nuclear factor (NF)- κ B. *J Exp Med* 1997;185(11):1897–1907. [PubMed: 9166419]
3. Aune TM, Mora AL, Kim S, Boothby M, Lichtman AH. Costimulation reverses the defect in IL-2 but not effector cytokine production by T cells with impaired IkappaBalpha degradation. *J Immunol* 1999;162(10):5805–5812. [PubMed: 10229814]
4. Corn RA, Aronica MA, Zhang F, Tong Y, Stanley SA, Kim SR, et al. T cell-intrinsic requirement for NF- κ B induction in postdifferentiation IFN- γ production and clonal expansion in a Th1 response. *J Immunol* 2003;171(4):1816–1824. [PubMed: 12902482]
5. Seetharaman R, Mora AL, Nabozny G, Boothby M, Chen J. Essential role of T cell NF- κ B activation in collagen-induced arthritis. *J Immunol* 1999;163(3):1577–1583. [PubMed: 10415062]
6. Tato CM, Villarino A, Caamano JH, Boothby M, Hunter CA. Inhibition of NF- κ B activity in T and NK cells results in defective effector cell expansion and production of IFN- γ required for resistance to *Toxoplasma gondii*. *J Immunol* 2003;170(6):3139–3146. [PubMed: 12626571]
7. Egawa T, Albrecht B, Favier B, Sunshine MJ, Mirchandani K, O'Brien W, et al. Requirement for CARMA1 in antigen receptor-induced NF- κ B activation and lymphocyte proliferation. *Curr Biol* 2003;13(14):1252–1258. [PubMed: 12867038]
8. Finn PW, Stone JR, Boothby MR, Perkins DL. Inhibition of NF- κ B-dependent T cell activation abrogates acute allograft rejection. *J Immunol* 2001;167(10):5994–6001. [PubMed: 11698479]
9. Zhou P, Hwang KW, Palucki DA, Guo Z, Boothby M, Newell KA, et al. Impaired NF- κ B activation in T cells permits tolerance to primary heart allografts and to secondary donor skin grafts. *Am J Transplant* 2003;3(2):139–147. [PubMed: 12603209]
10. Zhou P, Balin SJ, Mashayekhi M, Hwang KW, Palucki DA, Alegre ML. Transplantation tolerance in NF- κ B-impaired mice is not due to regulation but is prevented by transgenic expression of Bcl-xL. *J Immunol* 2005;174(6):3447–3453. [PubMed: 15749879]
11. Youssef AR, Otley C, Mathieson PW, Smith RM. Effector mechanisms in murine allograft rejection: comparison of skin and heart grafts in fully allogeneic and minor histocompatibility antigen-mismatched strain combinations. *Transpl Int* 2002;15(6):302–309. [PubMed: 12072901]
12. Zenke M, Hieronymus T. Towards an understanding of the transcription factor network of dendritic cell development. *Trends Immunol* 2006;27(3):140–145. [PubMed: 16406699]
13. Wu L, Liu YJ. Development of dendritic-cell lineages. *Immunity* 2007;26(6):741–750. [PubMed: 17582346]
14. Maraskovsky, E.; Pulendran, B.; Shortman, K. Lymphoid-related dendritic cells. Academic Press; NYC: 1999.
15. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, et al. The nature of the principal type 1 interferon-producing cells in human blood. *Science* 1999;284(5421):1835–1837. [PubMed: 10364556]

16. Ochando JC, Homma C, Yang Y, Hidalgo A, Garin A, Tacke F, et al. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat Immunol* 2006;7(6): 652–662. [PubMed: 16633346]
17. Allam JP, Novak N, Fuchs C, Asen S, Berge S, Appel T, et al. Characterization of dendritic cells from human oral mucosa: a new Langerhans' cell type with high constitutive FcεpsilonRI expression. *J Allergy Clin Immunol* 2003;112(1):141–148. [PubMed: 12847491]
18. Hacker C, Kirsch RD, Ju XS, Hieronymus T, Gust TC, Kuhl C, et al. Transcriptional profiling identifies Id2 function in dendritic cell development. *Nat Immunol* 2003;4(4):380–386. [PubMed: 12598895]
19. Merad M, Hoffmann P, Ranheim E, Slaymaker S, Manz MG, Lira SA, et al. Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. *Nat Med* 2004;10(5):510–517. [PubMed: 15098028]
20. Stoitzner P, Holzmann S, McLellan AD, Ivarsson L, Stossel H, Kapp M, et al. Visualization and characterization of migratory Langerhans cells in murine skin and lymph nodes by antibodies against Langerin/CD207. *J Invest Dermatol* 2003;120(2):266–274. [PubMed: 12542532]
21. Schuler G, Romani N, Steinman RM. A comparison of murine epidermal Langerhans cells with spleen dendritic cells. *J Invest Dermatol* 1985;85(1 Suppl):99s–106s. [PubMed: 3159809]
22. Mathers AR, Tkacheva OA, Janelsins BM, Shufesky WJ, Morelli AE, Larregina AT. In vivo signaling through the neurokinin 1 receptor favors transgene expression by Langerhans cells and promotes the generation of Th1- and Tc1-biased immune responses. *J Immunol* 2007;178(11): 7006–7017. [PubMed: 17513750]
23. Gorbachev AV, Fairchild RL. CD40 engagement enhances antigen-presenting langerhans cell priming of IFN-gamma-producing CD4+ and CD8+ T cells independently of IL-12. *J Immunol* 2004;173(4):2443–2452. [PubMed: 15294958]
24. Affleck DG, Bull DA, Albanil A, Shao Y, Brady J, Karwande SV, et al. Interleukin-18 production following murine cardiac transplantation: correlation with histologic rejection and the induction of INF-gamma. *J Interferon Cytokine Res* 2001;21(1):1–9. [PubMed: 11177575]
25. Franzoso G, Carlson L, Xing L, Poljak L, Shores EW, Brown KD, et al. Requirement for NF-kappaB in osteoclast and B-cell development. *Genes Dev* 1997;11(24):3482–3496. [PubMed: 9407039]
26. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation* 1973;16(4):343–350. [PubMed: 4583148]
27. Szot GL, Zhou P, Sharpe AH, He G, Kim O, Newell KA, et al. Absence of host B7 expression is sufficient for long-term murine vascularized heart allograft survival. *Transplantation* 2000;69(5): 904–909. [PubMed: 10755548]
28. Harlin H, Podack E, Boothby M, Alegre ML. TCR-independent CD30 signaling selectively induces IL-13 production via a TNF receptor-associated factor/p38 mitogen-activated protein kinase-dependent mechanism. *J Immunol* 2002;169(5):2451–2459. [PubMed: 12193714]
29. Chen L, Wang T, Zhou P, Ma L, Yin D, Shen J, et al. TLR engagement prevents transplantation tolerance. *Am J Transplant* 2006;6(10):2282–2291. [PubMed: 16970798]
30. Hannum LG, Haberman AM, Anderson SM, Shlomchik MJ. Germinal center initiation, variable gene region hypermutation, and mutant B cell selection without detectable immune complexes on follicular dendritic cells. *J Exp Med* 2000;192(7):931–942. [PubMed: 11015435]
31. Reed AJ, Noorchashm H, Rostami SY, Zarrabi Y, Perate AR, Jeganathan AN, et al. Alloreactive CD4 T cell activation in vivo: an autonomous function of the indirect pathway of alloantigen presentation. *J Immunol* 2003;171(12):6502–6509. [PubMed: 14662850]
32. Ochando JC, Krieger NR, Bromberg JS. Direct versus Indirect Allorecognition: Visualization of Dendritic Cell Distribution and Interactions During Rejection and Tolerization. *Am J Transplant*. 2006
33. Mayerova D, Wang L, Bursch LS, Hogquist KA. Conditioning of Langerhans cells induced by a primary CD8 T cell response to self-antigen in vivo. *J Immunol* 2006;176(8):4658–4665. [PubMed: 16585558]

34. Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ. Epidermal langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 2005;23(6):611–620. [PubMed: 16356859]
35. Szot GL, Zhou P, Rulifson I, Wang J, Guo Z, Kim O, et al. Different mechanisms of cardiac allograft rejection in wildtype and CD28-deficient mice. *Am J Transplant* 2001;1(1):38–46. [PubMed: 12095035]
36. McNerney ME, Lee KM, Zhou P, Molinero L, Mashayekhi M, Guzier D, et al. Role of natural killer cell subsets in cardiac allograft rejection. *Am J Transplant* 2006;6(3):505–513. [PubMed: 16468959]
37. Morelli AE, Rubin JP, Erdos G, Tkacheva OA, Mathers AR, Zahorchak AF, et al. CD4+ T cell responses elicited by different subsets of human skin migratory dendritic cells. *J Immunol* 2005;175(12):7905–7915. [PubMed: 16339526]
38. Kumamoto T, Huang EK, Paek HJ, Morita A, Matsue H, Valentini RF, et al. Induction of tumor-specific protective immunity by in situ Langerhans cell vaccine. *Nat Biotechnol* 2002;20(1):64–69. [PubMed: 11753364]
39. Fay JW, Palucka AK, Paczesny S, Dhodapkar M, Johnston DA, Burkeholder S, et al. Long-term outcomes in patients with metastatic melanoma vaccinated with melanoma peptide-pulsed CD34(+) progenitor-derived dendritic cells. *Cancer Immunol Immunother* 2006;55(10):1209–1218. [PubMed: 16331519]
40. Demirci G, Amanullah F, Kewalaramani R, Yagita H, Strom TB, Sayegh MH, et al. Critical role of OX40 in CD28 and CD154-independent rejection. *J Immunol* 2004;172(3):1691–1698. [PubMed: 14734751]
41. Rothstein DM, Sayegh MH. T-cell costimulatory pathways in allograft rejection and tolerance. *Immunol Rev* 2003;196:85–108. [PubMed: 14617200]

Figure 1. CARMA1-deficient mice accept cardiac but not skin allografts long term
BALB/c hearts (left panel) or skin (right panel) allografts were transplanted into the abdominal cavity of fully mismatched B6/CARMA1^{+/+}, CARMA1^{+/-} or CARMA1^{-/-} mice (n=5 for all groups except for CARMA1^{+/-} where n=2). Graft survival was assessed over time.

Figure 2. Skin rejection in IkBαΔN-Tg is not due to residual T cell-NF-κB signaling

A. B6/IkBαΔN-Tg and WT T cells were stimulated with anti-CD3- or anti-CD3 plus anti-CD28-coated beads (1 μg/ml each) for 12h. Nuclear extracts were prepared and used in an EMSA for detection of DNA-binding activity by NF-κB proteins. **B.** Sublethally irradiated RAG2-deficient mice were reconstituted with p50/p52-double deficient or control p50^{+/+}/p52^{-/-} bone marrow. Splenic T cells were purified 12 weeks later and stimulated with anti-CD3 and anti-CD28 mAb-coated beads, as described above. At 12 h of stimulation, nuclear extracts were prepared and assayed for the presence of NF-κB/DNA and AP-1/DNA complexes by EMSA. **C.** Full thickness BALB/c skin was grafted onto the flank of RAG2-deficient mice unmanipulated or reconstituted with either p50^{-/-}/p52^{-/-} or control p50^{+/+}/p52^{-/-} bone marrow and graft survival was assessed over time. (n = 4-5/group).

Figure 3. Skin allografts promote proliferation and IFN- γ production by NF- β B-impaired T cells
A. CFSE-labeled DO11.10 or DO11.10/IkBαΔN-Tg CD4⁺ T cells (2×10^6) were adoptively transferred into syngeneic BALB/c recipients. One day later, mice received skin or heart transplants from syngeneic BALB/c or OVA-Tg mice. After 5 days, proliferation of live DO11.10 T cells was examined by flow cytometry, using CFSE dilution and DAPI exclusion within CD4⁺/KJ1-26⁺ cells from spleen, axillary and mesenteric lymph nodes. This result is representative of two independent experiments. **B.** B6/IkBαΔN-Tg or WT mice were left untreated or were transplanted (Tx) with BALB/c skin. Mice were sacrificed 18 days later and cell suspensions of peripheral LN were prepared. Cell cultures were adjusted such that 5×10^5 responder T cells were co-cultured with 4×10^5 irradiated, syngeneic (B6, light gray bars) or donor (BALB/c, dark gray bars) stimulators. *** $p < 0.001$. Results represent the mean and SD of 6 determinations.

Figure 4. Rejection of skin devoid of LC is markedly delayed in IκBαΔI-Tg mice

A. Tail skin from Id2^{-/-} or Id2^{+/-} mice was stained for IA/IE (upper panels) or with the rat IgG isotype control (lower panels). The filled arrows point to epidermal LC that express IA/IE; the empty arrows point to dermal DC. **B.** Full thickness Id2^{+/+}, Id2^{+/-} or Id2^{-/-} skin (H-2^q) was grafted onto the flank of B6/WT and IκBαΔI-Tg mice and graft survival was assessed over time. (n=5 for all groups except for recipients of Id2^{+/-} grafts where n=3). p < 0.01 between survival of Id2^{-/-} and that of Id2^{+/-} or Id2^{+/+} in IκBαΔI-Tg mice). **C.** Mice from Panel B were sacrificed after skin allograft rejection and stimulation for detection of IFN-γ-producing alloreactive splenocytes was performed as for Figure 3B. *** p<0.001

between $\text{Id}2^{+/-}$ and $\text{Id}2^{-/-}$ in $\text{I}\kappa\text{B}\alpha\Delta\text{N-Tg}$ recipients. **D.** Weekly analysis of serum IgG specific for FvB splenocytes was determined in 3 mice per group.

Figure 5. LC are stronger immunostimulators of $\text{IkB}\alpha\Delta\text{N-Tg}$ T cells than splenic MDC/LDC or pDC

A. BALB/c epidermal LC and splenic CD11c^{hi}IA^d^{hi} (MDC and LDC) and CD11c^{int}IA^d^{int} (pDC) were obtained from the epidermis and spleen, as described in Materials and Methods. Cell suspensions were stained for CD11c (N418) and IA^d and analyzed by flow cytometry, previously to FACS-based sorting. In the upper inset: epidermal CD11c⁺IA^d⁺ cells were stained intracellularly for Langerin (gray line) or with control IgG (black line). In the middle inset, expression of CD11b and CD8 α by MDC/LDC. Lower inset: expression of mPDCA-1 and B220 by pDC. **B.** Proliferation of WT or $\text{IkB}\alpha\Delta\text{N-Tg}$ DO11.10 CD4⁺ T cells (10^5) stimulated with LC, splenic MDC/LDC or pDC (4×10^3) cultured with OVA protein in

the presence or absence of 10 ng/ml IL-1 β , was assessed by ^3H -thymidine incorporation. *
* $p < 0.01$; *** $p < 0.001$.

Figure 6. Donor antigen presentation, but not donor CD80/CD86 is required for normal rejection kinetics of skin allografts by IκBαΔN-Tg

A. Full thickness B6/WT or CD80/CD86 double knockout (DKO) skin was transplanted into the flank of BALB/c/WT and IκBαΔN-Tg mice and graft survival was assessed over time (n=7 for CD80/CD86 WT and n=8 for CD80/CD86 DKO). **B.** Full thickness MHC class II^{+/+} or MHC class II^{-/-} (H-2^b) skin allografts were grafted onto the flank of BALB/c/WT (n=4 for MHC II^{+/+} and n=8 for MHC II^{-/-}) and BALB/c/IκBαΔN-Tg (n=4 for MHC II^{+/+} and n=5 for MHC II^{-/-}) mice and graft survival was assessed over time. p< 0.01 for IκBαΔN-Tg recipients of MHC II^{-/-} grafts when compared with all other groups).

Figure 7. Donor LC are sufficient to induce cardiac allograft rejection in $\text{IkB}\alpha\Delta\text{N}$ -Tg mice
 $\text{IkB}\alpha\Delta\text{N}$ -Tg mice were transplanted with BALB/c hearts and left untreated or injected with FACS-based sorted epidermal LC (allo-LC) or $\text{CD11c}^{\text{hi}}\text{IA}^{\text{hi}}$ splenic DC (allo-SpDC) that had all been cultured for one day as a cell suspension (n=5 in each group with 2 mice receiving LC i.v. and 3 mice receiving LC s.c.). Graft survival was assessed over time. $p < 0.01$ between recipients of LC and splenic DC.