

Ly-6A is critical for the function of double negative regulatory T cells

Zhu-Xu Zhang¹, William L. Stanford² and Li Zhang¹

¹ Departments of Laboratory Medicine and Pathobiology, Immunology, University of Toronto, Multi Organ Transplantation Program, Toronto General Research Institute, University Health Network, Toronto, Canada

² Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, Canada

We have recently demonstrated that CD3⁺CD4⁻CD8⁻ double negative (DN) T cells can down-regulate allogeneic immune responses both *in vitro* and *in vivo* by killing activated syngeneic CD8⁺ T cells. The goal of this study was to identify molecules that are crucial for DN T cell-mediated suppression. We demonstrate that Ly-6A (Sca-1) is highly expressed on DN T cells. Incubation with IL-10 significantly reduced Ly-6A expression and the function of DN T cells. DN T cell-mediated killing was significantly reduced when Ly-6A was blocked. Ly-6A-deficient mice showed an accelerated allograft rejection when compared to wild-type controls. Furthermore we demonstrate that pretransplantation donor lymphocyte infusion (DLI) led to activation and proliferation of recipient DN T cells and prolongation of bm1→B6 skin allograft survival. However, when the recipients were deficient in Ly-6A, the beneficial effect of DLI on allograft survival was abolished. Moreover, deficiency in Ly-6A did not affect the activation and proliferation of DN T cells. Rather, it impaired the ability of DN T cells to kill activated anti-donor CD8⁺ T cells. Taken together, our data indicate that Ly-6A plays a crucial role in DN T cell-mediated regulation *in vitro* and *in vivo*, perhaps by enhancing DN-CD8⁺ T cell signaling.

Key words: Ly-6A / DN T cell / Regulatory / Transplantation

Received	23/1/02
Revised	25/2/02
Accepted	4/3/02

1 Introduction

There is a compelling evidence to support a role for regulatory T cells in the down-regulation of immune responses to self or allogeneic antigens [1–5]. However, the molecular mechanisms by which regulatory T cells exert their function remain unclear. Previous studies have demonstrated that single MHC class I locus mismatched pre-transplantation donor-lymphocyte infusion (DLI) can enhance donor-specific allograft survival [6–10]. Because none of the recipients were thymectomized, the function of newly released anti-donor T cells from the thymus must have been inhibited to maintain the allografts. Indeed, alloantigen reactive CD8⁺ T cells are inhibited [7–9, 11]. We have demonstrated that double negative (DN) regulatory T cells are able to specifically suppress anti-donor CD8⁺ T cells *in vitro* and *in vivo* by eliminating activated syngeneic CD8⁺ T cells through

direct cell-cell contact [12]. The regulatory function of DN T cells requires signals through both TCR-MHC and Fas-Fas ligand interaction [12]. As there is no detectable expression of conventional costimulatory molecules CD4, CD8, CD28, CD44 and CTLA-4 on DN T cells [12], the goal of this study was to identify accessory molecules that are important for the function of DN regulatory T cells. cDNA subtraction and differential screening of RNA between regulatory and non-regulatory DN T cell clones identified several genes, including Ly-6A, which may be involved in the function of DN regulatory T cells.

Ly-6A is a glycosyl phosphatidylinositol (GPI)-anchored cell surface protein belonging to the Ly-6 gene family. It is widely expressed on different cell types, including hematopoietic stem cells, most lymphocytes, thymocytes, monocytes, kidney epithelial cells and osteoblasts [13–16]. Ly-6A expression is up-regulated upon T cell activation [13–15, 17]. The function of Ly-6A remains controversial. Several studies have shown that cross-linking of Ly-6A can activate both CD4 and CD8 T cells [18–20]. The activation function of Ly-6A is directly through TCR signal transduction pathway [18, 19, 21, 22]. T cell activation via the TCR was substantially reduced if Ly-6A was mutated or inhibited by antisense

[1 22827]

Abbreviations: DN: Double negative DLI: Donor lymphocyte infusion GPI: Glycosyl phosphatidylinositol MST: Mean survival time

nucleotide [16, 23]. Furthermore, Ly-6A associates with the Src family tyrosine kinases, p56^{lck} and p59^{fyn}, that are known to be required for normal TCR signaling [22, 24]. Additional studies suggested that Ly-6A mediates cell-cell adhesion by binding to a ligand expressed on lymphoid cells [25]. These results suggest that expression of Ly-6A is important for TCR signaling and T cell activation. In contrast, others have reported that activation of T cells by using cross-linking antibodies to both Ly-6A and CD3 down-regulates IL-2 production [18, 19], suggesting an inhibitory role of Ly-6A in T cell activation.

Thus, Ly-6A appears to function differently depending upon the stimuli that are applied to T cells. We undertook a genetic approach to determine the function of Ly-6A in T cell activation. We have demonstrated that CD4⁺ or CD8⁺ T lymphocytes from Ly-6A null mice exhibit a higher and sustained proliferation both *in vivo* and *in vitro* in responding to antigen or mitogen stimulations that act through the TCR [26]. These data support the notion that the function of Ly-6A is to down-modulate proliferation of immune effector cells [18, 19, 26]. However, whether Ly-6A exerts its effect directly on CD4 and CD8 cells, or indirectly through other cells or molecules is not known. Moreover, whether Ly-6A has any effect on the function and mechanism of DN regulatory T cells has not been studied. Here we demonstrate that Ly-6A is a repressor for allogeneic immune responses, which exerts its role, at least partially, through enhancing the function of DN regulatory T cells.

2 Results

2.1 Treatment with IL-10 reduces the suppressive function of DN regulatory T cells

Previously, we have demonstrated that DN T cells possess regulatory function and can specifically kill syngeneic CD8⁺ T cells activated by the same antigen [12]. PCR analysis showed that DN T cells, unlike CD4 regulatory T cells reported by others [1–3], do not express IL-10 RNA at any time after activation [12]. To determine whether exogenous IL-10 could modulate the function of DN T cells, the DN regulatory T cell clone TN12 was pre-treated with IL-10 before being used as a suppressor cells in a standard suppression assay. As shown in Fig. 1, the ability of the DN T cells to suppress the proliferation of CD8⁺ T cells was significantly diminished after IL-10 treatment compared with non-treated TN12 DN T cells. The DN T cells were sensitive to IL-10 at concentrations as low as 10 ng/ml. Similar results were obtained when other DN regulator T cell clones (CN02 and CN04) were studied (data not shown). These data demonstrate that IL-10 inhibits the regulatory function of DN T cells.

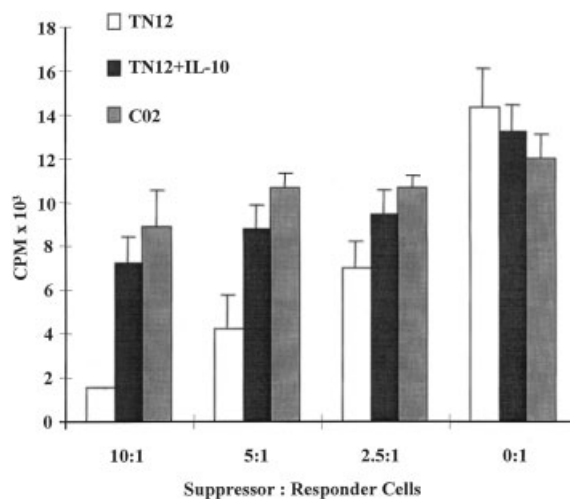
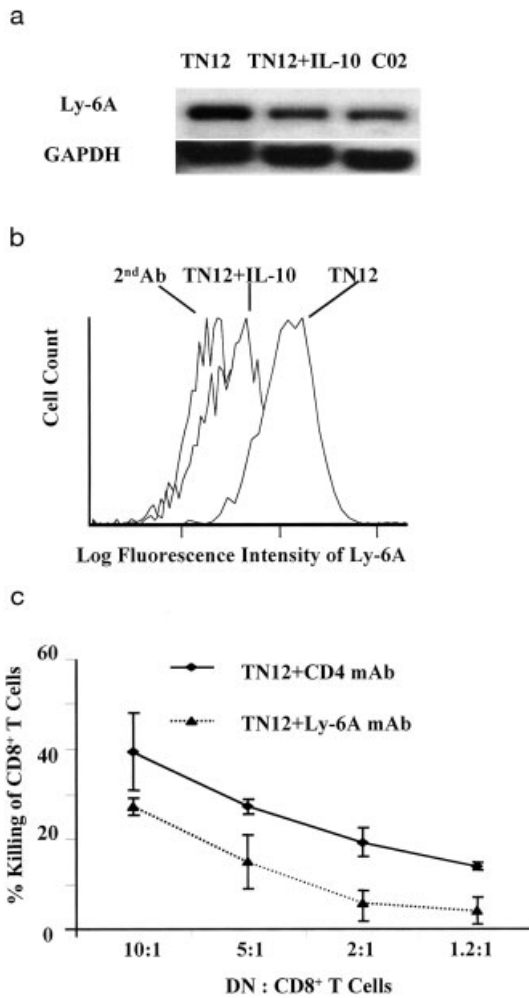


Fig. 1. Abrogation of 1B2⁺DN regulatory T cell-mediated suppression by IL-10. Purified naive CD8⁺ T cells from (2C \times dm2)_{F1} mice were used as responders and stimulated by irradiated (B6 \times BALB/c)_{F1} splenocytes. 1B2⁺DN regulatory T cell clone TN12 was either preincubated with IL-10 and washed (black bars) or left untreated (white bars) before being used as putative suppressor cells. A non-regulatory 1B2⁺CD8⁺ T cell clone (C02, grey bars) was used as a control. Responder and suppressor cells were co-cultured at ratio as indicated. Cell proliferation was measured by [³H]dThd incorporation. The results represent three independent experiments each with triple replicates. Similar results were obtained when other DN regulatory T cell clones (CN02 and CN04) were used.

2.2 IL-10 down-regulates Ly-6A expression and DN regulatory T cell-mediated cytotoxicity

To understand the molecular mechanisms by which IL-10 abrogates the function of DN regulatory T cells the gene expression pattern between IL-10-treated and non-treated DN TN12 clones was compared. The DN TN12 cells were incubated in the presence or absence of IL-10, and total mRNA was then purified. The cDNA from non-IL-10-treated TN12 cells was then subtracted by using the cDNA from IL-10-treated TN12 cells. The remaining cDNA species that were differentially expressed between the regulatory and non-regulatory DN T cell clones were cloned and sequenced. One of the identified gene fragments that demonstrated higher expression in IL-10-non-treated TN12 cells than in IL-10-treated cells was a 343-bp gene fragment of Ly-6A.

To confirm that Ly-6A gene expression is down-regulated by IL-10, the total mRNA was collected from non-treated or IL-10-treated TN12 clones as well as a non-regulatory CD8⁺ T cell clone C02. Northern blot analysis in Fig. 2a indicates that the regulatory DN T cells



express a higher level of Ly-6A mRNA than non-regulatory T cells. Treatment with IL-10 abolished the suppressive function as well as down-regulated Ly-6A mRNA expression in the DN T cells. Furthermore, immunofluorescence staining confirmed that Ly-6A protein expression on the DN T cells was also down-regulated after IL-10 treatment (Fig. 2b). In contrast, treatment with IL-4, another Th2 cytokine, did not alter the expression of Ly-6A on DN T cells (data not shown). These findings demonstrate that expression of Ly-6A RNA and protein is significantly higher on DN regulatory T cells than on the IL-10-treated non-regulatory DN or CD8⁺ T cells. To determine whether Ly-6A plays a role in DN T cell-mediated killing of CD8⁺ T cells, the DN regulatory T cell clone TN12 was pre-incubated with soluble anti-Ly-6A monoclonal antibody (mAb) to block the binding of Ly-6A to its ligand before being used as effector cells in cytotoxicity assays. We found that the DN T cells that were pre-treated with soluble anti-Ly-6A mAb significantly

reduced ability to kill activated syngeneic CD8⁺ T cells when compared with the cells that were incubated with the isotype-matched control mAb (Fig. 2c). Similar results were found when other DN regulatory T cell clones (CN02, and CN04) were used (data not shown). These results indicate that Ly-6A is an important molecule for the function of DN regulatory T cells *in vitro*.

2.3 Deficiency in Ly-6A accelerates allograft rejection

We have previously demonstrated that T cells from Ly-6A-deficient mice exhibited higher and sustained proliferation in response to alloantigen stimulation *in vitro* than T cells from wild-type littermate controls [26]. To determine the role and mechanism of Ly-6A expression in regulating allogeneic immune responses *in vivo*, we first studied whether lack of Ly-6A expression in recipients would accelerate allograft rejection. B6 wild-type and B6 Ly-6A-deficient mice were transplanted with allogeneic skin grafts from single MHC-class I locus-mismatched bm1 mice. The skin grafts were inspected daily. As shown in Fig. 3a, Ly-6A-deficient mice reject the bm1 skin grafts much faster than normal B6 mice [mean survival time (MST)=9.6 days, $n=6$ versus MST=15.6 days, $n=6$, $p<0.001$]. This result indicates that deficiency in Ly-6A in transplant recipients leads to accelerated allograft rejection.

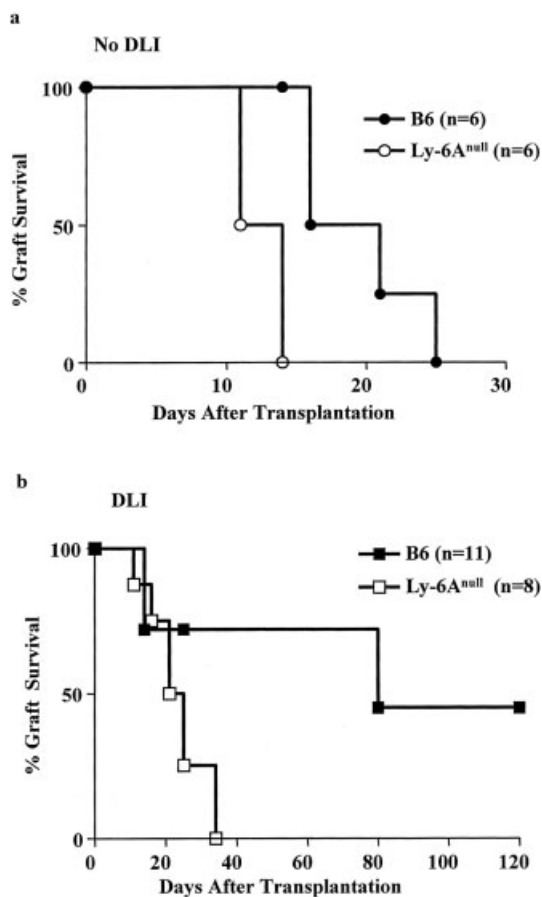


Fig. 3. Deficiency in Ly-6A in skin graft recipients accelerates allograft rejection. (a) B6 wide-type (filled circles) and Ly-6A null mice (open circles) were transplanted with allogeneic skin grafts from bm1 mice as described previously [7, 12]. The skin grafts were inspected daily until rejection. MST for B6 mice was 15.6 days and for Ly-6A null mice was 9.6 days ($p < 0.001$). (b) B6 (filled squares) or B6 Ly-6 null mice (open squares) were infused with viable spleen cells collected from bm1 mice. One week later, all mice were given skin grafts from bm1 mice. Survival of the skin grafts was monitored and scored for 120 days. MST for B6 mice was 80 days and for Ly-6A null mice was 18.5 days ($p < 0.0007$).

Previously, we and others have demonstrated that single MHC class I locus-mismatched pre-transplant DLI could enhance allograft survival [6–10]. Recently, we have further demonstrated in 2C-transgenic mice that DLI activates recipient DN regulatory T cells which can kill anti-graft CD8⁺ T cells ([12] and Young et al., submitted for publication). To test the hypothesis that the absence of Ly-6A may reduce DN regulatory T cell function and abrogate DLI-induced allograft survival, age- and sex-matched B6 and B6 Ly-6A-deficient mice were given DLI from bm1 mice. One week later, each recipient was transplanted with a bm1 skin allograft. As shown in Fig. 3b,

the bm1 skin graft survival was significantly prolonged in DLI-treated B6 mice (MST=80 days, $n=11$) compared to DLI-non-treated controls (MST=15.6 days, $n=6$), which is consistent with previous reports [8–10]. Of DLI-treated mice 40% permanently accepted bm1 skin allografts (>120 days). In contrast, all the bm1 skin grafts were rejected by DLI-treated Ly-6A null animals within 36 days with MST=18.5 days ($n=8$), which was significantly shorter than that seen in DLI-treated B6 wide-type skin graft recipients (MST=80 days, $n=11$, $p < 0.0007$, Fig. 3b). These data clearly demonstrate that the lack of Ly-6A expression in recipients leads to acceleration of allograft rejection in both non-treated and DLI-treated recipients.

2.4 Normal DN T cell proliferation and impaired depletion of CD8⁺ T cells after DLI

The accelerated allograft rejection seen in Ly-6A null mice could be due either to an enhanced proliferation of anti-donor T cells or reduced number and/or function of regulatory T cells as indicated by the sustained T cell proliferation in Ly-6A null mice [26] and the impairment of DN T cell-mediated killing of activated syngeneic CD8⁺ T cells by the pre-incubation of the DN regulatory T cell clones with soluble Ly-6A mAb (Fig. 2c). To understand the mechanism by which Ly-6A modulates allogeneic immune responses, we monitored the fate of recipient CD4⁺, CD8⁺ and DN T cells *in vivo*. B6 and Ly-6A null mice were given DLI followed by skin grafting from bm1 mice. At various time points after DLI, the spleens were harvested and the cells were triple-stained with anti-CD3-FITC, anti-CD4-PE and anti-CD8-Cy-chrom. The percentage of CD3⁺CD4⁺, CD3⁺CD8⁺ and CD3⁺DN T cells was analyzed using flow cytometry. No significant change in the percentage of CD4⁺ T cells was observed in B6 or B6 Ly-6A null mice (data not shown). However, there was a significant reduction in the percentage of CD8⁺ T cells in the spleens of B6 mice within the first 3 weeks after DLI (Fig. 4a). The percentage of CD8⁺ T cells gradually recovered at a later stage. In contrast, no reduction in the percentage of CD8⁺ T cells in the spleens of Ly-6A null mice was seen after DLI and transplantation (Fig. 4a). In fact, the percentage of CD8⁺ T cells in transplanted Ly-6A null mice was higher than what was seen in naive Ly-6A null mice. These data demonstrate that the lack of Ly-6A expression in DLI-treated recipients results in the accumulation of activated CD8⁺ T cells. Furthermore, the proportion of DN T cells in both B6 and B6 Ly-6A null mice was increased more than 2-fold one week after DLI (Fig. 4b). These results indicate that the lack of Ly-6A expression does not affect the proliferation of DN T cells in recipients, but leads to accumulation of peripheral CD8⁺ T cells.

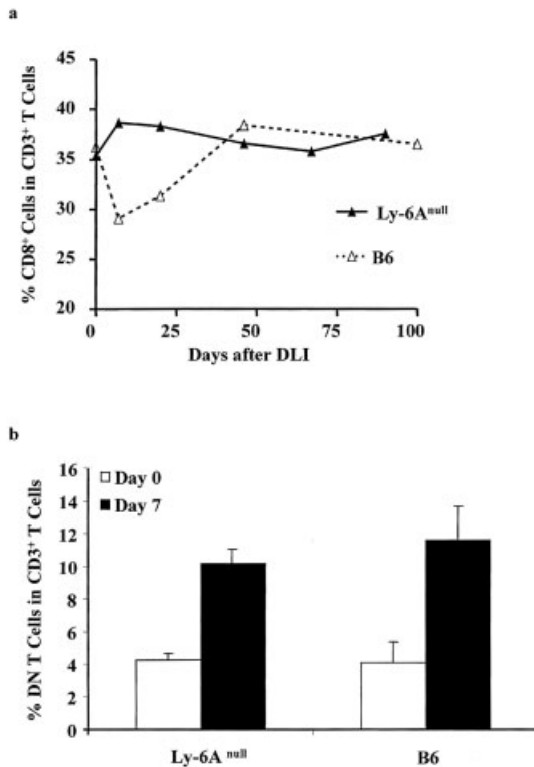


Fig. 4. Kinetics of CD8⁺ and DN T cells after DLI. B6 wide-type (dotted line) and Ly-6A null (solid line) mice were given DLI followed by skin grafting from bm1 mice. At various time points after transplantation, recipient mice were sacrificed and spleens were harvested. The splenocytes were triple-stained with anti-CD3-FITC, anti-CD4-PE and anti-CD8-Cy-chrom. The percentages of CD8⁺ T cells (a) and DN T cells (b) in CD3⁺ T cells were analyzed using flow cytometry. The percentages of CD8⁺ T cells (a) and DN T cells in the spleen of non treated B6 and Ly-6A null mice were also determined and served as day 0 controls. Each time point represents data collected from at least three mice.

2.5 Lack of Ly-6A expression reduces DN regulatory T cell-mediated cytotoxicity to syngeneic CD8⁺ T cells

There are at least two possibilities that may explain the increase in Ly-6A null and the decrease in B6 mice of CD8⁺ T cells after DLI. One could be that Ly-6A is involved in causing death of activated CD8⁺ T cells either by sending a negative signal directly or together with TCR to CD8⁺ T cells. The lack of Ly-6A expression therefore would result in the accumulation of CD8⁺ T cells in Ly-6A null mice. It is well known that cross-linking CD3 or TCR on activated CD8⁺ T cells can elicit activation induced cell death. To test this possibility, the activated CD8⁺ T cells from both wild-type and Ly-6A null B6 mice were purified and cross-linked by plate-bound anti-CD3

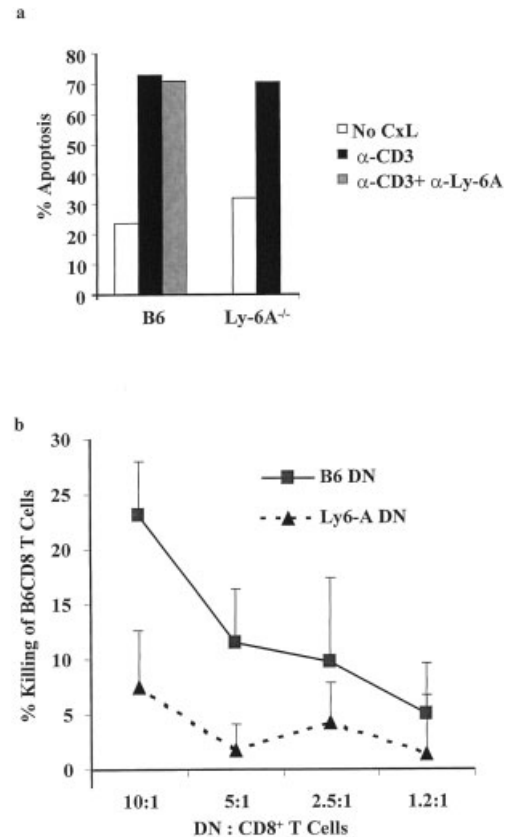


Fig. 5. (a) Ly-6A does not send negative signal to CD8⁺ T cells. Activated allogeneic CD8⁺ T cells (1×10^6 /well) from B6 wild-type or Ly-6A null mice were cultured in 24-well plates pre-coated with anti-CD3, anti-Ly-6A mAb or both for 3 h. Cells were harvested and stained with FITC-Annexin V and analyzed by flow cytometry. Data are expressed as percent apoptotic cells in the total population. Similar results were obtained after 8 h of cross-linking. (b) Ly-6A-deficient DN T cells have impaired cytotoxicity to syngeneic CD8⁺ T cells. DN T cells were purified from B6 (solid line) and Ly-6A null (dotted line) mice as described in Sect. 4, stimulated by irradiated bm1 spleen cells and used as effector cells. CD8⁺ T cells were obtained from B6 mice and stimulated by irradiated bm1 splenocytes and used as targets. Percent killing of B6 anti-bm1 CD8⁺ T cells by DN T cells was calculated as described in Sect. 4. The data are expressed as mean percent killing of three replicate cultures. The experiment was repeated four times and similar data were obtained.

mAb in the presence or absence of anti-Ly-6A mAb to mimic ligand binding [18–20]. Apoptosis of CD8⁺ T cells was analyzed by flow cytometry at 3 and 8 h after cross-linking. Apoptosis levels were not increased by either cross-linking of CD3 on Ly-6A^{-/-} CD8⁺ T cells or cross-linking both CD3 and Ly-6A on B6 CD8⁺ T cells when compared to cross-linking CD3 on B6 CD8⁺ T cells (Fig. 5a). These results indicate that it is unlikely that Ly-

6A sends a negative signal either directly or together with TCR to CD8⁺ T cells.

We have demonstrated that DN regulatory T cells can kill syngeneic CD8⁺ T cells that are activated by the same alloantigen [12], and that Ly-6A expression affects the function of DN regulatory T cell clones (Fig. 2c). To test the possibility that Ly-6A may modulate CD8⁺ T cells indirectly through regulating the function of DN T cells, DN T cells and CD8⁺ T cells were purified from B6 wild-type and Ly-6A null mice and stimulated with irradiated bm1 spleen cells. The activated DN T cells and CD8⁺ T cells were used as effector and target cells, respectively, in cytotoxicity assays. As shown in Fig. 5b, a significant loss of killing capability was observed when Ly-6A^{-/-} DN T cells were used as effector cells, compared to B6 wild-type DN T cell effectors. Similar results were obtained when the number of CD8⁺ T cells before and after coculture with DN T cells was assessed by flow cytometry (data not shown). These findings are consistent with the results using 1B2⁺ DN T cell clones (Fig. 2c) and demonstrate that Ly-6A is critical for DN T cell-mediated killing of activated syngeneic CD8⁺ T cells. These results together with the finding that DN T cells proliferated equivalently in both wild-type and Ly-6A null mice suggest that the high number of CD8⁺ T cells and accelerated skin graft rejection seen in Ly-6A null mice may be, at least partially, due to impaired killing of activated CD8⁺ T cells by DN T cells.

3 Discussion

IL-10 is a well-documented immunosuppressant that represses multiple activities of immune responses by inhibiting cytokine production [27, 28] and down-regulating surface protein expression of MHC class II, ICAM-1 and B7 [27, 29–31]. However, the function of IL-10 *in vivo* remains unclear. Studies have shown that administration of exogenous IL-10 prolonged murine cardiac graft survival [32, 33]. In addition, CD4⁺ regulatory T cells have been shown to secrete IL-10 and suppress CD4⁺ and CD8⁺ T cells that cause autoimmune diseases and transplant rejection [1, 2, 5]. In contrast, other studies have demonstrated that IL-10 does not prevent or even exacerbated graft-versus-host diseases and allograft rejection [34–36]. In the present study we found that IL-10 could down-regulate Ly-6A expression on DN regulatory T cells and impair the immunosuppressive function of DN T cells. This finding provides direct evidence that IL-10 can also inhibit the function of immune regulatory T cells that are involved in promoting immune tolerance, which helps to explain the contradictory effects of IL-10 reported by others [32–36].

We and others have demonstrated that pre-transplant infusion of single MHC class I locus-mismatched lymphocytes from allograft donors leads to significant prolongation of donor-specific skin graft survival [6–10, 12]. It was also shown that anti-donor CD8⁺ T cells were eliminated after DLI [7–9], and that regulatory T cells may be involved in DLI-induced donor-specific transplantation tolerance [12, 37]. However, the mechanism by which regulatory T cells mediate antigen-specific suppression remains unclear. In this study, we demonstrate that single MHC class I locus-mismatched DLI leads to a more than twofold increase in the number of DN regulatory T cells in both wild-type and Ly-6A null B6 mice (Fig. 4). This is a first report that DLI can elicit DN regulatory T cells activation that relates to donor-specific transplantation tolerance. However, elimination of CD8⁺ T cells and prolonged survival of skin allografts was only seen in wild-type, but not Ly-6A null mice (Fig. 3 and 4). These data indicate that Ly-6A is not important for activation and proliferation of DN regulatory T cells, but may be involved in DN regulatory T cell-mediated killing of activated CD8⁺ T cells. Indeed, we found that the ability to kill activated syngeneic CD8⁺ T cells by Ly-6A null DN T cells were markedly reduced *in vitro* (Fig. 5b). This may explain the observation that even though the number of DN T cells was increased after DLI, the anti-donor CD8⁺ T cells in Ly-6A null mice was not efficiently eliminated compared to that seen in wild-type mice (Fig. 4). These data, together with the results generated from transgenic DN T cell clones (Fig. 2c), demonstrate that Ly-6A plays an important role in regulating allogeneic immune responses, at least in part, by enhancing DN regulatory T cell-mediated killing of activated CD8⁺ T cells. Deficiency in Ly-6A in recipients impairs peripheral deletion and accelerates graft rejection in both untreated and DLI-treated mice.

The molecular mechanism by which Ly-6A enhances DN regulatory T cell-mediated suppression remains elusive. Our cross-linking results indicate that Ly-6A does not seem to send negative signals directly to CD8⁺ cells (Fig. 5a). Several studies have shown that Ly-6A can function as an accessory molecule to enhance cell-cell interaction, presumably through receptor-ligand interactions [38, 39]. Previously, we have demonstrated that direct cell-cell contact is necessary for DN T cell-mediated cytotoxicity to CD8⁺ T cells [12]. Because DN regulatory T cells do not express CD4, CD8, CD44, CD28 and other well-characterized accessory molecules [12], but do express a high level of Ly-6A (Fig. 2), it is plausible that Ly-6A functions as an important accessory molecule for DN T cells, perhaps through recognition of its ligand on target T cells [25, 39], which may increase adhesion between DN and CD8⁺ T cells, thereby indirectly increasing Fas-FasL interaction and T cell killing.

Another possibility could be that Ly-6A enhances the function of DN regulatory T cells indirectly by sending signals through other molecules or pathways. GPI-anchored proteins, including Ly-6A, are shown to mediate cell signaling during ligand-receptor engagement by interacting with receptors and secondary signaling molecules in glycosphingolipid cholesterol “rafts” that form microdomains within the plasma membrane [40, 41]. These microdomains function as platforms to coordinate the induction of signaling pathways. Analysis of TCR activation has demonstrated that following ligand binding, the activated TCR complex, the Src family kinases p56^{lck} and p59^{lyn}, the tyrosine kinases ZAP-70, Syk and Ras, and the adapter protein Shc co-localize in microdomains [24, 42, 43]. However, direct analysis of Ly-6A signaling cannot be performed until the ligand for Ly-6A is identified.

In summary, we have demonstrated for the first time that Ly-6A is an important molecule for DN regulatory T cell mediated inhibition of allogeneic immune response both *in vitro* and *in vivo*. The lack of Ly-6A expression and/or signaling leads to a significant reduction of DN regulatory T cell-mediated killing of activated syngeneic CD8⁺ T cells, and accelerated allograft rejection. Further investigation is required to determine whether Ly-6A can also play a role in modulating other regulatory T cells, and the molecular mechanism by which Ly-6A transduces its signal.

4 Materials and methods

4.1 Mice

C57BL/6 (B6, H-2^b), (B6×BALB/c)_{F1} (H-2^{b/d}), BALB/c-H-2-dm2 (dm2, a BALB/c L^d loss mutant), B6.C-H-2^{bm1} (bm1) mice were purchased from Jackson Laboratories (Bar Harbor, ME). B62C-transgenic mice express a transgenic TCR reactive against L^d class I MHC, which can be detected by a clonotypic mAb 1B2. B62C-transgenic mice were bred with dm2 mice and the subsequent (2C×dm2)_{F1} mice (H-2^{b/d}, L^d, anti-L^d TCR⁺) were used for studies. The mice were maintained in the animal facilities at the Ontario Cancer Institute or The Samuel Lunenfeld Research Institute.

4.2 Isolation of DN and CD8⁺ T cells

To isolate DN T cells, spleen and lymph node cells were obtained from B6 or Ly-6A null mice, depleted red blood cells, and passed through a nylon wool column to enrich the T cell population. The cells were then treated with depletion antibodies specific for murine CD4 (RL172, rat IgM) and CD8 (3.168.8, rat IgM) at 4°C for 45 min followed by the addition of rabbit complement (Cedarlane, Canada) at 37°C for

45 min. To isolate CD8⁺ T cells, only CD4 depletion antibody was applied. The viability and purity of cells were monitored by flow cytometry and were more than 96%. The purified DN or CD8⁺ T cells were stimulated with irradiated BALB/c or bm1 spleen cells in α -MEM supplemented with 10% FCS and 30 IU/ml IL-2 [12].

4.3 Treatment of 1B2⁺ CD8⁺ and 1B2⁺DN T clones with rIL-10

DN T cell clones TN12, CN02 and CN04 were prepared as previously described [12], and cultured in α -MEM supplemented with 10% FCS and 30 U/ml rIL-2, 30 U/ml rIL-4 and irradiated L^{d+} cells. For IL-10 treatment, 100 ng/ml recombinant IL-10 (Schering-Plough, NJ) was added to cultures for 5–8 days. T cells were then washed before being used as effector cells or for RNA subtraction.

4.4 cDNA subtraction

Total RNA was extracted with TriZol reagent (Gibco/BRL). cDNA was synthesized from Poly(A)⁺ RNA purified from total RNA using oligo(dT) cellulose chromatography (Pharmacia Biotech). The PCR-Select cDNA subtraction kit (Clontech) was used to subtract TN12 cDNA from cDNA isolated from IL-10 treated TN12 cells. The remaining cDNA was directly inserted into T/A cloning vector (Invitrogen). The cloned cDNA was sequenced (ABI Prism 377 DNA Sequencer) after plasmid purification using spin columns (QIAGEN). The sequences were analyzed using the Blast nucleotide research database (www.ncbi.nlm.nih.gov:80/Blast).

4.5 Northern blot analysis

The genes isolated from cDNA subtraction were confirmed by Northern blot analysis. The probes were synthesized using inserts isolated from the cloned plasmids with EcoRI and purified on agarose gels, then labeled with [³²P]dCTP (Amersham) by using T7 Quick Prime kit (Pharmacia Biotech). Total RNA (15 μ g) from the TN12 T cell clone was separated on 1.2% denatured agarose gels and transferred onto nylon membranes (Amersham). Hybridization and washing were carried out using standard protocols. The membrane was stripped between each hybridization and reprobbed with the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH).

4.6 Cell surface marker staining

T cell clones at various time points after activation were stained with fluorescence-conjugated mAb specifically recognizing the $\alpha\beta$ -TCR (1B2), CD3, CD4, CD8, CD28, $\gamma\delta$ -TCR, NK1.1 anti-Ly-6A/E mAb (E13–161.7), and Fas (all from

PharMingen). Data were acquired and analyzed on an EPICS® XL-MCL flow cytometer (Coulter Co. Miami, FL).

4.7 Mixed lymphocyte reaction (MLR) and suppression assays

Naive splenic (2C×dms)_{F1} CD8⁺ T cells (1,000 cells/well) were co-cultured in 96-well plates with irradiated (20 Gy) sex-matched splenocytes (3×10⁵ cells/well) from (B6×BALB/c)_{F1} mice in α -MEM supplemented with 10% FCS and 30 U/ml of rIL-2 and rIL-4. Varying numbers of purified DN regulatory T cells were added to MLR. After a 3-day incubation, 1 μ Ci of [³H]dThd was added to each well. Eighteen hours later, cells were harvested and counted in a beta scintillation counter. Cultures to which no DN T cells were added were used as controls.

4.8 Cytotoxicity assays

Target cell death resulting from co-culture with DN T cells was measured as previously reported [12]. Briefly, DN T cells were stimulated by irradiated allogeneic splenocytes for 2–3 days in the presence of IL-2/IL-4 (30 U/ml). Viable DN T cells were used as effector cells. CD8⁺ T cells were stimulated with appropriate antigens *in vitro* for 2–3 days, labeled with 10 μ Ci/ml of [³H]dThd at 37°C overnight and used as targets. After co-culture with the effector cells at 37°C for 18 h in the presence of fresh irradiated allogeneic splenocytes (20 Gy), the cells were harvested and counted in a beta scintillation counter. Specific cell lysis was calculated using the following equation: % Specific killing = (S–E)/S ×100, where E (experimental) is cpm of retained DNA in the presence of effector cells, and S (spontaneous) is cpm of retained DNA in the absence of effector cells.

4.9 DLI and skin grafting

B6 or B6 Ly-6 null mice were either infused with 5×10⁷–7×10⁷ viable spleen cells collected from bm1 mice or left untreated as controls. One week later, all mice were given skin grafts from bm1 mice as previously described [7, 12]. Survival of the skin grafts was monitored and scored for 120 days.

4.10 Cross-linking assay

The 24-well tissue culture plates were coated with anti-CD3, anti-Ly-6A mAb or both (2 μ g/ml, PharMingen) in PBS at 4°C overnight. The plates were washed with culture medium twice before being used. Activated allogeneic CD8⁺ T cells were added to the plates (10⁶/well). At various time points after culture, cells were harvested and stained with FITC-Annexin V (R&D) and analyzed by flow cytometry. Activated CD8⁺ T cells cultured in the same plate without cross-linking antibody were used as controls.

Acknowledgements: The authors thank C. Y. J. Li, K. Young and W. Chen for assistance in the animal experiments. Z. X. Zhang is the Canadian Institutes of Health Research (CIHR) post-doctoral fellow and W. L. Stanford is the Karyn Glick Memorial Special Fellow of the Leukemia and Lymphoma Society. This work is supported by CIHR (MOP-14431 to LZ).

References

- 1 **Zhai, Y. and Kupiec-Weglinski, J. W.**, What is the role of regulatory T cells in transplantation tolerance? *Curr. Opin. Immunol* 1999. **11**: 497–503.
- 2 **Roncarolo, M. G. and Levings, M. K.**, The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr. Opin. Immunol* 2000. **12**: 676–683.
- 3 **Zhang, Z. X., Young, K. and Zhang, L.**, CD3⁺CD4⁺CD8⁺ alphabeta-TCR⁺ T cell as immune regulatory cell. *J. Mol. Med.* 2001. **79**: 419–427.
- 4 **Zelenka, D., Adams, E., Humm, S., Lin, C. Y., Waldmann, H. and Cobbold, S. P.**, The role of CD⁺ T cell subsets in determining transplantation rejection or tolerance. *Immunol. Rev.* 2001. **182**: 164–179.
- 5 **Read, S. and Powrie, F.**, CD⁺ regulatory T cells. *Curr. Opin. Immunol* 2001. **13**: 644–649.
- 6 **Yang, L., DuTemple, B., Gorczyński, R. M., Levy, G. and Zhang, L.**, Evidence for epitope spreading and active suppression in skin graft tolerance after donor-specific transfusion. *Transplantation* 1999. **67**: 1404–1410.
- 7 **Yang, L., DuTemple, B., Khan, Q. and Zhang, L.**, Mechanisms of long-term donor-specific allograft survival induced by pre-transplant infusion of lymphocytes. *Blood* 1998. **91**: 324–330.
- 8 **van Twuyver, E., Kast, W. M., Mooijaart, R. J. D., Wilmink, J. M., Melief, C. J. M. and de Waal, L. P.**, Allograft tolerance induction in adult mice associated with functional deletion of specific CTL precursors. *Transplantation* 1989. **48**: 844–847.
- 9 **Azuma, T., Sato, S., Kitagawa, S., Hori, S., Kokudo, S., Hamaoka, T. and Fujiwara, H.**, Tolerance induction of allo-class I H-2 antigen-reactive Lyt-2⁺ helper T cells and prolonged survival of the corresponding class I H-2-disparate skin graft. *J. Immunol.* 1989. **143**: 1–8.
- 10 **Wong, W., Morris, P. J. and Wood, K. J.**, Pretransplant administration of a single donor class I major histocompatibility complex molecule is sufficient for the indefinite survival of fully allogeneic cardiac allografts: evidence for linked epitope suppression. *Transplantation* 1997. **63**: 1490–1494.
- 11 **Ferber, I., Schonrich, G., Schenkel, J., Mellor, A. L., Hämmerling, G. J. and Arnold, B.**, Levels of peripheral T cell tolerance induced by different doses of tolerogen. *Science* 1994. **263**: 674–676.
- 12 **Zhang, Z. X., Yang, L., Young, K. J., DuTemple, B. and Zhang, L.**, Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nat. Med.* 2000. **6**: 782–789.
- 13 **LeClair, K. P., Bridgett, M. M., Dumont, F. J., Palfree, R. G., Hämmerling, U. and Bothwell, A. L.**, Kinetic analysis of Ly-6 gene induction in a T lymphoma by interferons and interleukin 1, and demonstration of Ly-6 inducibility in diverse cell types. *Eur. J. Immunol.* 1989. **19**: 1233–1239.

- 14 **Shevach, E. M. and Korty, P. E.**, Ly-6: a multigene family in search of a function. *Immunol. Today* 1989. **10**: 195–200.
- 15 **Reiser, H., Oettgen, H., Yeh, E. T., Terhorst, C., Low, M. G., Benacerraf, B. and Rock, K. L.**, Structural characterization of the TAP molecule: a phosphatidylinositol-linked glycoprotein distinct from the T cell receptor/T3 complex and Thy-1. *Cell* 1986. **47**: 365–370.
- 16 **Yeh, E. T., Reiser, H., Bamezai, A. and Rock, K. L.**, TAP transcription and phosphatidylinositol linkage mutants are defective in activation through the T cell receptor. *Cell* 1988. **52**: 665–674.
- 17 **Dumont, F. J., Dijkmans, R., Palfree, R. G., Boltz, R. D. and Coker, L.**, Selective up-regulation by interferon-gamma of surface molecules of the Ly-6 complex in resting T cells: the Ly-6A/E and TAP antigens are preferentially enhanced. *Eur. J. Immunol.* 1987. **17**: 1183–1191.
- 18 **Codias, E. K., Rutter, J. E., Fleming, T. J. and Malek, T. R.**, Down-regulation of IL-2 production by activation of T cells through Ly-6A/E. *J. Immunol.* 1990. **145**: 1407–1414.
- 19 **Codias, E. K., Fleming, T. J., Zacharchuk, C. M., Ashwell, J. D. and Malek, T. R.**, Role of Ly-6A/E and T cell receptor-zeta for IL-2 production. Phosphatidylinositol-anchored Ly-6A/E antagonizes T cell receptor-mediated IL-2 production by a zeta-independent pathway. *J. Immunol.* 1992. **149**: 1825–1852.
- 20 **Malek, T. R., Ortega, G., Chan, C., Kroczek, R. A. and Shevach, E. M.**, Role of Ly-6 in lymphocyte activation. II. Induction of T cell activation by monoclonal anti-Ly-6 antibodies. *J. Exp. Med.* 1986. **164**: 709–722.
- 21 **Sussman, J. J., Saito, T., Shevach, E. M., Germain, R. N. and Ashwell, J. D.**, Thy-1- and Ly-6-mediated lymphokine production and growth inhibition of a T cell hybridoma require co-expression of the T cell antigen receptor complex. *J. Immunol.* 1988. **140**: 2520–2526.
- 22 **Lee, S. K., Su, B., Maher, S. E. and Bothwell, A. L.**, Ly-6A is required for T cell receptor expression and protein tyrosine kinase fyn activity. *EMBO J.* 1994. **13**: 2167–2176.
- 23 **Flood, P. M., Dougherty, J. P. and Ron, Y.**, Inhibition of Ly-6A antigen expression prevents T cell activation. *J. Exp. Med.* 1990. **172**: 115–120.
- 24 **Stefanova, I., Horejsi, V., Ansotegui, I. J., Knapp, W. and Stockinger, H.**, GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. *Science* 1991. **254**: 1016–1019.
- 25 **Bamezai, A. and Rock, K. L.**, Overexpressed Ly-6A.2 mediates cell-cell adhesion by binding a ligand expressed on lymphoid cells. *Proc. Natl. Acad. Sci. USA* 1995. **92**: 4294–4298.
- 26 **Stanford, W. L., Haque, S., Alexander, R., Liu, X., Latour, A. M., Snodgrass, H. R., Koller, B. H. and Flood, P. M.**, Altered proliferative response by T lymphocytes of Ly-6A (Sca-1) null mice. *J. Exp. Med.* 1997. **186**: 705–717.
- 27 **Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. R., Howard, M., Moore, K. W. and O'Garra, A.**, IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* 1991. **146**: 3444–3451.
- 28 **Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M. and O'Garra, A.**, IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* 1991. **147**: 3815–3822.
- 29 **Willems, F., Marchant, A., Delville, J. P., Gerard, C., Delvaux, A., Velu, T., de Boer, M. and Goldman, M.**, Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. *Eur. J. Immunol.* 1994. **24**: 1007–1009.
- 30 **de Waal, M. R., Haanen, J., Spits, H., Roncarolo, M. G., te, V.A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. and de Vries, J. E.**, Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med.* 1991. **174**: 915–924.
- 31 **Ding, L. and Shevach, E. M.**, IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. *J. Immunol.* 1992. **148**: 3133–3139.
- 32 **Qin, L., Chavin, K. D., Ding, Y., Favaro, J. P., Woodward, J. E., Lin, J., Tahara, H., Robbins, P., Shaked, A. and Ho, D. Y.**, Multiple vectors effectively achieve gene transfer in a murine cardiac transplantation model. Immunosuppression with TGF-beta 1 or vIL-10. *Transplantation* 1995. **59**: 809–816.
- 33 **Qin, L., Chavin, K. D., Ding, Y., Tahara, H., Favaro, J. P., Woodward, J. E., Suzuki, T., Robbins, P. D., Lotze, M. T. and Bromberg, J. S.**, Retrovirus-mediated transfer of viral IL-10 gene prolongs murine cardiac allograft survival. *J. Immunol.* 1996. **156**: 2316–2323.
- 34 **Krenger, W., Snyder, K., Smith, S. and Ferrara, J. L.**, Effects of exogenous interleukin-10 in a murine model of graft-versus-host disease to minor histocompatibility antigens. *Transplantation* 1994. **58**: 1251–1257.
- 35 **Blazar, B. R., Taylor, P. A., Smith, S. and Valleria, D. A.**, Interleukin-10 administration decreases survival in murine recipients of major histocompatibility complex disparate donor bone marrow grafts. *Blood* 1995. **85**: 842–851.
- 36 **Qian, S., Li, W., Li, Y., Fu, F., Lu, L., Fung, J. J. and Thomson, A. W.**, Systemic administration of cellular interleukin-10 can exacerbate cardiac allograft rejection in mice. *Transplantation* 1996. **62**: 1709–1714.
- 37 **Cobbold, S. P., Adams, E., Marshall, S. E., Davies, J. D. and Waldmann, H.**, Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8. *Immunol. Rev.* 1996. **149**: 5–33.
- 38 **Brakenhoff, R. H., Gerretsen, M., Knippels, E. M., van Dijk, M., van Essen, H., Weghuis, D. O., Sinke, R. J., Snow, G. B. and van Dongen, G. A.**, The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J. Cell. Biol.* 1995. **129**: 1677–1689.
- 39 **English, A., Kosoy, R., Pawlinski, R. and Bamezai, A.**, A monoclonal antibody against the 66-kDa protein expressed in mouse spleen and thymus inhibits Ly-6A.2-dependent cell-cell adhesion. *J. Immunol.* 2000. **165**: 3763–3771.
- 40 **Horejsi, V., Drbal, K., Cebecauer, M., Cerny, J., Brdicka, T., Angelisova, P. and Stockinger, H.**, GPI-microdomains: a role in signaling via immunoreceptors. *Immunol. Today* 1999. **20**: 356–361.
- 41 **Simons, M., Kramer, E. M., Thiele, C., Stoffel, W. and Trotter, J.**, Assembly of myelin by association of proteolipid protein with cholest. *J. Cell. Biol.* 2000. **151**: 143–154.
- 42 **Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, J. P., Pierres, M. and He, H. T.**, Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. *EMBO J.* 1998. **17**: 5334–5348.
- 43 **Xavier, R., Brennan, T., Li, Q., McCormack, C. and Seed, B.**, Membrane compartmentation is required for efficient T cell activation. *Immunity* 1998. **8**: 723–732.

Correspondence: Li Zhang, Multi Organ Transplantation Program, The Toronto General Hospital, University Health Network, CCRW2-809, 101 College Street, Toronto, M5G 2C4, Canada
 Fax: +1-416-597-9749
 e-mail: lzhang@transplantunit.org