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BIO Luminescence Imaging (BLI) Visualizes Activation of NF- κ B in Mouse Cardiac Transplantation

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Abstract

Background—The purpose of the current study is to evaluate the role of bioluminescence imaging (BLI) in the determination of NF- κ B activation in cardiac allograft rejection and ischemia-reperfusion injury.

Methods—To visualize NF- κ B activation, luciferase transgenic mice under the control of a mouse NF- κ B promoter (*NF- κ B-Luc*) were utilized as donors or recipients of cardiac grafts. Alternatively, *NF- κ B-Luc* spleen cells were adoptively transferred into *Rag2*^{-/-} mice with or without cardiac allografts. BLI was performed post-transplantation to detect luciferase activity that represents NF- κ B activation.

Results—The results show that luciferase activity was significantly increased in the cardiac allografts when *NF- κ B-Luc* mice were used as recipients as well as donors. Luciferase activity was also elevated in the wild-type (WT) cardiac allografts in *Rag2*^{-/-} mice that were transferred with *NF- κ B-Luc* spleen cells. CD154 mAb therapy inhibited luciferase activity and induced long-term survival of cardiac allografts. TLR-9 ligand, CpG DNA, enhanced luciferase activity and abrogated tolerance induction by CD154 mAb. Luciferase activity was also increased in ischemia-reperfusion injury of the cardiac grafts.

Conclusion—BLI using *Luc-NF- κ B* mice is a non-invasive approach to visualize the activation of NF- κ B signaling in mouse cardiac allograft rejection and ischemia-reperfusion injury. CD154 mAb can inhibit NF- κ B activation, which is reversed by TLR engagement.

Keywords

BLI; cardiac transplantation; TLR; NF- κ B; mice

INTRODUCTION

NF- κ B is a potent pro-inflammatory signal transduction molecule, and NF- κ B activation amplifies the expression of multiple chemokine and cytokine genes involved in the innate and

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adaptive immune responses (1,2). However, our knowledge of the role of NF- κ B in the mediation of alloimmune response remains limited due to the difficulties in obtaining *in vivo* data.

A quantitative method with transgenic mice for evaluating NF- κ B activation *in vivo* has been developed recently using bioluminescence imaging (BLI) (3–5). The transgenic mice have been engineered to possess a construct comprising the proximal 59 human immunodeficiency virus (HIV-1) long terminal repeat (LTR) driving the expression of *Photinus* luciferase complementary DNA (cDNA). The proximal HIV-LTR is a NF- κ B-responsive promoter (3), containing a TATA box, an enhancer region between 282 and 2103. In these genetically modified mice (referred to as *NF- κ B-Luc* mice), luciferase production and intracellular accumulation are dependent on NF- κ B-activated gene transcription; therefore, BLI using *NF- κ B-Luc* mice provides a useful *in vivo* reporter-based assay system in which to analyze NF- κ B enhancer activity in response to a variety of inflammatory signals including alloimmune response (3,6).

Previous reports show that the Toll-like receptor (TLR)/NF- κ B signaling pathway had been implicated in the innate and adaptive responses *in vivo* (7,8). After activation of TLR, MyD88, an adaptor, is recruited into the TLR complex that induces IL-1 receptor associated kinase (IRAK) and TNF receptor associated factor-6 (TRAF-6). Both IRAK and TRAF dissociate and activate the intracellular transcriptional factor, NF- κ B. In this manner, NF- κ B contributes to immunologically mediated diseases and alloimmune response (9,10). Immunosuppressants, such as cyclosporine A, can inhibit NF- κ B activation in a cardiac transplantation mouse model. Decreased mRNA levels of the p50/p65 subunits of NF- κ B delayed rejection to cardiac allograft (1,11,12). Recent reports suggest that TLR signaling mediates the alloimmune response. TLR engagement prevents tolerance induction and impairs tolerance maintenance induced by CD154 mAb treatment (7,11). The direct evidence that whether anti-CD154 monoclonal antibody (mAb) treatment can regulate NF- κ B signaling is still lacking.

Increasing evidence has also suggested that the innate immune response involving the TLR/NF- κ B pathway mediates tissue ischemia-reperfusion injury (13–15). TLR activation stimulates the downstream transcriptional factor, NF- κ B. Activation of NF- κ B induces gene programs leading to transcription of factors which promote inflammation, among them leukocyte adhesion molecules and cytokines, such as IL-1 β and TNF α . A quantitative method of NF- κ B assessment using transgenic *NF- κ B-Luc* mice has been developed (3,16,17), which provides a useful *in vivo* reporter-based assay system in which to analyze NF- κ B enhancer activity in response to a variety of inflammatory signals.

In this study, we used BLI for the detection of NF- κ B activation in a cardiac transplantation mouse model and tissue ischemia-reperfusion injury. Our results show that luciferase activity is elevated in the cardiac allografts and the cardiac grafts suffering from ischemia-reperfusion injury. CD154 mAb therapy has the capacity of inhibiting NF- κ B activation which can be reversed by TLR engagement with CpG DNA. The current study suggests that BLI using *NF- κ B-Luc* mice is a useful non-invasive approach for the detection of NF- κ B activation during allograft rejection and tissue ischemia-reperfusion injury.

MATERIALS AND METHODS

Mice and cardiac transplantation

C57BL/6, Balb/c mice, and *Rag2*^{-/-} were purchased from Jackson Laboratory, the National Cancer Institute (NIH, Frederick, MD), and Taconic (Hudson, NY). Mice expressing luciferase transgene under the control of a NF- κ B promoter (*NF- κ B-Luc*) on Balb/c (H-2d) and C57BL/6 (H-2b) were kindly provided by Dr. Tim Blackwell (17) and were utilized for the detection

of NF- κ B activation after cardiac transplantation using BLI. All mice were housed in pathogen-free conditions in the Division of Animal Care at Vanderbilt University Medical Center, according to the National Institutes of Health guidelines.

Heterotopic vascularized cardiac transplantation was performed using microsurgical techniques as described previously (18). The donor aorta was sutured to the recipient aorta in an end to side fashion using 10-0 sutures (USSC) and the inferior vena cava (IVC) to the recipient IVC in the same manner. Tolerance was induced by anti-CD154 mAb (MR1) at 1mg/mouse, i.v. at 0 d, +1 d, and +2 d relative to transplantation. The mAbs were purified in our laboratory following our previous protocol (7,19,20). For TLR engagement, CpG DNA (CpG-B ODN 1826, Coley Pharmaceutical Group Ltd, Ottawa, Ontario, Canada) was administered (100 μ g per mouse $\times$ 3 at day 0, day 1, and day 3). The day the graft stopped beating was defined as the time of rejection. Recently rejected and long-term surviving mice, engrafted with cardiac allografts, were sacrificed for histopathological examinations (20,21). The grafts were frozen rapidly in liquid nitrogen or stored in 10% formalin solution and four to six μ m sections were stained with H&E for the assessment of cell infiltration.

To visualize the NF- κ B activation in the cardiac grafts that suffered from ischemia-reperfusion injury, syngeneic *NF- κ B-Luc* (Balb/c) cardiac grafts were preserved in cold (4°C) University of Wisconsin (UW) solution for 16hr and then transplanted into WT Balb/c recipients. Syngeneic *NF- κ B-Luc* (Balb/c) cardiac grafts were transplanted immediately into WT Balb/c recipients as control (with very short ischemic time).

In vivo *bioluminescence imaging* (BLI) BLI was performed as previously described (3,5). After anesthesia with isoflurane, luciferin (Roche, Mannheim, Germany) was injected intravenously at a dose of 50mg/kg for *NF- κ B-Luc* mice as recipients and 100mg/kg for *NF- κ B-Luc* as donors. Mice were housed inside a light-tight box and imaged with an ICCD camera (Hamamatsu C2400-32, Xenogen Corp). Bioluminescence in control mice without transplantation was also assessed. Light emission through the ventral body was detected as photon counts over a standardized area of the organs of interest using the ARGUS-50 software for image processing expressed as Regions of Interest (ROI) (3,22,23). Since luciferase production is under the control of the NF- κ B promoter, luciferase activity represents NF- κ B activation and the value of ROI represents the density of luciferase activity. Bioluminescence is dependent on tissue penetration, and the intensity or light emission from the cardiac grafts may vary. To reduce the variability, all mice were positioned on their back, which allowed us to obtain signal intensity in a standard bed fashion.

Tissue luciferase activity tests

Luciferase in the spleen, draining lymph nodes (along with the aorta adjacent to cardiac graft), and cardiac grafts were determined by Luciferase Assay System (Promega, Madison, WI). The tissues were pelleted by centrifugation, resuspended in a lysis buffer, and clarified by centrifugation at 12,000 *g* for 2 min at 4°C. Luciferase activity was measured in the tissue by adding 100 μ l of freshly reconstituted luciferase assay buffer to 20 μ l of tissue lysate. Luciferase activity is expressed as relative light units (RLU) normalized for protein content measured by Bradford assay.

Splenocytes transfer

Whole splenocytes were obtained from *NF- κ B-Luc* mice. Briefly, the spleen was removed, and single-cell suspensions were prepared. The spleen cells were washed twice in PBS, and red blood cells were lysed with an ammonium chloride and potassium solution. The viable washed cells were counted using Trypan-blue exclusion. Different numbers of whole splenocytes (5×10^6 , 10×10^6 , and 20×10^6) were injected intravenously into *Rag2*^{-/-} mice on the day of

cardiac transplantation followed by the detection of BLI and luciferase activity post-transplantation. In another group, mice were transferred with *NF-κB-Luc* spleen cells alone, and the luciferase activity was assessed. One month after adoptive cell transfer the cardiac allografts were transplanted, and the luciferase activity was re-assessed.

Statistics

The graft survival days were presented as mean survival time $\pm$ standard deviation (MST $\pm$ SD) and evaluated for statistical significance by ANOVA analysis (Abacus Concepts, Berkeley, CA). *P*-value of < 0.05 or less was considered as significant.

RESULTS

BLI defines NF-κB activation in cardiac graft transplantation

NF-κB-Luc C57BL/6 mice (female, 22 to 24 grams) were used as recipients of WT Balb/c cardiac allografts, and BLI was examined at the different timepoints post-transplantation. Increased luciferase activity was observed in the cardiac allografts at 4 hr following cardiac transplantation (Fig. 1a). The highest luciferase activity in the recipient abdomen was on day 4. Tissue luciferase activity was significantly increased in the cardiac allograft and surrounding lymph nodes (Fig. 1b). Next, *NF-κB-Luc* C57BL/6 mice were used as donors of the cardiac allografts that were transplanted into WT Balb/c mice. Figure 2 (upper panel) showed that luciferase activity was elevated in the *NF-κB-Luc* cardiac allografts up to 10 days. The results suggest that NF-κB activation is increased in the cardiac allografts when *NF-κB-Luc* mice used as either recipients or donors.

The role of NF-κB activation in the mediation of tissue ischemia-reperfusion injury has been documented (2,8,24). Figure 2 (lower panel) shows that luciferase activity in the preserved cardiac graft was significantly increased, compared to that in the cardiac grafts immediately transplanted into recipients with very short ischemic time (less than 30 minutes, Fig. 2, upper panel). The results suggest that cardiac ischemia-reperfusion injury is associated with NF-κB activation that can be detected by BLI.

Either recipient- or donor-derived cells activate NF-κB signaling pathway

To define the specific role of NF-κB in the leukocyte compartment during allograft rejection, *NF-κB-Luc* (C57BL/6, 5×10^6 , 10×10^6 and 20×10^6) whole splenocytes were adoptively transferred into *Rag2*^{-/-} mice (C57BL/6) engrafted with WT Balb/c cardiac allografts on the day of transplantation. The results in Figure 3a show that Balb/c allografts were rejected in *Rag2*^{-/-} mice transferred with different numbers of *NF-κB-Luc* (C57BL/6) spleen cells. Tissue luciferase activity in the cardiac grafts was elevated up to 10 days post-transplantation with the transfer of 10×10^6 *NF-κB-Luc* (C57BL/6) spleen cells (Fig. 3b). On day 7, the selected mice were sacrificed and cardiac grafts, spleen, and lymph nodes were collected for tissue luciferase activity. Tissue luciferase activity was observed in the cardiac grafts and the lymph nodes surrounding cardiac allograft (Fig. 3c, Spl+Heart). However, increased tissue luciferase activity in the spleen was observed in mice transferred with spleen cells without cardiac transplantation (Fig. 3c, Spl only).

We hypothesize that NF-κB can be activated without recipient-derived lymphocyte activation since increased luciferase activity was observed in the syngeneic cardiac grafts. *NF-κB-Luc* (Balb/c) cardiac allografts were transplanted into T and B cell deficient *Rag2*^{-/-} mice to define if donor-derived cells involve activation of NF-κB. As expected, an increased luciferase activity was observed in the *NF-κB-Luc* grafts, which was diminished by day 7 (Fig. 3d); thus, donor-derived cells are enough to drive NF-κB activation independent of recipient-derived T

and B stimulator cells. The results suggest that either donor-or recipient-derived cells have the ability to activate NF- κ B signal in response to variety of stimulations.

Anti-CD154 mAb therapy inhibits NF- κ B activation which can be reversed by TLR engagement

Previous reports have demonstrated that CD154 mAb therapy induces long-term survival of cardiac allograft and decreases mRNA levels of NF- κ B and I κ B genes (2,20,25,26). TLR engagement with CpG DNA impaired tolerance induced by costimulatory blockade (7). In the current study, we analyzed the role of anti-CD154 mAb in the inhibition of NF- κ B activation in a cardiac transplantation mouse model. WT cardiac allografts (C57BL/6) were transplanted into *NF- κ B-Luc* (Balb/c) mice with or without anti-CD154 (MR1, 1mg per mouse $\times$ 3) treatment. The results in Figure 4a show that anti-CD154 mAb therapy induced long-term survival of cardiac allografts in *NF- κ B-Luc* recipients. Administration of CpG DNA on day of transplantation (100 μ g per mouse $\times$ 3, i.p.) abrogated tolerance induced by CD154 blockade, and all cardiac grafts were rejected within 20 days (Fig.4a MR1 + CpG1). In addition, CpG DNA treatment starting at 30d post-transplantation can also elicit allograft rejection and reverse the inhibition of NF- κ B activation by CD154 blockade (Fig.4a MR1 + CpG2).

To determine if anti-CD154 mAb can inhibit NF- κ B activation in donor-derived cell, *NF- κ B-Luc* (C57BL/6) cardiac allografts were transplanted into WT Balb/c mice. The results in Figure 4b show that anti-CD154 mAb induced long-term survival of cardiac allografts and inhibited luciferase activity; and additional CpG DNA elicited an elevation of luciferase activity in anti-CD154 mAb treated cardiac allografts (Fig. 4b and c). The data indicate that *NF- κ B-Luc* mice, when used as donors or recipients of cardiac allografts, have the same biological characterization with WT combination (19). Anti-CD154 mAb therapy can inhibit both recipient cell- and donor cell-derived NF- κ B activation, which can be reversed by TLR engagement.

DISCUSSION

NF- κ B is a rapid response transcription factor for genes whose products are critical for inflammatory reaction and alloimmune response (1,27). The availability of genetically modified transgenic *NF- κ B-Luc* mice provides a useful reporter-based assay system in which to analyze NF- κ B enhancer activity in response to a variety of inflammatory signals. Since luciferase production is under the control of NF- κ B promoter, NF- κ B activation is required for transcriptional activity of luciferase and luciferase activity represents the activation of NF- κ B. The current results suggest that luciferase activity can be detected in the cardiac grafts when *NF- κ B-Luc* mice are used as recipients. To characterize if donor-derived passenger leukocytes are involved in the NF- κ B activation, *NF- κ B-Luc* mice were used as donors of the cardiac allografts. The results show that luciferase activity is elevated in the cardiac allografts up to 10 days and the cardiac allografts are rejected within 7 – 10 days. Our observation of a significant increase of luciferase activity in the cardiac allografts is consistent with previous report by Tanaka (23), in which BLI visualized migration and proliferation of passenger leukocytes in both syngeneic and allogeneic recipients. The current results suggest that recipient-derived lymphocytes and donor-derived passenger cells have the ability to activate NF- κ B.

A growing body of the literature suggests that tissue ischemia-reperfusion injury activates TLR/ NF- κ B signaling pathway (13,28,29). Here, we report that long-term ischemia reperfusion (16 hr) of cardiac grafts will lead to significant increase of luciferase activity that is elevated up to 10 days. In the syngeneic cardiac grafts immediately transplanted into recipients with very short ischemic time, temporary increase of luciferase activity is observed that diminished within 4 days. The results indicate that antigen-independent insults, such as tissue ischemia-

reperfusion injury, can stimulate the innate immune response and elicit NF- κ B activation, and the visualization of NF- κ B activation by BLI is a useful approach to analyze, *in vivo*, tissue ischemia-reperfusion injury.

To define the source of cells involved in NF- κ B activation, *NF- κ B-Luc* spleen cells are adoptively transferred into *Rag2*^{-/-} mice on the day of cardiac allograft transplantation. *NF- κ B-Luc* spleen cells lodge in the cardiac allograft and the lymph nodes surrounding cardiac allograft. In the absence of cardiac allograft, these cells migrate to the other parts of the body, such as the feet, the tail, and the native spleen. To further confirm allograft-mediated activation of NF- κ B, the cardiac allografts are transplanted back to *Rag2*^{-/-} mice transferred with *NF- κ B-Luc* spleen cells alone two weeks later. As expected, luciferase activity in the cardiac allografts recurs by day 7 post-transplantation. The results indicate that *NF- κ B-Luc* spleen cells are recruited into the area where alloimmune response occurs resulting in NF- κ B activation.

We hypothesized that NF- κ B can be activated in the absence of recipient lymphocytes since luciferase activity is transiently increased in syngeneic cardiac grafts (*NF- κ B-Luc* Balb/c to WT Balb/c). A previous study has shown that cardiac grafts survive in T and B cell deficient *Rag2*^{-/-} mice indefinitely without any immunosuppressant (30). The current results suggest that luciferase activity in cardiac grafts is observed at 3 hr and maintains for up to 4 days in *Rag2*^{-/-} mice, suggesting that NF- κ B can be activated independent of recipient-derived T or B cell stimulation.

Anti-CD154 mAb therapy has been demonstrated to induce donor specific tolerance (19,31). Previous reports indicate that anti-CD154 mAb decreases mRNA level of NF- κ B (2,32). Our results show that anti-CD154 mAb reduces NF- κ B activation and induces tolerance to cardiac allograft evidenced by decreased luciferase activity when *NF- κ B-Luc* mice were used as recipients of cardiac allografts. The inhibition of NF- κ B activation occurs after 2 days of cardiac transplantation. Our previous results suggest that tolerance induced by anti-CD154 mAb therapy can be abrogated by additional TLR-9 ligand, CpG DNA (7). Indeed, the current data show that tolerance cannot be induced by anti-CD154 mAb when administration of CpG DNA is on the day of transplantation. CpG DNA can also abrogate tolerance induced by anti-CD154 mAb 30 days after transplantation and enhance NF- κ B activation.

We observed that anti-CD154 mAb therapy has the ability to inhibit not only recipient cell-derived but also donor cell-derived NF- κ B activation. NF- κ B activation is diminished in *NF- κ B-Luc* cardiac allografts when transplanted into WT mice treated by anti-CD154 mAb and this inhibition is reversed by TLR engagement. The current results collectively suggest that TLR engagement with CpG DNA abrogates anti-CD154 mAb-induced tolerance and restores the NF- κ B signaling pathway.

In conclusion, our model provides a useful approach to visualize NF- κ B activation non-invasively and quantitatively from passenger leukocytes and recipient lymphocytes in a cardiac transplantation mouse model. NF- κ B is activated in allograft rejection and tissue ischemia-reperfusion injury. TLR engagement prevents anti-CD154 therapy-mediated tolerance induction through activation of the NF- κ B signaling pathway.

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Figure 1.

Figure 1a BLI evaluates luciferase activity in *NF-κB-Luc* recipients engrafted with wild-type (WT) cardiac allografts.

NF-κB-Luc (C57BL/6) mice were used as recipients of WT Balb/c cardiac allografts.

Luciferase activity was analyzed by BLI at 4hr, 1d, 4d, 7d, and 10 d post-transplantation after i.v. injection of luciferin (50mg/kg). Increased luciferase activity (or light emission expressed as Regions of Interest, ROI) was observed in the abdomen engrafted with cardiac allografts compared to the mouse without cardiac transplantation (naïve control).

Figure 1b Tissue luciferase activity in *NF-κB-Luc* recipients engrafted with WT cardiac allografts.

Cardiac graft, spleen, and drainage lymph nodes surrounding cardiac graft were collected at 7d after the completion of BLI. Tissue luciferase activity was performed as described in the Materials and Methods. Increased luciferase activity (referred as relative light units, RLU) was found in the cardiac allograft ($X \pm SE = 38,900 \pm 8,909$) and lymph nodes ($X \pm SE = 35,600 \pm 6,654$, $n = 4$) compared to that in the spleen ($X \pm SE = 9,998 \pm 345$, $p < 0.05$).

Figure 2. Luciferase activity was elevated after ischemia-reperfusion injury

Syngeneic (Balb/c) *NF-κB-Luc* cardiac grafts with or without 16 hr preservation in UW solution were transplanted into WT Balb/c recipients, and luciferase activity was elevated after transplantation. The mean value of luciferase activity ($X \pm SE$) was $2.48e+05 \pm 5,850$, $4.88e+05 \pm 4,858$, $3.65e+05 \pm 7,125$, $1.85e+05 \pm 4,213$, and $1.22e+05 \pm 2,134$ in the syngeneic cardiac grafts without ischemia-reperfusion injury (Syngeneic cardiac graft without IRI, $n = 5$) at 3 hr, 2 d, 4 d, 7d, and 10 d post-transplantation respectively. Compared to syngeneic cardiac grafts without IRI, luciferase activity in the syngeneic cardiac grafts with IRI (Syngeneic cardiac graft with IRI, $n = 4$) was $4.48e+05 \pm 29,578$, $p < 0.05$; $2.85e+06 \pm 54,867$, $p < 0.05$; $1.85e+06 \pm 15,756$, $p < 0.05$; $2.05e+05 \pm 5,831$, $p > 0.05$; and $1.55e+05 \pm 4,428$, $p > 0.05$ at the same timepoint.

Figure 3. Adoptive transfer of *NF-κB-Luc* spleen cells

a) *NF-κB-Luc* (C57BL/6) spleen cells (20×10^6 , 10×10^6 , and 5×10^6) were transferred into *Rag2^{-/-}* (C57BL/6) mice on the day of transplantation of WT Balb/c cardiac allografts.

Allograft rejection was induced, and all cardiac allografts were rejected in a mean survival time (MST) of 9.8 days when transferred with 20×10^6 *NF-κB-Luc* spleen cells (MST $\pm$ SD = 9.8 ± 2.1 d, n = 4), 17 days with 10×10^6 *NF-κB-Luc* spleen cells (MST $\pm$ SD = 14.5 ± 3.8 d, n = 4), and 17.8 days with 5×10^6 *NF-κB-Luc* spleen cells (MST $\pm$ SD = 17.8 ± 5.1 d, n = 4).

b) Luciferase activity was elevated for up to 10 days after transfer of 20×10^6 *NF-κB-Luc*

(C57BL/6) spleen cells. Although the cardiac allografts were rejected when transferred with *NF-κB-Luc* spleen cells less than 20×10^6 (Fig 3a), luciferase activity can be only detected at d 4 or d 7 (data not shown). **c**) Tissue luciferase activity was measured as described in the Materials and Methods. When cardiac transplantation, in combination with spleen cells, was performed, the highest luciferase activity was observed in the cardiac allograft and surrounding lymph nodes (Heart+Spl). *NF-κB-Luc* spleen cells migrate to the spleen in the absence of cardiac allograft (Spl only). No luciferase activity was observed in cardiac transplantation only (HTx only, n = 3 in each group). **d**) *NF-κB-Luc* (Balb/c) cardiac allografts were transplanted into *Rag2*^{-/-} recipients (n = 5). Luciferase activity in the cardiac grafts was determined by BLI. The results show that luciferase activity was elevated up to 4 days post-transplantation and diminished by day 7 with the same dynamic change to that in syngeneic cardiac graft (Fig. 3a). The results suggest that donor-derived NF-κB activation is independent of recipient lymphocyte stimulation.

Figure 4. TLR engagement reverses CD154 mAb-induced tolerance and enhances NF-κB activation
a) When NF-κB-Luc (Balb/c) mice were used as recipients of WT C57BL/6 cardiac allografts, the untreated cardiac allografts were rejected within 9 days ($MST \pm SD = 7.5 \pm 0.5d$, $n = 6$). In anti-CD154 mAb-treated mice (MR1, 1mg per mouse $\times 3$), tolerance to cardiac allografts was induced ($MST > 100d$, $n = 5$); however, TLR engagement with TLR-9 ligand, CpG DNA ($100\mu g/mouse \times 3$ at 0d, +1d, and +3d relative to transplantation), reverses tolerance induction (MR1+CpG1, $MST \pm SD = 14.5 \pm 5.4d$, $n = 5$). In addition, when the recipients were received CpG DNA injection ($100\mu g$ per mouse $\times 3$) at day 30 following transplantation, tolerance was abrogated and 80% of cardiac allografts were rejected within 20 days after CpG DNA injection (MR1+CpG2, $MST \pm SD \geq 18.8 \pm 7.2d$, $n = 5$). **b)** When NF-κB-Luc (C57BL/6) mice were used as donors of cardiac allografts, the untreated cardiac allografts were rejected within 9 days ($MST \pm SD = 7.6 \pm 0.89d$, $n = 5$). Anti-CD154 mAb therapy induced tolerance to cardiac allografts ($MST > 100d$, $n = 5$). TLR engagement with CpG DNA ($100\mu g/mouse \times 3$ at 0d, +1d, and +3d relative to the transplantation) reverses tolerance induction (MR1+CpG, $MST \pm SD = 16.4 \pm 3.85d$, $n = 4$). **c)** We asked if donor-derived NF-κB signaling could be inhibited by anti-154 mAb and if CpG DNA can reverse this inhibition. *NF-κB-Luc* (C57BL/6) mice were used as donors of cardiac allografts treated with anti-CD154 mAb (MR1, 1mg per mouse $\times 3$) and CpG DNA. Anti-CD154 mAb therapy inhibits luciferase activity in the cardiac allografts (middle panel, Allograft+MR1, $n = 6$), compared to control that received cardiac transplantation without any immunosuppressant (upper panel, Control). CpG DNA ($100\mu g/$

mouse $\times$ 3) enhanced luciferase activity post-transplantation (lower panel, Allograft+MR1 +CpG, n =4).