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Engendering Allograft Ignorance in a Mouse Model of Allogeneic Skin Transplantation to the Distal Hind Limb

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Abstract

Objective—The aim of this study was to demonstrate lymphatic isolation in a model of hind limb lymph node (LN) excision, consisting of ipsilateral popliteal and inguinal LN excision and to evaluate the immunologic response to allogeneic skin transplanted onto this region of lymphatic isolation.

Methods—To study lymphatic flow, C57BL/6 mice underwent lymphadenectomy (n = 5), sham lymphadenectomy (n = 5), or no intervention (n = 5), followed by methylene blue injection. Mice were dissected to determine whether methylene blue traveled to the iliac LN. To study host response to skin transplantation, C57BL/6 mice underwent allogeneic skin transplantation with LN excision (n = 6), allogeneic skin transplantation alone (n = 6), or syngeneic skin transplantation (n = 4). Skin grafts were placed distal to the popliteal fossa and mice were euthanized at day 10. Grafts were stained for endothelial cell and proliferation markers (CD31 and Ki67, respectively). Secondary lymphoid tissues (spleen, ipsilateral axillary LN, and contralateral inguinal LN) were removed and rechallenged with BALB/c alloantigen in vitro with subsequent assay of interferon- γ and interleukin 4 cell expression using ELISPOT technique.

Results—Mice that underwent LN excision had no evidence of methylene blue in the iliac nodes; mice without surgical intervention or with sham LN excision consistently had methylene blue visible in the ipsilateral iliac nodes. Mice treated with allogeneic skin transplantation and LN excision had lower expression of interferon- γ and interleukin 4 in the secondary lymphoid tissues.

Conclusions—Lymph node excision completely interrupts lymphatic flow of the hind limb. This model of lymphatic isolation impairs the ability of the transplant recipient to acutely mount a Th1 or Th2 response to allogeneic skin transplants.

Keywords

antigen presentation; ELISPOT; lymph node; lymphadenectomy; lymphedema; methylene blue; sentinel node; skin transplant

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More than 70% of burn injuries involve the extremities; an increasing number of patients require treatment of burn or blast injuries involving the extremities. Although autologous tissue provides a reconstructive tool for these patients, this results in a donor site defect in an already devastated patient. Large burn and cutaneous wound patients often lack sufficient donor site tissue to allow for skin autograft coverage of all affected areas. Current techniques available for large cutaneous burns and wounds include cadaveric allograft, synthetic materials, and cultured epidermal autografts. Synthetic materials are temporary and often complicated by infection, and recent reports of squamous cell carcinoma after cultured epidermal autografts have led to significant concerns regarding patient's safety. Even after reconstruction, patients are left with nonfunctional limbs and may require prostheses with limited function. Transplantation of tissue from deceased donors offers a promising tool to restore both form and function. Burn patients may benefit from skin allografts procured from deceased donors, and patients with mutilating trauma to the extremities may benefit from entire limb allografts. However, skin or limb transplantation has only been used sparingly because of need for aggressive immunosuppressive therapy to prevent graft rejection.¹⁻⁵ Allograft take can be prolonged with immunosuppressive medications, but these medications result in severe systemic effects including infections, tumors, and chronic conditions including hypertension, diabetes, and renal failure. Therefore, there is a pressing need to investigate nonpharmacologic methodologies to prevent rejection of transplanted allografts.

The lymph node (LN) basin plays a significant role in regulating lymphatic flow, migration of recipient alloantigen-bearing antigen-presenting cells into the secondary lymphoid tissues, and the immune response to virus, tumor, and transplanted tissues.⁶⁻¹⁰ Allograft rejection is an antigen-specific response initiated by the presentation of antigen from the allograft via antigen-presenting cells to lymphocytes in the site-draining LNs.¹⁰⁻¹² Early studies showed that antigen from transplanted skin travels to draining LNs.^{12,13} Disruption of the lymphatic vessels, which allow for antigen-presenting cells to travel to LNs, was one of the early strategies used to delay allograft rejection. Delayed graft rejection was elicited by stripping dermal pedicles of their lymphatic vessels and transplanting skin onto these a lymphatic pedicles.¹⁴⁻¹⁷ However, these models were limited by the difficult design of the pedicled dermis, often resulting in graft death even after syngeneic transplantation.¹⁴⁻¹⁷ In addition, lymphatic stripping is not feasible in humans, limiting its clinical utility. Finally, evaluation of these models was limited to gross observation of the grafts, without characterizing the mechanism contributing to delayed rejection.

The importance of lymphoid tissue in mounting allograft rejection has been demonstrated by skin transplantation into knockout mice (aly/aly) lacking LNs. These grafts survive indefinitely because of immunologic "ignorance" of the transplanted graft.¹⁸ Although this model has provided insight into the role of LNs in the rejection process, this model cannot be applied clinically. Previously, removal of axillary, brachial, cervical, and inguinal LNs with skin allotransplantation to the flank in mice has been shown to delay skin graft survival; however, at that time, those were not shown to be the only nodes draining the flank.¹⁵ More recently, studies have demonstrated that removal of the submandibular LN may delay or prevent corneal rejection.¹⁹⁻²² These results have been difficult to duplicate

because of the variability of lymphatic drainage in the head/neck region. In addition, these studies did not characterize the mechanism of delayed graft rejection.

Studies have previously described the direct visualization of mouse LNs with the assistance of Evan's Blue Dye injection into the footpad (Fig. 1).²³ This technique identifies LNs involved in lymphatic drainage from the hind limb, with the popliteal, inguinal, and iliac LNs consistently visualized within 15 minutes after injection.

The aim of our study was to demonstrate lymphatic isolation in a model of hind limb lymphadenectomy and to evaluate the immunologic response to skin transplanted to the lower limb after disruption of LN drainage. We hypothesized that removal of the popliteal and inguinal LNs would inhibit lymphatic drainage to surrounding LNs in the mouse. In addition, given the known role of LNs in antigen presentation and T-cell proliferation, we hypothesized that removal of draining LNs will impair host Th1 and Th2 priming by the donor graft antigen.

MATERIALS AND METHODS

Mice

Male C57BL/6 and BALB/c mice aged 8 to 12 weeks were purchased from Charles River Laboratories International (Wilmington, MA). This study was approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan.

LN Excision With Methylene Blue Dye Injection

Three groups of mice were assessed for lymphatic flow: (1) naïve, (2) sham surgery, or (3) LN excision (n = 5 mice per group). In the group undergoing LN excision, each mouse was anesthetized with inhaled 3% isoflurane. Buprenorphine (0.1 mg/kg) was given in the dorsal subcutaneous tissue for extended pain relief, and 1 mL of 0.9% normal saline was injected into the dorsal subcutaneous tissue for fluid management. Loupe magnification was used to identify and remove the popliteal and superficial inguinal LNs from the right hind limb only. A vertical midline incision on the abdomen was made with lateral dissection superficial to the rectus muscles to access the inguinal node. The skin flap was elevated laterally leaving abdominal muscle and fat deep to the dissection; a separate horizontal incision extending laterally from the superior aspect of the vertical incision was made across the skin to allow the skin flap to be elevated and to allow for identification and removal of the superficial inguinal node. A separate incision was fashioned along the popliteal fossa in a longitudinal fashion and the popliteal LN was identified and removed. Incisions were then closed with a 4-0 nylon suture. Confirmation of successful LN excision was performed with water density testing.²¹ These nodes were then preserved in 10% formalin solution and sent for slide processing to confirm presence of lymph tissue.

Sham LN excision was performed in a method similar to that described for LN excision with incisions for both the superficial inguinal and popliteal LNs. Lymph nodes were visualized under loupe magnification but were not removed.

All methylene blue injections were given under anesthesia with isoflurane. For all 3 groups (control, sham, and lymphadenectomy), the mouse's right footpad was injected with a 50- μ L solution of 1% (10 mg/mL) methylene blue (Fig. 1). After this, the mouse was allowed to recover for 40 minutes.

LN Visualization

After methylene blue injection in the 3 groups described previously, mice were allowed to recover for 40 minutes and subsequently euthanized. Dissections were performed to visualize the expected locations of the ipsilateral popliteal, superficial inguinal, and iliac LNs. Photographs of these areas were taken to document presence or absence of blue-stained LNs.

Skin Transplantation With or Without LN Excision

Mice received (1) allogeneic skin transplantation with lymphadenectomy ($n = 6$), (2) allogeneic skin transplantation without lymphadenectomy ($n = 6$), or (3) syngeneic skin transplantation ($n = 4$). Full-thickness skin was harvested from the dorsum of a BALB/c donor mouse and sectioned into 1×1 cm grafts for allogeneic transplantation onto C57BL/6 mice. In addition, full-thickness skin was harvested from the dorsum of a C57BL/6 donor mouse and similarly sectioned into 1×1 cm grafts for syngeneic transplantation into C57BL/6 mice. All skin transplantation was performed on the right hind limb distal to the popliteal fossa. All skin grafts were affixed to the surrounding distal hind limb skin with 6–0 nylon suture. Bolsters were fashioned from Vaseline gauze and Telfa pads (Covidien, Mansfield, MA) and affixed to the hind limb overlying the skin transplant with 5–0 nylon sutures. The entire construct was then covered with clear tape dressing. Mice were monitored daily to ensure the integrity of the dressings; dressings that seemed to be disturbed (eg, dislodged dressing tape) were immediately corrected upon identification. All recipients were allowed to survive for 10 days. On day 10, the skin transplant, recipient spleen, contralateral inguinal node, ipsilateral axillary node, and blood were collected; ipsilateral, popliteal, and inguinal LNs were collected in mice without LN excision (Fig. 1).

Histology

Skin grafts were sectioned and sent for paraffin embedding and hematoxylin and eosin staining and immunohistochemistry staining for CD31 (endothelial cells) and Ki67 (cell proliferation) as previously described.^{24,25}

Cytokine Expression Assay

ELISPOT technique was used to assay cell expression of interferon- γ (Th1), interleukin (IL)-4 (Th2), and IL-17 (Th17).^{26,27} Briefly, splenocytes from Balb/c mice were irradiated and used as stimulators at a concentration of 4×10^6 /mL in RPMI/2% fetal calf serum for ELISPOT. Three sets of responder cells were evaluated: (1) spleen, (2) pooled contralateral inguinal/axillary nodes, and (3) pooled ipsilateral popliteal/inguinal nodes (mice without LN excision only). Plate wells were coated with 100 μ L of purified antimouse interferon- γ (BD Biosciences, San Jose, CA) diluted to 4 mg/mL. On the day of spleen and node harvest, plates were emptied of all solutions. Responder cells were resuspended to a concentration of

10⁷ cells per milliliter in RPMI/2% fetal calf serum. Wells were plated with 100 μ L of Balb/c splenocytes in RPMI, ConA in RPMI, or RPMI alone; 100 μ L of responder cells added to each well. Plates were incubated at 37°C with 5% CO₂ for 24 hours. Wells were emptied and 100 μ L of secondary antibody [biotinylated rat antimouse interferon- γ , rat antimouse IL-4, or rat antimouse IL-17 (BD Biosciences, San Jose, CA)] at 2 mg/mL was added to each well. Plates were incubated overnight at 4°C and washed. Antibiotin-alkaline phosphatase antibody was added to wells with antimouse interferon- γ and Streptavidin-horseradish peroxidase to wells with antimouse IL-4 or antimouse IL-17. Plates were washed after 90 minutes of incubation at room temperature. Nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolylphosphate solution was incubated with interferon- γ wells; 3-amino-9-ethylcarbazole-chromagen/hydrogen peroxide in IL-4 and IL-17 wells. Plates were dried and spots were counted. All experiments were performed in triplicate.

Statistical Analysis

Statistical analysis (Student *t* test) was performed using the GraphPad PRISM 4 software, and differences were considered significant when *P* < 0.05.

RESULTS

Popliteal and Inguinal LN Excision Prevents Lymphatic Drainage From Hind limb to the Iliac LN

Control mice received methylene blue injection with no incisions over the hind limb. After dissection, we observed consistent staining of the popliteal and inguinal LNs in 100% (5/5) of control mice. Upon further dissection, we also noted consistent blue staining of the ipsilateral iliac LN again in 100% (5/5) of mice (Fig. 2A). The stain was specific for LNs; only the tissue immediately surrounding the injection site was stained blue in addition to the discrete areas of LN staining.

Mice undergoing sham LN excision had incisions and dissection over the popliteal fossa, inguinal region, and midabdomen identical to the operation performed in the mice receiving actual LN excision. They subsequently received methylene blue injections in the footpad as previously described. In these mice, we noted consistent staining of the ipsilateral popliteal, inguinal, and iliac LNs in 100% (5/5) of mice (Fig. 2B).

Mice that received LN excision with removal of the popliteal and inguinal LN had no discrete staining in the popliteal or inguinal regions in 100% (5/5) of mice. Upon further dissection, the ipsilateral iliac LN was identifiable but remained *unstained* in 100% (5/5) of mice (Figs. 2C, D).

Skin Transplants Exhibit Survival After Transplantation to the Distal Hind Limb

Skin transplants were performed to the hind limb distal to the popliteal fossa. All skin transplants on mice with syngeneic skin alone (4/4), allogeneic skin alone (6/6), and allogeneic skin with LN excision (6/6) seemed to have survived on the basis of gross examination at day 10. Lymph node excision did not alter skin graft take or viability.

Skin transplants were removed on day 10 and stained with CD31 or Ki67 to assess endothelial cell presence and cell proliferation, respectively. We noted that all skin samples stained for CD31 and Ki67, regardless of whether they were from mice that had syngeneic skin alone, allogeneic skin alone, or allogeneic skin with LN excision, further providing evidence of graft survival (Figs. 3A, B).

Allogeneic Skin Transplanted to the Hind limb Primes the Draining LNs

Ipsilateral popliteal and inguinal LNs were collected in mice that did not undergo LN excision, 10 days after allogeneic and syngeneic skin transplantation. The ELISPOT technique was used to assess expression of interferon- γ by lymphocytes derived from these nodes after ex vivo antigen rechallenge. We found that there was a uniformly elevated expression of interferon- γ in mice with allogeneic skin transplants; however, mice with syngeneic skin transplants had significantly lower expression ($P < 0.0001$) (Fig. 4).

Hind limb LN Excision Prevents Th1/Th2 Priming of Secondary Lymphoid Tissue by Day 10

Spleen, ipsilateral axillary, and contralateral inguinal nodes were collected from all mice 10 days postoperatively; in addition, ipsilateral popliteal and inguinal nodes were collected in mice that did not undergo LN excision. Ipsilateral axillary and contralateral inguinal nodes were pooled and the ipsilateral popliteal and inguinal nodes were pooled for each mouse to provide an appropriate number of cells for assay. The ELISPOT technique was used to assess the number of cells expressing interferon- γ (spleen and LNs), IL-4 (spleen), and IL-17 (spleen). Expression of interferon- γ in splenocytes was undetectable in 4 of 6 mice receiving allogeneic skin transplants with LN excision; however, expression of interferon- γ in splenocytes was significantly elevated in 6 of 6 mice receiving allogeneic skin transplants without LN excision ($P = 0.001$) (Fig. 5A). Expression of interferon- γ in the pooled ipsilateral axillary/contralateral inguinal node samples was low or undetectable in 5 of 6 mice receiving allogeneic skin transplants with LN excision, compared with uniformly elevated levels (>1500 spots/ 10^6 cells) in 6 of 6 mice receiving allogeneic skin transplants without LN excision ($P = 0.0006$) (Fig. 5B). Expression of IL-4 in splenocytes was low or undetectable in 5 of 6 mice receiving allogeneic skin transplants with lymphadenectomy, compared with elevated levels in 6 of 6 mice receiving allogeneic skin transplants without LN excision ($P = 0.005$) (Fig. 5C). Finally, expression of IL-17 was uniformly low in all samples (Fig. 5D).

DISCUSSION

In this study, we describe the impact of sentinel LN excision in the mouse hind limb on lymphatic flow and graft alloantigen recognition on the basis of Th1 and Th2 cytokine production in secondary lymphoid tissues. Our model of skin transplantation to the hind limb serves as an efficient model for transplantation involving the extremities, as skin is known to be the most immunogenic tissue; whole limb transplantation in small animals requires microvascular instruments and expertise, which is both costly and difficult to reproduce in large numbers. By performing skin transplantation to the hind limb, we are able to mimic the clinical scenario involving transplantation to the extremities with a

reproducible model. Using methylene blue dye injection, we found that excision of the hind limb popliteal and inguinal LNs obstructs lymphatic flow to the iliac node. In contrast to mice that did not undergo hind limb LN excision, mice with LN excision did not have methylene blue dye in the iliac node. Blue dye has previously been shown to delineate LNs draining the hind limb region within 15 minutes after injection.²² We allowed up to 40 minutes for dye to travel in the experimental and control groups in our model, which is over twice the amount of time previously reported.²² Therefore, it is likely that removal of the popliteal and inguinal LNs results in discontinuity of the lymphatic chain and impedes lymphatic flow from the footpad. Although axillary LN excision has previously been described as a method to obstruct lymphatic flow in mice, these studies do not describe removal of the brachial node.^{28,29} With the brachial node intact, the resulting limb still has an obviously draining node capable of mounting an immunologic response against allografts transplanted to the limb. Our model of hind limb popliteal and inguinal LN excision provides a simple approach to developing a “node-less” limb that demonstrates lymphatic restriction.

The significance of local LNs in the immune response is well recognized, as LNs are a site of antigen presentation. Draining LNs are often assayed to study the immune response to locally introduced antigens. Our results demonstrating impaired lymphatic drainage using methylene blue led us to further question how lymphadenectomy may affect the immune response to locally transplanted grafts. We demonstrate that sentinel LN excision (eg, excision of ipsilateral popliteal and inguinal LNs) without immunosuppressive medications diminished antigen presentation to other secondary lymphoid tissues and impaired graft recognition. Our findings show that the interferon- γ and IL-4 production are significantly diminished in the remaining secondary lymphoid tissue (eg, spleen and distant LNs) in mice receiving allogeneic skin transplants and ipsilateral hind limb LN excision, when compared with mice receiving allogeneic skin transplants alone. These cytokines are expressed by T-cells exhibiting Th1 or Th2 effector function, respectively, and their diminished expression suggests impaired Th1 and Th2 priming.³⁰ Diminished priming of the secondary lymphoid tissues suggests that these secondary lymphoid tissues were not exposed to the offending antigen *in vivo*, before *ex vivo* antigen rechallenge. Therefore, LN excision provides immunologic ignorance early after skin transplantation to the hind limb.

Previous studies have shown that disruption of lymphatic drainage results in delayed skin transplant rejection in a guinea pig model.^{14,15} In these studies, allogeneic skin transplants were transplanted onto pedicled de-epithelialized skin paddles that had been stripped of their lymphatic drainage. Carbon injection studies were performed to confirm impaired lymphatic flow in their model. Additional studies performed similar experiments in a rat model, demonstrating prolonged allograft survival with eventual graft loss within several weeks.^{16,17} However, these studies were difficult to replicate and skin graft loss may have occurred because of factors aside from rejection. In addition, these models of alymphatic pedicled skin transplants do not directly alter the locally draining LNs and cannot be readily translated to humans. At that time, techniques such as cytokine analysis using ELISPOT had not yet been performed. Our study benefits from the reliability of our model and the ease of LN excision. Lymph node excision is performed as part of routine care for patients with

melanoma and breast cancer and can be readily translated to humans. Future studies will be needed to determine whether LN excision results in durable immunologic ignorance or whether immunologic recognition of grafts is eventually established, possibly via generation of collateral lymphatic vessels draining to surrounding nodes.

Allogeneic skin transplantation in the setting of cervical, axillary, brachial, and inguinal LN excision has also been described; in these studies, skin was transplanted onto the flank, with delay in rejection by 3 to 4 days.¹⁵ That report differs from ours in 1 key aspect—in our model, the allogeneic skin is transplanted directly onto the hind limb, which is devoid of lymphatic drainage. Skin transplantation onto the flank, despite excision of multiple draining LNs, may still result in drainage of antigen to other intact LNs because of the extensive lymphatic drainage patterns of the flank.

Our results are consistent with another study, which shows that knockout mice devoid of all LNs exhibit ignorance to the transplanted graft. Alymphoplastic (*aly/aly*) mice with intact spleen fail to produce antibodies against donor antigens.¹⁸ Future studies using our model in addition to splenectomy might offer additional information about the interdependence of the LNs and the spleen.

The immunologic impact of sentinel LN excision has also been previously described in corneal allograft models.^{19–22} These studies have noted prolonged corneal allograft acceptance after submandibular LN excision. However, actual priming of the lymphoid system by the corneal antigens has not been assessed. In addition, corneal transplants do not incur the high rates of rejection seen by other tissues because of the baseline immunologic privilege enjoyed by the cornea. Finally, these studies have been difficult to reproduce due to the variable head/neck lymphatic drainage patterns across species. Our model utilizes a highly immunogenic tissue—skin—to demonstrate ignorance after excision of 2 LNs in the hind limb. The consistency of lymphatic drainage patterns in the extremities across species makes this a model with promise for studies in large animals and potentially humans.

Our model has several limitations. Although we can visualize whole LNs using methylene blue dye, this method does not allow for precise identification of lymphatic channels in the mouse. This is likely due to the small caliber of the lymphatics. Future studies may benefit from use of more advanced imaging techniques to study lymphatic channels such as indocyanine green imaging using spectroscopy. In addition, we have limited these studies to assessing graft ignorance at 10 days after transplantation. It is likely that after a prolonged period of time, collateral lymphatics form allowing for drainage patterns to the intact ipsilateral axillary LN. However, further studies may benefit from administration of compounds that inhibit lymphatic growth such as Cox-2 inhibitors³¹ or anti-VEGF receptor antibodies.^{29,30} Finally, this study addresses 1 component of graft recognition—Th1 and Th2 priming. Interestingly, we found uniformly low graft-specific antibody formation in mice with or without sentinel LN excision (data not shown). Graft-specific antibodies are known to contribute to tissue rejection. It is unclear why skin transplantation to the hind limb would not result in graft specific antibody formation; however, this represented a clear difference when compared with mice receiving allogeneic skin transplants to the flank. This

suggests that location of skin transplantation on the extremities may alter the kinetics of graft recognition or rejection.

The results of our study suggest that hind limb LN excision results in impaired lymphatic drainage of the footpad. After LN excision, skin transplants placed in the area of lymphatic restriction may enjoy a period of ignorance from host recognition. These results suggest that LN removal may be valuable as an adjunct to minimize the risk of rejection. Lymph node excision is not without risks, the most recognized of which is chronic lymphedema. Chronic lymphedema is characterized by impaired lymphatic drainage from the limb and may cause limb pain, tightness, and decreased range of motion. Patients may experience diminished fine motor skills and difficulty with personal care; furthermore, patients with severe lymphedema are prone to developing wounds involving the affected limb. A large meta-analysis of lymphedema in the breast cancer population suggests that patients who have undergone a complete lymphadenectomy have a 20% risk of lymphedema; patients who have only the sentinel LNs removed have a 5% to 6% risk.

CONCLUSIONS

It should be noted that the findings presented here are short-term. Although it is certainly interesting that removal of LNs can modulate short-term allograft recognition, long-term studies will help determine the durability of this immunologic “nonresponse.” These long-term studies will be essential to determine whether sentinel LN excision is a viable technique for engendering site-specific immunologic “ignorance” for the purpose of tissue transplantation. In addition, future studies will be required to evaluate whether inhibitors of lymphatic channel growth, such as Cox-2 inhibitors or anti-VEGF receptor antibodies,^{29–31} may be used in conjunction with LN excision to modulate this effect. Studies involving larger animals will be informative, as de novo growth of lymphatic channels in larger animals may take longer; these channels may be less efficient in draining the limbs of larger animals due to the longer distances required for growth to surrounding LNs. Finally, we may also establish transplant models of tissues other than skin (eg, muscle and/or bone) in larger animals where these may be performed with relative ease than in smaller animals. Our results should be interpreted with caution, but they deserve further attention.

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FIGURE 1.

Lymphatic drainage of mouse footpad with experimental models. A, Lymphatic drainage of mouse footpad to the popliteal and inguinal nodes. Lymph then progresses to the iliac node consistently; lymphatic drainage to the axillary node occurs inconsistently. B, Excision of the inguinal and popliteal lymph nodes results in obstructed lymphatic flow to the iliac or axillary lymph nodes. C, Lymphatic restriction of the transplanted skin after inguinal and popliteal lymph node excision requires transplantation to the distal hind limb. (Adapted with modifications from Harrell et al.²³). Lymph nodes (oval), excised nodes and interrupted lymphatics ("x"), and skin graft (square) are shown. AX indicates axillary; IL, iliac; IN, inguinal; PO, popliteal; RE, renal.

FIGURE 2.

Lymph node staining with methylene blue after LN excision. A, Naïve mouse with no surgical intervention demonstrates methylene blue drainage to the ipsilateral popliteal, inguinal, and iliac lymph nodes after hind limb injection. B, Mouse with sham surgery demonstrates methylene blue drainage to the ipsilateral popliteal, inguinal, and iliac lymph nodes after hind limb injection. C, Mouse with popliteal and inguinal LN excision demonstrates no methylene blue drainage to the ipsilateral iliac LN. D, Mice receiving no intervention or sham surgery had blue dye in 5/5 popliteal (black), inguinal (light grey), and iliac (grey) nodes. Mice receiving popliteal and inguinal LN excision had blue dye in 0/5 iliac nodes. SLNEx indicates sentinel LN excision.

FIGURE 3.

A, Day 10 allograft appearance (10×). (Top) Syngeneic graft near 100% survival, CD31 stain shows capillary growth, and Ki67 stain shows graft cell proliferation. (Middle) Allograft without LN excision near 100% survival, CD31 stain shows capillary growth, and Ki67 stain shows graft cell proliferation. (Bottom) Allograft with hind limb LN excision near 100% survival, CD31 stain shows capillary growth, and Ki67 stain shows graft cell proliferation. Therefore, all skin grafts were able to heal onto the recipient site. Bar indicates distance of 100 μm. B, Day 10 allograft appearance (20×). (Top) Syngeneic graft near 100%

survival, CD31 stain shows capillary growth, and Ki67 stain shows graft cell proliferation. (Middle) Allograft without LN excision near 100% survival, CD31 stain shows capillary growth, and Ki67 stain shows graft cell proliferation. (Bottom) Allograft with hind limb LN excision near 100% survival, CD31 stain shows capillary growth, and Ki67 stain shows graft cell proliferation. Therefore, all skin grafts were able to heal onto the recipient site. Bar indicates distance of 100 μm . SLNEx indicates sentinel LN excision.

FIGURE 4.

Secondary lymphoid tissues are primed for response against alloantigen after skin allotransplantation. Elevated IFN- γ expression in lymphocytes from ipsilateral popliteal and inguinal LNs in mice that did not undergo LN excision. Mice that had undergone LN excision did not have popliteal or inguinal LNs that could be assayed. IFN- γ indicates interferon- γ .

FIGURE 5.

Secondary lymphoid tissue Th1/Th2 priming in mice with LN excision is diminished. A, Decreased IFN- γ expression in splenocytes after skin allograft with LN excision. B, Decreased IFN- γ expression in lymphocytes from pooled ipsilateral axillary/contralateral inguinal LNs after skin allograft with LN excision. C, Decreased IL-4 expression in splenocytes after allogeneic skin graft with hind limb LN excision. D, Low levels of IL-17 expression in splenocytes from all mice. IFN- γ indicates interferon- γ , SLNEx, sentinel LN excision.