

# Pretransplant Kidney-Specific Treatment to Eliminate the Need for Systemic Immunosuppression

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**Background.** Despite significant side effects, chronic systemic immunosuppression remains the backbone of clinical transplantation. We investigated the feasibility of preventing early allorecognition in canine renal allografts using a nonsystemic pretreatment.

**Methods.** The renal vasculature was treated with a bioengineered interface consisting of a nano-barrier membrane during 3 hr of ex vivo warm perfusion.

**Results.** Preliminary feasibility of the immunocloaking technology was established by the following criteria: it is possible to achieve approximately 90% coverage of the vasculature with nano-barrier membrane after 3 hr of ex vivo warm perfusion; covering the luminal surfaces prevents allorecognition as determined by mixed lymphocyte-vascular endothelial reaction; covering the luminal surfaces does not negatively affect renal function as determined by auto-transplant outcomes; and graft rejection is significantly postponed in canine kidneys treated with the immunocloaking technology. In the absence of systemic immunosuppression, untreated control dogs experienced a mean onset of rejection on day 6, whereas in the treated dogs with modified renal vascular luminal surfaces, the mean onset of rejection was significantly delayed until day 30.

**Conclusions.** The ability to postpone, or eventually eliminate, the allorecognition that occurs immediately on reperfusion could provide a new window of opportunity to introduce adjunct therapies to support tolerance induction. To our knowledge, this is the first time significantly prolonged canine renal allograft survival has been achieved in the absence of systemic immunosuppression or immunologic manipulation of the recipient.

**Keywords:** Renal transplantation, Immunocloaking, Kidney, Warm perfusion, Tolerance.

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Activation of recipient polymorphonuclear cells, antigen-presenting cells, and lymphocytes that is inherent in allorecognition is known to trigger effector mechanisms in the graft vascular endothelium. This endothelial activation, particularly in the postcapillary vessels, results in alterations of both its structure and function (1–4). The structural and functional changes result in hyperpermeability, modulation

of endothelial cell coagulant properties, increased polymorphonuclear adhesion, fibrin deposition, and intravascular coagulation. Vascular endothelial cell (VEC) activation amplifies the proinflammatory and procoagulant pathways leading to enhanced transendothelial migration of recipient immune cells into the allograft.

Although passenger leukocytes and dendritic cells are known to play roles in the sensitizing milieu that contributes to the overall antigenicity of an allograft, the major target of the immune response is the vascular endothelium (5). This phenomenon holds true whether the immunologic assault is manifested hyperacutely, acutely, or even chronically. The intima of the renal vasculature is the site of recruitment of monocytes, T cells, and also of antibody deposition. The antigenicity of vascular endothelium is related to the surface expression of many antigen systems including the major histocompatibility complex class I and II, ABO blood group antigens, and a long list of antigens with restricted tissue expression that provides for its organ-specific functions (6). Therefore, reducing the antigenicity of the vascular endothelium may represent a logical approach to ameliorating alloresponsiveness to engraftment.

The goal of this study was to investigate whether it would be feasible to “immunocloak” the vasculature within canine kidney allografts using a receptor-mediated bioengineered nano-barrier membrane (NB-LVF4) as a barrier

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method of immunomodulation. The research questions addressed included:

1. Would coverage of the vascular endothelium in fact prevent allorecognition as assessed in a mixed lymphocyte-VEC reaction (MLER)?
2. Is it feasible to apply NB-LVF4 to the vasculature of the kidneys, resulting in adequate coverage of the luminal surfaces?
3. If successfully applied, would covering the renal vasculature adversely affect renal function?
4. If application of NB-LVF4 to the renal vasculature did not adversely affect function, would such immunocloaking provide protection against early allograft rejection?

## RESULTS

### Mixed Lymphocyte-Vascular Endothelial Cell Reaction

The traditional mixed lymphocyte culture has been long used as sensitive test for evaluating cellular compatibility within the major histocompatibility class II complex in transplantation (Table 1). The results were interpreted as “mini-graft” where a stimulation index (SI) of less than 5.0 was considered negative, indicating there was no significant proliferative response to the allogenic cells. Similarly, a SI more than 5.0 was considered positive where an aggressive immunogenic response could be anticipated. In this study, VEC were used as the stimulator cell in place of lymphocytes. The

**TABLE 1.** Mixed lymphocyte-vascular endothelial cell reaction<sup>a</sup>

Combination	CPM	SI <sup>b</sup>	Inhibition (%)
Canine mononuclear cells	385	—	—
Canine mononuclear cells+allogenic VEC	19,200	49.8	—
Canine mononuclear cells+NB-LVF4-treated allogenic VEC	409	1.1	99.9
Canine mononuclear cells+NB-LVF4	280	0.7	—

<sup>a</sup> Results are represented as the mean of the three experiments.

<sup>b</sup> SI=mean CPM test/mean CPM negative control.

CPM, counts per minute of the mean; VEC, vascular endothelial cell; NB-LVF4, nano-barrier membrane; SI, stimulation index.

canine lymphocytes proliferated when stimulated by allogenic VEC, resulting in a SI of 49.78. However, when NB-LVF4 was applied to the surface of the confluent VEC, the lymphocytes proliferation was essentially eliminated with a 99.98% inhibition of the proliferative response to the VEC. Supporting the immunocloaking effect is the observation that canine lymphocytes also did not respond to NB-LVF4 alone. Therefore, NB-LVF4 is not immunogenic, does not elicit a proliferative response by itself, and results in an immunocloaking effect.

### Ability to Immunocloak the Renal Vasculature (Group 1)

Deposition of NB-LVF4 at a concentration of 66  $\mu\text{g/g}$  of kidney provided ubiquitous coverage in approximately 90% of the vascular luminal surface including both small and large blood vessels. No occlusion of vessels was observed immunohistochemically. This observation was supported by stable perfusion pressures and vascular flow rates throughout the 3 hr of ex vivo warm perfusion after NB-LVF4 application. Similarly, although there were isolated areas of kidney vasculature not covered, these areas were focal and randomly dispersed. All other areas seemed to be uniformly immunocloaked (Fig. 1).

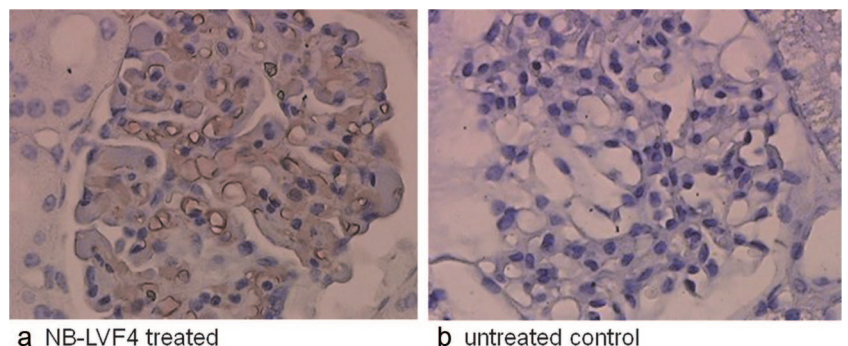
### Renal Function After Deposition of NB-LVF4 (Group 2)

After NB-LVF4 application at the selected concentration listed above during the 3 hr of ex vivo warm perfusion, the kidneys were autotransplanted with contralateral nephrectomy. All four treated autotransplanted kidneys provided normal serum chemistries, normal electrolyte profiles, and normal hematology (Table 2). The normal function that was observed throughout the posttransplant period in terms of the stable serum creatinine values (Fig. 2) and the various parameters listed in Table 2 provides evidence that NB-LVF4 treatment did not adversely affect renal function.

### Feasibility of Immunocloaking (Group 3)

After NB-LVF4 application in the same manner as described earlier, the kidneys were allotransplanted with nephrectomy of the native kidneys. Untreated controls were similarly warm perfused and then allotransplanted with nephrectomy of the native kidneys. No systemic immunosuppression was administered to either group. In the untreated control dogs, the mean onset of rejection oc-

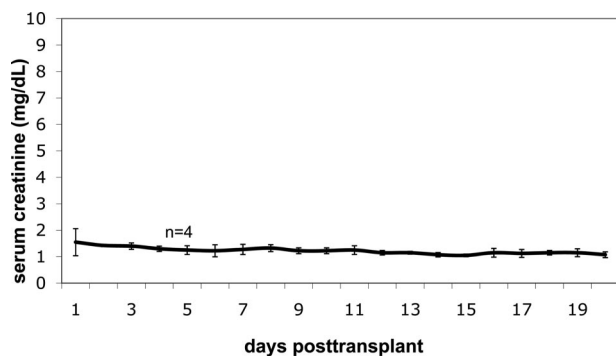
**FIGURE 1.** Application of nano-barrier membrane (NB-LVF4). High-power view of (a) biotin-avidin immunoperoxidase staining using primary rabbit antibody to the membrane of 2- $\mu$  section of kidney treated with 66  $\mu\text{g/g}$  of NB-LVF4 during 3 hr of subsequent near-normothermic perfusion. (b) Immunoperoxidase staining of control kidney that was warm perfused but not treated with NB-LVF4.



**TABLE 2.** NB-LVF4-treated canine kidney autotransplants

	Pretx <sup>a</sup>	Posttx <sup>b</sup> day1	Posttx day 7	Posttx day 14	Posttx day 180	Normal range
Blood chemistry						
BUN (mg/dL)	16.3 ± 3.5	33.3 ± 6.9	17.8 ± 2.6	16 ± 3.2	16 ± 1.4	6–27
Total protein (g/dL)	6 ± 0.3	4.5 ± 0.3	6.2 ± 0.3	6.3 ± 0.4	6.3 ± 0.4	5.0–7.5
Sodium (mEq/L)	148 ± 2.2	139 ± 2.9	148.7 ± 3.3	148.8 ± 1	149.8 ± 2.2	145–158
Chloride (mEq/L)	113.8 ± 1.5	101.5 ± 2.4	113 ± 1.2	113.5 ± 1	113.5 ± 1.3	105–122
Potassium (mEq/L)	5.1 ± 0.4	5.2 ± 0.4	5.2 ± 0.4	5.2 ± 0.3	5.1 ± 0.1	3.5–5.8
Calcium (Eq/L)	10.3 ± 0.1	10.4 ± 0.1	10.4 ± 0.1	10.4 ± 0.1	10.4 ± 0.1	9.0–11.8
Hematology						
RBC (M/ $\mu$ L)	6 ± 0.8		5.2 ± 1			4.0–6.0
HGB (g/dL)	13.5 ± 1.4		12.1 ± 2			11.0–18.0
HCT (%)	40 ± 5		36 ± 7.7			35.0–60.0
MCV (fL)	67.6 ± 0.7		67.6 ± 0.4			80.0–99.9
CHC (g/dL)	33.6 ± 2		34.5 ± 1.3			33.0–37.0
RDW (%)	13 ± 0.8		12.6 ± 0.3			11.6–13.7
PLT (K/ $\mu$ L)	301 ± 149		273 ± 153			150–450
WBC (K/ $\mu$ L)	10.7 ± 3.8		10.4 ± 3.7			4.5–10.5

Pretx, pretransplant; Posttx, posttransplant; BUN, blood urea nitrogen; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; CHC, cellular hemoglobin concentration; RDW, red cell distribution width; PLT, platelets; WBC, white blood cell.

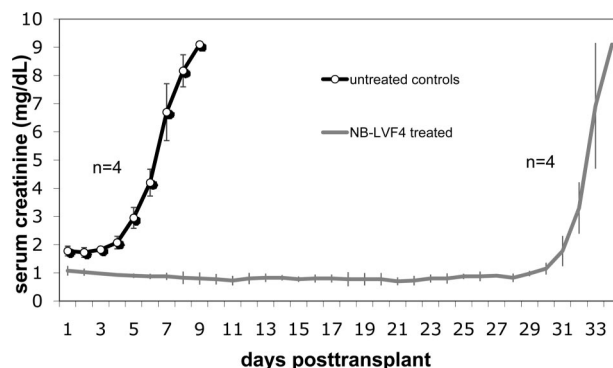


**FIGURE 2.** Autotransplantation after nano-barrier membrane (NB-LVF4) application. Mean serum creatinine concentrations with standard deviations after renal autotransplantation of kidneys treated with 66  $\mu$ g/g of NB-LVF4 therapy.

curred on day 6. In contrast, in the treated dogs where the luminal surfaces of the renal vasculature had been modified, the mean onset of rejection was significantly delayed occurring on day 30 (Fig. 3).

## DISCUSSION

To our knowledge, this is the first documented case of prolonged canine renal allograft survival in the absence of any type of systemic immunosuppression or immunologic manipulation of the recipient. These preliminary results demonstrate that it may become feasible to bioengineer the luminal surfaces within renal allografts without adversely affecting function. Our approach to graft immunocloaking entailed coating the vascular surface within kidneys with a nano-barrier membrane, NB-LVF4, that is composed of laminin, vitronectin, fibronectin, and type IV collagen. Several integrin complexes are involved in the attachment of NB-LVF4 to the surface of VECs. Blocking studies using antibodies to the  $\beta$ 1



**FIGURE 3.** Allotransplantation in the absence of systemic immunosuppression. Mean serum creatinine concentrations with standard deviations after renal allotransplantation of kidneys treated with 66  $\mu$ g/g of nano-barrier membrane (NB-LVF4) compared with the untreated control kidneys that were similarly warm perfused.

and  $\beta$ 3 class of receptors demonstrate receptor-ligand interactions are involved in the strong binding of NB-LVF4 to the surface of VEC, particularly the functional domain of the arginine-glycine-aspartate sequence (data not shown). The VEC receptors are diffusely distributed along the cell membrane and are freely mobile by means of a contact redistribution mechanism that is cytoskeletal independent.

NB-LVF4 provides an apical surface that remains non-thrombogenic and nonimmunogenic. The goal was to interrupt the normal interface by providing a physical barrier between recipient immune cells in circulation and the renal allograft vasculature. We postulate that the network of adhesive molecules within the polymerized NB-LVF4 functions as a barrier to cellular migration while simultaneously allowing the free diffusion of nutrients and oxygen. A possible mechanism for the preliminary efficacy demonstrated in this study

is the observation that leukocyte transmigration into tissue occurs only where there is a direct contact with VEC (7). The presence of NB-LVF4 on the luminal surfaces of the renal vasculature may function by interrupting the leukocyte/VEC interaction required for the transmigration of immune cells into the graft. This interruption of leukocyte/VEC interaction may be important in the unique organization found at sites of postcapillary venules that is particularly permissive for leukocyte trafficking (8). The results of the MLERs where NB-LVF4 prevented a proliferative response in comparison with untreated controls provides initial evidence for this hypothesis.

Previous attempts to alter the luminal surface of an allograft have not been successful, in large part, because the modification was attempted during the period of profound hypothermia that is used in clinical organ preservation (9). Hypothermic preservation is predicated on the cold-induced inhibition of metabolism, where basal metabolism is inhibited by more than 96% (10, 11). Therefore, the ability to alter the luminal surface in the cold is severely hampered by the lack of substantial cell metabolism.

Encapsulating materials made from biocompatible polymers that have been used for camouflaging a variety of proteins and isolated cells are of limited application in the case of an intact solid organ. Of these polymers, the most studied is poly(ethylene-glycol) (PEG) that has been covalently bonded to a variety of proteins, carbohydrates, lipids, and cells since the 1970s. PEG has been used in several applications, including reducing red blood cell (RBC) antigenicity in both blood group mismatched and xenotransfusion, coupled with hemoglobin to develop blood substitutes, and treatment of isolated pancreatic islets (12–15). However, when PEG treatment was used to attempt chemical camouflage of the endothelial cells lining, the luminal surfaces of a heart xenograft during perfusion at 4°C, no protection from hyperacute rejection was observed (11). This finding was in contrast to studies by the same group where PEG treatment of isolated endothelial cells during cell culture (37°C) prevented antibody-antigen interactions. Although RBC PEGylation is useful for some blood group antigen coverage, the ABO blood group antigens A and B have been difficult to mask (16–19). Interestingly, monomethyl polyethylene glycol has been shown to be stably attached to RBC membranes for 30 days, even in the case of hemolysis (20). In light of this finding, the 30-day allograft survival observed in NB-LVF4-treated kidneys in this study may reflect the duration bioengineered materials are retained on cell membranes before degradation.

This study was designed to address initial issues pertaining to feasibility. Inasmuch, these results demonstrate prolonged protection from rejection in the presence of NB-LVF4 that immunocloaks the vascular surface within a kidney allograft. The near-normothermic perfusion that was used to apply the NB-LVF4 has been shown in previous studies to be nondamaging. The warm perfusion technology has also demonstrated the ability to maintain kidneys *ex vivo* for 48 hr, ameliorate reperfusion injury, overcome warm ischemic damage of as much as 2 hr, and to support reparative processes (21, 23–25). Therefore, the application of NB-LVF4 before transplantation during a non-damaging near-normothermic perfusion allows for optimal polymerization and binding to the VEC.

With the onset of rejection in the treated kidneys, it was not possible to determine whether the NB-LVF4 was still present 30 days posttransplant, or whether its deterioration

lead to the rejection. The acute onset of the rejection was similar to rejection in the cyclosporine era with widespread coagulation and necrosis. The goal of this study was to determine whether it is feasible to modify the luminal surface of the vasculature within an allograft without compromising renal function and whether protection from early allograft rejection in the absence of system immunosuppression could be achieved. It was beyond the scope of this initial feasibility study to determine the underlying mechanism of protection, particularly in light of our concerns of disrupting the NB-LVF4 coverage of the luminal surface by taking a sufficient number of biopsies to be representative of the kidney milieu.

Further studies are underway to determine how long the NB-LVF4 remains intact in the face of vascular blood flow post-transplant. It is anticipated that an understanding of the stability of NB-LVF4 application will help to elucidate the underlying protective mechanisms involved in immunocloaking. An understanding of the *in vivo* retention of NB-LVF4 along with its underlying protective mechanism will help to develop treatment strategies that could 1 day lead to enhanced graft survival without the traditional dependency on chronic systemic immunosuppression. Similarly, an understanding of the NB-LVF4 impact on costimulatory pathways involved in allorecognition and activation will help to optimize the treatment and lead to further enhancement in graft survival.

The results of this initial feasibility study demonstrate that treatment with NB-LVF4 delays the onset of renal allograft rejection. Modifying the antigenicity of the vascular wall in the absence of any systemic immunosuppression represents an organ-specific therapy that may function by interrupting early allorecognition, at least temporarily. The ability to postpone, or eventually eliminate, the allorecognition that occurs immediately on reflow could provide a new window of opportunity to introduce adjunct therapies to support tolerance induction.

## MATERIALS AND METHODS

### NB-LVF4

NB-LVF4 is manufactured from canine proteins consisting of laminin, vitrogen, fibronectin, and type IV collagen from extracellular basement membranes produced by endothelial cells in culture. The components are polymerized into a tridimensional transparent membrane that is 200 nm in thickness. Laminin polymers serve as the template for the NB-LVF4 assembly. The cross-linked type IV collagen provides the structural integrity, and the disulfide bonds stabilize the components in the membrane. Because NB-LVF4 is permeable to small molecular weight compounds, free transport of nutrients and oxygen is unaffected, and the graft tissue remains viable.

### Application and Detection of NB-LVF4

The process involves solubilizing NB-LVF4 by acidification at 4°C. The NB-LVF4 is neutralized just before use and then applied to the vasculature of canine kidneys during an acellular warm perfusion at 32°C for 3 hr (24). The solubilized NB-LVF4 is introduced in the arterial line at a rate of approximately 100 µg/min. Varying concentrations of NB-LVF4 have been previously tested, and the optimal dosage that was used for this study was 66 µg/g of kidney because lower doses resulted in inadequate coverage of the luminal surfaces, while higher doses resulted in thick deposition that in some cases obstructed the glomeruli.

Kidneys were evaluated for the presence of NB-LVF4 using a biotin-avidin immunoperoxidase assay with a rabbit primary antibody to canine laminin. Two-micron sections from five areas of each kidney were fixed and tested in duplicate.

## Mixed Lymphocyte-Vascular Endothelial Cell Reaction

The MLER was performed using canine allogenic cells. Canine VEC were isolated from blood vessels using collagenase digestion. Allogenic lymphocytes were isolated from whole heparinized blood on ficoll hypaque gradients and then centrifuged at 500g for 30 min. The lymphocytes were collected and washed twice, and then resuspended at a concentration of  $1 \times 10^6$ /mL. The VEC isolated from multiple vessels from a single donor were pooled and seeded into 96-well tissue culture plates. The VEC cultures were maintained until confluence at 37°C, 5% CO<sub>2</sub>. Once confluence was established,  $2.5 \times 10^5$  of responding allogenic lymphocytes isolated from the peripheral blood were added to each triplicate well. The cultures were maintained at 37°C for an additional 4 days and then pulsed with 0.6  $\mu$ Ci <sup>3</sup>H-thymidine and harvested 18 hr later. DNA synthesis was evaluated by scintillation counting of the harvested cells. In the wells where NB-LVF4 was tested, the cold solubilized membrane was neutralized and then applied gently to cover the bottom of the wells or the confluent layer of VEC. The treated wells were incubated at 37°C for 3 hr before introducing the allogenic mononuclear cells that were layered onto the newly polymerized NB-LVF4. The test combinations included:

- Negative control—canine mononuclear cells alone.
- Positive control—canine mononuclear cells+allogenic VEC.
- Test—canine mononuclear cells+NB-LVF4-treated allogenic VEC.
- Test—canine mononuclear cells+NB-LVF4 alone.

The testing was performed in triplicate on three separate days, and the results represented as the mean of the three experiments using the mean of the counts per minute (CPM) from each experiment. The SI was calculated as: mean CPM test/mean CPM negative control. The effectiveness of NB-LVF4 treatment was determined by the percent of inhibition it provided.

## Canine Transplantation Procedure

The transplantation experiments were performed on foxhounds weighing 20 to 30 kg. The animals demonstrated normal renal function before the start of the study. All experiments were performed following the principles of laboratory animal care according to the National Institutes of Health standards. Donor and recipient foxhounds were purchased from different vendors to ensure that the donors and recipients were unrelated. Canine kidneys were nephrectomized, a cannula was placed in the renal artery, flushed of blood, and then placed on warm perfusion as described previously (24, 25). After treatment and subsequent 3 hr of warm perfusion to allow for adequate polymerization of NB-LVF4, the kidneys were reimplanted with nephrectomy of the contralateral kidney in the autotransplants and the two native kidneys in the allotransplants. The onset of rejection was defined as an increase in serum creatinine of more than or equal to 1.0 mg/dL overnight accompanied by a continued increase of more than 8.0 mg/dL or clinical symptoms requiring euthanasia.

## Treatment Groups

### Feasibility of Applying NB-LVF4 to the Vasculature of the Kidneys

Group 1 (n=5) canine kidneys were warm perfused for 3 hr at 32°C after administration of 66  $\mu$ g/g of kidney of the barrier membrane. The kidneys were then sectioned and evaluated in a blinded fashion for luminal surface coverage.

### Effect of NB-LVF4 on Renal Function

Group 2 (n=4) ability to retain normal renal function after application of NB-LVF4 was assessed using a canine autotransplant model with contralateral nephrectomy of the untreated kidney.

### Immunocloaking—Protection Against Early Allograft Rejection

Group 3 (n=8) efficacy of the immunocloaking treatment was assessed in a canine allotransplant model by comparing outcomes of treated allografts

(n=4) with untreated control allografts (n=4) in the absence of systemic immunosuppression.

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