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Divya Haridas Date

USE OF INTEGRIN BLOCKADE TO INHIBIT CD8$^+$ MEMORY T CELLS IN SOLID ORGAN TRANSPLANTATION

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By

Divya Haridas
B.S., University of Alabama in Huntsville, 2009

Advisor: Mandy L. Ford, Ph.D.

An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
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Program in Immunology and Molecular Pathogenesis

2011

ABSTRACT

USE OF INTEGRIN BLOCKADE TO INHIBIT CD8$^+$ T CELLS IN SOLID ORGAN TRANSPLANTATION

By: Divya Haridas

Although calcineurin-based inhibitors have come a long way in inhibiting organ rejection, the associated toxicities have led to the continued quest for better immunosuppressants. Two potential candidates are belatacept and efalizumab. Belatacept is a costimulation blocker that has been shown to have higher acute rejection rates in kidney transplant patients compared to traditional calcineurin inhibitors. Here we hypothesize and show that belatacept had no effect on the cytokine production of memory CD8$^+$ T cells. Efalizumab binds the integrin LFA-1 and blocks the LFA-1-ICAM-1 interaction crucial for T cell activation and trafficking. We show here that efalizumab was effective in inhibiting alloreactive memory CD8 T cell responses. We also found that graft-elicited CD8$^+$ T cells were inhibited by anti-LFA-1 but not pathogen-elicited T cells. Thus, the susceptibility of T cells to different immunosuppressants is different based on their priming conditions and this warrants further investigation.

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I wish to thank Mandy Ford for her amazing mentorship, patience and encouragement during my research and studies at Emory University.

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Introduction

The history of transplantation can be traced back to 1908 though the writings of Alexis Carrel who attempted to understand the physiological and pathological character of the functions of transplanted kidneys (1). He went on to develop a technique that connected blood vessels. In the 1940s, Peter Medawar performed groundbreaking work in the field of immune tolerance. He was a physician who observed that burn victims from World War II were rejecting donor skin and began using experimental skin transplants on animals to understand rejection. In his research article he showed that exposure of animals to foreign antigens before the animals have fully developed their immunological responses leads to tolerance rather than a heightened resistance (2). Dr. Joseph Murray performed the first successful solid organ transplant in 1954 when he transplanted a kidney from one identical twin to the other (3). Although worldwide, the kidney is the most transplanted organ, other organs like the heart, liver, pancreas, lungs, intestine and thymus can also be transplanted. Tissues like skin and cornea are also transplanted.

One of the major problems in transplantation is immune-mediated transplant rejection. During transplant rejection, the body rejects the transplanted organ from the donor as "foreign" and mounts an immune response against the transplanted organ. This leads to organ rejection.

Types of Graft Rejection

There are 3 types of rejection –hyper-acute, acute and chronic rejection (4). Pre-formed antibodies in the recipient that are specific against donor tissue cause hyper-acute rejection. These antibodies called "allo-antibodies" can be found in unsensitized individuals but can also be formed in the recipient through various ways including blood transfusions and pregnancy. Often these allo-antibodies can reject the organ in a few

days. In acute rejection, the transplant appears successful at first but after a few days, the organ begins to show the first symptoms of rejection. If not treated with proper immunosuppression (5), the organ will lose functionality over time and will be rejected. The immune cells involved are both B and T cells. In the third type of rejection known as chronic rejection, organ rejection occurs over a long period of time and results in the deterioration of the organ and loss of function. Immunosuppression is now used to prevent organ rejection (5).

History of Immunosuppression

The introduction of calcineurin-based immunosuppressive drugs reduced graft rejection and enhanced engraftment (5). Other drugs were discovered over the years. Azathioprine and corticosteroids were shown to have synergistic effects in renal transplant patients in the 1960s (6). The discovery of cyclosporine by researchers at Sandoz Ltd at Bazel, Switzerland marked the beginning of the modern immunosuppression era. Cyclosporine works by engaging cyclophilin and thereby disrupting the activation of calcineurin. By 1996, around 200,000 patients were relying on it to prevent organ rejection (7). In the 1980s, clinical trials were started to study the effects of monoclonal antibodies against T cells and in 1987, the anti-CD3 monoclonal antibody *Orthoclone OKT-3* was approved by the Food and Drug Administration (8). Immunosuppressive drugs work either by depleting or diverting the T and B lymphocytes involved in rejection or by blocking the signal response pathways. The drugs used so far in the clinics have had serious side effects.

Important Immunosuppressive Drugs and Their Side Effects

In spite of development of a large number of immunosuppressants, the associated toxicities of these drugs lead to a continued quest to develop better drugs. A number of important immunosuppressive drugs and their side effects are listed below (5)-

(a) Cyclosporine-The main side effect of this drug is nephrotoxicity, hypertension, hirsutism, post-transplantation diabetes mellitus and hyperlipidemia (9).

(b) Tacrolimus-This macrolide antibiotic binds to FKBP12 and inhibits T cell activation by binding to calcineurin phosphatase. The toxic side effects are similar to cyclosporine but with lower incidence of hypertension (10).
(c) Azathioprine-This drug inhibits purine synthesis and the side effects include delayed wound healing, pneumonitis and interstitial lung disease (11).
(d) Rituxumab-This chimeric monoclonal antibody binds to CD20 on B cells and mediates lysis. Its side effects are mainly sensitivity (12).
(e) Steroids- Transplant patients are needed to take steroids such as prednisone as part of the immunosuppressive regimen to prevent rejection. There are a number of side effects related to steroids including hypertension, diabetes, cholesterol, etc (13).
(f) Rapamycin- This drug was first discovered from soil samples in Easter Island. It blocks the mammalian target of rapamycin (mTOR) pathway. Impaired wound healing, pneumonitis and aphthous ulcer formation are some of the disadvantages of this immunosuppressant (14).

The side effects associated with the drugs listed above reveal a clear need for better immunosuppressive drugs. Traditionally, there are three "signals" required to activate naïve T cells (15), and each of these could serve as a possible target for immunosuppression. The first signal is the direct interaction of the T cell receptor with the peptide-MHC on the antigen-presenting cell (APC) (15). The downstream signaling cascade associated with this binding is a target for immunosuppressants. The second is the costimulation necessary to activate T cells, which is the binding of the CD28 on the T cell with the B7.1 and B7.2 on the APC (15). And finally, the third signal required for naïve T cell activation is the cytokines secreted by innate immune cells that help in the activation of T cells (16).

Development and efficacy of Belatacept

Scientists at Bristol-Myer Squibb in collaboration with scientists at Emory Transplant Center and several other centers across the US have developed a new immunosuppressant drug named belatacept which binds the B7 on the APCs and prevents

them from delivering costimulation to the T cells, thus preventing T cell activation (17). Recently, the five-year safety trial of belatacept was published which found that belatacept was associated with lower nephrotoxicity than traditional calcineurin inhibitors but had higher acute rejection rates in the first year compared to traditional calcineurin inhibitors (18). We hypothesized that this rejection may be mediated by alloreactive memory $CD8^+$ T cells, which are known to have lower requirements for costimulation compared to naïve T cells. Adult humans have about ~50% memory T cells and this is a potential cause of mediating acute rejection (19). One potential candidate to inhibit alloreactive memory $CD8^+$ T cells is efalizumab. Efalizumab (trade name Raptiva) is a monoclonal antibody that binds to the CD11a portion of lymphocyte function-associated antigen-1 (LFA-1) in humans (20) . LFA-1 is an integrin found on T cells, B cells, macrophages and neutrophils and helps in the activation and trafficking of immune cells through the high endothelial venules (HEV) from the bloodstream into tissues (20). LFA-1 binds to inter-cellular adhesion molecule-1 (ICAM-1) found on endothelial and other immune cells like dendritic cells (DCs). The LFA-1 and ICAM-1 interaction is an important part of the peripheral supramolecular activation cluster (p-SMAC) found in immunological synapses that activate T cells (21). LFA-1 and ICAM-1 interactions are also responsible for the trafficking of T cells into tissues (22). Efalizumab was initially developed as a treatment for psoriasis, an autoimmune disease in which faulty signals by the body's immune cells speeds up the growth of skin cells (23). Efalizumab is a recombinant, humanized, monoclonal IgG1 antibody and by targeting the initial activation and trafficking of lymphocytes, alleviates the pathogenesis of psoriasis. Efalizumab was approved by the Food and Drug Administration (FDA) in November 2003 and by the European Medicines Evaluation Agency in September 2004 for the treatment of patients with moderate to severe cases of psoriasis. After greater than three years on efalizumab, three patients developed progressive multifocal leukoencephalopathy which led to the drug being voluntarily withdrawn from the market (24).

Progressive multifocal leukoencephalopathy (PML) is a disease characterized by the reactivation of the JC virus (20). JC virus is a polyoma virus that is found in about 70% of the healthy, adult population (25). It is a persistent infection that is kept under

check by a healthy immune system. Under conditions of immunosuppression (such as HIV infection, pregnancy or during immunosuppression following transplantation), uncontrolled reactivation of JC virus can lead to axonal demyelination of the brain. This is progressive, untreatable and eventually fatal (26). Newer drugs like efalizumab, rituximab and natalizumab seem to carry a higher risk of reactivating the JC virus to cause PML in patients. However, efalizumab has not been tried as a transplant immunosuppressant. We now propose that a therapeutic window for LFA-1 blockade may exist, wherein we can inhibit graft-elicited T cell responses but not pathogen-elicited T cell responses.

In a small pilot study at the Emory Transplant Center, efalizumab was used as an immunosuppressant on four patients post-islet transplantation. All four patients were insulin-independent for nine months post transplantation (27). Unfortunately, these patients had to be taken off efalizumab because efalizumab had been withdrawn from the market voluntarily by Genentech after 3 out of 46,000 patients on efalizumab for psoriasis treatment developed progressive multifocal leukoencephalopathy (PML) (24). In a similar pilot study at the Transplant Surgery Center at the University of California in San Francisco, eight patients with type I diabetes received allogeneic islet transplants and were treated with efalizumab and sirolimus or mycophenolate (28). All eight patients were insulin independent and had no further hypoglycemic events. Efalizumab was well tolerated and no serious adverse events were reported (28) but the long-term follow-up was limited by the discontinuation of efalizumab from the market (24).

There is a need to further understand why LFA-1 antagonism inhibits graft-specific T cell responses 100% of the time but JC-virus specific responses only ~0.005% of the time. We believe that understanding this difference may lead us to a therapeutic window wherein we can inhibit graft-specific T cell responses while maintaining some pathogen-specific protective immunity.

METHODS:

In vitro Allostimulation Assay

Different pairs of human responders and stimulators were drawn for blood after informed consent in accordance to IRB protocols. PBMCs were obtained after Ficoll-Paque gradient centrifugation. Stimulators were irradiated and the responders and stimulators were plated together in a 1:1 ratio (~10^6 total cells/well) in a 96 well plate. The cells were left untreated or treated with belatacept at 100 μg/ml (from Bristol-Myer Squibb), anti-LFA-1 (TS-1) at 250 μg/ml (from BioXcell) or both and the cells were incubated for 6 hours in a 37^0C incubator with 5% carbon dioxide.

Intracellular cytokine staining for the production of IFNg and TNF was assessed via flow. Cells were divided into naïve, effector memory, central memory and T_{EMRA} subsets based on the expression of CD45RA and CD197 (CCR7). Memory cells were gated as CD45RA negative and CD197 high (central memory) or CD197 low (effector memory).

Adoptive Transfers and LM-OVA infections

Two groups of naïve B6 mice were adoptively transferred with 10^6 OT-I T cells given through i.v. injections. One group of mice was grafted with mOVA skin grafts (~ 1 cm * 1 cm in area) from the ears and tail skin of mOVA mice that express OVA ubiquitously (29) and the other group was given i.p. injections of *Listeria Monocytogenes*-OVA infection (10^4 cfu/mouse). Anti-murine LFA-1 (M17/4, BioXCell, West Lebanon, NJ) was given to the appropriate groups through i.p. injections on days 0, 2, 4 and 6 at 250 μg/mouse. After 10 days, the mice were sacrificed and splenocytes were obtained.

Intra-Cellular Cytokine Staining

Intra-cellular cytokine stimulation was performed for 5 hours with SIINFEKL peptide at 1nM concentration and Golgi Plug at 10μg/ml. 2 X10^6 cells were plated out per well and after 4 hours, surface and cytokine staining was performed and the cells were run on a LSR machine to gauge both surface molecule expression and cytokine production.

RESULTS:

Anti-LFA-1 inhibits IFNγ release in human alloreactive memory CD8$^+$ T cells unlike Belatacept

In order to assess the impact of CD28 and LFA-1 blockade on cytokine secretion by allo-reactive memory T cells in response to allostimulation, we isolated PBMC from normal healthy donors. Donors were MHC typed for at least two HLA I and HLA II mismatches and the PBMCs obtained were used to perform an *in vitro* allostimulation assay. Briefly, the responder and stimulator cells were either left untreated or were treated with belatacept and/or anti-LFA-1 for 6 hours and the cytokine production was assessed using intra-cellular surface staining (ICCS). We gated on CD8$^+$ T cells alloreactive memory T cells using CD45RA and CD197 expression. Memory T cells are CD45RA negative cells. After gating on the alloreactive memory CD8$^+$ T cells, we found that anti-LFA-1 (TS-1) inhibited IFNγ production ($p<0.03$) but not TNF production. Belatacept however, did not inhibit IFNγ or TNF production. A combination of anti-LFA-1 and belatacept also was effective in inhibiting the IFNγ production in these cells (Figure 1).

LFA-1 blockade inhibits graft-elicited CD8$^+$ T cells but not LM-OVA elicited CD8$^+$ T cells

We hypothesized that there may be a difference in the susceptibility of T cells to LFA-1 blockade based on whether they were primed by a graft vs. a pathogen. In order to test this we primed OT-I T cells (which are a CD8$^+$ TCR transgenic T cells specific for chicken ovalbumin) either with a skin graft (29) or with a pathogen (30) (both engineered to express the ovalbumin antigen recognized by OT-I cells) and studied the susceptibility of both these T cells to LFA-1 blockade. Briefly, we adoptively transferred 10^6 OT-I T cells into naïve, C57BL/6 mice. Two days after the transfer, the mice were grafted with a mOVA skin graft (which ubiquitously expresses membrane-bound ovalbumin) (29) or given LM-OVA infection (a genetically-engineered *Listeria monocytogenes* bacteria which expresses ovalbumin) (30). They were either left untreated or administered anti-LFA-1 on days 0, 2, 4 and 6. The mice were sacrificed on day 10 and the spleen, lymph nodes and blood were collected. We found that the LFA-1 blockade inhibits the OT-I T

cells in the skin graft-elicited T cells (Figure 2), an inhibition that was also evident in the decrease in absolute numbers of OT-I T cells in the spleen on day 10 (Figure 3). In stark contrast, we found that anti-LFA-1 treatment did not inhibit the expansion of OT-I T cells after pathogen infection with LM-OVA (Figures 4 and 5).

Pathogen-elicited CD8$^+$ T cells exhibit higher expression of LFA-1 than graft-elicited CD8$^+$ T cells

In order to further explore the differential effects of LFA-1 blockade on graft versus pathogen-elicited responses, we endeavored to determine if there were any fundamental differences in the LFA-1 expression between pathogen-elicited and graft-elicited CD8$^+$ T cells. In order to do so, we adoptively transferred OT-1 T cells into B6 mice. The B6 mice were divided into two groups: one group received skin graft from mOVA mice while the other group was infected with LM-OVA. The mice were then sacrificed on day 10 and splenocytes obtained. We found that the pathogen-elicited CD8$^+$ T-cells exhibited higher LFA-1 expression than the SG-OVA –elicited CD8$^+$ T cells (Figure 6). Another difference we observed was that the LM-OVA elicited CD8$^+$ T cells produced more IFNγ and TNF than the graft-elicited T-cells. Specifically, while the pathogen-elicited T-cells were mostly double producers of IFNγ and TNF, the graft-elicited T-cells were both single and double producers of IFNγ and TNF (Figure 7).

LFA-1 is equivalently blocked in both LM-OVA-elicited and graft-elicited CD8$^+$ T cells

We then asked whether this difference in LFA-1 expression might be the reason that efalizumab was able to inhibit graft-elicited CD8$^+$ T cells and not pathogen-elicited CD8$^+$ T cells. The higher expression of LFA-1 on the LM-OVA elicited CD8$^+$ T cells may have led to an incomplete blockade by anti-LFA-1, thus preventing the inhibition of LM-OVA elicited T cells in the infection model. In order to determine if the anti-LFA-1 was completely blocking the LFA-1 expression on both LM-OVA elicited and graft-elicited OT-I T cells, we compared the MFIs of LFA-1 in both groups and found that the LFA-1 was equivalently blocked in both groups (Figures 8 and 9). Thus, the incomplete inhibition of pathogen-elicited T cell responses by LFA-1 blockade cannot be fully

attributed in differences in LFA-1 surface expression between graft-elicited and pathogen-elicited T cells.

The mechanisms underlying the differential susceptibility of graft- vs. pathogen-elicited T cells to LFA-1 blockade are currently unknown, and this remains an important area of future investigation. However, it is interesting to speculate that this fundamental difference between pathogen-elicited and graft-elicited T cells may give us a "therapeutic window" wherein we can inhibit graft-elicited T cells while preserving some of the pathogen-elicited T cells, thus preserving protective immunity in transplant recipients.

DISCUSSION:

The fundamental differences between pathogen-elicited and graft-elicited $CD8^+$ T cells suggest that there may be a "therapeutic window" wherein protective immunity in the patient can be maintained while graft-rejection could be prevented. Efalizumab is a possible transplant immunosuppressant as it binds to the CD11a subunit of LFA-1 and prevents T cell activation and trafficking. Here we have shown unlike belatacept (a CD28 antagonist that will likely soon be FDA-approved for use in clinical transplantation), anti-LFA-1 was effective in inhibiting the cytokine responses of human alloreactive memory CD8+ T cells. Importantly, pilot clinical trials of efalizumab in transplantation were highly encouraging. For example, efalizumab successfully prolonged the survival of islet transplants in 4 patients, all of whom were insulin-free for the duration of the trial and all of whom experienced minimal side effects compared to a control group treated with conventional immunosuppressants (27). Unfortunately, 3 out of 46,000 patients who were being treated for psoriasis developed PML and the transplant patients had to be taken off of efalizumab (24). This early clinical experience highlights the critical need to describe the impact of LFA-1 blockade on graft-elicited versus pathogen-elicited T cell responses.

Our hypothesis was that graft and pathogen-elicited T cells are differentially susceptible to anti-LFA-1 blockade. In order to clarify the unique differences between these T cell populations, we employed an OT-I based experimental system, in which the epitope recognized by the T cells on both the mOVA skin grafts and the LM-OVA pathogen infection were identical, thereby enabling us to focus exclusively on the impact of antigen context on LFA-1 susceptibility. T cells that were primed in the presence of a graft were inhibited in the presence of anti-LFA-1 whereas T cells primed in the presence of a bacterial pathogen were not inhibited.

We thus found that anti-LFA-1 has differential effects on T cells based on the priming conditions of the T cells. To determine the mechanisms that underpin this differential susceptibility, we first focused on differences in LFA-1 surface expression between these different subsets of primed OT-I T cells. We found that LFA-1 is expressed more on

pathogen-primed T cells as compared to graft-primed T cells. Considering this differential LFA-1 surface expression, we hypothesized that efalizumab may not have succeeded in completely blocking all the LFA-1 on the pathogen-elicited T cells, resulting in incomplete inhibition of the pathogen-elicited T cell responses. However, further experiments demonstrated that the mean fluorescent intensity (MFI) of LFA-1 in the animals treated with anti-LFA-1 was equivalent regardless of whether the animal had received an mOVA skin graft or an LM-OVA infection, thereby suggesting that LFA-1 on both the graft and pathogen-elicited T cells had been equivalently blocked. One possibility to explain the differences that we have observed between the LM-OVA elicited T cells and the graft-elicited T cells is the difference in the strength of the ligand-T cell receptor binding (31). However, this is not the case in these experiments as the mOVA skin graft and LM-OVA pathogen both present the same epitope (SIINFEKL) to a monoclonal population of T cells (OT-I) thereby removing this variability. We speculate that antigen persistence may also play a role. In the context of the *Listeria monocytogenes* infection, the antigen persists for approximately 5 days while in the setting of an allograft, antigen is continuously being processed and presented until full rejection (32). Also, while the LM-OVA is a systemic infection, the SG-OVA is a localized inflammation. Lastly, pathogens like LM-OVA trigger Toll like Receptors (TLRs) while the skin graft is not known to trigger any TLRs. *Listeria monocytogenes* is an intracellular gram-positive bacterium that is known to trigger to TLR 2 (33) and TLR 4 (34). Since this bacterium is an intracellular bacterium it also triggers TLR 9 in the endosome (35). The activated TLRs deliver signals to adaptor molecules like MyD88, TRIF and TRAM which act as important messengers to activate downstream kinases (IKK complex) and transcription factors like NFκB and AP-1 which produce effector molecules including cytokines, chemokines and inflammatory enzymes (36). In this way, the cytokine milieu generated by the pathogen may be considerably different than the allograft and this influences the priming conditions of the T cell. Thus, there are fundamental differences in the priming of $CD8^+$ T cells that warrant further studying, in order to understand the effects of different drugs on T cells.

FIGURES

Fig. 1. Anti-LFA-1 unlike belatacept inhibits IFNγ release in human alloreactive memory CD8+ T cells

Human PBMCs were obtained from responders and stimulators. Irradiated stimulators were plated with responders and either left untreated or treated with belatacept and/or anti-LFA-1 (TS-1). Intracellular cytokine staining was performed and the production of IFNγ and TNF was assessed in the CD45RA negative $CD8^+$ T cell memory subset.

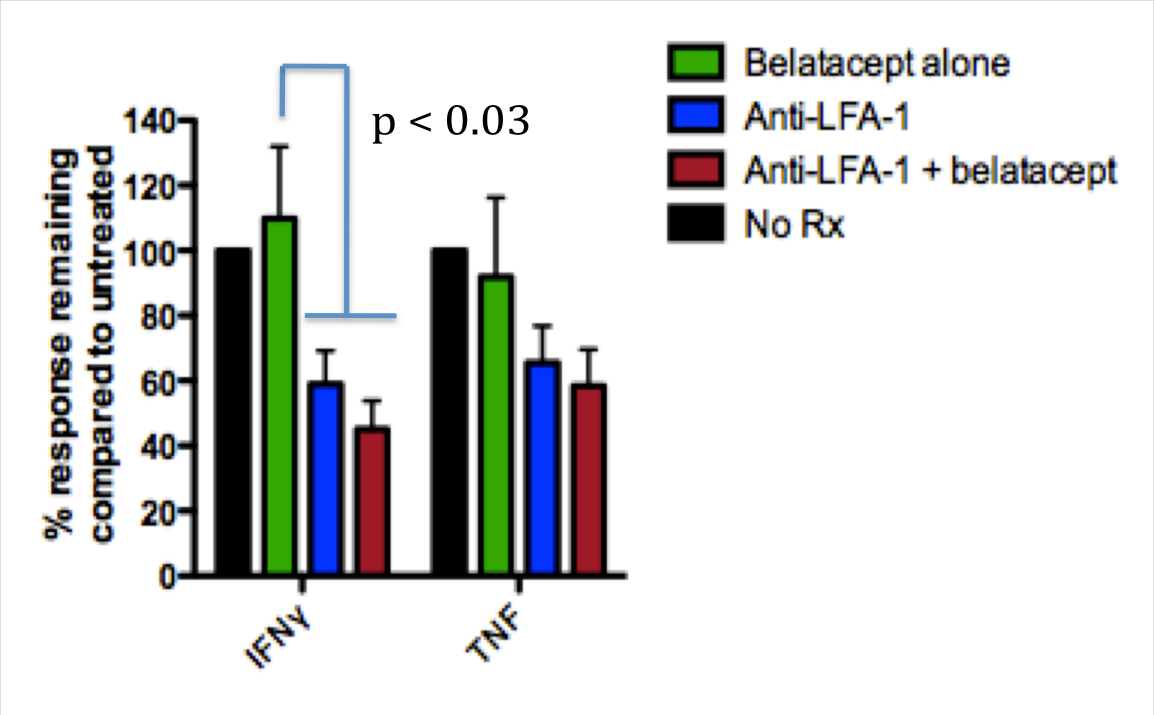


Fig. 2. Anti-LFA-1 inhibited graft-elicited CD8$^+$ T cells

Two groups of naïve B6 mice were adoptively transferred with OT-I T cells. One group was grafted with mOVA skin grafts and the other group was injected with LM-OVA. Both groups of mice were treated with anti-LFA-1. On day 10, splenocytes were obtained and the frequency of OT-I T cells was determined. Anti-LFA-1 inhibited the mOVA graft-elicited CD8$^+$ T cells.

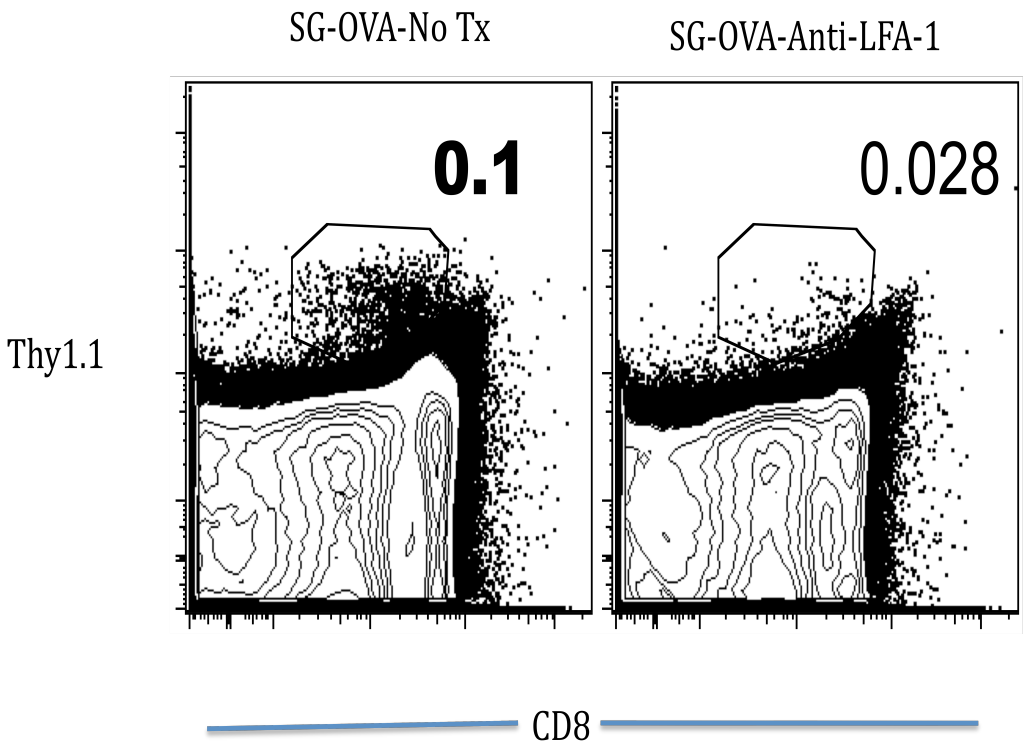


Fig. 3. Anti-LFA-1 reduced the absolute number of graft-elicited CD8$^+$ T cells
Two groups of naïve B6 mice were adoptively transferred with OT-I T cells. One group was grafted with mOVA skin grafts and the other group was injected with LM-OVA. Both groups of mice were treated with anti-LFA-1. On day 10, splenocytes were obtained and the absolute numbers of OT-I T cells were determined. Anti-LFA-1 inhibited the absolute number of graft-elicited CD8$^+$ T cells.

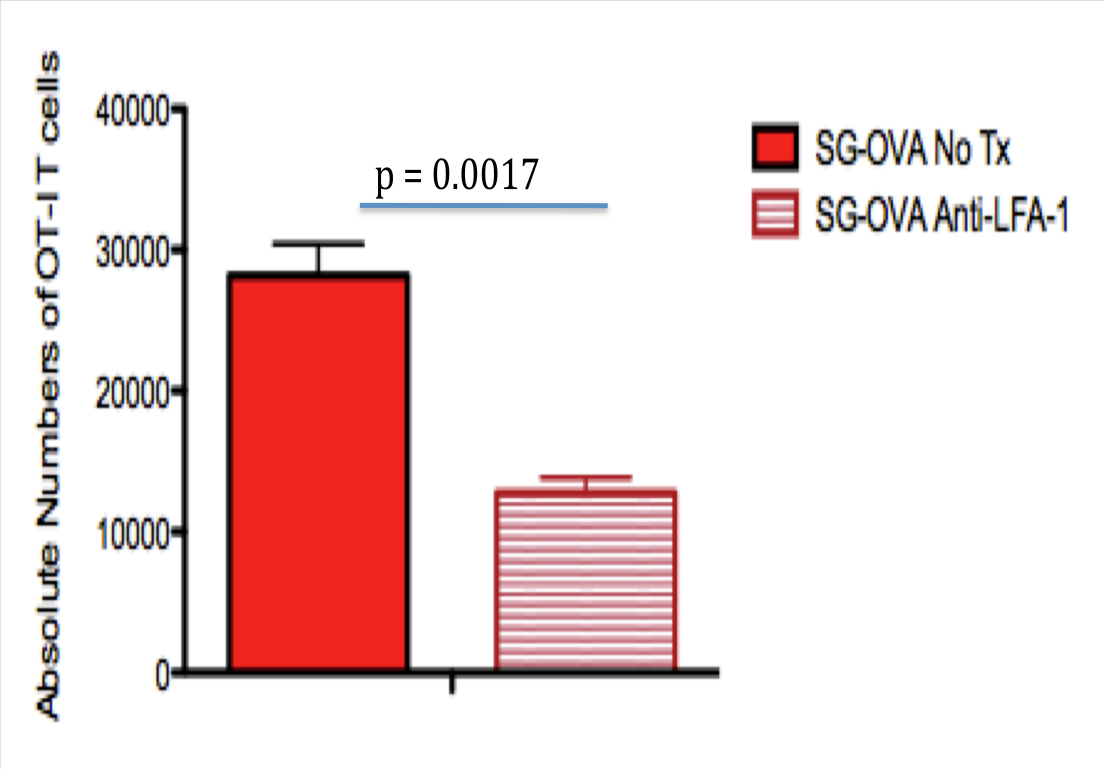


Fig. 4. Anti-LFA-1 did not inhibit LM-OVA elicited CD8$^+$ T cells

Two groups of naïve B6 mice were adoptively transferred with OT-I T cells. One group was grafted with mOVA skin grafts and the other group was injected with LM-OVA. Both groups of mice were treated with anti-LFA-1. On day 10, splenocytes were obtained and the frequency of OT-I T cells was determined. Anti-LFA-1 did not inhibit the frequency of LM-OVA elicited CD8$^+$ T cells.

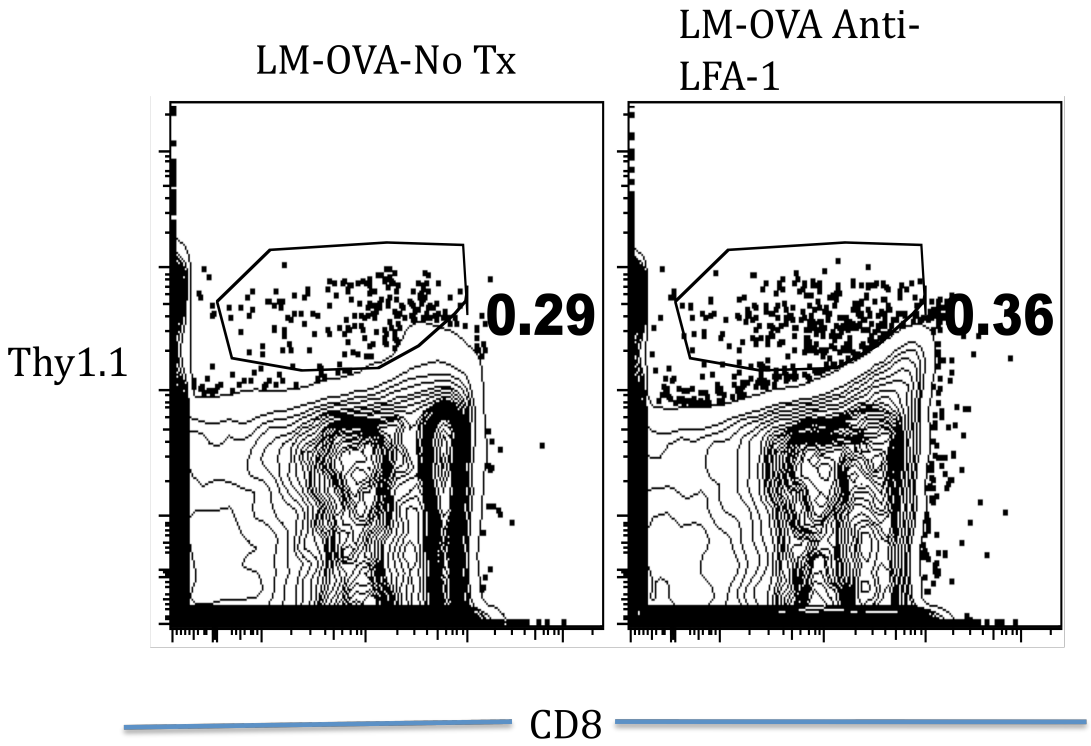


Fig. 5. Anti-LFA-1 did not reduce the absolute number of LM-OVA elicited $CD8^+$ T cells

Two groups of naïve B6 mice were adoptively transferred with OT-I T cells. One group was grafted with mOVA skin grafts and the other group was injected with LM-OVA. Both groups of mice were treated with anti-LFA-1. On day 10, splenocytes were obtained and the absolute number of OT-I T cells was determined.

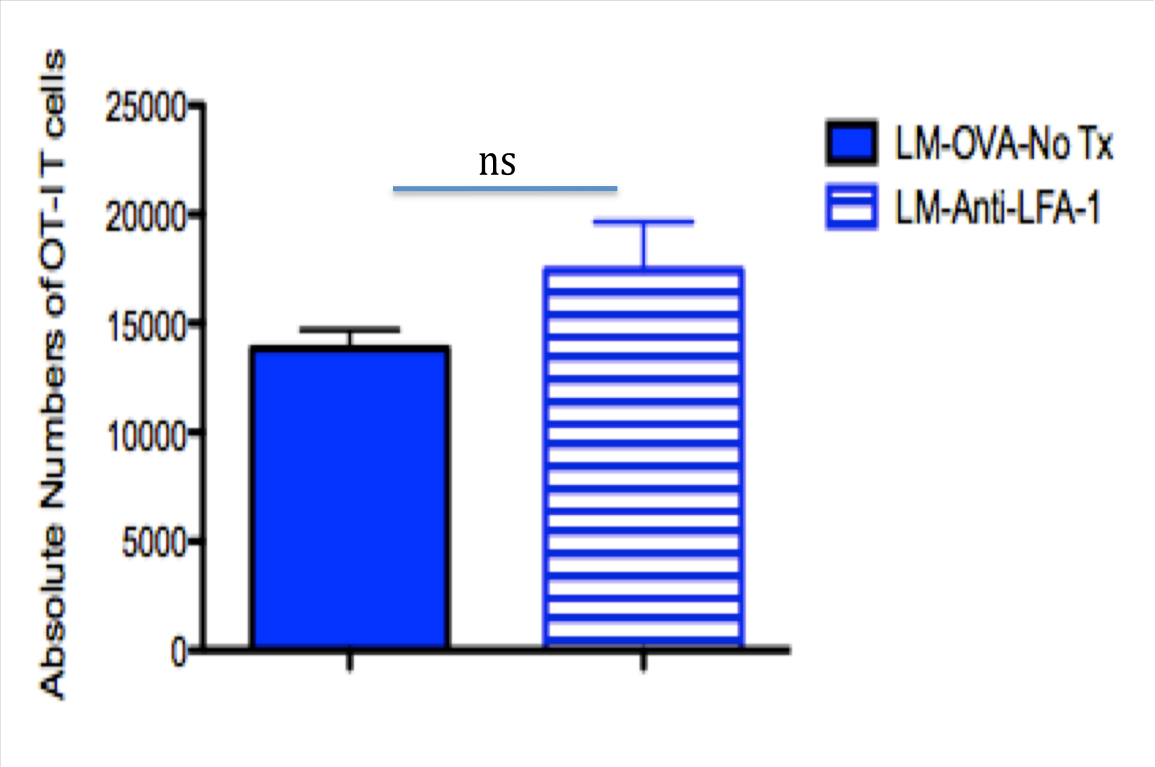


Fig. 6. LM-OVA elicited CD8+ T cells had higher LFA-1 expression than graft-elicited CD8$^+$ T cells

Two groups of naïve B6 mice were adoptively transferred with OT-I T cells. One group was grafted with mOVA skin grafts and the other group was injected with LM-OVA. On day 10, splenocytes were obtained and the expression of LFA-1 was determined on OT-I T cells.

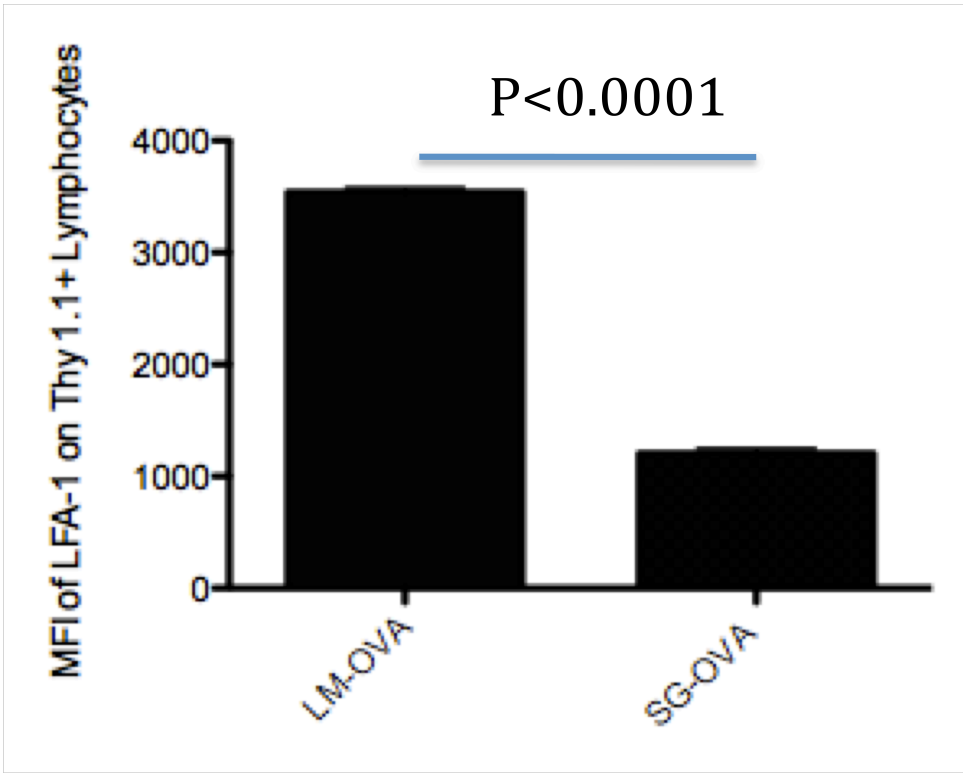


Fig. 7. LM-OVA elicited CD8+ T cells were mostly double-producers of IFNγ and TNF whereas SG-OVA elicited CD8+ T cells were both single and double-producers
Two groups of naïve B6 mice were adoptively transferred with OT-I T cells. One group was grafted with mOVA skin grafts and the other group was injected with LM-OVA. On day 10, splenocytes were obtained and production of IFNγ and TNF was assessed via ICCS.

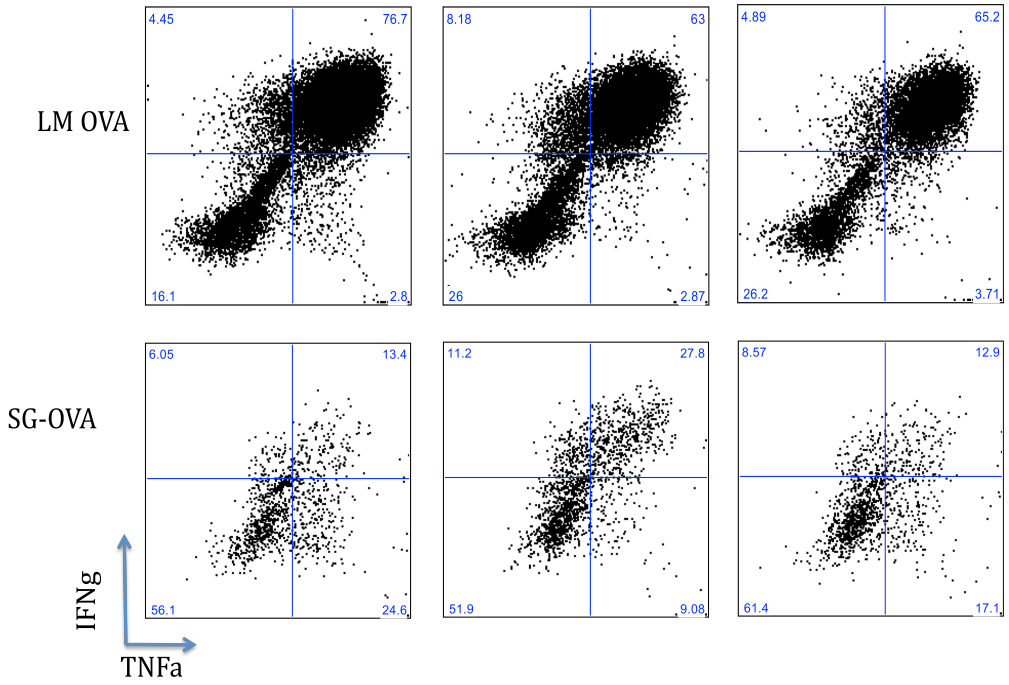


Fig. 8. LFA-1 was blocked in both LM-OVA elicited and SG-OVA elicited $CD8^+$ T cells in the LFA-1 blockade groups compared to the untreated groups

Four groups of naïve B6 mice were adoptively transferred with OT-I T cells. Two groups were grafted with mOVA skin grafts and the other two groups were injected with LM-OVA. Among the two skin graft groups, one group was left untreated and the other was treated with anti-LFA-1. Similarly, between the two LM-OVA infected groups, one group was left untreated and the other was treated with anti-LFA-1. On day 10, splenocytes were obtained and the expression of LFA-1 was determined.

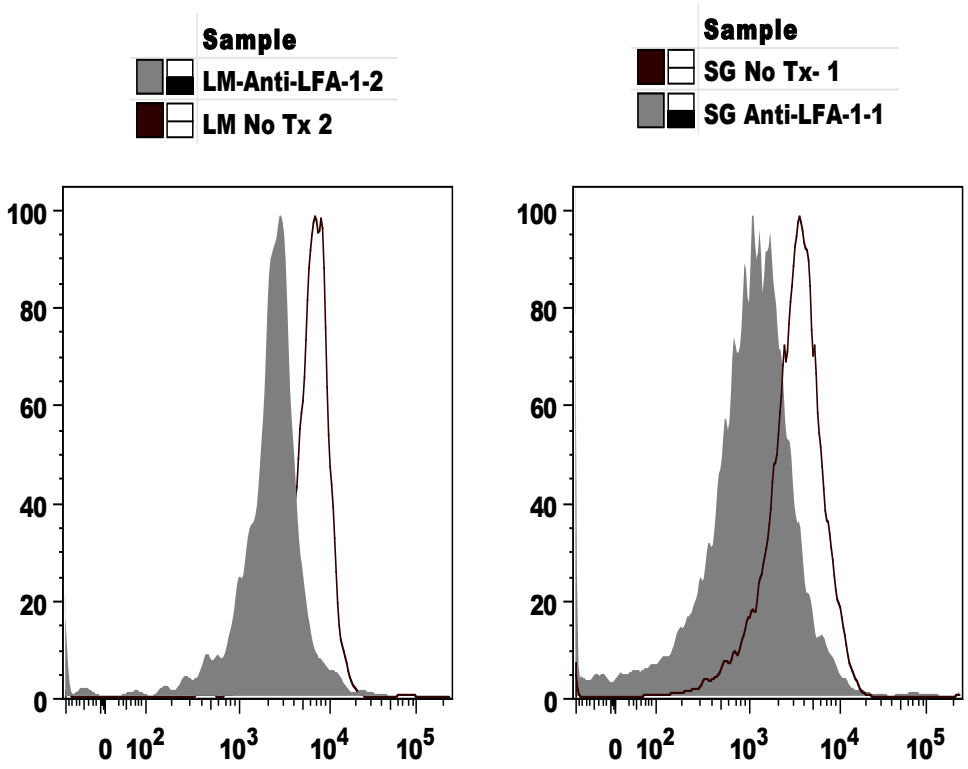
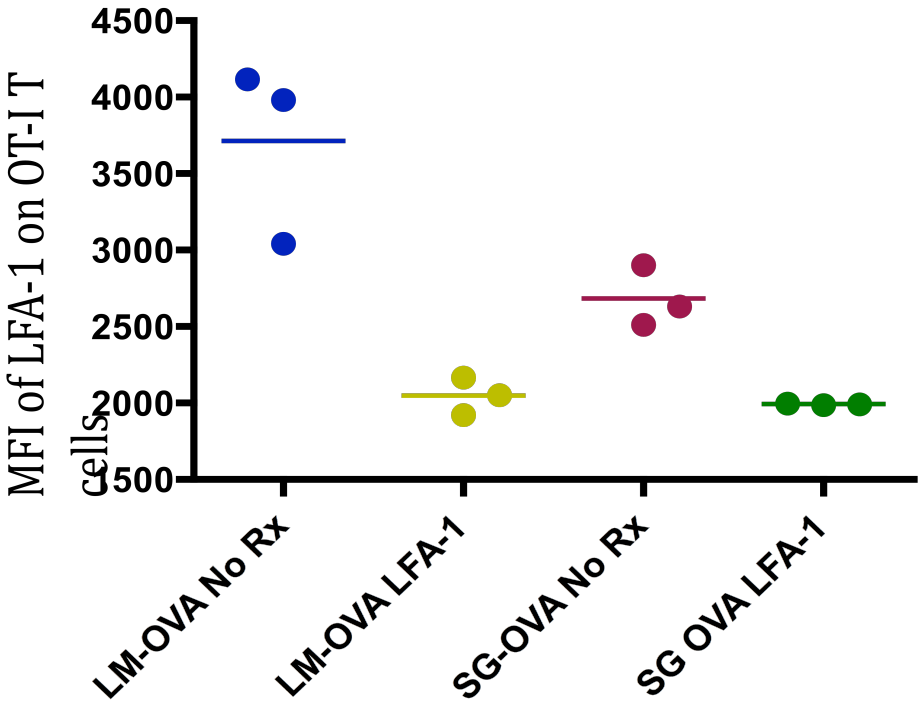


Fig. 9. The MFI of LFA-1 was equivalent in the LM-OVA and SG-OVA elicited OT-I T cells that have been blocked with anti-LFA-1

Four groups of naïve B6 mice were adoptively transferred with OT-I T cells. Two groups were grafted with mOVA skin grafts and the other two groups were injected with LM-OVA. Among the two skin graft groups, one group was left untreated and the other was treated with anti-LFA-1. Similarly, between the two LM-OVA infected groups, one group was left untreated and the other was treated with anti-LFA-1. On day 10, splenocytes were obtained and the MFI of LFA-1 was determined among all four groups.



References

1. Carrel A. 1908. Transplantation in Mass of the Kidneys. *J Exp Med* 10: 98-140
2. Billingham RE, Brent L, Medawar PB. 1953. Actively acquired tolerance of foreign cells. *Nature* 172: 603-6
3. Murray JE. 2005. The first successful organ transplants in man. *J Am Coll Surg* 200: 5-9
4. AJ M. 1994. Chronic Rejection--definition and correlates. *Clinical Transplantation* 8: 162-7
5. Halloran PF. 2004. Immunosuppressive drugs for kidney transplantation. *N Engl J Med* 351: 2715-29
6. Starzl TE, Marchioro TL, Waddell WR. 1963. The Reversal of Rejection in Human Renal Homografts with Subsequent Development of Homograft Tolerance. *Surg Gynecol Obstet* 117: 385-95
7. Tribe H. 1998. The discovery and development of cyclosporine. *Mycologist* 12
8. Friedman J, Barnes L, Sheahan M, Tsai H, Goldstein G. 1987. Orthoclone OKT3 treatment of acute renal allograft rejection. *Transplant Proc* 19: 46
9. Clase CM, Mahalati K, Kiberd BA, Lawen JG, West KA, Fraser AD, Belitsky P. 2002. Adequate early cyclosporin exposure is critical to prevent renal allograft rejection: patients monitored by absorption profiling. *Am J Transplant* 2: 789-95
10. Oz HS, Hughes WT. 1997. Novel anti-Pneumocystis carinii effects of the immunosuppressant mycophenolate mofetil in contrast to provocative effects of tacrolimus, sirolimus, and dexamethasone. *J Infect Dis* 175: 901-4
11. Elion GB. 1993. The George Hitchings and Gertrude Elion Lecture. The pharmacology of azathioprine. *Ann N Y Acad Sci* 685: 400-7
12. Becker YT, Becker BN, Pirsch JD, Sollinger HW. 2004. Rituximab as treatment for refractory kidney transplant rejection. *Am J Transplant* 4: 996-1001
13. 1995. Placebo-controlled study of mycophenolate mofetil combined with cyclosporin and corticosteroids for prevention of acute rejection. European Mycophenolate Mofetil Cooperative Study Group. *Lancet* 345: 1321-5
14. Gonwa T, Mendez R, Yang HC, Weinstein S, Jensik S, Steinberg S. 2003. Randomized trial of tacrolimus in combination with sirolimus or mycophenolate mofetil in kidney transplantation: results at 6 months. *Transplantation* 75: 1213-20
15. Chappert P, Schwartz RH. 2010. Induction of T cell anergy: integration of environmental cues and infectious tolerance. *Curr Opin Immunol* 22: 552-9
16. Curtsinger JM, Mescher MF. 2010. Inflammatory cytokines as a third signal for T cell activation. *Curr Opin Immunol* 22: 333-40
17. Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blancho G, Lang P, Grinyo J, Halloran PF, Solez K, Hagerty D, Levy E, Zhou W, Natarajan K, Charpentier B. 2005. Costimulation blockade with belatacept in renal transplantation. *N Engl J Med* 353: 770-81
18. Vincenti F, Blancho G, Durrbach A, Friend P, Grinyo J, Halloran PF, Klempnauer J, Lang P, Larsen CP, Muhlbacher F, Nashan B, Soulillou JP,

Vanrenterghem Y, Wekerle T, Agarwal M, Gujrathi S, Shen J, Shi R, Townsend R, Charpentier B. 2010. Five-year safety and efficacy of belatacept in renal transplantation. *J Am Soc Nephrol* 21: 1587-96

19. De Paoli P BS, Santini GF. 1988. Age-related changes in human lymphocyte subsets: Progressive reduction of the CD4 CD45R (suppressor inducer) population. *Clinical Immunology and Immunopathology* 48: 290-96
20. Kitchens WH, Larsen CP, Ford ML. 2011. Integrin antagonists for transplant immunosuppression: panacea or peril? *Immunotherapy* 3: 305-7
21. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. 1999. The immunological synapse: a molecular machine controlling T cell activation. *Science* 285: 221-7
22. Setoguchi K, Schenk AD, Ishii D, Hattori Y, Baldwin WM, 3rd, Tanabe K, Fairchild RL. 2011. LFA-1 Antagonism Inhibits Early Infiltration of Endogenous Memory CD8 T Cells into Cardiac Allografts and Donor-Reactive T Cell Priming. *Am J Transplant* 11: 923-35
23. Gottlieb AB, Krueger JG, Wittkowski K, Dedrick R, Walicke PA, Garovoy M. 2002. Psoriasis as a model for T-cell-mediated disease: immunobiologic and clinical effects of treatment with multiple doses of efalizumab, an anti-CD11a antibody. *Arch Dermatol* 138: 591-600
24. Gluck T. 2011. [Rare viral infections during immunosuppressive therapy : A "normal" side effect?]. *Z Rheumatol*
25. White MK, Khalili K. 2011. Pathogenesis of progressive multifocal leukoencephalopathy--revisited. *J Infect Dis* 203: 578-86
26. Talamonti M, Spallone G, Di Stefani A, Costanzo A, Chimenti S. 2011. Efalizumab. *Expert Opin Drug Saf* 10: 239-51
27. Turgeon NA, Avila JG, Cano JA, Hutchinson JJ, Badell IR, Page AJ, Adams AB, Sears MH, Bowen PH, Kirk AD, Pearson TC, Larsen CP. 2010. Experience with a Novel Efalizumab-Based Immunosuppressive Regimen to Facilitate Single Donor Islet Cell Transplantation. *Am J Transplant*
28. Posselt AM, Bellin MD, Tavakol M, Szot GL, Frassetto LA, Masharani U, Kerlan RK, Fong L, Vincenti FG, Hering BJ, Bluestone JA, Stock PG. 2010. Islet transplantation in type 1 diabetics using an immunosuppressive protocol based on the anti-LFA-1 antibody efalizumab. *Am J Transplant* 10: 1870-80
29. Ehst BD, Ingulli E, Jenkins MK. 2003. Development of a novel transgenic mouse for the study of interactions between CD4 and CD8 T cells during graft rejection. *Am J Transplant* 3: 1355-62
30. Pope C, Kim SK, Marzo A, Masopust D, Williams K, Jiang J, Shen H, Lefrancois L. 2001. Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection. *J Immunol* 166: 3402-9
31. Hogg N, Patzak I, Willenbrock F. 2011. The insider's guide to leukocyte integrin signalling and function. *Nat Rev Immunol* 11: 416-26
32. Ferrer IR, Wagener ME, Robertson JM, Turner AP, Araki K, Ahmed R, Kirk AD, Larsen CP, Ford ML. 2010. Cutting edge: Rapamycin augments pathogen-specific but not graft-reactive CD8+ T cell responses. *J Immunol* 185: 2004-8

33. Torres D, Barrier M, Bihl F, Quesniaux VJ, Maillet I, Akira S, Ryffel B, Erard F. 2004. Toll-like receptor 2 is required for optimal control of Listeria monocytogenes infection. *Infect Immun* 72: 2131-9
34. Cluff CW, Baldridge JR, Stover AG, Evans JT, Johnson DA, Lacy MJ, Clawson VG, Yorgensen VM, Johnson CL, Livesay MT, Hershberg RM, Persing DH. 2005. Synthetic toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. *Infect Immun* 73: 3044-52
35. Perry AK, Chen G, Zheng D, Tang H, Cheng G. 2005. The host type I interferon response to viral and bacterial infections. *Cell Res* 15: 407-22
36. Jeong E, Lee JY. 2011. Intrinsic and extrinsic regulation of innate immune receptors. *Yonsei Med J* 52: 379-92