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Yoav Karpenshif 4/14/2011

Tracking Alloreactive B-cell Responses

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Abstract

Tracking the Alloreactive B-cell Responses
By Yoav Karpenshif

Modern medicine has become increasingly aware of the detrimental effects that the B-cell mediated humoral response has on allografts. Unfortunately, the lack of knowledge about the signals and mechanisms that govern this response has impeded the development of an effective clinical treatment to counter it. This research presents a proof of concept of a murine transplant model system that is well suited to observe the B-cell mediated humoral response to donor tissue. The system utilizes a combination of advanced flow cytometric techniques, ELISA, and ELISPOT assays to phenotype donor specific B-cells, track the kinetics of the response, and quantify as wells as qualify donor specific antibodies. The use of the model system has made it possible to map the kinetics of the humoral response to donor antigen and demonstrate that a difference in duration of antigen exposure the long-term humoral alloreactivity of an organism. Furthermore, the model system has allowed for the phenotyping of the donor specific memory B-cells and has allowed for the identification of an IgM expressing subset of memory B-cell that has yet to be identified in a murine system. Lastly, the system was applied to T-cell receptor knock out transgenic mice to demonstrate that a long-term humoral response to allograft occurs in the absence of T-cell help.

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Introduction

Organ transplantation represents a triumph for the field of medicine. Over the last sixty years, the field has matured from being considered an unattainable dream to being a reality with an impressive infrastructure to support it. Immunosuppression, tissue typing, and advancements in surgery have all significantly contributed to the success of the field. However, in the past two decades, the field has struggled to extend the lifespan of allografts [1, 2]. This has lead to a reassessment of the factors that contribute to organ health and damage.

Though the suppression of the T-cell mediated cellular immune response has made organ transplantation a reality, recent evidence has shown that medicine's ability to promote long-term graft survival has plateaued. Meier-Kriesche *et al* challenged the accepted notions that the best predictor for long-term graft survival was a reduction in early acute rejection rates and that the minimization of such rejection would lead to better long-term tolerance of grafts. By analyzing the long-term data of the patients who underwent their first renal transplant between 1995-2000, they were able to show that the reduction of acute rejection during that time period did not lead to longer graft survival[1]. In a separate paper, Meier-Kriesche *et al* used a similar analysis for patients from 1988-1995 and showed that the actual half-life of grafts was significantly shorter than the clinically accepted projection. The authors used these data to argue that it was time to reconsider the therapeutic strategies being used in the field of transplantation[2]. These papers imply that though

medicine has the ability to reduce the severity and occurrence of acute rejection by pharmaceutically suppressing the T-cell mediated response, other factors detrimentally affect the long-term prognosis of transplanted organs.

Recent studies have increasingly shown that the B-cell mediated alloantibody humoral response is a major factor for organ damage and loss. The response is characterized by antibody production that is specific for donor major histocompatability complex (MHC; also Human Leukocyte Antigen or HLA in human subjects), ABO blood groups, or other donor specific antigen expressed on allograft tissue. Patients can present with this type of humoral response even if their T-cell driven cellular response is well controlled[3, 4]. However, the mere presence of donor specific antibody (DSA) does not necessarily predict a detrimental humoral response to the graft. In fact, in the phenomenon known as accommodation, DSA is well tolerated in the graft and may actually be beneficial for graft survival[5]. Still, the mechanisms that lead to accommodation are not well understood.

There are three clinically relevant categories of B-cell mediated humoral responses to allografts that detrimentally affect patient prognosis. Hyper-acute rejection (HAR) occurs in patients that are pre-sensitized, i.e. have pre-formed antibodies to donor specific antigen. In HAR, the immune response reacts vigorously to donor MHC class 1 or ABO blood group antigens and leads to graft loss within hours of transplantation. Pre-transplantation screening for DSA has largely reduced the occurrence of HAR [6]. Antibody mediated rejection (AMR) results in the high production of de novo DSA, residual but undetected levels of

pre-transplant DSA, which leads to a rapid loss of function and a high risk of graft loss. AMR is highest in patients who are likely to be pre-sensitized, like those who have received previous transplants, have had blood transfusions, or have previously been pregnant [3, 7]. Lastly, chronic levels of DSA, which are present in patients' blood serum but do not result in acute rejection, have been shown to be associated with an increased risk of allograft loss[7].

Though the increasingly sensitive techniques used to determine whether a transplant patient is pre-sensitized to donor antigen have greatly reduced the incidences of HAR and AMR, studies have shown that a de novo antibody response is also linked to acute rejection episodes. Crespo *et al.* studied 81 patients who presented with episodes of acute rejection within 3 months of kidney transplantation. The patients who responded to steroid immunosuppressant treatments did not produce any DSA, implying that their rejection was caused by a T-cell mediated cellular response. However, 37% of patients with steroid-insensitive acute rejection had DSA. Of those patients, 95% had widespread peritubular C4d staining in their allograft biopsies, which stains for a complement split product and serves as indirect evidence of an antibody mediated humoral response [3, 8]. Similarly, Piazza *et al.* screened 120 unsensitized kidney transplant recipients for DSA at 1 yr post-transplant by flow cytometry cross-match and FlowPRA (panel reactive antibody) bead assays. 24.2% of the patients developed DSA, with most of them being detected within the first 3 months after transplantation. Patients with DSA had a higher incidence of acute rejection episodes (62% *versus* 13%), more allograft failure

(34% *versus* 1%), and higher creatinine levels, a marker that is inversely proportional with kidney function, at 2 years after transplantation compared with patients without DSA [9]. Chronic DSA, a de novo antibody response, has also been associated with graft damage and loss [10]. It is therefore clear that controlling the humoral immune response to transplantation can play a crucial role in prolonging graft survival.

The recent plateau of graft survival rates [1, 2] can therefore be at least partially described by medicine's failure to control the DSA responses. Worthington *et al* compared a group of patients who had lost their grafts to a group of patients with graft survival. They found that the majority of patients who lost their grafts had an alloantibody response, while only a small number (1.6%) of patients with graft survival developed DSA. The authors were able to conclude that the presence of anti-HLA DSA was a strong predictor for graft loss[11]. Therefore, it is clear that controlling the humoral immune response to grafts is a key to improving long-term graft survival.

Unfortunately, the mechanisms that govern the B-cell mediated humoral response are not well understood. It has been shown that resting naïve B-cells are primarily found in secondary lymphoid tissue and are stimulated when their B-cell receptors (BCR) encounter and bind to an antigen. In the case of AMR, the antigen is usually class I or II of MHC or the ABO blood group antigens. Allo-specific activated B-cells become the seed of a germinal center (GC), a structure that allows the quick proliferation of allo-specific B-cells. In the GC, the proliferating B-cells are aided by helper T-cells and are surrounded

by a ring of B-cells. The activated B-cells inside the GC can then proceed through two different developmental pathways. The extrafollicular activated B-cells leave the GC and proliferate into short-lived plasma cells that maintain the initial humoral response. The germinal center activated B-cells differentiate into memory B-cells and high affinity long-lived plasma cells that maintain long term humoral immunity[12]. However, many of the specifics of the signal pathways and cell interactions of these responses, which could be potential targets for therapy, have yet to be determined.

Study into the nature of the B-cell response, however, has revealed unexpected results. The finding that B-cell proliferation requires "help" from T-cells was a great step forward in terms of understanding the mechanisms that produce acquired humoral immunity [13, 14]. Recent studies have shown that a subset of CD4 positive T-cells, named follicular B helper T-cells or T follicular helper cells (T_{fh}), are the subset that are functionally responsible for aiding in the formation of the humoral antibody response. The marker that serves as the identifying feature of this subset is the chemokine receptor CXCR5, which is one of the factors that promote the localization of the T-cells into the follicles of the GCs [15-18]. Once in the GC, these cells provide directed help to naïve antigen specific B-cells by aiding in such processes as somatic hypermutation, class switch recombination, and selection of high-affinity B cells. Recent experiments have shown that these T-cells are required for the formation of the GC, and that they provide the developmental signaling required for the formation of memory

B-cells and the high affinity plasma cells, which are responsible for the maintaining of a sustained and long-term humoral immunity [19, 20].

The ultimate purpose of our research was to be able to track and type the antigen specific B-cell populations throughout a rejection episode. We hoped to gain insight into the signals and pathways that govern the response. However, in order to conduct such experiments, we have had to develop a model system that allows for the detailed observation of donor specific B-cells and antibodies in a controlled environment. This research presents our murine transplant model system, which utilizes advanced flow cytometric techniques and specifically designed assays to gain a comprehensive and detailed perspective of the response. In developing this model system, we have been able to make meaningful observations about the kinetics of the immune response, the effect of antigen load on humoral memory, and the phenotypes of the populations of donor specific B-cells that govern the memory response. These results do not only serve as a proof of concept for our model system, but they also have expanded our knowledge about the antigen specific B-cell response to allografts.

Our model has the capability of tracking the B-cell response to a known antigen under physiologically and clinically relevant inflammatory conditions, while maintaining a very high-resolution view of the response. We therefore believe that our model can be adopted by immunological studies outside of transplantation to confirm, challenge, and expand on the immunological knowledge obtained from other systems. Furthermore, we believe that our

system can be modulated in a number of different ways in order to gain insight into the many facets of the humoral response. One such modification of the basic experiment is presented in this research, in which we tracked a long term B-cell mediated humoral response in transgenic mice without functioning T-cells. Since it tracks a clinically relevant inflammatory response, we also believe that this system would lend itself well to future studies that account for the effect of immunosuppression on the humoral response.

Methods

Mice

Grafting protocol: C57BL/6 and Balb/C mice were obtained from Jackson labs (Bar Harbour, ME). At day 0, skin and heart grafts were procured from C57BL/6 mice and surgically transplanted into BALB/C mice. Heart grafting was done by a microsurgeon. Mice were bled and sacrificed at days 15, 30, 60, 90, and 150 for ELISA, ELISPOT and flow cytometric analysis.

T-cell Receptor Knock Out Mice: T-cell Receptor Knock Out (TCRKO) from Jackson labs (Bar Harbour, ME) mice were used to track the T-cell independent B-cell response to allografts. A microsurgeon, performed heart transplants at day 0 of the experiment. Mice were sacrificed and bled on d>120.

H-2 Tetramer preparation

Highly concentrated biotinylated H-2 (murine MHC) solution was obtained from the Emory Tetramer Core Facility. The solution can consist of either H-2K^b or H-2K^d molecules. Conjugation of MHC and fluorochrome begins with 6.25μl of MHC solution aliquoted into eppendorf tubes. Subsequent additions of 1.08μl of concentrated APC-streptavidin (Invitrogen, San Diego, CA) (allogenic tetramer) or APC-CY7 (Invitrogen, San Diego, CA) (syngenic tetramer) is added to eppendorf tube ten times, with tubes being placed in dark for 10 minutes between each addition to allow for conjugation.

Flow Cytometry

Cell processing: Mice were sacrificed at specified time points. Spleens were extracted and crushed through 100μm filter (BD Falcon, Franklin Lakes, NJ) into 10ml of RPMI with 2% FBS (R2 buffer). Cells were then spun in a centrifuge for 5 minutes at 15000 rpm. The supernatants were then aspirated from the samples leaving just a cellular pellet. Cells were suspended in 5ml of High Yield Lysing Buffer (Invitrogen, San Diego, CA) in order to lyse red blood cells. After 5 minutes 5ml of R2 were added to the samples to suspend the lysing process. Cells were then centrifuged for 5 minutes at 15000 rpm. Supernatants were aspirated and cells were suspended in 10ml of R2 and counted using a cell counter. Cells were then centrifuged for 5 minutes at 15000 rpm.

Live Dead Staining: Processed and counted cells were resuspended in 1% BSA (by mass in PBS) so that the total concentration of cells was $40x10^6$ cells/ml. 200μl of cell solution were then aliquoted in clear FACs tubes. Cells were then washed once with 500μl of PBS at room temperature and then resuspended in 1ml PBS. 2μl of live dead reagent (Invitrogen, San Diego, CA) were mixed into each sample and left to incubate on ice in the dark for 30 minutes. Cells were then washed one in 500μl of pbs at room temperature and once in 500μl of 1%BSA.

Cell Surface Staining: Cells were resuspended into 300μl of 1% BSA and transferred into a 96 well U-bottom plate. 5μl of FC block were mixed into each sample. Cells were then surface stained for the appropriate cell surface markers

and incubated in the dark and on ice for 20 minutes. Cells were then washed twice with 200μl of FACS. Cells were then resuspended in 300μl of Cytofix/Cytoperm (Pharmingen San Diego, CA) and incubated on ice and in the dark for 30 minutes. Cells were washed 3 times with 300μl of 1x Wash/Perm (Diluted from stock 3X wash perm with PBS) and resuspended in 250μl of 1x Wash/Perm. Cells were then stained for APCCy7 by adding 10μl of 1:400 of a fluorescently tagged syngenic MHC tetramer. Cells were incubated on ice in the dark for 20 minutes. Cells were then stained for APC by adding 10μl of 1:400 solution of a fluorescently tagged allogenic MHC tetramer. Cells were then incubated on ice and in the dark for 40 minutes. Cell were then washed 3 times in 1X Wash/Perm and resuspended in 300μl of FACs. Flow cytometry was performed using a LSRII flow cytometer and data were analyzed using FlowJo software (Palo Alto, CA).

Enzyme Linked Immunosorbent Assay-ELISA

Plate preparation: Flat bottom (Thermo Scientific, Rockford, IL) plates were coated by diluting antigen in phosphate buffered saline (PBS) (1:1000 of 1x stock solution) and adding 50μl of diluted antigen solution to each well of the plate. The plates were covered with cellophane and left in 4°C over night. The different coating antigens included a monomer mix of MHC1 H-2D^{d} H-2K^{d} (D monomer mix) a monomer mix of MHC1 H-2D^{b}, H-2K^{b} (B monomer mix). In order to prevent non-specific binding of antibodies, plates were blocked 150μl of 3% BSA (3% by mass bovine serum albumin diluted in PBS) were added to

each well on top of the coating solution. Plates were covered with cellophane and were either returned to 4°C overnight or left at room temperature for two hours. After blocking, plates are washed using an Amersham Bioscience Biotrak II Plate Washer. Each cycle of washing consisted of completely aspirating the wells, adding washing solution, and aspirating the washing solution from the wells. Plates were first washed 6 times with a solution of PBS and the detergent tween (.05% by volume in PBS), and then 6 times with PBS. This same procedure was repeated in all subsequent wash steps.

Serum extraction and preparation: Mice were bled at specified time points following transplantation. Approximately .2ml of blood was collected from each mouse. Samples were then left at room temperature for 2-3 hours to partially congeal. The samples were then spun in an eppendorf micro-centrifuge at 15,000rpm for 10 minutes in order to separate serum from cell mass. Serum was then extracted from the samples and frozen in -20°C until ready to use.

Sample and secondary addition: Thawed serum samples were serially diluted in 1% BSA in V-bottom dilution plates (BD Falcon, Franklin Lakes, NJ). Sample concentration differed based on the antigen with which the plates were coated. 50μl of diluted samples were transferred into the washed plates using a multichannel pipette, covered with cellophane, and left at room temperature for 2 hours. Plates were then washed.

Secondary antibodies specific for different types of immunoglobulin and tagged with a developing reagent were diluted in 1% BSA to a concentration of 1:1000 compared to the stock solution. 50μl of secondary antibody solution was then

added to the wells. Plates were covered with cellophane and left for 1 hour at room temperature**.** Plates were then washed.

Plate Development: The development solution was prepared by adding 1 phosphotase tablet and 10µl of 1M $MgCl_2$ solution per 5ml of development buffer (.65ml 1M Na_2CO_3 and 1.85ml 1M $NaHCO_3$ diluted with deionized water into 50ml). 50µl of development solution were added to each well. Plates were then read at 5-minute intervals using a Spectramax 340PC plate reader (Molecular Devices Sunnyvale, CA) set to read at absorption of 405nm. Data was collected using SoftmaxPro software and analyzed in Microsoft Excel.

Enzyme Linked Immunosorbent Spot-ELISPOT

Multiscreen filter plates (Millipore Billerica, MA) were coated with 100µl of 1:1000 goat-anti-mouse IgG or IgM (Invitrogen, San Diego, CA) stock solution diluted in PBS. Plates were then left at 4°C overnight. Plates were washed 3 times with PBS+tween and 3 times with PBS. Every subsequent wash step was carried out in the same fashion. After washing, 200µl of Complete RPMI with 10% FBS (R10 buffer) nutrient solution were added to each well of the plate to block. Plates were then placed in an incubator set at 37°C for 2 hours. Splenocytes, harvested and processed as described in the flow cytometry protocol, were serially diluted in a dilution plate, so that each sample had a diltution of 2, 1, and .5 million cells in 150µl of R10. Samples were then aliquoted onto coated filter plates and incubated in 37°C for 6 hours. Plates were then washed, and coated with 1:1000 dilution of stock H-$2K^b$ or H-$2K^d$

monomer diluted in 1% BSA. Plates were then left at room temperature for 2hrs and then washed as above.

To develop the plates, 100μl of developing solution (20ml sodium acetate, 330μl AEC, and 100μl H_2O_2) were added to each well. Plates were left for 15 minutes at room temperature. Media was removed and plates were rinsed with running tap water.

Results

Novel methods to track donor specific humoral immunity:

In order to fully understand the effect on the B-cell mediated humoral response on transplanted organs, the mechanisms that stimulate and govern the response must be well understood. The key to generating such an understanding is to develop a model system that is able to track the development of the specific populations of cells that produce DSA, quantify them, and to correlate the activation of those cells with serum level of DSA. We have been able to develop a model system that differs from other models previously used in immunology in that it allows us to track the humoral antibody response in a physiologically relevant model.

Monitoring and Quantification of donor-specific B cell populations by Flow Cytometry: We have developed a new murine model systems that allows us to track endogenous donor specific B-cells in vivo. The system utilizes H-2 (the mouse designation of MHC) mismatched strains of mice, C57BL/6 (H-2k^b) and BALB/c (H-2k^d), as skin or heart donors and recipients. In order to track H-2 reactive B-cells, we use fluorescently tagged H-2k^b or H-2k^d tetramers. Tetramers are made by binding four identical H-2 molecules that are biotinylated to a streptavidin molecule that is tagged to a molecule of the fluorochrome allo-phycocyanin (APC). The use of a tetramer allows for more efficient binding of the BCR to the antigen.

For this system to be sensitive enough to track the small population of memory B-cells, we used an important control tetramer. This was done because the staining approach that we utilized is complicated by non-specific binding interaction due to the very low frequency of certain populations of antigen specific cells, such as the memory B-cell populations. In order to overcome this difficulty, we stained for syngenic H-2 tetramer, which is similarly conjugated, by using biotin-streptavidin interactions, to a tagged fluorochrome, APC-Cy7 instead of APC. This control is effective because it accounts for non-donor specific interactions, like anti-biotin or anti-streptavidin activity, and "sticky" anti-H2 reactivity (specific for non-polymorphic sections of the molecule), which may be present in the naïve and activated B-cell populations.

Figure 1 shows how this staining strategy is employed. In this assay, normal BALB/c (H-2k^d) recipient mice received cardiac allografts from fully mismatched C57BL/6 (H-2k^b) donors on Day 0 of the experiment. Anti- H-2k^b reactivity of the donor-specific B cells in allograft recipients were identified using APC- H-2k^b tetramers. On Day 15 of the experiment, mice were sacrificed; splenocytes were harvested, separated, stained, and analyzed using flow cytometry. Populations of lymphocytes that were negative for CD4 or CD8 (T-cell markers), F480 (a macrophage marker), and 7AAD (viability) were selected for by negative gating (1A-1B). Cells that are CD19 positive (B-cell marker) and negative for the control tetramer (non-specific binding interactions) were then gated for (1C). The resulting population was then analyzed by gating for cells

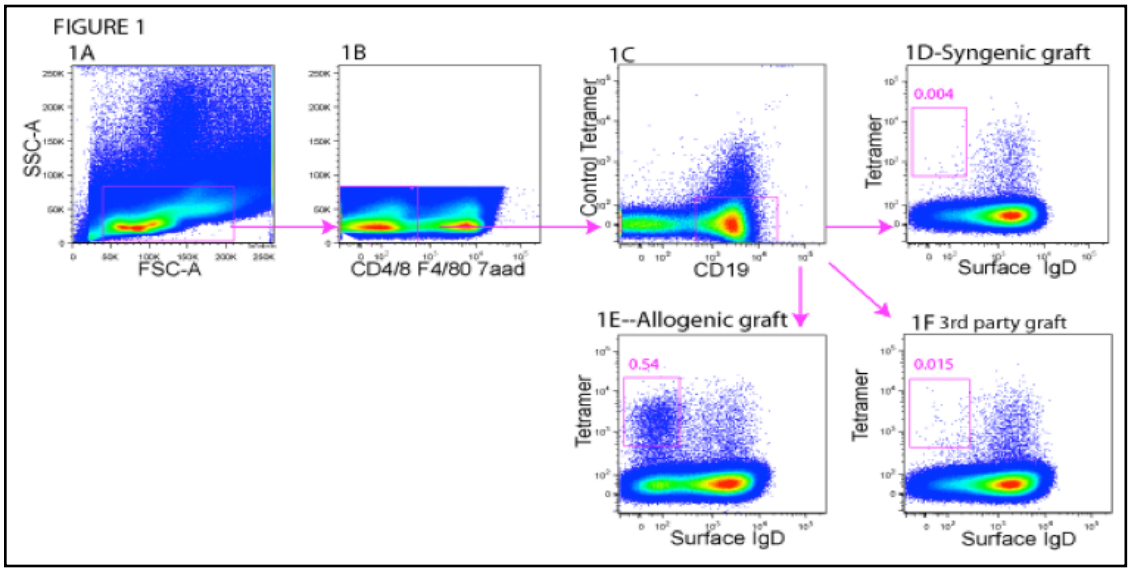

Figure 1: TRACKING DONOR-SPECIFIC B-CELLS Normal C57BL/6 (H-2K^{b}) donors and BALB/c (H-2K^{d}) recipient as fully mismatched cardiac donor and recipients. Anti-H2Kb reactivity of the donor specific B-cells in allograft recipients was assessed using fluorescently labeled H-2Kb tetramers. Splenocytes were isolated on day 15 and analyzed using the following gating strategy: 1A represents a generous lymphocyte gate based on size and granularity; 1B gated for cells that were negative for CD4,CD8, F480, and 7AAD; 1C gated for cells that were positive for CD19 and negatibe for H-2Kd tetramer; 1D, 1E, and 1F represent populations that are positive for allogenic tetramer and express low surgace IgD in syngenic, allogenic, and third party grafted samples respcetidly .

that are negative for surface IgD (previous antigen stimulation) and positive for allogenic tetramer (donor specific reactivity). Syngenic grafts, used as a negative control, develop a very small population of this cell type (1d). However, allogenic grafts show a marked upregulation of donor specific B-cells (1e). A third party allograft (H-2K^{k}) was used as a specificity control in order to show that the B-cells are produced in response to donor antigen, as opposed to being generically produced as part of the inflammation response to tissue grafts (1f).

Quantification of donor-specific memory B cell and plasma cells frequencies generated by in vitro stimulation: Studies have shown that vaccine-specific

memory B cells and plasma cells remain constant over multiple decades after vaccination, which suggests that monitoring these populations over time should be an effective gauge of the long-term B-cell humoral immunity [21]. Using an approach that was adopted from assays used to test pathogens specific responses, we have developed transplantation related donor-specific enzyme linked immunosorbent spot (ELISPOT) assays to measure donor-specific antibody-secreting cells in mice using MHC Class I tetramers. ELISPOT analysis allows us to quantify the number of donor-specific plasma cells by their functional capacity to produce antibody directly *ex vivo* by virtue of their ability to spontaneously secrete antibody. Donor-specific antibody secreting plasma cells appear during the contraction phase and persist for more than 150 days by residing specifically in the bone marrow compartment. An example of an ELISPOT output is shown in Figure 2.

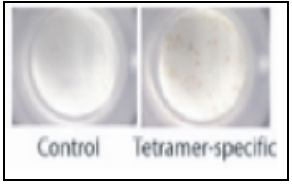

Figure 2: EXAMPLE OF ELISPOT OUTPUT Example of donor-specific ELISPOT assays to measure donor-specific antibody-secreting cells in mice using plate-bound MHC Class I tetramers on D50 of experiment.

Measurement of DSA by ELISA. As antibodies are the ultimate effectors of the humoral response, gauging DSA levels is important for understanding the progression of the immune response. In order to measure circulating serum levels of DSA, we use the enzyme linked immunosorbent assay (ELISA). This assay is commonly used to monitor the serum levels of CMV, EBV, influenza, tetanus toxoid, measles virus, and vaccinia virus-specific IgG antibodies in humans. Using a similar approach with plate-bound MHC Class I monomers in

an ELISA plate format, we have developed donor-specific ELISA assays to monitor donor-specific antibodies in mice. Our ELISAs compliment the experiments described above, to obtain a comprehensive view of donor-reactive B-cells (Figure 3).

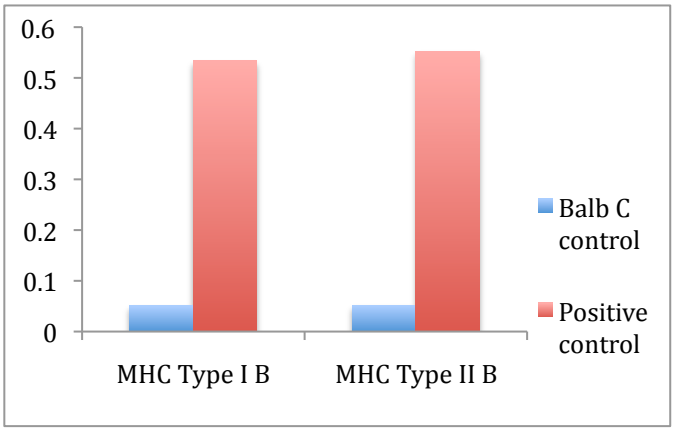

Figure 3: EXAMPLE OF ELISA DATA BALB/c mice were challenged with C57BL/6 mismatched skin allografts (red). The assay tested for the presence anti-MHC type I and anti MHC type II DSA levels. Y axis represent OC values for absorption at 405nm, which is directly proportional to concentation. Naïve BALB/c mice (blue) were used as a negative control.

Defining the Donor-specific Memory B-Cell Response

Kinetics of the donor-specific memory B-cell response: Our studies allowed us to establish a system of kinetics for donor specific memory B cell response in both skin and cardiac grafted mice. As shown in figure 4A, MHC tetramer binding in our flow cytometric analysis peaks at approximately day 15 post transplant. In skin grafted mice, tetramer binding cells rapidly decreased from day 15 to day 30, decreased at a slower rate from day 30 to day 90, and remained constant after day 90. Heart grafted mice showed a slowed rate of decrease in tetramer binding cells in the contraction phase (Figure 4A). This can partially be explained by the fact that heart tissue and its antigens remain in the system after rejection, while skin grafts usually fall off the mice. The resting levels

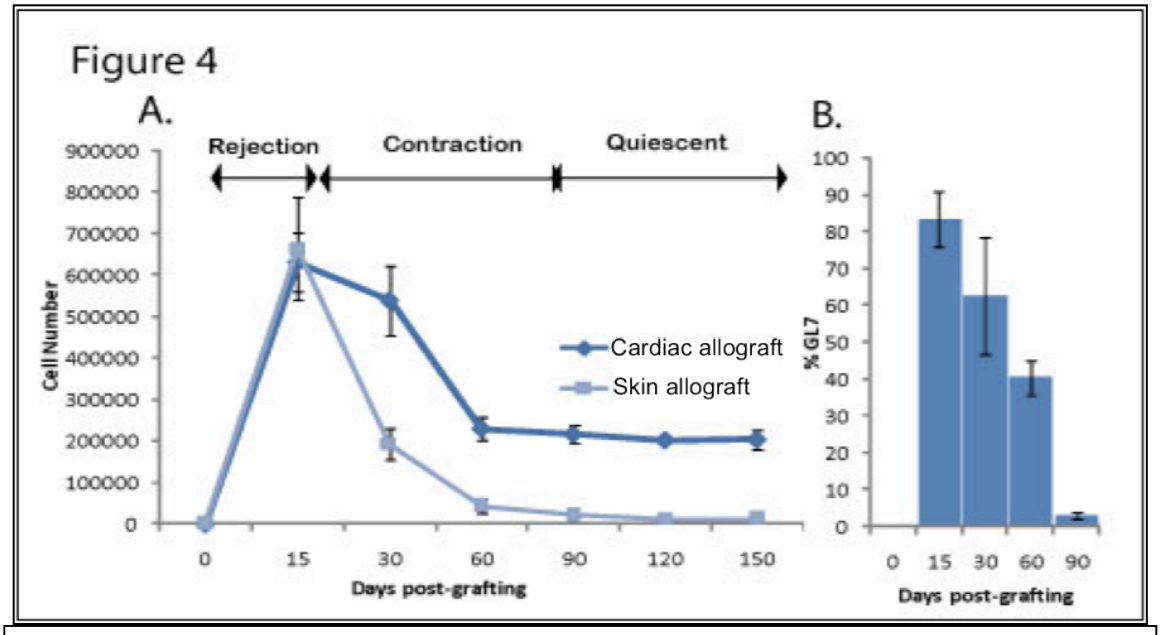

FIGURE 4: KINECTICS DONOR-SPECIFIC B CELLS: A. Normal C57BL/6 (H-2k^b) donors and BALB/c (H-2k^d) recipients mice as fully mismatched donors (Skin and heart) and recipients. Splenocytes were isolated and analyzed by flow cytometry on d15, d30, d60, d90, d120, and d150. N=6 for each timepoint. B. GL7 expression of donor-specific B-cells

of donor-reactive cells in cardiac-grafted mice were approximately 10 fold higher than in skin-grafted mice. We reason that the increased levels of tetramer binding cells are due to the increased levels of antigen associated with the heart graft and the longer duration of antigen exposure. In cardiac grafts the GL7, a germinal center activity marker, is expressed on tetramer specific B-cells until day 90 post graft (4B). Based on this evidence, we estimate that the antigen from a cardiac graft stays present until somewhere between day 80-90.

These results have helped us divide the donor specific development of B-cells into three distinct phases (Figure 4A): Rejection phase (days 0-15) when donor specific B-cells build up to a peak, Contraction Phase (days 15-90) when the levels decline, and the quiescent memory phase (from day 90 on) when the populations of donor specific memory B-cells remain constant. Experiments

that challenge murine systems with non-physiological antigens have been shown to yield about $1x10^3$ to $5x10^4$ memory B-cells per immunized spleen [22, 23]. In contrast, our experiments with cardiac grafts yielded about 3-4 times as many cells in the quiescent phase of memory B-cell development.

Identification of novel IgM and IgG donor-specific memory B-cells: Our goal for establishing this model system was to be able to define specific sub-populations of memory B-cell and determine their phenotype and physiological function during an immune response. The system described above produces an adequate quantity of donor specific memory B-cell and allows us to study them with sufficient specificity and resolution to determine their phenotype and function. Therefore, we were able to begin analyzing donor-specific memory B-cell development in response to a fully mismatched cardiac graft (C57BL/6 grafts to BALB/c mice) throughout the rejection, contraction, and quiescent phases. Our initial phenotype for which we gated for the analysis of the population of donor specific memory B-cells was $7AAD^-CD4^-CD8^-CD19^+tetramer^+IgD^{lo}B220^+$.

We initially analyzed the previously identified murine memory B-cell markers, CD38, CD95, CD80 and CD73, during the rejection (day 15) and quiescent (day 150) phases. Our intentions were to identify a stable memory marker. In order to do this, we compared the expression of donor-specific cells in the quiescent phase ($7AAD^-CD4^-CD8^-CD19^+tetramer^+IgD^{lo}B220^+GL7^-$) as compared to effector ($7AAD^-CD4^-CD8^-CD19^+tetramer^+IgD^{lo}B220^+GL7^+$) and naïve B-cells ($7AAD^-CD4^-CD8^-CD19^+IgD^+B220^+GL7^-$.) Our definition of naïve B-cells is based on the upregulation of IgD and the lack of expression of the

germinal center marker GL7. The only marker that shows differential expression from both naïve and effector subsets of B-cells was CD80 (figure 5).

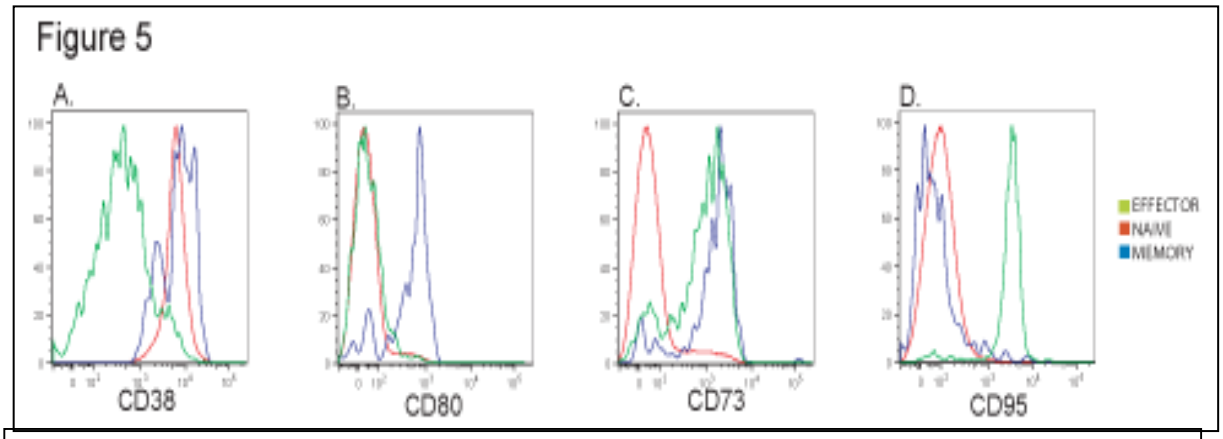

Figure 5: Murine memory B-cell markers : Expression patterns of memory phenotypes on donor-specific B-cell during the rejection phase (green) and the quiescent phase (blue) and naïve cells (red).

We next analyzed the surface immunoglobulin expression on cells that upregulate CD80 during the contraction and quiescent phases of the response. In order to stain for surface IgG/IgA, which indicate a class shift, we used a cocktail of anti-IgG1, IgG2a, IgG2b, IgG3 and IgA that were all conjugated to the same fluorochrome. Interestingly, donor specific memory B-cells can be clearly divided into two distinct subpopulations (figure 6). The IgG/IgA positive population resembles the typical class shifted reactive memory B-cell population, while the IgG/IgA negative population expresses a phenotype that, to our knowledge, has yet to be described as a murine B-cell subpopulation. This unique population is especially interesting considering that, during the quiescent phase, it makes up about 50% of the CD80 positive memory B-cell population. Also, there seems to be an increase in the proportion of the IgG/IgA negative population between day 60 and day 90 of the immune response, which

signifies the progression between the contraction and quiescent phase (6B/C). Testing the IgG/IgA positive cells for expression of IgM showed no such expression, which is consistent with the current theory of recombinant class switching. However, IgG/IgA negative cells did express low level of IgM (6d).

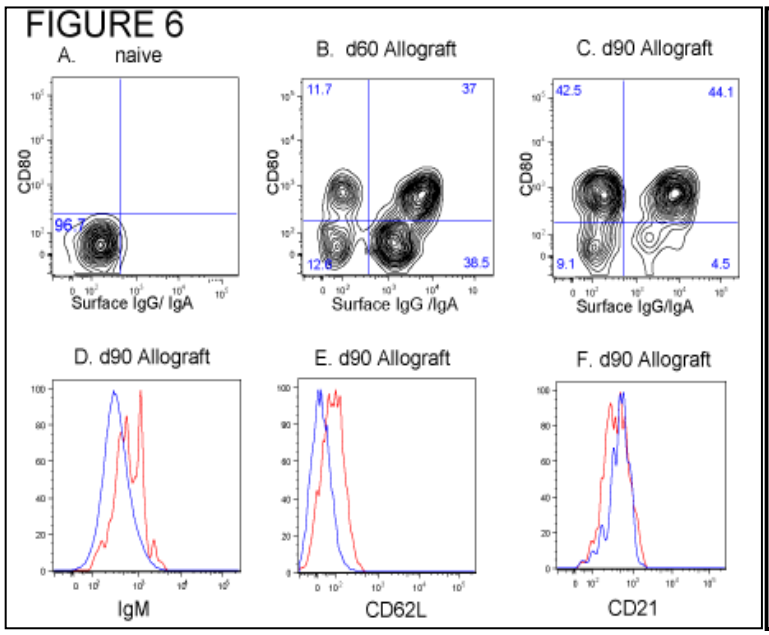

Figure 6: Murine memory B-cell markers : Expression patterns of surface IgG on donor-specific B-cell during the contraction phase (B. d60) and the quiescent phase (C. d90) and naïve cells(A). D. IgM expression on IgG negative (blue) and positive cells (red). E. CD62L expression on IgG negative (blue) and positive cells (red). F. CD21expression on IgG negative (blue) and positive cells (red).

Applying the model system-Tracking the T-cell independent response

TCR KO transgentic mice-An application of the model system: Recent studies have shown the importance of T-cell help in the B-cell mediated humoral response. T_{fh} cells are associated with GC formation, somatic hypermutation, class shift, and other factors that mediate the long-term effects of the B-cell directed response. Transplant patients are treated with heavy immunosuppression; therefore, their T-cell activity is greatly inhibited. It is thus important to be able to track donor specific B-cell responses that are T-cell independent. To do this, we applied the same methods used in our model system, but to T-cell receptor knock out (TCRKO) mice. As Figure Y shows, we

were able to find upregulation of tetramer specific B-cells in quiescent phase mice as opposed to a negative control. This cell population differs from the ones described above in that still has an upregulation of IgD (Figure 7b), and does not class shift from IgM to IgG (7d).

Figure 7

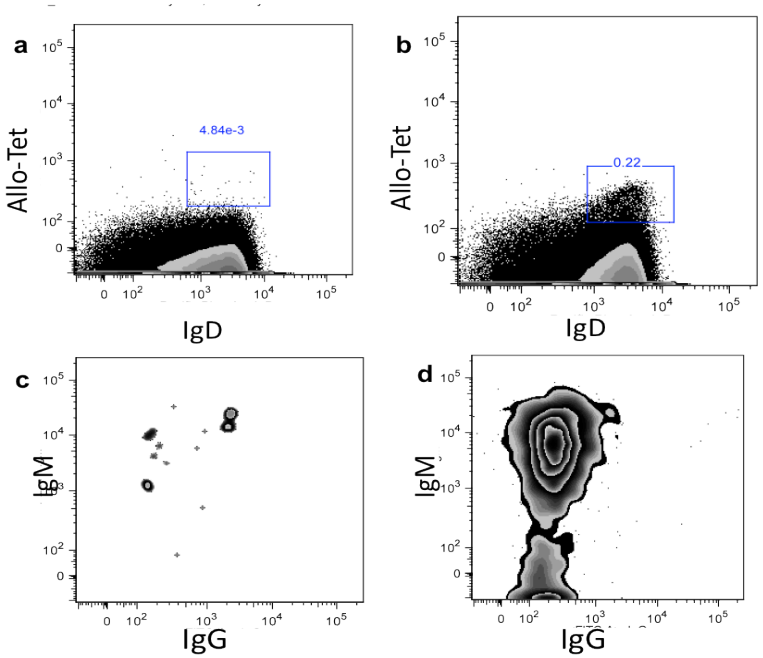

Figure 7: TCRKO MURINE MODEL: TCRKO transgenic BALB/c (H-2K^{d}) mice (b,d) were challenged with C57BL/6 (H-2K^{b}) cardiac grafts, compared to naïve BALB/c mice (a,c) a,b: Gating for allogenic H-2 reactivity and expressed surface IgD. c,d: Analysis of gated population for expression of IgM and IgD.

Discussion

A new model system-Its virtues and proof of concept

The system developed by our lab to track donor-specific B-cell development allows for an in depth and holistic analysis of the B-cell mediated humoral response. The strength of the system comes from the three-pronged approach to tracking the donor specific B-cell activity: which utilizes flow cytometry, the ELISA, and the ELISPOT assay. Each of these provides insight into a different facet of the immune response but is also complimentary to the other assays, which allows for the corroboration of observations. The complimentary approach allows for each technique to fill in the gaps of knowledge left by the others.

The use of flow cytometry, our most powerful technique, allows for the quantification and qualification of donor specific B-cells. An advantage of our equipment is that we can stain for as many as eight different channels per assay. However, through negative gating techniques, it is possible to account for more than eight phenotypic markers. For instance, our dump channel stains for the markers of the lymphocytic populations that we are not examining (T-cells, macrophages, dead cells) with the same fluorochrome. Upon analyzing results, cells that do not stain with that fluorochrome are selected for. This frees up the other channels for the examination of other phenotypic markers or groups of markers. By carefully designing our staining strategies, we can gather meaningful phenotypic information from each marker that we examine. For

example, if a cell population is expressing high surface IgD it shows that it is either a naïve population of B-cells or that it has not undergone class shifting (like in the TCRKO results). It is important to note that the flow cytometric technique that was utilized is somewhat limited; its phenotypic distinctions can only be made by staining for cell surface markers. However, since the immune response utilizes cell surface markers for recognition of antigen (i.e. BCR), signal receptors, and localization of cell populations (i.e. GL7), flow cytometry is able to provide a relatively comprehensive phenotypic view of B-cells.

Our MHC tetramer staining protocol, which combines syngenic tetramer stains with allogenic tetramer stains, allows for a high-resolution view of the cellular component of the humoral response. As discussed above, syngenic tetramer staining accounts for non-donor specific interactions, like specificity for non-polymorphic sections of the antigen or specificity for the biotin-streptavidin complex used in preparing the tetramer. With large populations of antigen specific cells, such interactions do not tend to skew results. However, when examining very small populations of cells, like memory B-cells, non-specific binding interactions can prevent the gathering of any meaningful data. Our transplant model system is therefore well suited for the study of the mechanism of B-cell memory, given its resolution in tracking antigen specific memory B-cells.

The ELISA and ELISPOT assays are more limited in their capabilities than flow cytometry, but seriously supplement and reinforce flow cytometric data. The ELISA allows for the direct tracking of serum antibody levels. As previously

stated, it is important to quantify serum DSA because antibodies are the ultimate effectors of the humoral response. However, the ELISA's purpose is not only to quantify DSA but also to qualify it. The use of secondary antibodies that are specific for different classes of secreted immunoglobulin allows for the corroboration of observations about class shift obtained in flow cytometry. Similarly, the ELISPOT allows for the quantification of donor specific B-cells outside based upon their antibody-secreting ability.

In the process of developing a system that provides a physiologically relevant model for transplant rejection, we have also established a new method to track the humoral immune response and assess the currently accepted knowledge of basic B-cell biology. The main advantage of our system over others is its ability to track the immune response effectively under clinically relevant conditions. Previous work on the B-cell immune response typically utilized BCR transgenic mice, which do not express any polymorphism for their BCR, or the use of haptenated antigen studies, which track the response to a known but physiologically irrelevant antigen (i.e. nitrophenol) [24-26]. These studies have lead to a greater understanding of memory B-cell development, but in order to advance the field, models utilizing different antigens and which develop an immune response under different conditions, must be used in order to confirm, challenge and hopefully extend our current understanding of the response. Our system has the ability to track polyclonal alloantigen-specific B-cell responses in the context of a physiologic inflammatory response. Therefore, we believe that the model system described through this research is an

excellent complement to the study of haptenated antigens and BCR transgenic mice, and that the tools developed in our lab are comparable to those used in other systems. Thus, aside from developing a system that is an accurate model of allograft rejection, we developed a system that has the potential to provide vital information about the basics of B-cell biology.

Our system has already yielded meaningful results into the nature of the donor specific humoral immune response. First, our initial experiments with fully mismatched grafts on wild type strains of mice have provided insight into the kinetics of the immune response. Our data have allowed us to break up the immune response into three phases based on the levels of donor specific antibody: rejection, contraction, and quiescence. Next, our data also provided insight into how different antigen loads affect the development of a long-term response. This was achieved by varying the type of grafted tissue. Since skin grafts fall off after being rejected, it does not leave a significant amount of antigen residue. On the other hand, we showed strong evidence, using GL7 as a marker for GC activity, that cardiac grafts leave residual levels of antigen up to d80-90 post transplantation (4B). This information helps to partially explain the higher levels of donor specific B-cells in cardiac-grafted mice during the quiescent phase (4a). Finally, our data do not only serve as a proof of concept for our model's ability to track donor specific memory B-cells, but we were also able to use our novel techniques to describe a subset of the memory B-cell population that has yet to be characterized in a murine model system.

IgM memory B-cells

Using our model system we were able to track two distinct subpopulations of memory B-cells: The IgG/IgA positive set and the IgG/IgA negative set. Mature, but naïve, B-cells express high levels of IgD/IgM on their plasma membranes. If these cells bind antigen, they become activated and, with the help of T-cells undergo class switching. The expression of any of the IgG isotypes (IgG1, IgG2a, IgG2b, IgG3) or the IgA isotype on the plasma membrane shows that the population has underwent isotype class switching. The IgG/IgA positive population of memory B-cells could likely trace their lineage back to cells that developed within the GC and in the presence of T_{fh} cells. The lack of expression of IgM on IgG/IgA positive memory cells also indicates class shifting. Because of their high expression of IgG/IgA we termed this subpopulation IgG memory B-cells cells. IgG/IgA negative memory B-cells express low levels of IgM, and therefore we termed this subpopulation the IgM memory B-cells.

The IgM memory phenotype suggests that the subpopulation developed outside of the GC and without the help of T_{fh} cells. That IgM subpopulation exists in a relatively equal proportion to the IgG subpopulation is striking. Though further research into the IgM subpopulation is necessary to describe its complete development, this research provides some interesting data. The IgM memory cells generated by our model is antigen dependent and exists within the quiescent phase of development that is absent of antigen specific GC activity. Therefore, we believe that IgM and IgG donor-specific memory B-cells subpopulation originate from separate lineages and IgM subset preferentially

survives during contraction. Further research is needed to confirm or deny this prediction.

Though the topic remains controversial, there have been studies that have provided evidence that population of IgM and IgM/IgD memory B-cells could be clearly differentiated in humans [27, 28]. These studies, however, are severely limited. This is due to their inability to assess the age of these populations of cells, their inability to assess whether their development is antigen dependent or independent, and their inability to track these cells based on a specific antigen. It is impossible to conduct the type of controlled experiment needed to address these problems in human subjects. Furthermore, due to these limitation it would surely be very difficult to determine whether the IgM memory population described in our research matches up with populations described in human studies. We believe that the controlled, yet clinically applicable, conditions of our system offer much less ambiguous assessment of the immune response, and could potentially explain the lineage of the populations described in human studies.

Applying the model-TCRKO mice

The model discussed so far tracks an unmodified immune response to fully mismatched allograft. Tracking the B-cell directed response in this system is therefore akin to tracking the antibody-mediated component of a un-immunosuppressed rejection episode in human patients. We do not doubt that further investigation into this unmodulated model will yield clinically relevant results. However, the main advantage of our model system is its ability to track

specifically known antigen under physiological and clinically relevant conditions. By modifying are system, we hope to be able to model other clinically relevant rejection episodes. Our TCRKO experiments have already yielded relevant results that shed light on the nature of the humoral immune response.

The purpose of the TCRKO experiments was to gain insight into how T-cell help affects the B-cell response. As discusses in the introduction, T-cell help is critical in B-cell the differentiation and proliferation processes. The signaling process relies on costimulatory signal cascades between CD4+ helper T-cells and antigen specific B-cells that are not well understood. Research has shown that the coordination and the timing of the different signals have profound effects on the maturation of B-cell populations [29-31]. We hoped that by effectively eliminating the contributions of helper T-cells to the immune response using TCRKO mice, we would be able to observe how the lack of T-cell help alters our base model system.

Our data indicated that a humoral response is initiated against the mismatched graft in the absence of T-cell help. The response is characterized by a substantial population of donor specific B-cells, which express IgD and IgM, and do not express high levels of IgG. Given the absence of T-cell help in class switching, these results are expected. It is worth noting, that even though these results were gathered >120D post transplantation, they do not necessarily point to a memory population. In the absence of a T-cell response, the cardiac graft was not destroyed. In fact, while extracting the spleen for processing, we

observed that most of the TCRKO mice had beating cardiac grafts. The humoral response alone was not sufficient at that time point to cause functional graft failure. It is, therefore, safe to assume that there must have been a significant and persistent antigen load at the time of analysis.

That data gathered from our TCRKO experiments is promising, but it is clear that further experiments are needed in order to enhance our knowledge of T-cell independent lineages of B-cells. Our experiments conclusively show that antigen specific lineages of B-cells develop in response to mismatched donor MHC, and that, using our model system, it is possible to track these responses. Further research should examine the T-cell independent pathways in greater detail using a variation of staining strategies to type the cells. Also, the TCRKO system presented here could be modified to examine if a memory population develops by surgically removing the graft at d80-90, which would simulate the kinetics that were established by our base model system. We believe that the T-cell independent response is clinically relevant considering that modern immunosuppressant regiments target T-cells.

Future research

For our base model to truly simulate the rejection episodes experienced by organ transplant patients, it would be necessary to treat grafted mice with clinically proportional doses of immunosuppressant. Future research can explore the effects of commonly used immunosuppressants, like the mTOR inhibitor rapamycin or the calcineurin inhibitor tacrolimus, on the immune response by comparing the kinetics, development, and phenotype of

immunosuppressed donor specific B-cell populations to the data gathered by our baseline model system. These experiments should use physiologically and clinically applicable dosing of the drugs. We predict that immunosuppression would affect the development of B-cells either by directly targeting B-cells or by indirect effects caused by a reduction in T-cell help.

Also, since previously sensitized patients are at the highest risk for AMR [3, 7], we can establish a system that aims to model that response. By re-challenging mice with fully mismatched allograft during the quiescent phase of development, we can use our methods to track the second, more vigorous, humoral response. Such experiments can be done without immunosuppression, in order to track the cell populations that are responsible for the sensitized response, and with immunosuppression, in order to observe how these processes are altered by the drugs. We predict that the both the IgM and IgG subpopulations of memory B-cells both have a profound role in the memory response.

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