MOLECULAR CHARACTERIZATION OF THE HUMAN ALLOIMMUNE RESPONSE

by

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DISCLAIMER

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MELODY SMITH

DISSERTATION

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ABSTRACT

MOLECULAR CHARACTERIZATION OF THE HUMAN ALLOIMMUNE RESPONSE

Publication No. 1

Melody Smith

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Supervising Professor: Robert Collins, MD

When T lymphocytes from two individuals are placed in a mixed lymphocytic reaction (MLR) and allowed to stimulate each other a powerful proliferative response is elicited, reflecting the degree of incompatibility at the major histocompatibiltiy loci. Such *in vitro* proliferations translate *in vivo* into graft rejection of stem cell and solid organ transplants and to graft versus host disease (GvHD) in recipients of allogeneic stem cell transplants. Defining the clonal T cell responses elicited in an MLR between two individuals is complicated by the multiplicity of T cell HLA- antigen interactions.

We used flow cytometry to investigate the MLR between mismatched individuals to better characterize responder T cell proliferation and the subsets involved in the alloresponse. After observing proliferation in the setting of a completely mismatched one- way MLR and ensuring that the design for the MLR was viable, we sought to characterize clonal T cell responses in a simple system. We attempted to elicit T cell responses to autologous antigen-presenting cells (APC) transfected with a single

mismatched HLA-A2 molecule. We failed to detect any alloresponding T cells using a flow cytometric system, except when dendritic cells were used.

We then used an HLA-A2 negative responder pre-stimulated in an MLR with an HLA-A2 positive individual for re-challenge by the responder's own APC transfected with an HLA-A2 plasmid. In this way, we sought to identify an expanding HLA-A2 specific T cell population which could be further characterized by T cell receptor cloning. Nevertheless, we failed to detect an alloimmune response in this T cell line stimulated with HLA-A2 transfected APCs three times over a 21- day culture period.

These results suggest that either the frequency of alloreactive T cells was below the limit of detection by these methods or that the particular stimulator-responder pairs used in these experiments were non-reactive. Future work will extend the study to more HLA mismatched stimulator-responder pairings to better define the characteristics of alloreactive T cells.

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PRIOR PUBLICATIONS & PRESENTATIONS

Abstract Accepted for Presentation at 35th Annual Meeting of the European Group for Blood and Bone Marrow Transplantation (EBMT), Göteborg, Sweden
March 2009

- Title: BOTH NAÏVE AND MEMORY LYMPHOCYTES PARTICIPATE IN ALLORESPONSES TO HLA MISMATCHED STIMULATORS- IMPLICATIONS FOR SELECTIVE DEPLETION OF GVHD ACTIVITY
- Authors: J. Barrett, M. Smith, Z. McIver, N. Hensel, J. Melenhorst (National Institutes of Health, Bethesda, US)
- Bone Marrow Transplantation 43(S1):S304; 2009

Oral Presentation at the NIH Clinical Research Training Program for Medical and Dental Students 2007-2008 Scientific Presentations, Bethesda, Maryland
May 2008

- Title: MOLECULAR CHARACTERIZATION OF THE HUMAN ALLOIMMUNE RESPONSE

Poster Presentation at the Pfizer Global Research and Development Facility, Groton, Connecticut
May 2008

- Title: MOLECULAR CHARACTERIZATION OF THE HUMAN ALLOIMMUNE RESPONSE

Poster Presentation at the UT Southwestern 45th Medical Student Research Forum, Dallas, Texas,
January 2007

- Title: DEVELOPMENT OF A METHOD TO USE PENTOSTATIN TO REDUCE GRAFT VERSUS HOST ACTIVITY IN ALLOGENEIC T- CELLS

Oral Presentation of Summer Research for American Society of Hematology (ASH) 48th Annual Conference, Orlando, Florida
December 2006

- Title: MECHANISM FOR THE IMMUNO- SUPPRESION ACTIVITY OF PENTOSTATIN: SELECTIVE APOPTOSIS OF NAÏVE T- CELL SUBSETS

Poster Presentation at American Society of Hematology (ASH) 47th Annual Conference, Atlanta, Georgia
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- Title: EX VIVO FLUDARABINE SELECTIVELY DEPLETES HUMAN NAÏVE T- CELL SUBSETS: A STEP TOWARD MODIFYING DONOR LYMPHOCYTE INFUSION

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LIST OF ABBREVIATIONS

APC- antigen presenting cell

CD- cluster of differentiation

INF- interferon

MLR- mixed lymphocytic reaction

PBMC- peripheral blood mononuclear cells

SEB- Staphylococcal Enterotoxin B

TCL- T cell line

TNF- tumor necrosis factor

LIST OF DEFINITIONS

Antigen presenting cell (APC)- a cell that presents antigenic material on its cell surface via the major histocompatibility complex (MHC)

CD38- a marker for T cell activation

Carboxyfluorescein diacetate, succinimidyl ester (CFSE or CFDA-SE)- a dye that can be used to delineate cellular proliferation, as appreciated by its serial dilution with each cell division

Major histocompatibility complex (MHC)- complex by which self and nonself antigens can be presented on the cell surface of T cells

Mixed lymphocyte reaction (MLR)- a measure of histocompatibilty at the HLA locus, where peripheral blood lymphocytes from two individuals are mixed together in tissue culture for several days

One way MLR- an MLR in which the lymphocytes from one of the donors in the pair have been inactivated (i.e, with radiation) so that the only non- radiated remaining population of cells is able to proliferate in response to the foreign histocompatibility antigens

pCI- empty plasmid

Responder- the cells in an MLR that are responding to the antigenic stimulation

Stimulator- the cells in an MLR that are proliferating in response to antigenic stimulation

Transfection- the process of introducing DNA into cells by non-viral methods, such as electroporation.

CHAPTER ONE

Introduction

When the T lymphocytes from two different individuals are mixed together in tissue culture, they stimulate each other. This encounter of incompatible lymphocytes induces a powerful proliferation response. The intensity of the aforementioned reaction is a reflection of the level of histocompatibility at the HLA loci. Clinically this incompatibility between cells at the major histocompatibility complex (MHC) is reflected in the mixed lymphocytic reaction (MLR). The alloimmune response *in vivo* can be noted by the rejection of solid organ or bone marrow transplants and graft versus host disease in recipients of allogeneic bone marrow transplants or from passenger lymphocytes in solid organ transplants (2).

On the other hand, in the setting of HLA-identical and matched unrelated donor allotransplantation, donor T cells may recognize antigens expressed by recipient tissues, causing graft-versus-host disease (GvHD). The mechanism of alloreactivity is not fully understood, but appears to be for the most part mediated by T cells recognizing a peptide presented in the context of self- or allogeneic MHC. If the transplant was carried out in an HLA-identical setting, the only antigens that may cause GvHD are minor histocompatibility antigens (mHAg). If the transplant was carried out across MHC barriers, however, both minor and major histocompatibility antigens may be at the heart of the GvHD (5, 8).

Assuming that the alloreactive T cells arise in an MHC mismatched setting, such as in matched unrelated donors (MUD) or haplo-identical transplants, these cells may

recognize host peptides presented in the mismatched MHC molecule. This form of antigen presentation is known as direct alloreactivity. Alternatively, the mismatched MHC molecule may be processed intracellularly into constituent peptides, to be presented to the alloreactive T cells on MHC molecules shared by both parties. This form of processing antigenic material prior to presentation on the cell surface is referred to as indirect alloreactivity. The relative contribution of both pathways to alloreactivity is not clearly delineated.

As noted above, two pathways of allorecognition, the direct and the indirect, mediate the immune response that the major MHC provokes. The direct pathway of antigen presentation occurs when intact MHC molecules on the recipient's T cells recognize the antigens presented at the surface of the donor antigen-presenting cell (APC) within the graft and vice versa, in the case of an allogeneic stem cell transplant. The quantity of the precursor frequency of alloreactive T cells that recognize the allogeneic MHC molecules, as well as the direct binding of the T- cell receptor (TCR) to the allogeneic MHC molecules are two distinct aspects of the direct alloimmune response (3).

The indirect pathway represents another route for the presentation of foreign antigens to the MHC. In this mechanism, allogeneic MHC molecules and other donor alloantigens are first processed into peptides, which are then incorporated into the HLA molecules of the recipient APC. It has been postulated that T cells sensitized by the direct pathway initially dominate the rejection process. Thus, T cells sensitized by the indirect pathway might contribute to the chronic damage to the graft after the direct alloreactive T cells have become hyporesponsive (3).

In an effort to further elucidate the immune response that occurs in the setting of an allogeneic bone marrow transplant, we investigated which pathway of allorecognition, direct or indirect, is employed in antigen recognition. We also evaluated what the T cell perceives as foreign on an MHC mismatched stimulator APC. In this setting, any of the following might be recognized as foreign: the neo-self peptides (sequences that are only presented via the foreign MHC and therefore new to the responder), the foreign MHC molecule itself, the foreign MHC/peptide complex as a whole, or the foreign peptides derived from the mismatched MHC molecule presented by other (compatible) HLA molecules of the stimulator APC (i.e., indirect MHC recognition). Thus, we sought to demonstrate which element the cell perceives as foreign. Finally, we aimed to characterize which population of T cells recognizes the antigen, the naïve (CD45RA+) or the memory (CD45RA-) T lymphocytes.

We hypothesized that both the direct and indirect pathways of antigen presentation mediate the alloresponse to a foreign MHC molecule and that the response is modulated by the memory T cell population. Although methodology will be discussed in detail subsequently, the primary techniques used in the project were transfection of MHC molecules by electroporation and flow cytometry analysis following cell surface antibody, intracellular cytokine, or proliferation staining.

The experiments were performed using a whole MHC molecule to underscore the direct and the indirect pathways. Subsequent studies will use a truncated MHC molecule lacking the intramembrane portion and not presented on the cell surface to elucidate the indirect pathway only. In the process of evaluating the presentation of foreign MHC molecules to the immune system, we aimed to further elucidate the role of both the direct

and indirect pathways of allorecognition. Although one pathway may not necessarily be more prominent than the other, their respective importance could vary temporally. For example, the direct pathway might play a more pivotal role in the early response to a foreign MHC molecule, since the processing involved in the indirect response would lead to a delay in the presentation of a given antigen. Finally, further studies were performed using BAPCs and dendritic cells (DCs) to determine if they differed as stimulators from TAPCs.

CHAPTER TWO

Mixed Lymphocytic Reaction (MLR)

BACKGROUND

In the HLA- matched allogeneic setting there are a variety antigens that modulate the response. These include the minor histocompatibility antigens (i.e., H-Y), tissue restricted minor histocompatibilty antigens (i.e., HB-1), and antigens that are overly or aberrantly expressed by malignant cells (i.e., PR-1 and WT-1) (6). The *in vitro* recruitment and characterization of alloreactive T cells between HLA- identical siblings has long been a challenge because such T cells are present at very low precursor frequencies (6). Therefore, we created a model whereby we could evaluate this response in stimulator-responder pairs, mismatched at the HLA- A2 locus. By focusing on a single mismatch at the HLA-A2 locus, better characterization of the Class I antigen presentation pathway, which entails the processing of self-protein by the cell and its subsequent presentation to the T cell, could be achieved.

In an effort to identify the precursor frequency of T cells that would respond in the setting of a mismatch at the HLA-A2 locus, donor pairs were utilized in which one donor sample was HLA-A2 positive and the other was HLA-A2 negative. In this manner, one donor sample served as the stimulator, while the other served as the responder in the pair. Among these pairs, all other loci between the stimulator and responder were matched.

Additionally, in an effort to generate a more vigorous response, the HLA- A2 negative responder was chosen from those alleles that were most disparate from the

HLA- A2 locus. For example, as noted in Table 1, the alleles that are most distant from the 0201 allele are 11, 01, and 03, whereas alleles 68 and 69 are more phylogenetically similar to 0201. We postulated that donor pairs with a greater level of convergence from HLA- A0201 would generate a larger T cell response. Thus, these responder samples were used preferentially in our HLA-A2 mismatched MLRs. Finally, HLA-A0201 was selected as the locus of study as it is the most predominant MHC Class I allele in humans. Furthermore, its plasmid had already been generated in the laboratory; so it was readily available for use in experiments.

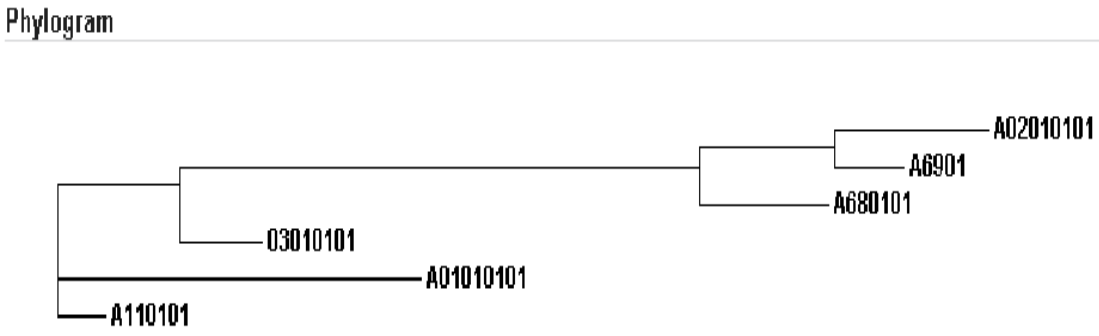

Table 1. HLA- A2 Phylogenetic Tree. Demonstrates the level of genetic divergence between selected alleles at the HLA- A locus. In particular, it highlights that alleles 68 and 69 are most similar genetically to the 02 allele, while alleles 11, 01, and 03 are more genetically different from the 02 allele.

In this phase of the project, we also sought to delineate the optimal ratio of stimulators to responders that should be used in the MLR. Previous studies had shown that the ratios for the stimulator-responder pairs varied depending upon which antigen presenting cell was used to generate the response. Thus, we utilized this data, using the following ratios: 8:1 for dendritic cells, 4:1 for B cells, and 1:1 for T cells (6).

METHODS

Culture of TAPCs- Using an established protocol, T lymphocytes were expanded from thawed PBMCs using OKT-3 and IL-2 (7). The cells were grown for 3 three days prior to being inspected for yield and quality of the culture.

Culture of BAPCs- B lymphocytes were cultured from thawed PBMCs or elutriated lymphocytes. The cells were grown in culture with CpG and 10 % NABS with RPMI-complete media. The cells were checked every other day for growth, and the media was changed as needed. The BAPCs were usually available for use in experiments after 6 to 7 days in the culture medium.

Culture of DCs- The dendritic cells were cultured from fresh or thawed monocytes. The cells were Ficolled, if necessary, and then placed in 1% NABS in RPMI complete media with IL-4 and GM- CSF. Three days later the cells were centrifuged and re- suspended in fresh media. Two days later, the cell media was changed again and TNF-α was added.

Transfection of APCs- The cultured APCs were electroporated $\geq 10^7$ cells, with a control or empty plasmid (pCI) and the same number of cells with a plasmid containing the HLA-A2 allele, pCI-HLAA2/FL (full length). An Amaxa electroporation protocol was used to achieve the transfection of the plasmids. Transfection of the plasmid was performed with an anticipated transfer of the MHC molecule greater than 95%. To evaluate the appropriate transfer of the plasmid, flow cytometry with a monoclonal antibody for HLA- A2 was used as confirmation. In this manner, the cells that received

the control plasmid did not fluoresce while the cells that received the full- length plasmid did. The transfected cells were allowed to rest overnight prior to being used in the experiment the next day. The viability of the cells post- transfection was generally about 20 percent of what they had been prior to undergoing electroporation. The transfceted cells were the stimulators in the MLR.

CFSE Staining- Peripheral mononuclear cells were thawed and rested overnight to be used as the repsonders in the MLR. The responder cells were stained with 25 ul of a 10 μM working solution of cells that were at a concentration 10^6 viable cells/ml in PBS. The cells were incubated in this suspension for 7 minutes at 37^0C in a water bath. After this time, the cells were removed from the water bath and washed with fetal calf serum and complete media three times. After the third wash, the cells were re-suspended at a concentration of 10^6 cells/ml in complete media. Special care was also taken during the staining process to shield the cells and CFSE from the light.

MLR Design- The stimulators and responders were counted and centrifuged. All centrifugation steps were carried out at room temperature for 5 minutes, 900 *g*, so as not to cause undue stress on the cells. Once an accurate cell count was known, the cells could be prepared for the mixed lymphocytic reaction.

The APCs were re-suspended at 10^6 cells/ml in complete media, which consisted of RPMI 1640 supplemented with 10% NABS, HEPES, glutamine, penicillin, and streptomycin. The cells, which would serve as the stimulators, were irradiated one cycle

(25 Gy) to ensure that they would not continue to expand. In this manner, we created a one- way MLR.

The responder cells were labeled with 0.25 μM CFSE and washed, as per protocol. Some of these responder PBMCs were left unlabeled and in culture so that they could be used to calibrate the flow cytometer at the end of the MLR when we analyzed the allorepsonse. After the responder peripheral blood mononuclear cells had been stained, the CFSE- labeled cells were suspended at 10^6 cells/ml and aliquoted 5 ml to two separate 14 ml round bottom tubes and 1 ml to two separate 4 ml (10 x 75 mm) round bottom tubes. The appropriate APC was then added to the tube at the ratios mentioned above, 8:1 for dendritic cells, 4:1 for B cells, and 1:1 for T cells (6). The caps of the tubes were left loose so that oxygen could still enter them. Although we initially used 14 ml round bottom tubes, we subsequently switched to 6- well plates to provide for more cellular interaction between the stimulators and responders. The MLRs were kept in the incubator so that they could culture for 6-7 days. We aimed to generate a powerful response in an MLR of the transfected cells with peripheral blood mononuclear cells.

MLR Controls- Also, at this time, adequate controls were prepared. There were three negative controls that were initially used: one with cells that were transfected with a blank plasmid, one with cells that had not been transfected, and one with an HLA- A2 individual whose cells have been transfected with HLA-A2. These negative controls were established as controls that should not induce proliferation in the mixed lymphocytic reaction. However, the only negative that was consistently used was the one in which the cells were transfected with a blank plasmid. Peripheral blood mononuclear cells in

culture with Staphylococcal Enterotoxin B (SEB), which is a powerful inducer of proliferation, were used as the positive control.

Staining Cells and Flow Cytometry Analysis - Once the T cells had proliferated in an MLR, the cells were characterized using IL-2, CD-4, CD-8, and CFSE to reveal proliferation, CD-38 to show the presence of granzyme, and ultimately with CD45RA to classify memory versus naïve T cells. Finally, when this descriptive information had been ascertained, we planned to utilize some of the more informative samples for Vβ analysis, which would delineate the clonality of the T cells that have been stimulated (1).

Thus, at the end of this culture period, the cells were centrifuged and transferred to 4 ml FACS tubes (polystyrene) and washed twice with plain PBS. The cells were stained with ViViD, as per protocol (i.e. add 50 µl of the 10^4-fold dilution to all the tubes and incubate for at least 15 min at room temperature), to ascertain the amount of cell death. The cell surface markers were then stained using a cocktail of monoclonal antibodies and analyzed using flow cytomertry. In this manner, we were able to assess which class of T cells is involved in the response to a foreign MHC molecule, and attempt to identify the major clones that proliferate in this setting. Since the memory subset of T cells has been implicated in graft versus host disease, it was anticipated that this population would play a more significant role in the response to foreign MHC molecules than the naïve T cells.

RESULTS

Negative and positive controls were prepared for this portion of the experiment. To have validity in the mixed lymphocytic reaction, the negative controls should generate minimal proliferation, whereas the positive control should be a powerful inducer of proliferation in the cell culture.

As cells proliferate following CFSE staining there is a serial dilution of the dye. Thus the quadrant on the flow cytometry graph representing proliferation is the CFSE Dim/ CD38 positive quadrant. Such cells have proliferated and are also activated. The large cell population on the right side of the flow cytometry figures are the CFSE high non- proliferating cells.

Ideally, the negative controls should elicit no proliferation or activation. As shown in Figure 1, which illustrates the proliferative response in peripheral blood mononuclear cells that were placed in culture alone, the negative control gives minimal proliferation. This background proliferation that is expressed, occurs at a low level in both the CD4 and CD8 T cell populations. This lack of response is worth noting as it demonstrates that there are no antigens in the culture that are causing proliferation at baseline.

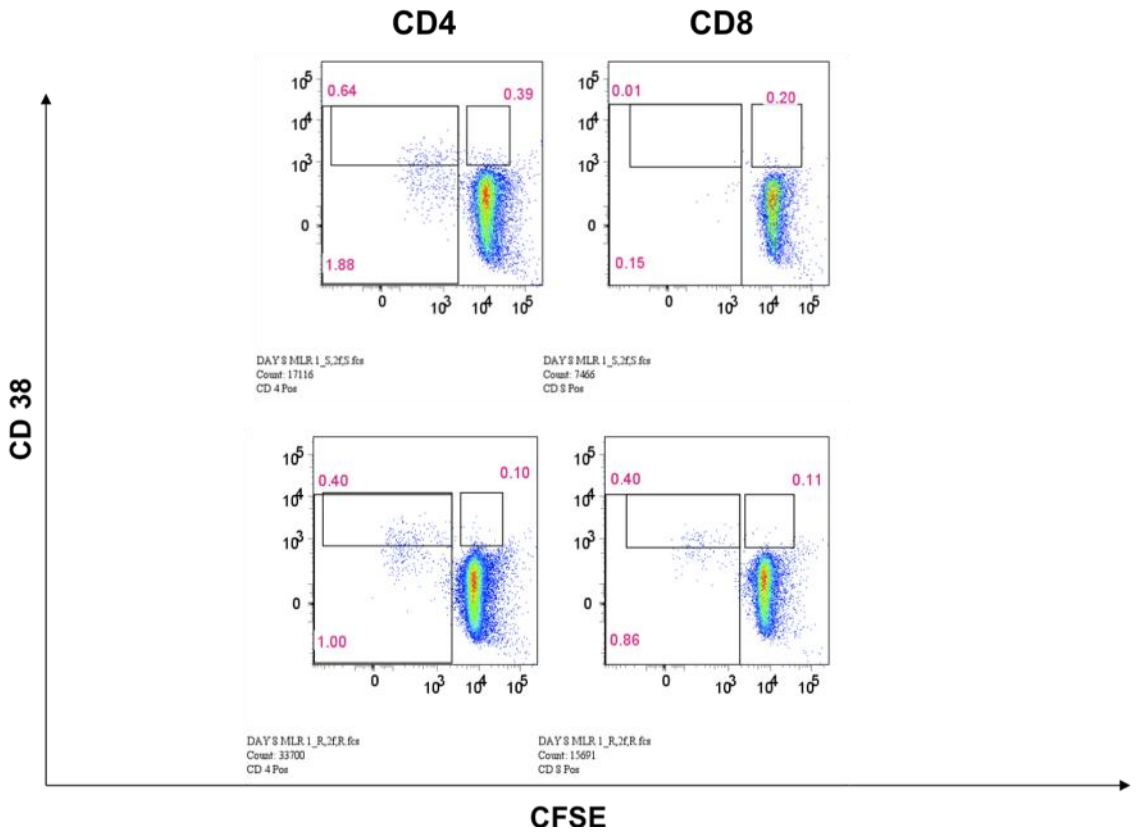

Fig. 1. MLR Day 8 Negative Controls. There was minimal proliferation of CD 4 and CD 8 T cells as evidenced by CFSE Dim cells.

The cells that are represented graphically in Figure 2 are the positive control. These cells were cultured with SEB, a bacterial antigen that should induce proliferation. As seen below, there was a greater degree of proliferation at Day 8 in the positive controls than in the negative controls. Thus, this result helps us to ensure that the cells have the capacity to proliferate. For instance, if the cells had not demonstrated this response in the presence of SEB, then this could have raised questions in regards to the fitness of the cells and their ability to proliferate in another setting.

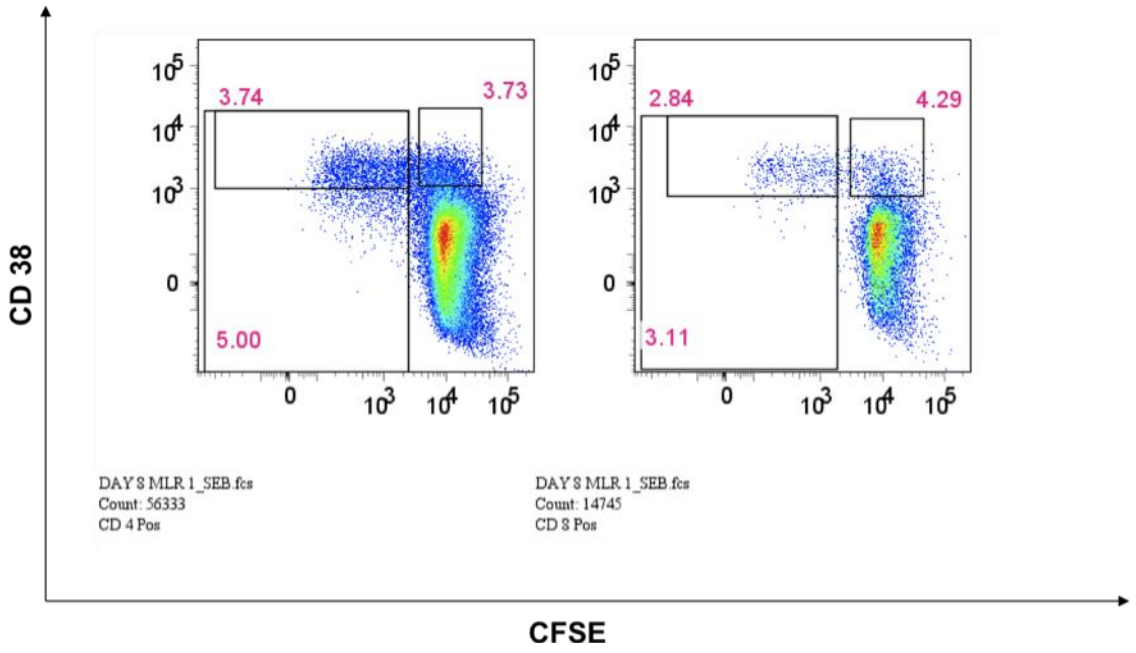

Fig. 2. MLR Day 8 Positive Controls. There was proliferation of the CD 4, and to a lesser degree, the CD 8 T cells in the presence of SEB.

Finally, Figure 3 demonstrates that we were able to generate a powerful proliferation response in a one- way mixed lymphocytic reaction with cells that were completely mismatched. Although the methodology section does not outline how a mismatched MLR is set- up, it is similar to the HLA- A2 mismatched MLR. The key difference is that the cells were not transfected. Thus, the negative control is of cells in culture alone, as opposed to cells in an MLR after transfection with an empty plasmid. Also, the donor pairs were completely mismatched, as opposed to being mismatched at only one locus, and were at a 1:1 ratio. The data from the completely mismatched MLR was essential to show that the MLR design is functional.

When comparing the response in the CD4 and CD8 T cells in the CFSE Dim/ CD38 positive quadrants across the negative and positive controls and the stimulator/

responder MLRs, there was a marked difference in the cellular response. The positive control in Figure 2 showed five percent proliferation in the CD4 T cells and three percent proliferation in CD8 T cells. On the other hand, the stimulator- responder pairs in Figure 3 had proliferation on the order of 31 percent and 23 percent in the CD4 and CD8 T cells, respectively. These results demonstrate that this technique can be used to elucidate the precursor frequency T cell population.

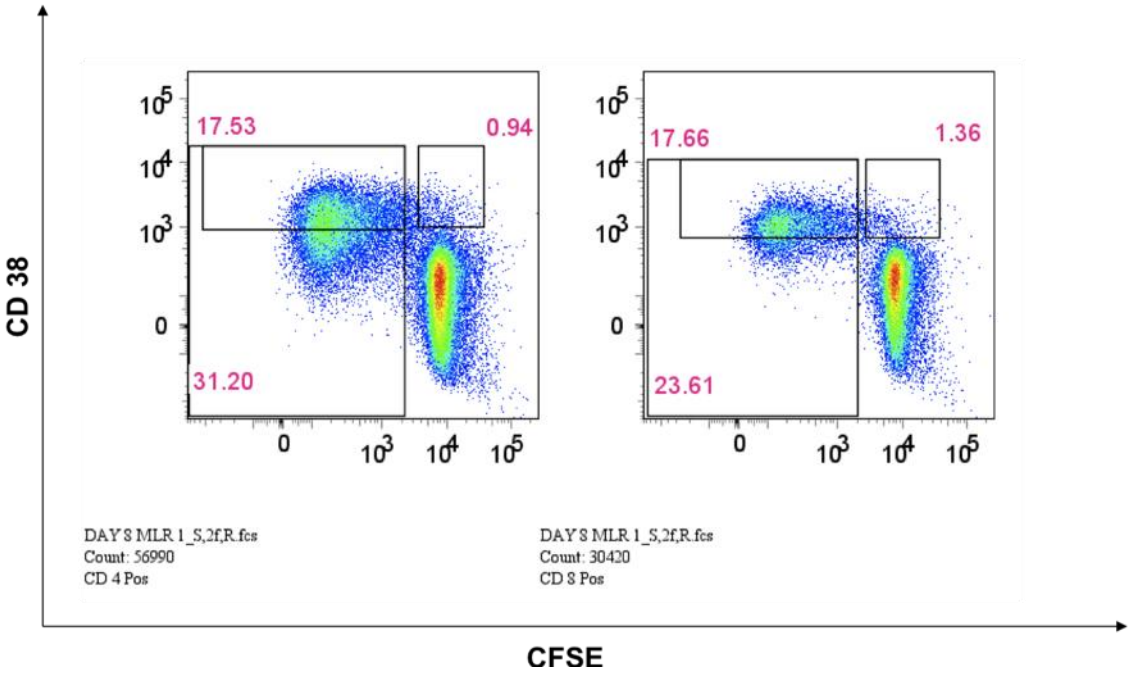

Fig. 3. Completely Mismatched MLR Day 8 Stimulators- Responders. The CD 4 and CD 8 T cells showed a high degree of proliferation in this one- way MLR.

Since one of the goals of this project was to determine which antigen-presenting cell would best generate the alloimmune response in a HLA-A2 mismatched MLR, the subsequent figures will illustrate data obtained from the MLRs of BAPCs and DCs. The donor sample 12113 used in the following experiments is HLA-A01. This designation is

of note in that this locus is phylogentically distant from the HLA-0201 locus, as noted in Table 1. This donor sample, 12113, was used for this reason, as we postulated that HLA-A alleles with a greater level of divergence from HLA- A0201 would be more likely to generate an alloimmune response when placed in an MLR mismatched only at the HLA-A locus. Although not shown below, the positive controls for this set of data were performed using SEB, as outlined above, and proliferation was observed.

The BAPCs were placed in a one- way MLR, as described for the TAPCs, where the stimulators were irradiated and the responders were CFSE labeled with the exception that the BAPC MLR was at a 4:1 ratio of stimulators to responders.

Figure 4 is a representation of the negative control for this donor sample. These cells were transfected with an empty plasmid, pCI. As noted, the CD4 T cells had a proliferation rate of 0.7 percent, whereas the CD8 T cells had a proliferation rate of 0.2 percent. Although this proliferation rate was not particularly high, when compared to the proliferation in the BAPCs that were transfected with an HLA-A2 plasmid it was much less.

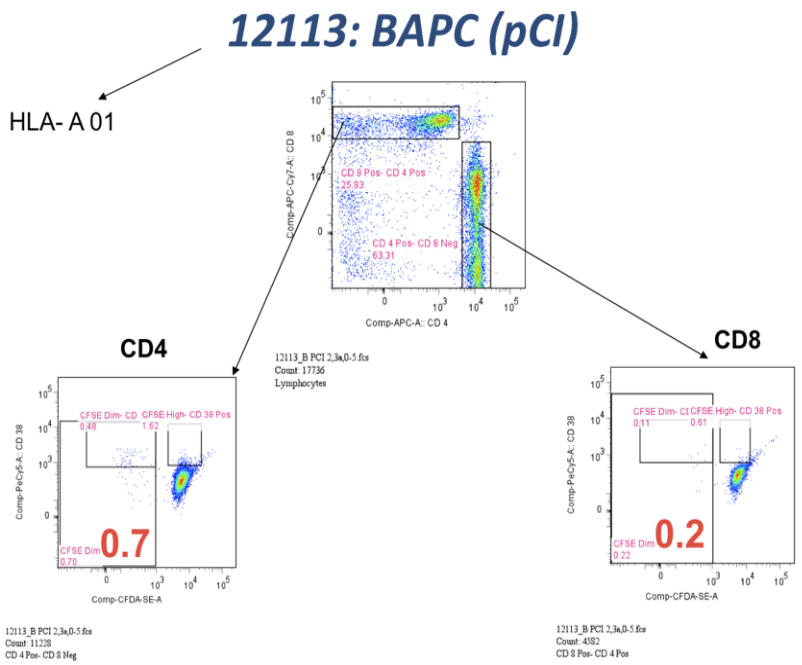

Fig. 4. Control MLR with BAPCs of Donor Sample 12113. The negative control, an MLR with cells transfected with an empty plasmid, produces almost no proliferation.

In the HLA-A2 mismatched MLR, as shown in Figure 5, there was a proliferation rate of 0.4 percent in the CD4 T cells and 0.5 percent in the CD8 T cells. Thus, the CD4 T cells in the control MLR had a higher proliferation than those in the HLA-A2 mismatched MLR, with 0.7 as compared to 0.4 percent proliferation. On the other hand, the CD8 T cells expressed an increase in proliferation of 0.3 percent from the control to the experimental MLR. This difference, however, was not marked enough to be considered significant. Therefore, this data denotes that in the mismatched HLA- A2 MLR for BAPCs, there was essentially a lack of proliferation. This same result was observed in several stimulator- responder pairs for BAPCs from different donor samples.

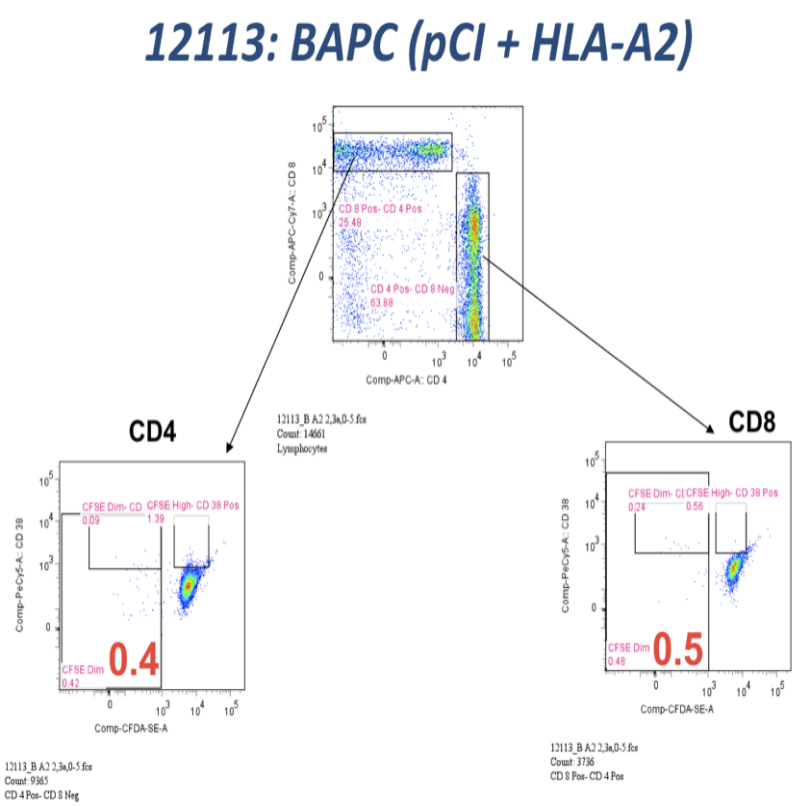

Fig. 5. HLA- A2 Mismatched MLR with BAPCs of Donor Sample 12113. There is a relative lack of proliferation in the CD 4 and CD 8 T cells in response to stimulation by the BAPCs that have been transfected with HLA- A2 plasmid.

The data for the dendritic cells HLA-A2 mismatched MLR, however, showed more of a response than that observed in the BAPCs. The DCs were placed in an MLR at a ratio of 8 stimulators to 1 responder. The control MLR showed minimal proliferation as observed in Figure 6, with a proliferation rate of 0.4 and 0.3 percent in the CD4 and CD8 T cells respectively. Thus, once again the background proliferation was minimal in the control sample.

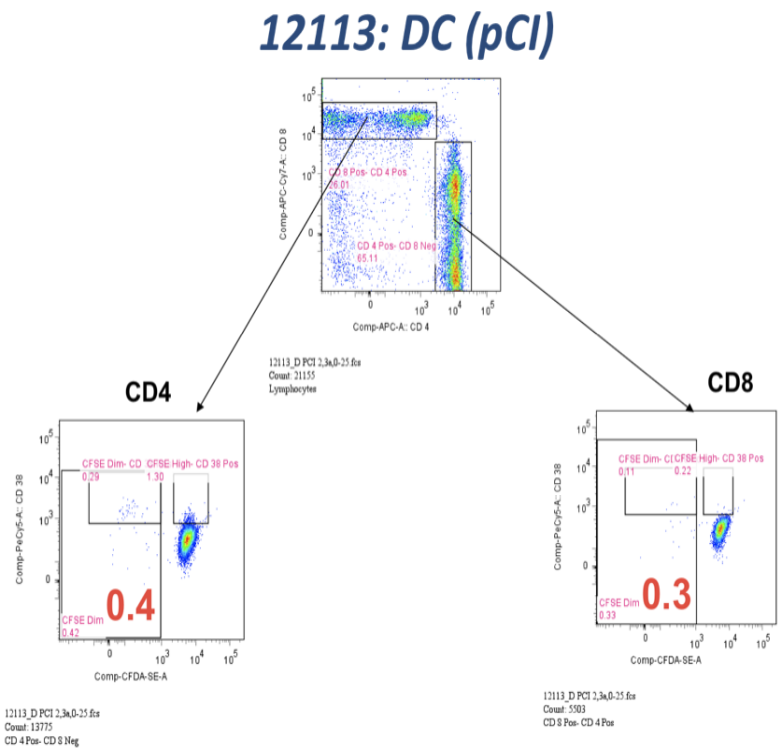

Fig. 6. Control MLR with DCs of Donor Sample 12113. The negative control, an MLR with cells transfected with an empty plasmid, produces almost minimal proliferation.

The result illustrated in Figure 7 was the strongest level of proliferation observed in any of the stimulator- responder pairs with DCs. With the low level of proliferation in the negative control as a baseline, the result in the HLA-A2 mismatched DC MLR, particularly within the CD8 T cell population, is noteworthy. The CD4 T cells demonstrated proliferation at rate of 0.9 percent, which was five percent greater than the baseline established by the negative control. However, the CD8 T cells proliferated at a rate of 2.3 percent, which was two percentage points above the baseline.

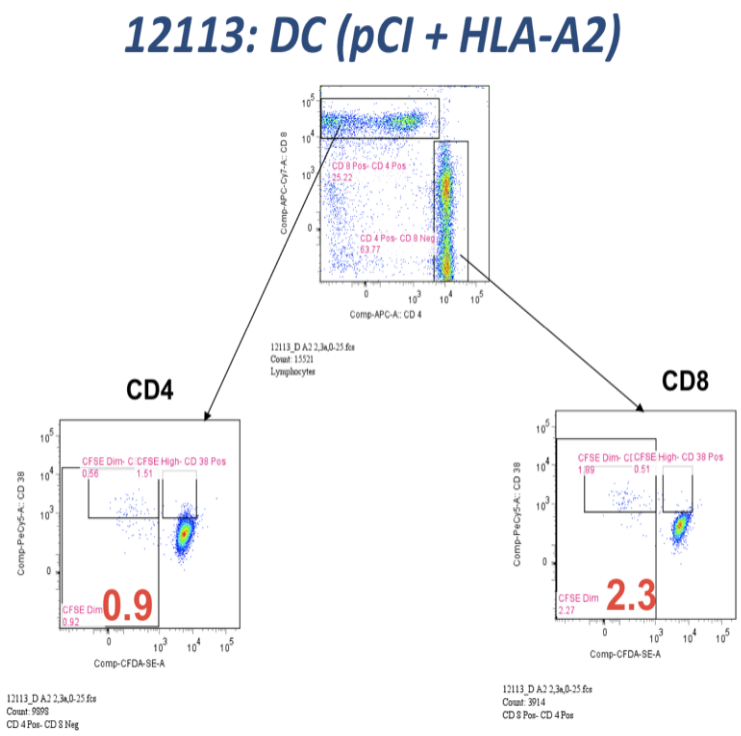

Fig. 7. HLA-A2 Mismatched MLR with DCs of Donor Sample 12113. There is a strong proliferative response, particularly in the CD 8 T cells, although the CD 4 T cells also undergo proliferation in response to the HLA- A2 mismatch.

DISCUSSION

In order to determine the relative contribution of direct versus indirect alloreactivity, we sought to generate alloreactive T cells specific for an MHC molecule by introducing an HLA gene into the APC of a donor that lacked expression of that particular allele, i.e. HLA-A*0201, and generating T cell responses against this molecule. The resultant T cells could then be screened for the mechanism of alloantigen recognition, i.e. whether the T cells recognized peptides presented in the context of the allogeneic HLA (direct alloreactivity), or whether peptides derived from the allogeneic HLA were presented by other HLA molecules of the APC (indirect alloreactivity).

Indirect alloreactivity could be identified by stimulating the alloreactive T cells with an APC expressing a signal sequence-minus HLA-A2 variant ($A2^{-SP}$). This HLA-A2 variant would be expressed within the cell. However, due to the missing signal sequence, the molecule stays inside the cell and would not be expressed on the cell surface. This molecule would then be degraded and presented in the context of the HLA molecules expressed by the autologous APC. In contrast, direct alloreactivity could be identified by using an APC that expresses the HLA molecule initially used to generate the response against, e.g. HLA-A2. Next, this same APC could be transfected to express the full length HLA molecule but is mismatched for all other HLA molecules expressed by the responder.

Although we sought to better describe the involvement of direct and indirect alloreactivity in the alloresponse, we first had to identify donor pairs that would generate a measurable alloresponse to HLA-A2. The first step in this process was to generate the various antigen-presenting cells that would be utilized for this purpose. Dendritic cells were cultured from monocytes, whereas the B- and T- APCs were cultured from peripheral blood mononuclear cells (PBMCs) or elutriated lymphocytes. After culturing the APCs, each of them were phenotyped using monoclonal antibodies against their respective cell surface markers. This was done as a quality control to ensure that the cells that were generated were actually what we purported them to be. For example, as observed in Figure 8, these TAPCs were progressively gated using flow cytometry to ensure that they expressed the markers that are on the cell surface of T cells, namely CD3 and CD4.

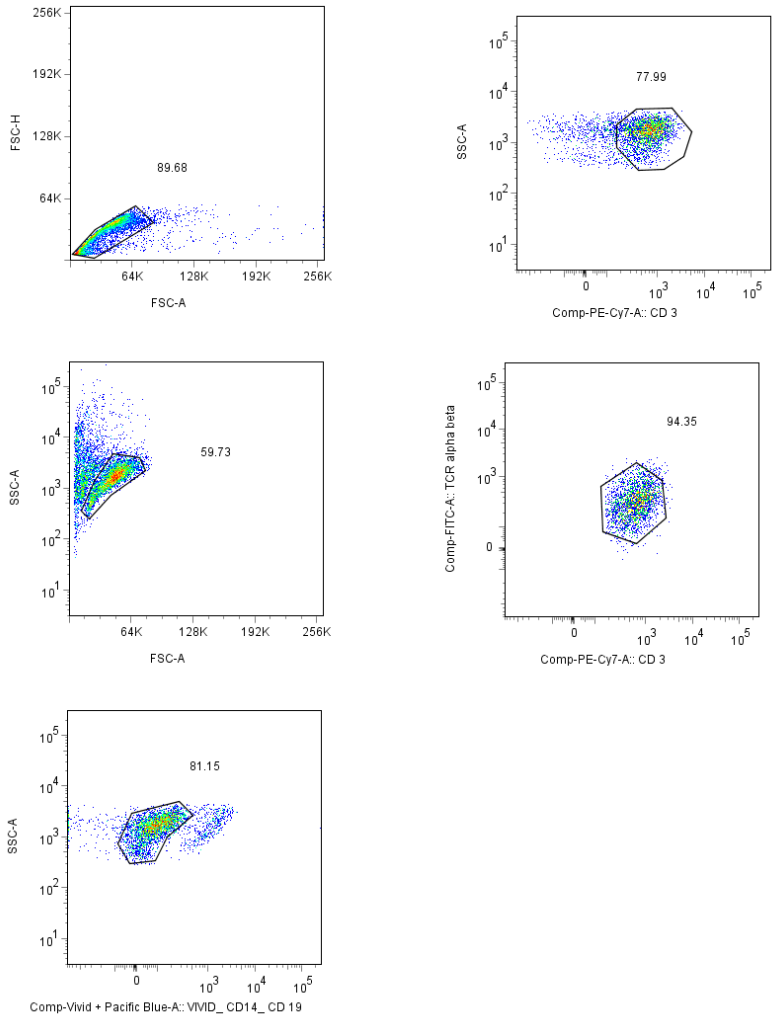

Fig. 8. Phenotyping of T APCs. Starting at the top and following the panels from right to left the cells were progressively gated in the following manner: Singlets (FSC-H/ FSC-A)> CD 3 positive> Lymphocytes (FSC-A/ SSC-A)> CD 3 positive/ TCR (T cell receptor) positive> Dump (Vivid, CD 14, C19 positivity) negative.

The phenotyping of the cultured dendritic cells was not consistently reliable. Similar to the TAPCs, we attempted to phenotype the DCs by using monoclonal antibody to the cell surface markers of immature and mature dendritic cells. However, due to the degree of variability across repeated phenotyping of the cultured DCs, it was dubious as to whether immature or mature DCs had been generated. There was internal consistency, however, with the phenotyping of the BAPCs. Finally, there was also

difficulty in the achieving a good yield with regards to the viability of the DCs and BAPCs after culture.

The flow cytometric figures displayed in the results section were all analyzed in a similar manner. Although three different classes of antigen presenting cells were used in the HLA- A2 mismatched MLRs, for the purposes of analysis, the proliferation and activation of the precursor frequency population of T cells was the population of interest. Therefore, the same staining cocktail was used on all of the MLRs after they had been cultured for seven days. The staining cocktails contained fluorescently tagged monoclonal antibodies to the following cell surface markers: CD 3, CD 4, CD 8, CD 14, CD 19, CD 38. Prior to being stained with the cocktail, however, the cells were stained with Vivid to differentiate those that were viable from those that died during the culture period. As the responder cells had been stained with CFSE prior to being placed in culture, CFSE dilution was used to demonstrate proliferation.

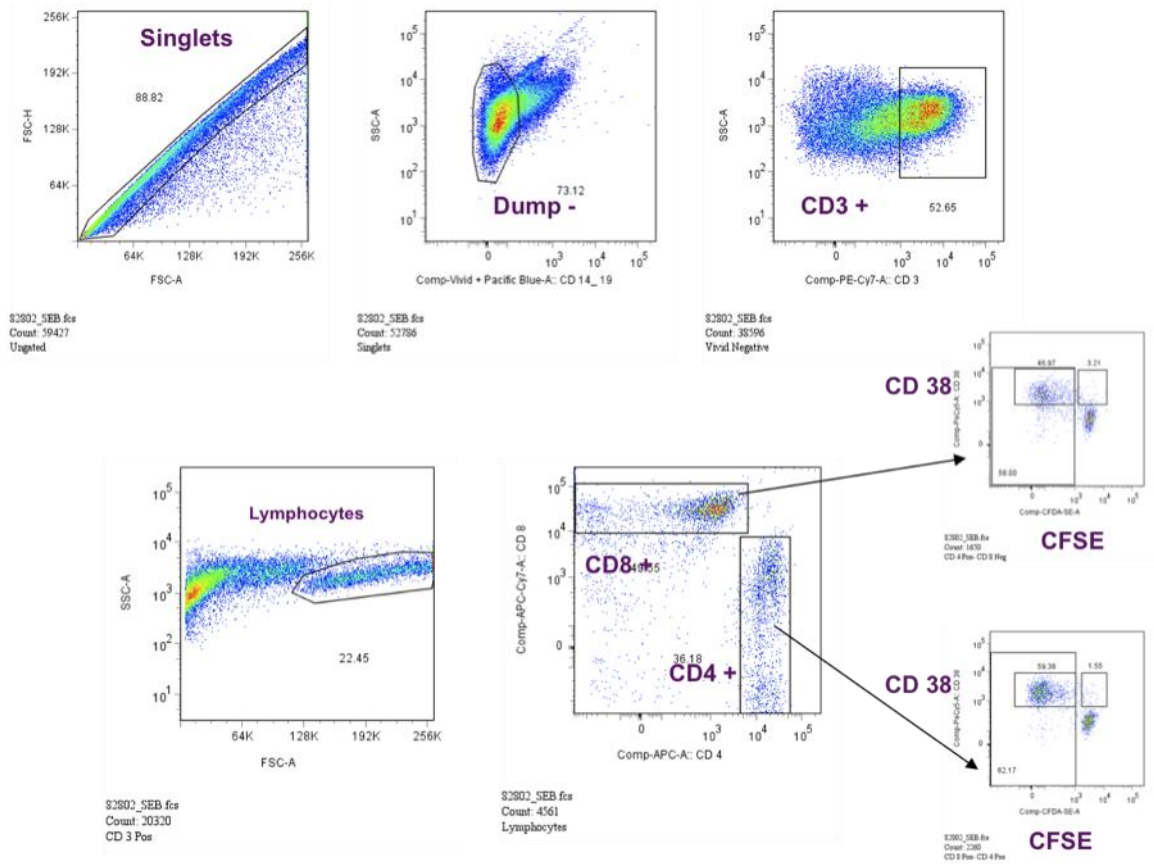

Fig. 9. Sample Gating of MLR Samples. This is an illustration of the manner in which the flow cytometry data was analyzed to identify the population of interest. The gating schema was: Singlets> Dump negative> CD 3 positive> Lymphocytes> CD 4 positive and CD 8 positive> their respective CD38 positive/ CFSE Dim populations.

Additionally, we also used antibody capture beads instead of cells to set up the compensation for the flow cytometer. Compensation is an essential initial step in the analysis of cell samples using flow cytometry to ensure that the flurochromes, which are being used to stain the cell surface markers, are fluorescing in the appropriate range with minimal overlap between the different colors. These compensation beads are coated with an antibody against mouse Ig kappa (Igκ), since the vast majority of monoclonal antibody that we used in flow cytometry experiments are of the Igκ isotype. Therefore, the beads

are used to set up the machine instead of using cells. The use of beads has some advantages over the use of cells. For instance, especially under circumstances where the quantity of cells is scarce and would more efficiently be used for analysis, the beads provide an avenue whereby the cells do not have to be used to set up compensation. Additionally, the fluorescence intensity obtained from beads is more than that which is seen when using cells, which is quite helpful in more clearly demarcating a distinct signal during the stage of data analysis.

Using CFSE, Vivid, and the aforementioned monoclonal antibodies in their respective flurochromes, the culture samples were progressively gated in order to ensure systematic analysis of the cellular response, as illustrated in Figure 9. Using forward scatter height (FSC- H) versus forward scatter area (FSC-A), the cells that were singlet were selected. This gate thereby removed cells fragments, debris, or clumps of cells from subsequent analysis.

Then, additional cell types that we desired to exclude were eliminated with the "Dump" gate. Three classes of cells were included in this gate: Vivid positive, CD 14 positive, and CD 19 positive cells. Once cells have died or undergone damage to their cellular membrane they can produce artifacts in immunophenotyping analysis by flow cytometry. For example, dead cells can nonspecifically bind monoclonal antibody conjugates, potentially leading to erroneous conclusions, particularly when cell frequencies are low. Thus, Vivid positivity was used as a marker for dead cell exclusion. CD 14, which is cell surface marker for monocytes, and CD 19, which is a marker for B cells, were respectively used to exclude these cell types. Each of these cell surface markers were stained with a flurochrome that fluoresced within the same range, namely

Vivid and Pacific Blue for CD 14 and CD 19. In this way, the cells from these three classes were pooled together and excluded as an aggregate. For the purposes of gating, the Dump negative cells are those that were selected.

The cells that were CD 3 positive were then gated, as this is a marker for all lymphocytes. The lymphocyte population was then more clearly isolated using FSC versus SSC to separate this population from cells other than those of interest that had not already been excluded. Next, using CD 4 versus CD 8, the cells were further separated into helper and cytotoxic T cells, respectively.

Finally, these two T cells populations were independently assessed for proliferation and activation using CD 38 positivity and CFSE dilution (CFSE Dim). A myriad of markers exist that can be used to assess for the activation of T cells. However, we chose to use CD 38, which is a cell surface marker that is located on the cell surface of many immune cells, including CD 4 and CD 8 T cells; it denotes the presence of granzyme.

CFDA SE or CFSE (carboxyfluorescein diacetate, succinimidyl ester) is a substance that passively diffuses into cells. It is colorless and non-fluorescent until its acetate groups are cleaved by intracellular esterases to yield highly fluorescent, amine-reactive carboxyfluorescein succinimidyl ester. The succinimidyl ester group reacts with intracellular amines, forming fluorescent conjugates that are well- retained and can be fixed with aldehyde fixatives. Excess unconjugated reagent and by-products passively diffuse to the extracellular medium, where they can be washed away. The dye–protein adducts that form in labeled cells are retained by the cells. The label is inherited by daughter cells after cell division and is not transferred to adjacent cells in a population. Thus, CFSE dye dilution can be used to categorize a proliferating population of cells as

the serial halving of CFSE occurs with each cell division (4). This technique allows for the visualization of eight to 10 discrete cycles of cell division. Hence, through the series of gates the cell population of interest was those that were CD 38 positive and CFSE dim.

Based upon the results from the completely mismatched one- way MLR, the set-up that we used for our MLR did work. We were able generate a strong proliferative response in several donor pairs. A representative of this response is depicted in Figure 3, which shows proliferation in the CD4 and CD8 T cells above twenty percent after eight days in culture. Although this data does not directly address our hypothesis, it reinforces the validity of the MLR design. Therefore, we could be confident that the small precursor frequency of T cells elicited in HLA-A2 mismatched MLR would be detected.

Subsequently, after analysis of the HLA-A2 mismatched MLRs, we found very low frequencies of proliferating cells in response to the transfected HLA-A2. This result was consistent across the three different antigen-presenting cells, TAPCs, BAPCs, and DCs. Results for the HLA-A2 mismatched MLR for the TAPCs were not displayed in the results section because they were similar to that obtained in the BAPCs; a proliferative response was not generated.

Although T cells and B cells can both present antigens, one of the main functions of dendritic cells is to process antigenic material and present it on its surface to other cells of the immune system. Hence, it was anticipated that the dendritic cells were the only ones that showed a proliferative response, albeit low, in the HLA- A2 mismatched MLR. Once the background proliferation in the negative control was subtracted from the stimulator- responder pair, 2% proliferation in the CD 8 cells was revealed.

One of the factors that may have contributed to the low level of proliferation in the HLA-A2 mismatched MLRs was the limited number of precursor T cells that responded to the antigenic stimulus. Various researchers have postulated as to the frequency of this T cell population in the alloimmune response. Data suggests that the precursor frequency in complete mismatch is 1/1,000, in partial mismatch 1/10,000, and 1/250,000 in matched siblings (1). While the precursor frequency of responding T cells have been underscored in these scenarios, it is unclear what it is in the case of a single transfected mismatched MHC. It could be assumed that the frequency would fall somewhere between the ranges of partial mismatch and matched siblings. Thus, it is possible that less than 1/100,000 T cells may respond.

With such a small population of cells potentially responding to a mismatch at only one of the HLA loci, it is understandable that the proliferative response, if present, was minimal. Furthermore, of note, there are other systems to determine the precursor frequency of the alloreactive T cells. These include tritiated thymidine ($[^3H]T$) uptake and cytotoxic T- lymphocyte precursor (CTL_P) assays, which were not evaluated in our investigations. These assays may have been more sensitive in detecting the alloreactive T cells than the flow cytometric- based method that we utilized.

CHAPTER THREE

T Cell Line (TCL)

BACKGROUND

A diversity of techniques with varying degrees of sensitivity exist that may detect the alloimmune response of the responding T cells at the level of individual cells. One of the primary methods analyzes the cytokine production by CD 4 and CD 8 T cells directly *ex vivo*. In this manner, the qualitative and quantitative characteristics of the alloantigen-specific T cell repertoire may be elucidated without the introduction of *in vitro* artifacts. Nevertheless, as the Barrett Lab in the Stem Cell Allo- Transplantion (SCAT) section of the Hematology Branch within NHLBI has shown with BK virus responses, the precursor frequency may be too low to detect any responses **(**Jongming Li, unpublished observations**)**.

Therefore, it is plausible that the sensitivity of the aforementioned MLR assay could be enhanced. This may be accomplished by setting up a proliferation-based assay that specifically detects the memory cell population of T cells. There are two methods to identify the memory population: an MLR can be allowed to proliferate for 7 days, after which time the memory T cell responses are analyzed. Alternatively, a primed lymphocyte test (PLT) could be established, where the response of memory T cells can be assessed after 4 days of proliferation. The assay can be enhanced by first boosting the responses *in vitro* by expansion, followed by a flow cytometry-based detection of intracellular cytokine production (ICS). This last technique to enhance the detection of the precursor T cell frequency is typically performed by stimulating the T cells three

times and testing the response at the end of the culture period. The release of intracellular cytokine could thus be assessed after each stimulation.

To describe the alloimmune response in the setting of a mismatch at the HLA-A2 locus so that the pathway for Class I antigen presentation might be further elucidated, the technique required further refinement. The antigen presenting cells used for this set of experiments were TAPCs. The cells were transfected with the respective genes encoding full length HLA-A2 for the generation of a T cell line, and empty plasmid for the control MLR.

In the anti-HLA-A2 alloresponse data reported thus far, we used an MLR to determine whether the anti-A2 response could be detected in the 7- day assay using dendritic cells (DC) or activated and expanded B- and T cells, B- and T-APCs respectively, transfected with HLA-A2. With this modification of the project, however, on the same day as the MLR is started we plan to also initiate the culture of a T cell line using the PBMCs of the same HLA-A2 negative donor stimulated with the HLA-A2-transfected APC. We preferably used dendritic cells for the initial stimulation, as they generated the strongest proliferative response in the previous tests with the HLA-A2 mismatched MLR. However, TAPCs were used for the subsequent stimulations. The cell lines were re-stimulated twice, for a total of 3 stimulations. At the end of the culture period the cells were assessed for the release of intracellular cytokines using CFSE-labeled responder cells. The CFSE served as a marker to allow for the visual discrimination of the APCs and responders in the flow cytometric analysis.

METHODS

Study Design for T Cell Line- The APCs, DCs and T APCs, were generated as described in the *Methods* section of Chapter 2. Following transfection with HLA- A2, the DCs and TAPCs were transferred to OKT3-coated T25 flasks in 10 ml IL-2 medium.

On ***Day 0,*** the PBMCs were counted and 10^7 cells were labeled with CFSE. The transfected DCs were counted and irradiated with 1 cycle, which equals 25 Gy. Then, the MLR was set up as described previously, although some cells were preserved so that they could be used to set up the flow cytometer. The T cell line was started by stimulating 5 x 10^6 unlabeled responder PBMCs with 0.6 x 10^6 or 1.25 x 10^6 irradiated (25 Gy) HLA-A2-transfected DCs in a 24-well plate, 1 well per condition, 2 ml complete medium per well.

On ***Day 3***, IL-7 (5 ng/ml final concentration) was added to the T cell line and two more T75 flasks were coated with OKT3. On ***Day 4***, the TAPCs were transferred to the two newly coated T75 flasks and continued in culture at 0.5 x 10^6 cells/ml in IL-2 medium. These cells would be transfected on day 6. The MLR was also harvested and analyzed by staining for Vivid, CD3, CD4, CD8, CD14, CD19, and CD38. On ***Day 5***, IL-2 was added to the T cell line to a final concentration of 50 IU/ml. If the color of the medium and the cell density warranted at this time, then the cells were transferred to a 6-well plate. On ***Day 6***, the TAPCs were transfected with HLA- A2 so that they could be used to re-stimulate the T cell line at a 5:1 TCL: APC ratio. On ***Day 7***, a cell count of the T cell line was obtained after half of the old medium had been removed and the cells had been re-suspended. A cell count was also performed on the transfected T-APCs from day

6. The transfected TAPCs were irradiated at 25 Gy and re-suspended at 2 x 10^6 cells/ml complete medium with 5 ng/ml IL-7. These TAPCs were then used to re-stimulate the TCL with HLA-A2-transfected, irradiated T-APCs at a responder to stimulator ratio of 5:1, if possible.

On ***Days 8*** and ***15***, IL-2 was added to the TCL at a concentration of 50 IU/ml. Also, three more T75 flasks were coated with OKT3 in preparation to make more TAPCs. The TCL was checked regularly to ensure that the medium did not turn yellow, denoting that it was old and needed to be changed. On ***Days 11 and 18***, a minimum of 5 x 10^7 cells TAPCs were re-stimulated on 2-3 OKT3-coated T75 flasks at a cell concentration of 0.5 x 10^6 cells/ml. On ***Days 13*** and ***20***, these same TAPCs were transfected with HLA-A2 antigens and with control plasmid. For the ICS, we aimed to have 0.5 x 10^6 (control and HLA-A2) transfected T-APCs, which meant that we would need 10-fold more cells in the transfections to generate the APC for the ICS alone. For the re-stimulation, we used an APC expressing full length HLA-A2.

Finally on ***Days 14*** and ***21***, the TCL line was re-stimulated. On day 21, the intracellular cytokine staining was performed. For the ICS, 9 x 10^6 cells from the TCL were first labeled with CFSE. Some of the unlabeled cells as well as some of the labeled cells that were unstimulated were kept aside so that they could be utilized to set up the flow cytometer. Then, the following conditions, all of which would occur in a in 1 ml complete medium containing 1 μl Brefeldin A/ml medium in a sterile 10x75 mm polystyrene Falcon tube, were created.

1. The cells (2 x 10^6) alone as a control.

2. The cells (2 x 10^6) from the TCL stimulated with 0.5 x 10^6 25 Gy irradiated empty plasmid-transfected T-APC.
3. The cells (2 x $10^{6)}$ from the TCL stimulated with 0.5 x 10^6 25 Gy irradiated HLA-A2 transfected T-APC.
4. The cells (2 x $10^{6)}$ from the TCL stimulated with SEB.

In order for them to be used a control for setting up the flow cytometer, 10^6 CFSE-labeled TCL cells were set aside. Likewise, an equal number of unlabeled cells from the TCL were used as an auto fluorescence control in the FACS experiment. The cells were incubated in the same FACS tubes for 6 hours at 37^0C. After this incubation, they were placed on ice in a Styrofoam box stored in the cold room overnight. The next day, they were stained with Vivid, cell surface markers (CD14, CD19 Pacific Blue; CD3 Cy7PE, CD4 Cy5.5PE, CD8 Ax750APC), and cytokines (TNFα PE; IFNγ APC).

RESULTS

Prior to starting the T cells lines, approximately one dozen HLA- A2 mismatched MLRs were performed in an effort to observe and characterize the alloimmnune response. Of the many HLA-A2 negative donor samples that were used, two in particular were able to generate a strong proliferative response. The HLA-A2 mismatched MLR results of one of these donor samples, 12113, were shown in the second chapter. As was noted at that time, 12113 expressed the HLA-A01 phenotype. On the other hand, the

other donor pair that yielded positive results in the HLA- A2 mismatched setting was donor 42502, which was HLA-A03, 11.

As was emphasized before, both of these donor samples express alleles at the HLA-A locus that are phylogentically distant from HLA-A0201. Hence, a response in these samples concurs with our hypothesis that more distant alleles phylogenetically from the HLA- A0201 allele would be more apt to elicit a response. Furthermore, we postulated that the donor samples 12113 and 42502, which expressed a proliferative response in the mismatched HLA-A2 MLR, would generate an enhanced response after being primed in a T cell line.

Thus, the responder cells were stimulated three separate times with HLA-A2 transfected APCs. The first time, DCs were used as the stimulators. However, all subsequent stimulations were performed using TAPCs. The response to the HLA-A2 mismatch was evaluated using intracellular cytokine staining for interferon (IFN) gamma and tumor necrosis factor (TNF) alpha.

Figure 10 demonstrates that the cells alone did not release the intracellular cytokines TNF alpha or IFN gamma. This result is that which was anticipated, as these cells were not stimulated with any HLA-A2 transfected APCs. Furthermore, the results are quite comparable in the CD4 and CD8 T cell populationa.

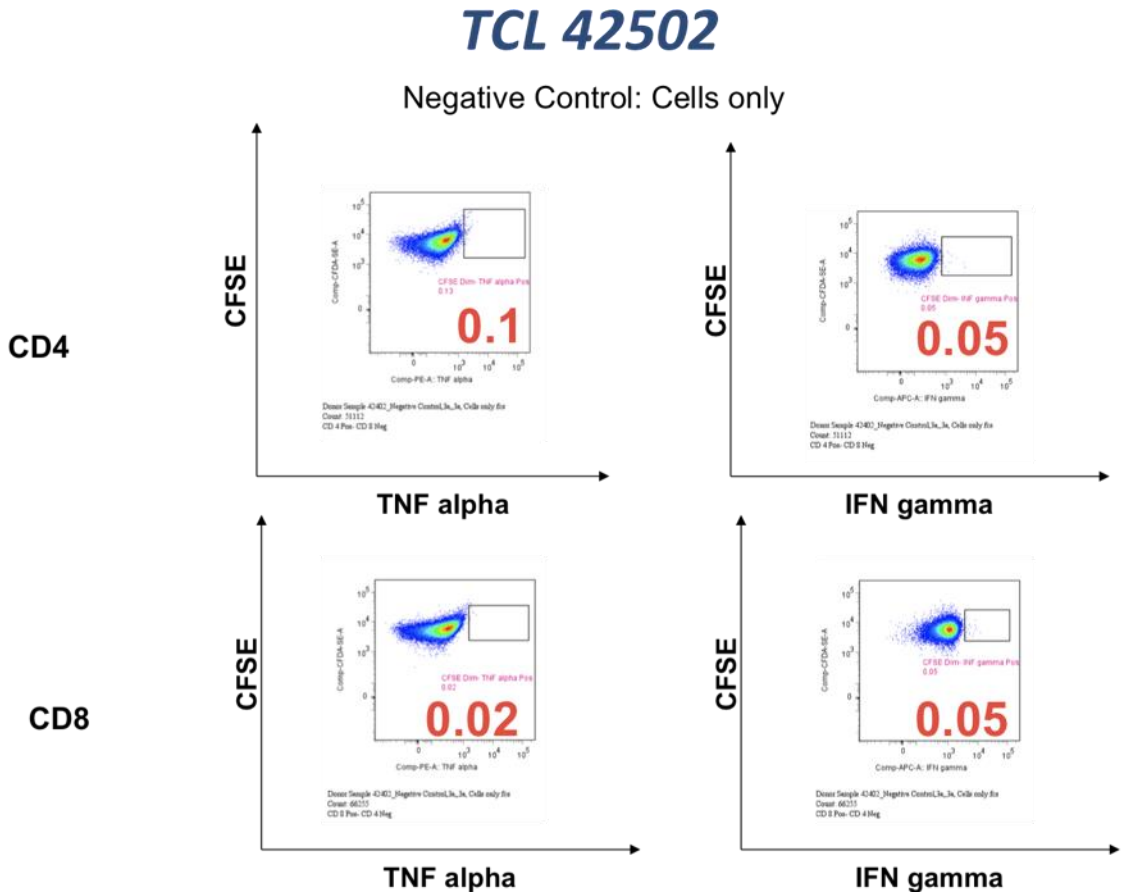

Fig. 10. T Cell Line (TCL) Negative Control with Cells Only. There is a negligible amount of cytokine release in these this un-stimulated cell culture.

On the other hand, the positive control with SEB, shown in Figure 11, reveals that the bacterial antigen was able to induce the release of the intracellular cytokines that were under observation. The amount of cytokine released by the CD 4 T cell population was greater than three times that released by the CD 8 T cells, when measuring for both TNF alpha and IFN gamma.

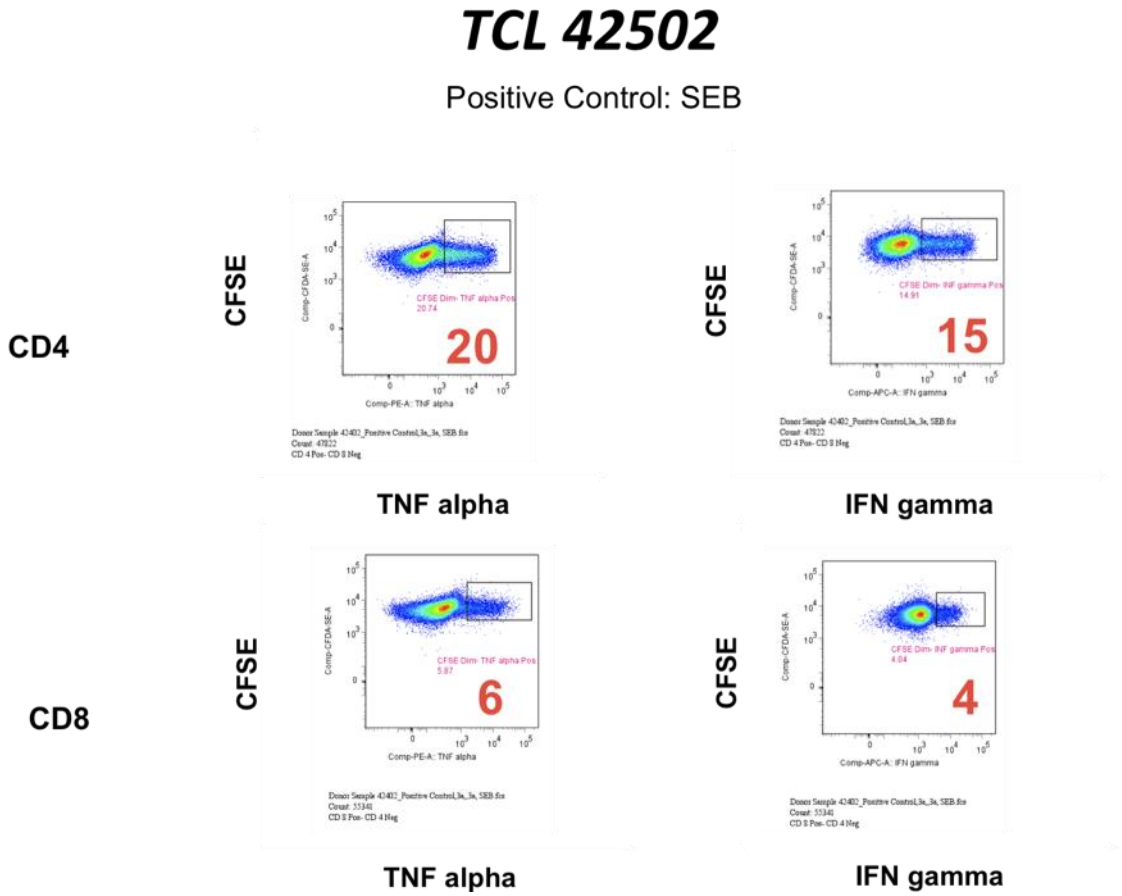

Fig. 11. T Cell Line (TCL) Positive Control with SEB. The cytokine release was much greater in the CD 4 T cell population than the CD 8 T cell population after stimulation with SEB.

In contrast, despite three distinct stimulations with HLA-A2 transfected APCs, the experimental T cell line, showed intracellular cytokine release on par with that which was observed in the negative control of cells alone. Neither interferon gamma nor tumor necrosis factor alpha was released to a significant degree from the CD 4 or CD 8 cells after three primings.

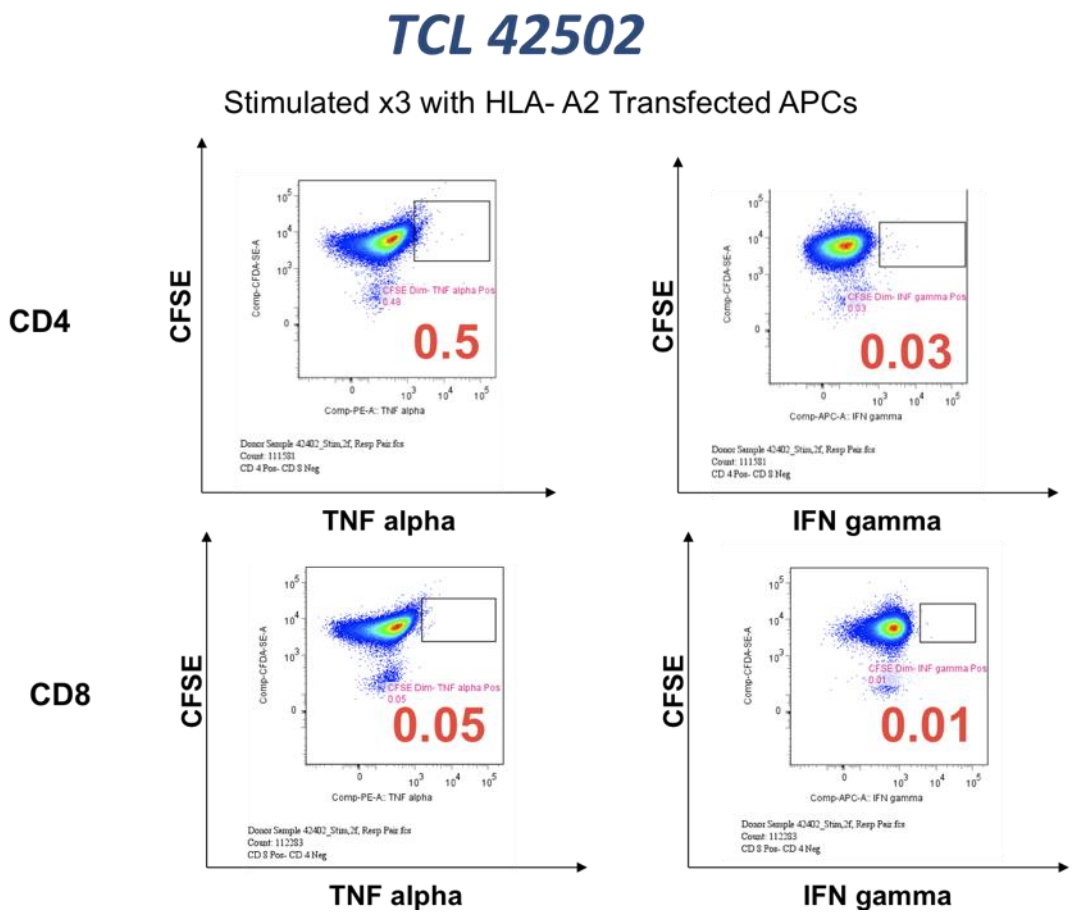

Fig. 12. T Cell Line (TCL) Stimulated with HLA-A2 Transfected APCs. Although we anticipated a boost in the alloimmune response after multiple stimulations, this TCL shows almost no cytokine release of interferon gamma or tumor necrosis factor alpha.

Thus, the data from the T cell lines did not produce the enhanced response that had been anticipated. Twenty- one days in culture with three stimulations was not sufficient to produce an alloimmune response that could be characterized by intracellular cytokine release.

DISCUSSION

The following flow cytomertic graphs for the T cell lines were progressively gated in the same manner as those for the MLRs, as outlined in Chapter 2. Figure 14 illustrates the selection of the singlets, Dump negative, lymphocytes, CD 3 positive, and CD 4 and CD 8 T cells. The analysis diverged, however, at the level of the CD 4 and CD 8 T cells. At this stage, the two T cell populations were individually graphed for CFSE versus INF gamma and CFSE verus TNF alpha. These final figures are those that were displayed in the results section of this chapter.

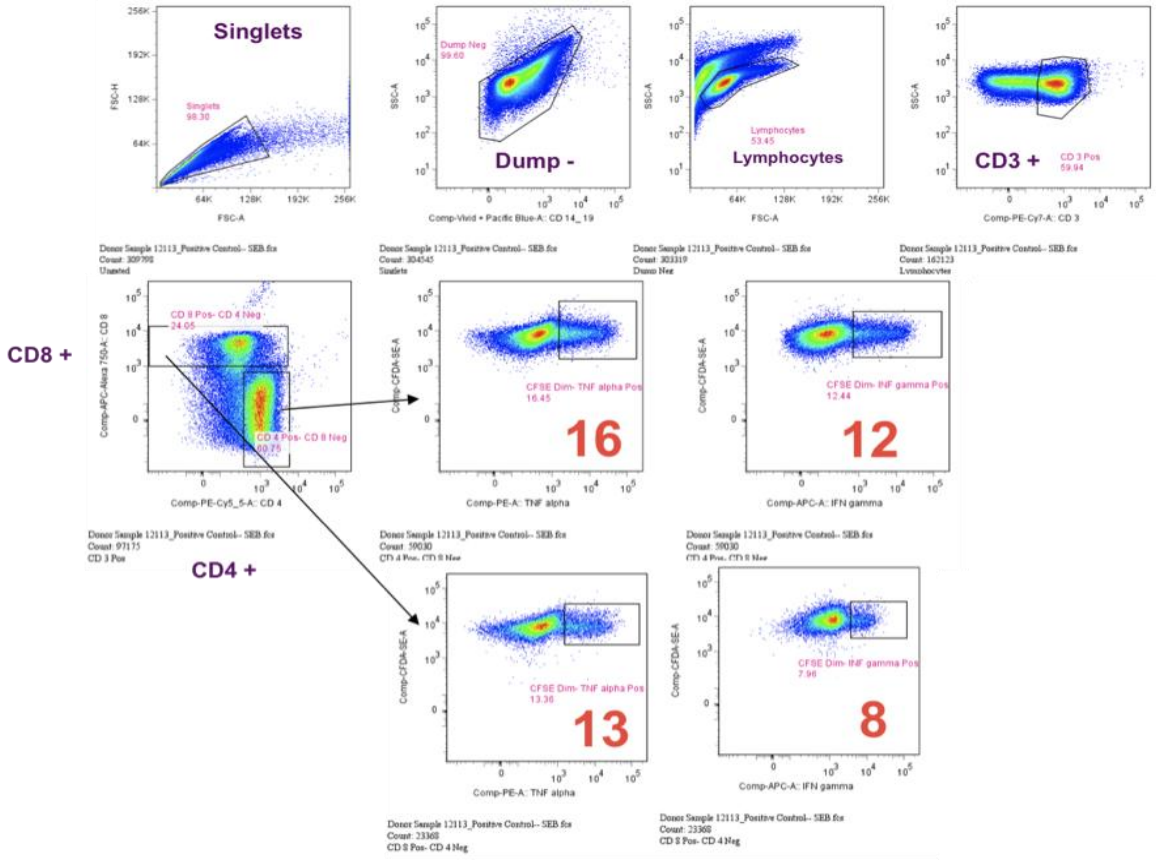

Fig. 13. Progressive Gating for T Cell Line (TCL). This is an illustration of the schema that was used to analyze the flow cytometry data from the TCL.

Although the T cell line was cultured for twenty- one days, no alloimmune response was generated. Intracellular cytokine was not released after three stimulations with HLA- A2 transfected APCs in the experimental sample. Nevertheless, one distinction between the experimental T cell line and the negative and positive controls was observed in Figure 12. Although it was not quantitatively measured, it is clear that there was a distinct proliferating population of T cells in the CD 4 and CD 8 T cells. This proliferating population is the small cluster of cells that is below the larger cluster of cells in that same figure. These cells are CFSE dim, and probably proliferated in response to the antigenic stimulation from the HLA-A2 molecule. This CFSE dim population is absent from the positive control data shown in Figure 11. Hence, even though intracellular cytokine is not released after the three HLA-A2 transfected APC stimulations, proliferation does occur. This unexpected finding warrants further investigation.

CHAPTER FOUR

Conclusions & Recommendations

Mismatched MLR

Conclusions- The completely mismatched mixed lymphocytic reaction data demonstrates that we were able to induce significant proliferation in a fully HLA- mismatched setting. This data was replicated in several stimulator- responder pairs and revealed that the set-up of our one-way MLR was indeed valid.

Recommendations- These studies established that a strong proliferative response could be generated after only eight days in culture. Although all of the data from these associated experiments was not shown, we initially analyzed the mismatched MLR at several different time points. We performed flow cytometry on the cells after Days 4, 6, 8, and 10. From these studies, we found that Day 8 is the optimal time point to analyze for proliferation and activation. In addition to requiring less time, the majority of the MLRs showed decreased proliferation between Days 8 and 10. Furthermore, we felt confident that 7 days was enough time for the cells to proliferate in the MLRs. Thus, all of he HLA-A2 mismatched MLRs were analyzed at this time point.

HLA- A2 Mismatched MLR

Conclusions- Most of the cells did not proliferate in the setting of HLA-A2 mismatch in the MLRs. The most promising, although limited, response was observed on the HLA-A2 mismatched MLR where dendritic cells were used as the stimulator. We postulated that the low precursor frequency of T cells limited the ability to detect proliferation and activation in the mismatch at the HLA- A locus only.

Recommendations- The data from the HLA-A2 mismatched MLRs suggests that further efforts to enhance the characterization of the precursor frequency T cells needs to be implemented. For example, future experiments may require a larger number of cells to increase the likelihood of being able to phenotype them. Also, as the dendritic cells showed the most promising data in the role of antigen presentation, perhaps they should be preferentially used over BAPCs and TAPCs in future work. Additionally, only after the alloimmune response is elicited to a significant degree can we proceed to evaluate the relative contribution of the memory and naïve T cell subsets, as well as identify the clonality of the responding T lymphocytes using Vβ analysis.

TCL

Conclusions- In regards to the T cells line, we need to further develop a technique to prime the T cell response so that proliferation and activation can be more clearly

elucidated. It would then be possible to study the contribution of the direct and indirect pathways of antigen presentation.

Recommendations- As suggested for the HLA-A2 mismatched MLRs, it may be optimal for the T cell line to be stimulated each time with dendritic cells as opposed to using dendritic cells only for the first stimulation. It could be that stimulation with dendritic cells at three discrete time points could boost the alloimmune response to a level where the release of intracellular cytokines might be detected. Additionally, an increase in the initial cell count used in the culture of the T cell lines might provide a greater chance of characterizing the cells of interest.

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VITAE

Melody Smith was born in Dallas, Texas on March 26, 1981, as the sixth of seven children to Arthur and Barbara Smith. After graduating from John Tyler High School in Tyler, Texas with an International Baccalaureate Full Diploma in 1999, she entered Vanderbilt University in Nashville, Tennessee. Throughout her undergraduate education, she was involved in research, although it was not until the fall of her senior year in college, when she was involved in a cancer drug trial at the Vanderbilt University Medical Center, that she saw the union of her interest in research and passion toward cancer medicine. Her experience as an undergraduate established a solid foundation and was also the impetus for her desire to seek out other clinical research avenues. Melody graduated from Vanderbilt in 2003 with a Bachelor of Science in Spanish and Biology. She worked as a fifth grade bilingual teacher in Tyler, Texas for one year prior to matriculating to medical school at the Universidad Central del Caribe School of Medicine in Bayamón, Puerto Rico. Under the auspices of the American Society of Hematology (ASH), she conducted research at the Emory University Winship Cancer Institute during the summers following her first and second years of medical school. As a result of this experience, she decided to transfer to the University of Texas Southwestern Medical Center in Dallas, Texas in the fall of 2006 so that she could have a greater level of exposure to research during her medical school training. Then, between her third and fourth years of medical school she took time off to conduct a year of research in the Hematology Branch of the National Heart, Lung and Blood Institute at the National Institutes of Health (NIH) through the Clinical Research Training Program (CRTP). This fellowship exposed her to all facets of the research process, providing both basic and translational perspectives. Furthermore, the investigations performed during this research year are presented in this thesis. Melody will complete her Doctorate of Medicine, with a Distinction in Research in June 2009. She will conduct her internship and residency in Internal Medicine at the University of Texas Southwestern Medical Center in Dallas, Texas, and then plans to complete a fellowship in adult Hematology- Oncology.