CHAPTER 4

DISCUSSION

Project I: Clonal composition of neuroantigen-specific CD8+ and CD4+ T cells in multiple sclerosis

In this study, we characterized the clonal composition of myelin-specific T cells in MS patients by performing an unbiased anchored PCR assay on the TCR BV chains of sorted CD4+ and CD8+ T cells. While there have been prior descriptions of TCR usage by myelin-specific T cells in MS, most of these are based either on long-term T-cell lines or on analysis methods that utilize CDR3 spectratyping (length) followed by detailed sequencing only of "major" peaks. Several unique features of our approach improve upon prior analyses. First, we utilized flow-cytometric sorting of CFSE-labeled PBMC cultured for seven days with pools of myelin peptide fragments spanning the entire length of both MBP and PLP. Thus, after relatively short term cultures, we were able to obtain antigen-specific CD4+ and CD8+ T cells from MS patients and healthy subjects. We have shown previously that these sorted cells represent highly enriched, HLA-restricted, antigen-specific T-cell populations (38). Second, by utilizing SMART-Race cDNA amplification in which an anchored RT-PCR is performed using a SMART switching mechanism in conjunction with a TCRβ constant region primer for the PCR we obviated the need for multiple TCR Vβ family primer sets and avoided any bias that may have arisen due to differing reaction efficiencies (52,53). Third, the direct sequencing of the CDR3 regions allowed us to formulate the clearest picture of the clonal distribution of myelin-specific T cells

without any presumption regarding the true sequence of the CDR3 region, based simply on TCRBV usage and CDR3 length. Indeed, numerous instances can be found where the N-D-N regions are of the same length, but contain completely different amino acid sequences. Fourth, with regard to the circulating peripheral population of T cells in MS patients, the clones of interest may not always be located in the dominant peak of a spectratype. Thus, we were able to generate a dataset containing over 500 TCR sequences from myelin-specific T cells. Finally, this approach has allowed me to provide the first detailed descriptions of myelin antigen-specific CD8 TCR sequences from MS patients and healthy subjects.

In general, populations of CD4+ T cells responding to MBP were polyclonal. Although two of the healthy subjects, H333 and H267 had some prominent clones peaking at 22.6% and 28.6% respectively, the CD4+ T cells from the MS patients possessed no single outgrowth greater than 16%. PLP-specific CD4+ T cells were also polyclonal with none of the clones having more than 10% representation in any MS patient or healthy subject's response. In contrast, MBP-specific CD8+ T cells from all three healthy subjects were less polyclonal than their CD4+ counterparts, showing clear prominence of some clones. This finding correlates with previous reports showing healthy subjects with oligoclonality in their CD8+ T-cell populations (60). However, the MBP-specific CD8+ T cells from MS patients (especially M210 and M250) were truly polyclonal, similar to our prior finding that glatiramer acetate (GA)-reactive CD8+ T cells from MS patients are polyclonal, distinct from those of healthy subjects (53). This difference in clonal distribution is intriguing, especially in the context of previous findings that, similar to GA-specific CD8+ T cells (61) CNS-specific CD8+ T cells also appear to have an important immune regulatory role during

CNS demyelinating disease (42,43). Thus, the oligoclonal myelin-specific CD8+ T cells from healthy subjects appear to have robust immune regulatory properties, which are lacking in the polyclonally distributed CD8+ T cells of MS patients, especially during clinical relapses (43). Therefore, while we did not observe specific TCRBV usage differences in the CNS-specific CD8+ T-cell responses between healthy subjects vs. MS patients, just the differences in the clonal distribution may indicate the lack of an important regulatory CD8+ T-cell subset in baseline MS. While prior studies have found some global CDR3 distribution differences in CD8+ T cells in MS patients vs. healthy subjects (62), this difference does not seem to represent CNS-specific CD8+ T cells. These findings correlate with a previous study finding little or no difference in the clonal composition of myelin antigen-reactive T cells between MS patients and healthy subjects (25). Possibly a more detailed breakdown of the CD8+ T-cell population would provide more insights, such as one performed by Somma et al., where they found a skewed repertoire in 3 pairs of MS discordant twins when they divided the CD8+ T-cell fraction into CCR7 negative (effector memory) and CCR7 positive (central memory, naïve) fractions (63).

Reports of skewed Vβ usage in MS patients have been controversial. No difference in peripheral blood TCR Vβ chain expression was found between MS patients and healthy subjects when investigated with fluorochrome conjugated antibodies to Vβ segments (48). However, two studies confirmed skewed repertoires with respect to Vβ23 by using other methods (37,63). Other studies have reported that Vβ5-family CDR3 gene segments were oligoclonally expanded in MS patients when compared to healthy subjects (49,64). Another recent study performed laser micro-dissection of brain autopsy specimens from multiple sclerosis patients and found expanded Vβ5

family TCR from CD8+ T cells in 3 of 4 patients studied (57). In our findings, MBP-stimulated CD4+ T cells from MS patients showed high levels of clonally expanded BV5 family TCR (21.7%) when compared to their healthy counterparts (6.3%). When looking at TCRBV5 family usage among the PLP-reactive T cells we found an increase in the CD4+ T cells from patient M210 (21.2%) when compared to the CD4+ T cells from healthy control H333 (14%). Thus, the higher representation of specific CD4+ T cell clones may reflect the inability of regulatory mechanisms to control the expansion of pathogenic clones in the context of MS.

Using our newly generated TCR database, we were able to query the Genbank database for matches of interest. Although MS patients and healthy subjects possess myelin-specific T-cell responses at comparable magnitudes (38), the TCR involved in these responses are quite distinct, in that TCR from healthy subjects did not generate any near-matches in previously published MS patients. In contrast, a number of TCR sequences from MS patients showed matches with sequences obtained from MS patients in prior studies (36). In fact, even matches that we deemed marginal (3 consecutive amino acids inside the hyper-variable region) were not found between this healthy cohort and previous MS sequences.

Interestingly, patient M971 possessed MBP-reactive CD8+ T cells that have very similar TCR sequences to CNS-infiltrating CD8+ T cells found in micro-dissected lesions from the study by Babbe et al (36). The clone containing the N-D-N sequence LAGQG (Table 9) was a close match for two clones from the Babbe et al., study, one of which matched the Vβ5.1 used and one that did not. This particular clone represented 12.5% of total clones (4/32). A second clone with N-D-N sequence TRTGSG was a close match for yet another clone from the same previous study and

both used Vβ5 family gene segments. This clone represented 6.25% of total sequences (2/32). We believe these findings of near-matching TCR from CD8+ T cells is both very rare and quite interesting when considered in light of other observations such as those made in a previous study by Skulina et al. in which they found clonally expanded CD8+ T cells in MS brain lesions. These exact clones were also found in the peripheral blood and were found to persist for years (65). The precise function of such expanded clones during the disease process (pathogenic vs. regulatory) will be of important consequence in understanding the underlying pathogenesis and devising better immunotherapeutic strategies in the future.

DISCUSSION

Project II: Reduction of CD147 surface expression on primary T cells leads to enhanced proliferation

CD147 is a pleiotropic molecule that is widely expressed in a variety of cell types. Perhaps some of the difficulty in illuminating the myriad possible functions and associations of CD147 lie in the very fact that they are so numerous. In this study we have tried to shed some light on the possible functions of CD147 in primary T cells. As this is the first report showing a transient knockdown of CD147 in primary T cells that I am aware of, I started with the most basic parameters with regard to T-cell function; proliferation upon activation and expression of surface markers and cytokines.

The expression levels of CD147 were clearly upregulated when T cells were activated and expression levels of CD25 and CFSE proliferation confirmed the activation state of the T cells (Figure 8). Both superantigen stimulation and direct stimulation using anti-CD3 mAb coupled with costimulation from anti-CD28 mAb produced similar results (Figure 9). The increased expression levels upon activation maybe linked to some function of CD147 dependent upon the formation of homo-dimers. It has been shown that CD147 will accumulate in lipid rafts upon T-cell activation (115) and Yoshida et al showed that the formation of these homo-dimers was dependent largely upon the N-terminally located Ig-like domain of the extracellular portion. In fact if the disulfide bond in this domain was destroyed by mutation CD147 could not form the homo-dimer. In contrast mutations in the C-terminal Ig-like domain or N-glycosylation sites didn't affect the association (94).

Further evidence for the significance of the homo-dimer comes from a study which found that a certain mAb. MEM-M6/6 is able to significantly reduce anti-CD3 mediated activation of T cells (up to 80%) only when administered in a certain dosage range. High dosage and low dosage do not produce the effect. The author postulates that this is because at high concentrations the antibody may bind monovalently due to the saturating conditions and at a low dose the antibody will bind but there may not be enough bound to pull together a significant number of CD147 dimers (115).

Furthermore, a recent study found that the CD147 Ig1 domain has been found to form a highly stable 'swapped dimer', where one C-terminal β-strand (residues 94-103) folds into the other monomer (125). However, there is no evidence of a monomer/dimer equilibrium for CD147 and all extracellular forms have been found to be monomeric. It remains to be seen whether this phenomenon is an artifact or misfolding of recombinant Ig-like domains, or there is some as yet undiscovered mediator of this domain swapping.

It is also worthy of note that the entire CD147 ectodomain as well as the individual Ig-like domains are cleaved from the cell surface by many of the same MMP's that are secreted due to CD147 stimulation, and these extracellular CD147-derived peptides maintain their stimulatory activity (84, 129).

Where others have had success in establishing a CD147 knockdown in Jurkat, my experience with the cell line did not produce significant results. Since other researchers have already performed these experiments and published results I did not wish to pursue experiments using the Jurkat cell line. The time I spent working with Jurkat was only meant to be used to titrate siRNA concentrations, and there was no need to pursue a perfect knockdown in Jurkat once the experiment had outlasted it usefulness.

While we expected an increase in CD25 levels to go hand-in-hand with the increase in proliferation observed in the knockdown cultures, such was not the case. More often than not there was an increase in CD25 expression in the knockdown culture, but this increase did not correlate with the amount of CD147 knockdown achieved. Also, in some cases there was no change in CD25, and even a few cases in which this trend was reversed. When these data are viewed (Figure 17) it can be seen that there is in fact, no significant difference in CD25 expression that relates to CD147 knockdown on the surface of T cells. With the exception of the outlier that had 3-fold higher expression of CD25, most of the data points are grouped at or around 1.00, no matter what the level of CD147 reduction.

Our finding that a transient reduction in CD147 expression resulted in enhanced proliferation is indeed very interesting. Currently there are limited reports that comment on this very subject and they have achieved opposing results. Igakura et al found that CD147 knockout mice are characterized by enhanced mitogenic response of T lymphocytes in mixed lymphocyte reactions (126). In addition to the support this report lends to our findings, another study concludes that CD147 is an integral component of the T-cell immune synapse and that its overexpression leads to the inhibition of NF-AT and this phenomenon is mediated by the intracellular tail of CD147. Also, CD147 knockdown in Jurkat cells promoted higher levels of NF-AT stimulation and Pak1 phsophorylation upon TCR cross-linking. They concluded that CD147, via selective inhibition of specific downstream elements of the Vav1/Rac1 route, contributes to the negative regulation of T-cell responses (124). In yet another study T cells from SLE patients were found to have increased expression of CD147, and these T cells showed a reduction in tyrosine phosphorylation levels when activated (113).

In contrast to these findings, another study claims that CD147 knockdown reduced proliferation and activation of the Jurkat T-cell lymphoma (112). Our thoughts are that possibly the level of detail in this study was not sufficient. Figure 18 shows the correlation between the reduction of surface CD147 and the subsequent increase in proliferation. This result can be compared with experiments in which a reduction in CD147 was not achieved. Failing to knockdown CD147 resulted in equal levels of CD25 and no change in proliferation. This could be explained by a possible failure to achieve activation in the culture. However, while the proliferation by CFSE was not strong (which is an expected result of the harsh transfection conditions) the level of CD25 upregulation shows that the cultures were indeed activated.

In light of the theory that CD147 density on the cell surface may indeed be critical for the MEM-M6/6 antibody to produce it's suppressive effect by signaling through CD147 dimers combined with the findings in the mouse knockout model, the SLE patients and the detailed findings regarding NF-AT phosphorylation, we support a model where CD147 does indeed have some negative regulatory effect on primary T cells. More studies are needed to address this phenomenon in primary T cells at the same level that it has previously been investigated in cell lines.

A significant difference in IFNγ levels was seen in two of the three knockdown experiments making use of intracellular cytokine staining. A 60% and 54% knockdown produced a 52% and 42% increase in IFNγ levels, however a third experiment which achieved a 35% reduction in CD147 did not show a significant change in IFNγ levels. Possibly this level of knockdown was not sufficient to affect a change in normal cellular function. The other cytokines IL-10 and IL-4, which were also tested intracellularly, showed vast changes however they were divergent so we cannot comment on the validity of these findings. A similar result was found when

CBA was used to measure culture supernatants for the presence of Th1/Th2 cytokines. Where it appeared that the assay was partially effective as it was possible to see drastic differences between activated/non-transfected and non-activated/non-transfected cultures, no solid conclusions could be drawn from the experimental data due to the high signal-to-noise ratio of the results.

CONCLUSIONS AND FUTURE DIRECTIONS

T-cell Receptor Sequencing in MS

Utilizing a novel combination of short-term culture, CFSE-based sorting and anchored PCR, we evaluated clonal compositions of neuroantigen-targeting T cells from relapsing remitting MS (RRMS) patients and controls. These results demonstrate distinct TCR usage by MS patients in their polyclonal CNS-specific CD4+ T-cell responses. In contrast to MS patients, healthy subjects exhibit oligoclonal CD8+ TCR distribution, which may reflect important differences in overall regulatory ability between MS patients vs. healthy controls.

Future studies are needed to address the functional role of these specific TCRBV myelin-specific T cells and address the question of whether these cells are playing a part in the pathogenic process of this autoimmune disease. This could be accomplished by focusing on the T cells circulating within the CNS of MS patients, such as the study by Skulina et al which found expanded CD8+ clones in the cerebral spinal fluid (CSF) of ms patients (65). If access to CSF was possible a combination of single-cell PCR methods similar to those used by Maryanski et al which found oligoclonality in the CD8+ compartment (137), and quantitative real-time PCR such as that used by Gillet et al while examining enhanced IL-18 receptor expression in the CSF of MS patients (138) could be used to better understand the character (inflammatory or suppressive) of CNS-resident T cells in MS.

Recent advances in the techniques used in this study have spawned a new generation of TCR clonality studies. High throughput sequencing has recently been used to gain new insight in the clonal diversity of T-cell subsets. This novel technology has expanded standard capillary electrophoresis-based sequencing to encompass a combination experimental/computational approach to measure TCR diversity based

on single-molecule DNA sequencing using the Genome Analyzer. This advance has made it possible to view millions of CDR3 regions per individual and researchers have found both more diversity in the TCR repertoire than expected and also less diversity between individuals than previously expected with haplotype-sharing individuals possessing up to 14% similarity in their TCR repertoires (139, 140, 141).

Understanding the role of CD147 expression on T cells in health and disease

In this study we were able to achieve transient reduction in CD147 gene expression in primary T cells. While obtaining consistent secondary readouts proved difficult I believe there is reason to continue this work given ample time and funding to transfect even larger pools of samples. I believe these experiments are important because all previous reports of CD147 knockdown in T cells were performed in cell lines. Indeed, there are many markers that may be investigated in primary T cells with the use of the Nucleofector if you have the time, money and patience to transfect a small number of cells per reaction many times in succession. The harsh reaction conditions necessitate transfecting a large population of cells to offset cell death while maintaining enough proliferating cells to let the experiment run for as many time points as possible. An ample supply of fresh whole blood will be needed and experiments will need to be repeated several times as it is difficult to transfect more than 8 tubes at a time while adhering to the standard of not allowing the cell culture to reside in the Nucleofection solution for more than 20 minutes.

Other researchers have reported similar difficulties with cell death, sometimes running as high as 50% when using the Nucleofector (127), however there have been reports of gene transfer into T cells causing apoptosis mediated by the TNF alpha receptor pathway even when other methods are involved (128). Because of the cost of the reagent for the Nucleofector we tried using PBS as a transfection solution based

on the advice of a colleague, however the results were unsatisfactory. We also tried a protocol using the Biorad machine with specific parameters, and weren't able to achieve the success we had with the Nucleofector. As of this date, the methods we used for the completion of these experiments are the best for achieving gene knockdown in primary T cells with siRNA, despite the costs and limitations of the system.

To continue these experiments, it would be very interesting to discover whether CD147 knockdown affects the formation of the immunological synapse by visualizing the cultures with confocal microscopy. Also a detailed analysis of tyrosine kinase cascade-protein phosphorylation would be very interesting to see in the same conditions as we ran these experiments.

A link between TGF-β and CD147 has been shown in corneal fibroblasts in which the addition of TGF-β increased the expression of both the protein and RNA levels of CD147. Interestingly the addition of TGF-β-blocking antibodies reduced CD147 levels (109). The link between the two was further bolstered by the fact that siRNA-mediated reduction of CD147 reduced downstream effects of TGF-β in this system.

TGF-β is generally known for its suppressive roles based on its ability to suppress IL-2 production (130) which has far-reaching implications in the immune response, and its ability to induce a master transcription factor of regulatory T cells, FoxP3 (131). The suppressive influence of TGF-β is also seen on B cells and NK cells and it also inhibits macrophage activation as well as the secretion of MMP-12 by macrophages (136). Additionally TGF-β has been shown to reduce the expression of MMP-1 and MMP-3 in dermal fibroblasts (135).

However, as it has recently been proposed that CD147 is a regulator of T-cell transmigration into the CNS in autoimmune disease by way of its ability to induce

MMP's, it is interesting to contemplate the associations between CD147, MMP's and TGF-β. Recent studies have found TGF- β is an essential element for the production of Th17 cells which play roles in inflammation and autoimmunity. Th17 cells and T cell-produced TGF-β1 have been shown to be critical for the induction of EAE (132). Additionally TGF-β secreted by breast cancer cells induce stromal fibroblasts to secrete MMP-9 (133), also TGF-β induces MMP-9 production in rat brain astrocytes (134) and it has long been established that CD147 induces MMP-9 (83), as well as several other MMP's. Based on this evidence an association between CD147 and TGF-β is sure to exist, but the relationship will likely be equally as complicated and diverse as the TGF-β story has become.

In conclusion, I have laid a foundation for the study of primary T cells with transiently reduced expression of CD147 by making preliminary investigations into proliferation, CD25 expression and cytokine profiles. Further investigation into more detailed alterations of T-cell biology are needed to better understand the role of CD147 in T-cell biology.