Title
B cells drive lymphocyte activation and expansion in mice with the CD45 wedge mutation and Fas deficiency

Author
Gupta, Vikas

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B cells drive lymphocyte activation and expansion in mice with the CD45 wedge mutation and Fas deficiency

by

Vikas Gupta

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Abstract

The role of CD45 in the immune system has been studied for many years. Our lab has proposed a model for CD45 regulation in which CD45 activity is inhibited by homodimerization. This inhibition is mediated by a structure called the "wedge," and mutation of the wedge prevents inhibition, resulting in background dependent autoimmunity. To study the contribution of the CD45 wedge mutation to autoimmunity on the C57BL/6 background, we crossed the wedge mutation to two other autoimmune prone strains of mice on this background, the complement *C4* knockout and the *Fas* mutation *lpr*. While combination of C4 deficiency with the wedge mutation did not alter the phenotype of mice compared to single mutant controls, the wedge and lpr mutations cooperated, significantly enhancing lymphoproliferation and autoantibody production. The lymphoproliferation consisted of both activated T and B cells. Restricting T cells to the ovalbumin peptide specific OT2 TCR transgene eliminated T cell activation and reduced lymphoproliferation, suggesting that signaling through the TCR was leading to T cell activation. Furthermore, both T and B cells were necessary for lymphoproliferation, as genetic deletion of either cell type also prevented lymphoproliferation. However, B cell activation remained increased even in the absence of T cells, suggesting that wedge/lpr mutant B cells are intrinsically activated and may be responsible for initiating the process of lymphoproliferation by activating T cells.

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Chapter 1: Background

Autoimmunity

One of the pitfalls of the adaptive immune system is the potential for randomly rearranged antigen receptors to be generated that recognize self-antigens and initiate an autoimmune response. Multiple mechanisms have evolved to prevent such a scenario from occurring. These mechanisms include both central processes that are used during development of T and B cells, and peripheral processes that maintain tolerance. Many of the inherited, highly penetrant, autoimmune diseases are caused by a single genetic lesion resulting in a major defect in one of these processes. However, for the pathogenesis of most autoimmune diseases, single genetic lesions are rarely sufficient. Instead, multiple interacting loci, each causing a relatively small defect in tolerance on its own, are likely to be necessary for most common autoimmune disease. Studying how these genetic modifiers interact and break tolerance is therefore critical to our understanding of autoimmunity.

One of the central processes responsible for eliminating a majority of auto-reactive cells is negative selection (Goldrath and Bevan, 1999). During their development, T and B cells are constantly exposed to self-antigen. Cells must be able to react weakly to these self-antigens to fully mature and survive. However, cells that respond too vigorously run the risk of causing autoimmunity and, therefore, are negatively selected by induction of programmed cell death. Two essential requirements for negative selection of T cells are the presentation of a broad range of self-peptides or proteins and appropriate signal transduction processes that induce death of developing cells. One of the molecules that is important for antigen presentation in the thymus is the protein AIRE, which has been shown to induce expression in the thymus of a broad range

of peptides normally found in peripheral tissues (Mathis and Benoist, 2007). The expression of these peptides ensures that the T cells that recognize this subset of antigens are deleted. In the absence of AIRE, these cells are not negatively selected and emerge into the periphery, where they encounter self-antigen and initiate an autoimmune response. For this reason, humans that lack AIRE develop a multi-organ autoimmune disease called autoimmune polyendocrinopathy with candidiasis and ectodermal dysplasia (APECED).

The other requirement for negative selection is appropriate signal transduction. Multiple molecules are involved in the transmission signals from the TCR (Kane et al., 2000). Negative selection occurs in response to strong signals, and therefore any defect in one of these molecules that reduces signal strength may prevent a cell with an auto-reactive TCR from being deleted. This is one hypothesis to explain the autoimmunity observed in mice carrying a spontaneously occurring ZAP-70 mutation (Sakaguchi et al., 2003). This mutation reduces the ability of ZAP-70 to interact with the TCR and to transmit signals, resulting in the survival of cells that would normally be deleted by negative selection. The surviving cells then induce arthritis in these mice under certain conditions.

Even when negative selection is working normally, it is not 100% efficient, and auto-reactive cells do escape into the periphery (Kim et al., 2007; Merrell et al., 2006). However, there are additional layers of protection designed to keep these cells quiescent and maintain tolerance. One way of preventing auto-reactive cells from becoming activated is to keep them from ever encountering their antigen on mature antigen presenting cells. Self-antigens are constantly produced by cell death, and thus clearance

of dead cell debris is thought to be one way of maintaining tolerance (Wakeland et al., 2001). A number of molecules have been implicated in this process. Complement proteins such as C1q and C4 are believed to bind to and aid in phagocytosis of apoptotic bodies; serum amyloid P component, DNase I, and DNase II may be responsible for the degradation and removal of nuclear components such as chromatin and DNA; and Axl, Mer, and Tyro3 are a family of kinases involved in macrophage phagocytic function (Lemke and Lu, 2003; Wakeland et al., 2001). Mice deficient in any one of these molecules develop autoimmunity. Furthermore, C1q and C4 deficiency are strong predisposing factors for lupus in humans, and some lupus patients appear to have reduced levels of DNase I, suggesting these factors are relevant for human disease as well (Manderson et al., 2004; Tsukumo and Yasutomo, 2004).

Regulatory T cells mediate a second method of tolerance maintenance (Zheng and Rudensky, 2007). These cells represent a special T cell subset that suppresses the activation of other auto-reactive T cells. The differentiation and maintenance of regulatory T cells requires the transcription factor FoxP3. FoxP3 deficiency causes an autoimmune disease in humans called immune dysregulation, polyendocrinopathy, X-linked (IPEX), and is responsible for the phenotype of scurfy mice, stressing the importance of regulatory T cells in prevention of autoimmunity. Although the exact mechanism of suppression is unclear, regulatory T cells secrete inhibitory cytokines, such as TGFβ, and loss of these cytokines can also result in autoimmunity (Rubtsov and Rudensky, 2007).

In addition to antigen clearance and suppression by regulatory T cells, proper control of signaling pathways also keeps potentially auto-reactive cells quiescent.

Antigen receptor bearing cells are constantly sampling their environment and being exposed to self-antigen. The cells depend on the low signals induced from these self-antigens for their survival and homeostasis (Surh and Sprent, 2005). Any alteration that enhances signaling may convert a survival signal into an activation signal. Many of the molecules in these signaling pathways have been identified and studied in mouse knockout models. Deletion of inhibitory receptors such as CTLA-4, PD-1, CD22, and FcγRIIb, or inhibitory signaling molecules such as SHP-1, SHIP, Cbl-b, Lyn, to name a few, cause robust autoimmunity in mice (Wakeland et al., 2001). Likewise, overexpression or hypermorphic alleles of activating molecules, the E613R CD45 wedge mutant for example, can also lead to autoimmunity (Hermiston et al., 2005; Majeti et al., 2000).

Another vital mechanism for suppressing auto-reactive cells is through peripheral apoptosis (Bidere et al., 2006). The opposing effects of carefully regulated pro-survival and pro-apoptotic factors control this process. Pro-survival factors include the Bcl-2 family, while pro-apoptotic factors include the BH3-only protein Bim and death receptor Fas. The Bim and Fas pathways control different aspects of cell death, but disruption of either can cause autoimmunity (Strasser and Pellegrini, 2004). Bim appears to be responsible for clearing cells deprived of survival cytokines; Fas initiates a process called activation induced cell death, which is believed to kill cells following their activation (Nagata and Golstein, 1995; Strasser, 2005). Defects in Fas are also associated with the human disease autoimmune lymphoproliferative syndrome (ALPS) and are responsible for the phenotype of lpr mice (Straus et al., 1999).

Although studies using knockout mouse models for autoimmunity provide insight into the function of immune regulators, rarely do adult humans have disease resulting from such complete loss of function (Davidson and Diamond, 2001). Instead, most human autoimmune disease is likely to be caused by allelic variants of these regulators that have very small effects on their own, but in combination can break tolerance. A similar phenomenon occurs in mice. Phenotypes of many knockout mice are highly background dependent (Bickerstaff et al., 1999; Bolland and Ravetch, 2000; Bygrave et al., 2004). Some strains are susceptible to autoimmunity, while others are resistant. Genetic modifier screens of mice from susceptible and resistant backgrounds have begun to identify loci that are predisposing or protective (Wakeland et al., 2001). Many studies have used spontaneous mouse models of autoimmunity resembling lupus such as the NZB/NZW F1 hybrid and NZM2410 to dissect the contributions of different loci. The NZM2410 strain has been most extensively characterized, and both sensitizing and suppressing loci have been identified. Two of the loci have been mapped to polymorphisms in *Cr2* and the SLAM/CD2 locus (Boackle et al., 2001; Wandstrat et al., 2004). Although known to modulate the immune response, knockouts of *Cr2* and SLAM family members have not been reported to cause autoimmunity, highlighting the value of such genetic screens for identifying new disease risk conferring alleles of previously characterized genes (Chan et al., 2006; Chen et al., 2000; Wu et al., 2002). Of importance to transgenic models of autoimmunity, two reports have identified loci from the 129 background that when transferred on to the C57BL/6 background predispose mice to autoimmunity (Bygrave et al., 2004; Carlucci et al., 2007). Most transgenic mice

are created on mixed C57BL/6 x 129 backgrounds, and thus these results suggest that the phenotype of these mice can be heavily influenced by loci other than the targeted one.

Fas

Regulated cell death and survival are critical aspects of immune homeostasis (Bidere et al., 2006; Marrack and Kappler, 2004). Disruption of the delicate balance between these two opposing forces can lead to lymphoproliferation or lymphopenia. Fas belongs to the TNF receptor family and is one of the receptors inducing cell apoptosis upon binding of its ligand, FasL (Nagata, 1997). Multiple tissues, including thymus, heart, lung, kidney, and ovary, express Fas, while FasL expression is limited to activated T cells and "immune privileged" sites such as the eye and testis (Nagata and Golstein, 1995). The importance of Fas is demonstrated by the occurrence of a lethal autoimmune disease in mice and the human disease autoimmune lymphoproliferative syndrome (ALPS) in the absence of either Fas or FasL (Straus et al., 1999).

Phenotype of Fas deficient mice

Before Fas had even been identified, a spontaneous mutation arose in the MRL mouse strain resulting in a severe lupus-like autoimmune disease (Cohen and Eisenberg, 1991). The unknown gene at the time was referred to as *lpr*. The MRL/lpr mice develop significant lymphadenopathy and splenomegaly with expansion of activated T and B cells. Multiple autoantibodies are produced, including anti-ssDNA, anti-dsDNA, anti-chromatin, rheumatoid factor, and anti-Smith, leading to immune complex mediated glomerulonephritis and eventual renal failure. Other autoimmune manifestations include

necrotizing arteritis and erosive synovitis. The life span of these mice is dramatically shortened, with 50% mortality at 5 months of age.

The phenotype of lpr mice is highly background dependent. In particular, on the C57BL/6 background lymphadenopathy and splenomegaly are greatly reduced, autoantibodies only appear in older mice, and there is no evidence of glomerulonephritis or arthritis (Cohen and Eisenberg, 1991; Hang et al., 1985; Izui et al., 1984). This dramatic difference in phenotype suggests that background genes have a significant influence on disease. Genome wide scans of MRL x C57BL/6 F2 crosses have begun to identify some of the modifying loci (Santiago-Raber et al., 2007; Vidal et al., 1998). The human disease ALPS is also sensitive to genetic modifiers. Although most Fas mutations are autosomal dominant, parents of ALPS patients have been reported to carry the same mutation without any clinical manifestations, arguing for the presence of other influencing loci (Straus et al., 1999).

A characteristic feature of lpr mice regardless of background is the accumulation of two unique T cell subsets, a large $CD3^+$ $B220^+$ $CD4^-$ $CD8^-$ double negative (DN) population that expands with age, and a smaller $CD3^+$ $CD4^{low}$ $B220^+$ group. In addition to B220, which is a CD45 isoform typically expressed on B cells, the DN population also expresses CD138 syndecan, a marker of plasma cells (Seagal et al., 2003). Despite the expression of B cell markers, these cells have a polyclonal repertoire of rearranged $\alpha\beta$ TCRs and show no evidence of immunoglobulin rearrangement, and are thus unquestionably T cells. They are believed to represent activated cells that have failed to undergo normal apoptosis, but have downregulated their CD4 or CD8 coreceptor instead, although whether they originate from CD4 or CD8 cells remains controversial. Arguing

for a TCR signaling requirement, lpr mice carrying various TCR transgenes do not generate DN cells, and in addition show much reduced lymphoproliferation and autoimmunity (Perkins et al., 1996; Sytwu et al., 1996). Use of Cre/lox technology to specifically delete Fas only in T cells has shown that development of DN cells is cell autonomous and does not require Fas deficiency in other cell types (Hao et al., 2004; Stranges et al., 2007). Interestingly, although they may have been activated at some point in their lifetime, DN and $CD4^{low}$ $B220^{+}$ cells are anergic in vitro. These cells do not proliferate or secrete cytokine in response to anti-CD3 stimulation (Giese and Davidson, 1992). Finally, the contribution of these cells to the pathology of lpr mice is also unknown, and they may simply be an unrelated phenomenon of Fas mutation. Transgenic expression of Fas only in T cells of MRL/lpr mice prevented the development of DN cells, but not autoantibodies and glomerulonephritis, suggesting T cells deficient in Fas are not necessary for disease (Fukuyama et al., 1998).

Fas Function

Fas was first identified as the target of antibodies that induced rapid cell death (Nagata and Golstein, 1995). It was also found to be responsible for death induced by TCR crosslinking in hybridomas. Anti-CD3 or phobol ester stimulation induced expression of both Fas and FasL, resulting in cell autonomous death, which was blocked by a Fas-Fc fusion protein (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). Fas mediated death required prior activation and IL-2 exposure (Lenardo, 1991), and was thus termed activation induced cell death. B cells were also found to undergo Fas

mediated death, and were sensitized by CD40 stimulation, while BCR stimulation produced resistance (Rothstein et al., 1995).

These observations led to the hypothesis that Fas may play a role in shutting down immune responses by deleting activated cells. A number of in vivo experiments seemed to confirm this idea. Although Fas deficient T cells developed normally and were still sensitive to negative selection in the thymus, they were not eliminated in response to immunization with cognate antigen or superantigen in the periphery (Mogil et al., 1995; Singer and Abbas, 1994; Van Parijs et al., 1998a). Other groups, however, found that Fas deficient cells were in fact eliminated normally after superantigen exposure (Hildeman et al., 2002; Scott et al., 1993). Instead, these groups proposed a role for the proapoptotic Bcl-2 family member Bim in contraction of cells after activation. In this model, clearance of antigen results in reduced levels of survival cytokines, which cause upregulation of Bim and cell death (Strasser and Pellegrini, 2004). Another hypothesis suggested that while the Bcl-2 pathway is involved in deletion of cells responding to foreign antigen, Fas deletes auto-reactive cells responding to self-antigen (Van Parijs et al., 1998b). This model was tested by adoptive transfer of HEL-specific TCR transgenic cells into mice endogenously expressing low levels of soluble HEL to mimic self-antigen. In these mice, Fas deficient cells accumulated more than wildtype cells, suggesting Fas was allowing increased survival. However, closer examination of the data shows that the increased accumulation was caused by a greater initial response and that the contraction of Fas deficient cells was actually equivalent to wildtype. Thus, Fas does not appear to be required for clearance of auto-reactive T cells in this context.

To the test the role of Fas in B cells, an approach similar to the T cell experiments was taken, but using HEL-specific BCR transgenic cells. Rather than adoptively transfer cells, though, mice were generated that were Fas deficient and co-expressed both the transgenic BCR and HEL in soluble (sHEL) or membrane bound (mHEL) form (Rathmell and Goodnow, 1994). This system had been previously used to show that sHEL induced anergy of HEL specific B cells, while mHEL resulted in their negative selection and death (Cornall et al., 1995). In Fas deficient mice, mHEL induced efficient deletion of HEL-specific B cells, indicating that Fas is not necessary in this processes. Fas deficient B cells were also anergized normally in sHEL expressing mice, although some older mice appeared to break tolerance and produce anti-HEL antibodies. This result suggests Fas may be controlling tolerization of auto-reactive B cells, but because the loss of tolerance only occurred in older mice, other Fas related events in non-B cells could be contributing to the phenotype (Rathmell and Goodnow, 1994). The same group went on to publish a series of follow up studies delineating a multi-step pathway for the deletion of tolerized B cells by T cells. In this system, HEL-specific BCR transgenic cells were cotransferred with HEL-specific TCR transgenic cells into sHEL expressing recipients. Transfer of naïve B cells resulted in their proliferation, while tolerized B cells from sHEL expressing mice disappeared in the presence of HEL specific T cells. Tolerized Fas deficient B cells, on the other hand, did not disappear (Rathmell et al., 1995). This killing required the interaction of multiple molecules. B cell presentation of MHC/peptide antigen induced T cells to express CD40L and FasL. Binding of CD40L to CD40 on B cells then induced the B cells to express Fas. The outcome of T cell FasL interacting with B cell Fas depended on the strength of signal from the BCR. The higher

levels of BCR on naïve cells generated a stronger signal that protected the cells from Fas mediated death, while in tolerized B cells the signal was muted and did not prevent apoptosis (Rathmell et al., 1996). Crosslinking of biotinylated HEL on tolerized B cells, however, did provide a strong enough signal to rescue these cells from deletion (Foote et al., 1998). B cells could also be rescued by expression of costimulatory molecule B7.2, possibly through induction of cytokine production from T cells (Foote et al., 1998; Rathmell et al., 1998). These studies suggest Fas may limit autoimmunity by T cell FasL mediated destruction of auto-reactive B cells expressing Fas.

In addition to control of T and B cells, Fas may control other antigen presenting cells such as dendritic cells. Overexpression of an anti-apoptotic protein in DCs and DC specific deletion of Fas both resulted in autoimmunity (Chen et al., 2006; Stranges et al., 2007). In both of these models, activated DCs accumulated and were able to promote activation and expansion of T cells. These mice also developed autoantibodies and lymphocyte infiltrates in multiple organs. Based on their results, the authors from these studies argue that deletion of antigen presenting cells presenting auto-antigens may be an important mechanism for maintaining tolerance.

CD45

Lymphocyte development and activation are controlled, in part, by the balance between protein tyrosine phosphorylation and dephosphorylation in signaling pathways (Hermiston et al., 2002). Protein tyrosine kinases catalyze the addition of phosphate to tyrosines, while protein tyrosine phosphatases catalyze its removal. Both kinases and phosphatases can have either net positive or negative effects on the outcome of these

pathways. Some of the most proximal molecules in lymphocyte signaling are the Src family kinases (SFK) and the receptor-like protein tyrosine phosphatase CD45, which regulates the SFKs.

SFK activity is determined by the phosphorylation of two key tyrosines, an inhibitory tyrosine near the C-terminus and an activation loop tyrosine (Palacios and Weiss, 2004). Phosphorylation of the inhibitory tyrosine by the C-terminal Src kinase (Csk) allows it to engage in an intramolecular interaction with the SFK SH2 domain. Binding of the phosphotyrosine to the SH2 domain results in a conformation that inhibits kinase activity. Dephosphorylation of this inhibitory tyrosine allows the SFK to adopt an open conformation that primes it for activation by phosphorylation of the activation loop tyrosine. Dephosphorylation of the activation loop tyrosine shuts down kinase activity. One of the primary targets of SFK activity in lymphocytes is the immunoreceptor tyrosine-based activation motif (ITAM) family of receptors, which includes antigen, Fc, and natural killer cell receptors (Chow and Veillette, 1995). Phosphorylation of ITAMs initiates a cascade of signaling events leading to lymphocyte activation. CD45 exerts its regulatory function by dephosphorylating Src family kinases, and thus regulates ITAM signaling as well. A synthesis of genetic, cell line, and biochemical data suggests that overall, CD45 is a positive regulator of SFK activity, but may also have a negative role in some contexts (Ashwell and D'Oro, 1999; Hermiston et al., 2003).

The first evidence for CD45 function in lymphocytes came from antibody studies. Experiments testing the effect of anti-CD45 antibodies on lymphocyte activation produced mixed results, with some finding a positive role for CD45, and others an inhibitory (Trowbridge and Thomas, 1994). As with many antibody studies, uncertainty

about whether the antibodies blocked or enhanced CD45 activity added an additional layer of complexity. A clearer picture emerged from work with CD45 deficient cell lines and mice, and eventually the identification of patients lacking CD45. T cells not expressing CD45 failed to induce ITAM or downstream phosphorylation, flux calcium, produce IL-2, and proliferate in response to APC/antigen or anti-CD3 stimulation (Koretzky et al., 1991; Koretzky et al., 1990; Pingel and Thomas, 1989). CD45 deficient B cells had a similar inability to flux calcium, while a CD45 deficient CD8 T cell line also had impaired cytotoxic activity (Justement et al., 1991; Ogimoto et al., 1995; Weaver et al., 1991; Yanagi et al., 1996). Restoration of CD45 in deficient cell lines reversed their abnormalities, showing that loss of CD45 was in fact responsible for the observed defects, and furthermore that CD45 is likely to be positively regulating T and B cell activity (Hovis et al., 1993; Koretzky et al., 1992; Pao et al., 1997; Pao and Cambier, 1997; Volarevic et al., 1993).

Studies of CD45 deficient cell lines led to the biochemical role of CD45 when the Src family kinase Lck was found to have reduced in vitro kinase activity in the absence of CD45 and was also hyperphosphorylated on its inhibitory tyrosine, Y505 (Cahir McFarland et al., 1993; Ostergaard et al., 1989). Addition of immunoprecipitated CD45 to lck in vitro led to dephosphorylation of the inhibitory tyrosine and increased lck activity in vitro (Mustelin and Altman, 1990; Mustelin et al., 1989). Other studies extended these observations to Fyn, another T cell SFK (Mustelin et al., 1992; Shiroo et al., 1992). However, multiple groups have shown that in some CD45 deficient cell lines, lck in vitro kinase activity is unchanged or even increased, despite hyperphosphorylation of Y505 (Burns et al., 1994; D'Oro et al., 1996; Shiroo et al., 1992). Differences in lck

activity also depend on what pool the lck was isolated from. In one study, the kinase activity of total lck was higher in CD45 deficient cells compared to CD45 expressing cells, while the activity of CD4 associated lck was reduced (Biffen et al., 1994). Furthermore, in some CD45 deficient cell lines the lck activation loop tyrosine, Y394, was also slightly hyperphosphorylated, and CD45 was able to dephosphorylate this tyrosine and inhibit lck activity (D'Oro et al., 1996). Like T cells, CD45 deficient B cell lines also showed hyperphosphorylation of the B cell SFK, Lyn, usually at both tyrosines (Katagiri et al., 1995). However, in some lines lyn activity was decreased, while in others it was increased. The consensus from these studies is that in the absence of CD45, antigen receptor signaling is inhibited and SFKs are hyperphosphorylated on their inhibitory tyrosine, arguing that CD45 is necessary for dephosphorylating this inhibitory tyrosine and permitting signaling to proceed. At the same time, though, there is evidence that CD45 also dephosphorylates the activation loop tyrosine and may downregulate SFK activity.

Consistent with the defective signaling in CD45 deficient cell lines, deletion of CD45 in mice by gene targeting also revealed a profound block in T cell development and B cell activation (Byth et al., 1996; Kishihara et al., 1993; Mee et al., 1999). In the T cell compartment of CD45 deficient mice there is a significant reduction in the number of mature peripheral CD4 and CD8 cells as well as a two fold reduction in the total number of thymocytes. Closer inspection of thymic subsets showed that the block in T cell development occurs at two stages, the DN3 to DN4 transition and the DP to SP transition. The number of CD4 and CD8 SP cells is most affected, with a 5-10 fold reduction in absolute count. The two disrupted transitions represent checkpoints for TCRβ and TCRα

rearrangement and require signaling through the pre-TCR and mature TCR, respectively, arguing that CD45 activity is necessary to initiate these signaling events. Use of fetal thymic organ culture and TCR transgenics also demonstrated impaired positive and negative selection (Byth et al., 1996; Mee et al., 1999). Finally, similar to CD45 deficient cell lines, thymocytes from CD45 knockout mice failed to manifest increases in intracellular free calcium when stimulated with anti-CD3 (Stone et al., 1997).

Examination of the B cell compartment in these mice revealed normal numbers of B cells, although there appears to be a partial block in development at the pro-B cell stage, and peripheral B cells display a more immature phenotype, being IgM high and IgD low (Benatar et al., 1996; Fleming et al., 2004). More significantly, these B cells had defective activation of the MAP kinase Erk, decreased intracellular free calcium flux, and reduced proliferation in response to anti-IgM or anti-IgD stimulation (Benatar et al., 1996; Cyster et al., 1996; Huntington et al., 2006). Like T cells, CD45 deficient B cells also showed altered positive and negative selection (Cyster et al., 1996). B cells expressing a transgenic BCR specific for hen egg lysozyme (HEL), but lacking CD45, did not accumulate to normal numbers compared to CD45 expressing cells, which was interpreted as defective positive selection. Additionally, when HEL binding B cells that express CD45 are exposed to soluble HEL as an auto-antigen, they downmodulate their BCR and show reduced survival as part of tolerization process (Cornall et al., 1995). Rather than showing reduced survival, however, CD45 deficient HEL binding B cells accumulate in response to soluble HEL and downregulate their BCR to a lesser degree, suggesting that the reduction in signaling caused by lack of CD45 rescues them from anergy induction (Cyster et al., 1996). In addition, after immunization with foreign

antigen, CD45 deficient mice showed decreased B cell survival and persistence of germinal centers, arguing that CD45 is also necessary for maintenance of the immune response (Huntington et al., 2006).

Further mouse work has supported the role for CD45 in regulating SFK activity. Correlative evidence came from the lck knockout, which showed a block similar, but more severe, to that in the CD45 knockout, suggesting that CD45 and lck are in the same pathway (Molina et al., 1992). Moreover, the block in thymocyte development seen in CD45 knockout mice was rescued by transgenic expression of an active lck in which the inhibitory tyrosine 505 was mutated to phenylalanine (Y505F) (Pingel et al., 1999; Seavitt et al., 1999). These data provided the most convincing proof of the requirement for CD45 desphosphorylation of lck Y505 in thymocyte development. However, like some cell line studies, lck activity was actually increased in CD45 deficient thymocytes despite hyperphosphorylation of the inhibitory tyrosine (Seavitt et al., 1999). Loss of CD45 also caused the Y505F mice to develop aggressive thymomas, whereas CD45 sufficient mice did not, suggesting that CD45 was inhibiting the activity of Y505F Lck, most likely by dephosphorylating the activation loop tyrosine (Baker et al., 2000). Indeed, Y394 was hyperphosphorylated in the absence of CD45 and lck activity was two fold higher.

In addition to T and B cell defects in CD45 deficient mice, defects in signaling from ITAM bearing receptors in natural killer (NK) and mast cells have been reported as well. CD45 deficient mast cells differentiated and expanded normally in vitro, but failed to increase cytoplasmic free calcium or degranulate following crosslinking of the high affinity IgE receptor (Berger et al., 1994). Similarly, CD45 was required for ITAM

receptor mediated cytokine production in NK cells, but not cytotoxicity (Hesslein et al., 2006; Huntington et al., 2005). Upon receptor ligation, these CD45 deficient NK cells also failed to increase calcium and showed reduced phosphorylation of multiple signaling proteins, including the kinase Syk, MAP kinases ERK and JNK, and the guanine nucleotide exchange factor Vav.

Collectively, these studies suggest that CD45 helps determine the strength of signal from antigen receptors. It does so by dephosphorylating the inhibitory tyrosine of SFK and potentially the activation loop tyrosine as well. The net effect of this activity may be to create a pool of SFK that is in an open, primed conformation. This primed conformation has little kinase activity, but is capable of being rapidly activated upon receptor engagement. As such, CD45 may serve as a rheostat in lymphocytes by determining the size of the primed SFK pool. Alterations in CD45 activity could alter the size of the pool: reduced CD45 activity, as in CD45 knockout mice, reduces the amount of primed SFK, preventing effective antigen receptor signaling; enhanced CD45 activity increases the pool of primed SFK, possibly enhancing signaling. In this way, CD45 may be controlling the balance between positive and negative selection or tolerance and autoimmunity, and therefore studying its regulation could have important implications for overall immune regulation.

CD45 Regulation

Since CD45 is a transmembrane receptor-like phosphatase, it is natural to ask whether it may have a ligand. Numerous groups have spent years attempting to answer this question without any definitive results, although a number of potential ligands have

been proposed. CD22, galectin, and macrophage galactose-type lectin (MGL) have all been found to bind the extracellular domain of CD45 (Stamenkovic et al., 1991; van Vliet et al., 2006; Walzel et al., 1999). While CD22 and MGL were reported to inhibit CD45 phosphatase activity, the data were not compelling. The experiments failed to show that equivalent amounts of CD45 were being assayed or that the inhibition was specific to CD45 and not other co-immunoprecipitating phosphatases. Furthermore, lectins bind numerous glycosylated receptors, of which CD45 is only one, and thus the functional effects of these putative ligands may not be mediated exclusively through CD45 (Daniels et al., 2002).

In order to bypass the need for a physiological ligand and study the effect of dimerization on CD45 function, a chimeric molecule was created containing the extracellular domain of the EGF receptor fused to the intracellular portion of CD45 (Desai et al., 1993). This molecule allowed the use of EGF as an artificial ligand. The EGFR-CD45 chimera was expressed in CD45 deficient cells and shown to be able to rescue signaling stimulated by anti-CD3 antibody. Surprisingly, use of EGF to induce dimerization of the chimeric molecule blocked TCR signaling, suggesting that CD45 dimerization inhibits its activity. The crystal structure of a related phosphatase, RPTPα, revealed a possible mechanism for this inhibition (Bilwes et al., 1996). In this crystal, domain 1 of RPTPα formed a symmetric dimer in which a helix-turn-helix motif, termed the "wedge", from one molecule inserted itself into the catalytic site of the opposing molecule. The insertion of the wedge obstructed the catalytic site, potentially preventing it from accessing phosphorylated substrate. In order to test the function of the putative wedge of CD45, a glutamic acid reside at its tip was mutated to arginine (E624R) and

introduced into the EGFR-CD45 chimera (Majeti et al., 1998). Now, in the presence of the E624R wedge mutation, addition of EGF did not prevent TCR signaling, arguing that the wedge was mediating the inhibition caused by dimerization. These results had very important implications for the regulation of CD45. They suggested that CD45 activity could be modulated by its dimerization. Increased dimerization would reduce CD45 activity, while decreased dimerization would enhance it. This altered activity might then translate into altered SFK activity and lymphocyte signaling. The prediction from these experiments, then, is that the CD45 wedge mutation would behave like a hypermorphic allele, and in the context of the immune system could lead to hyperactivation and possibly autoimmunity. Mutation of the equivalent wedge amino acid (E613R) in the endogenous mouse CD45 locus seemed to confirm this hypothesis (Majeti et al., 2000). The CD45 E613R wedge mutant mice on a mixed C57BL/6 x 129 background developed a lupus-like syndrome, which included a pronounced lymphoproliferative disease, autoantibodies, and immune complex-mediated glomerulonephritis. However, as is the case with many autoimmune phenotypes, when the CD45 wedge mutation was backcrossed onto a homogenous C57BL/6 background, much of the disease resolved (Hermiston et al., 2005). The CD45 wedge mutation still caused a much milder lympoproliferation and elevated IgM levels, but autoantibodies and glomerulonephritis were absent. B cell development was altered, with a shift towards increased B1 B cell production, which is associated with increased BCR signaling. Consistent with this phenotype, B cells from CD45 wedge mutant mice were hyper-responsive to BCR crosslinking, CD40 ligation, and LPS stimulation. These cells showed elevated calcium increases and ERK phosphorylation in response to stimulation. Together these results

suggest that dimerization mediated-inhibition of CD45 by the wedge is indeed a physiologic mechanism of CD45 regulation.

Evidence for dimerization

Following the crystallization of RPTPα D1 as a dimer and the description of wedge mediated inhibition of phosphatase activity, a search began for physiologic RPTP dimers. The first demonstration that dimers might exist in vivo came from the use of RPTPα cysteine trapping mutants (Jiang et al., 1999). In these mutants, unpaired cysteines were introduced into the extracellular juxtamembrane region at different locations. If RPTPα molecules formed a dimer, the unpaired cysteines could form an intermolecular disulfide bond, trapping the dimer, and allowing it be visualized on a non-reducing gel. Expression of the cysteine trapping mutants indicated that RPTPα did indeed dimerize in vivo. Moreover, this dimerization inhibited phosphatase activity, as assessed by dephosphorylation of the Src inhibitory tyrosine. Mutation of the wedge abrogated this inhibition. Similar experiments using cysteine trapping mutants with RPTPσ (Lee et al., 2007) and CD45 (Majeti et al., 2000) have revealed that they may also be present as dimers. Chemical crosslinking and FRET experiments using fluorescent protein tagged molecules have provided additional evidence for dimer formation of both RPTPα (Jiang et al., 2000; Tertoolen et al., 2001) and CD45 (Dornan et al., 2002; Xu and Weiss, 2002). For RPTPα, the intracellular and extracellular domain contributed to dimerization, but the transmembrane domain was found to be sufficient. In fact, the transmembrane domains of a number of phosphatases, including CD45, were capable of homodimerization (Chin et al., 2005). For CD45, the extent of dimerization depended on

isoform usage in the heavily glycosylated extracellular domain (Xu and Weiss, 2002). Larger, more glycosylated isoforms had less of a tendency to dimerize, while small isoforms did so more efficiently. Usage of these different isoforms in lymphocytes is regulated by cell type and activation state and could thus modulate CD45 dimerization at various stages of development and differentiation. The full length and cytoplasmic forms of another related phosphatase, RPTPε, were also shown to dimerize in vivo (Toledano-Katchalski et al., 2003). Although CD45 dimers have been detected on the cell surface by crosslinking, dimers of the full-length cytoplasmic domain of CD45 have not been observed in vitro under normal conditions (Xu and Weiss, 2002). Dimers of domain 1 alone, however, do occur (Felberg 1998). These data suggest that many phosphatases, including CD45, are present as dimers under physiologic conditions, but evidence for dimerization in vitro is still lacking.

CD45 Structure

The recent publication of the CD45 crystal structure has provided another tool for understanding CD45 regulation (Nam et al., 2005). All together, the structures of the cytoplasmic portions of five RPTP have been solved, including RPTPα, RPTPμ, RPTPσ, and LAR. Of these molecules, only the first domain of RPTPμ (Hoffmann et al., 1997) and RPTPσ (Eswaran et al., 2006) were crystallized, while D1 (Bilwes et al., 1996) and D2 (Sonnenburg et al., 2003; Yang et al., 2007) of RPTPα were crystallized separately, and only for LAR (Nam et al., 1999) and CD45 (Nam et al., 2005) were the proteins crystallized with both tandem domains intact. Comparison of the five D1 proteins shows a remarkably strong conservation of the tertiary structure. However, unlike the RPTPα

D1 crystal, RPTPμ and RPTPσ did not crystallize as dimers, suggesting that RPTPα dimer conformation may not be a universal property of phosphatases. All five crystal structures did reveal the presence of the wedge, which also appears to be present in a modified form in the second domain of LAR and CD45, the difference being the presence of four extra amino acids in the turn portion of the wedge (Nam et al., 1999). This would argue that the helix-turn-helix is a structure inherent to the phosphatase domains of RPTPs and not a unique structure of the juxtamembrane region.

The presence of the wedge in the CD45 crystal structure validates its potential role in dimerization mediated regulation of CD45 activity (Figure 1-1A). At the same time, though, the crystal structure poses a significant problem to a dimerization based model. As in the LAR structure, the orientation of CD45 D1 and D2 would preclude the wedge from participating in dimer formation (Nam et al., 2005). Arranging two CD45 molecules such that their first domains mimic the RPTPα domain 1 dimer results in a significant amount of steric clash between the second domains (Figure 1-1B). In addition, the extensive area of contact between domain 1 and domain 2 suggests that their interaction is very stable (Nam et al., 2005). How do we reconcile these observations with the existing in vitro and in vivo evidence for the role of the wedge?

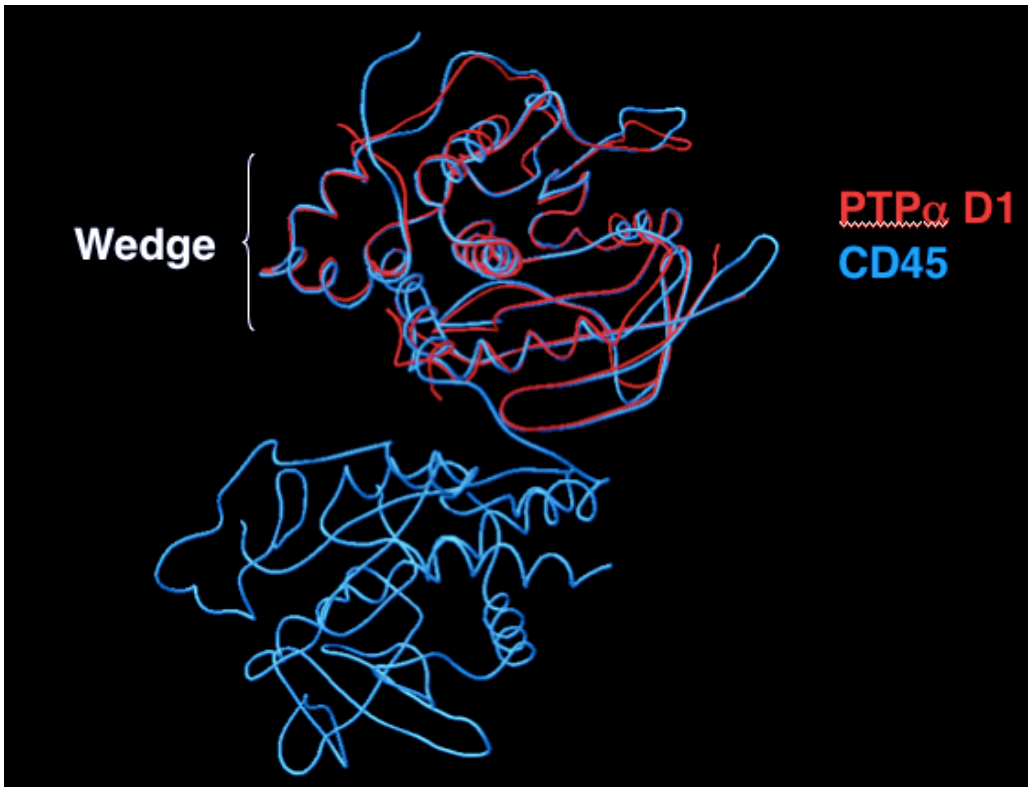
The most likely explanation is that there is in fact more flexibility between D1 and D2 than revealed in the crystal structure. Crystal structures represent a snapshot of protein conformation, but of course in solution proteins can adopt a number of conformations. For example, only the closed, inactive structures of full-length Src family kinases have been solved. The active conformations are likely to be too flexible to crystallize. For CD45 and other two domain phosphatases, it may be the active

Figure 1-1: CD45 structure and dimerization

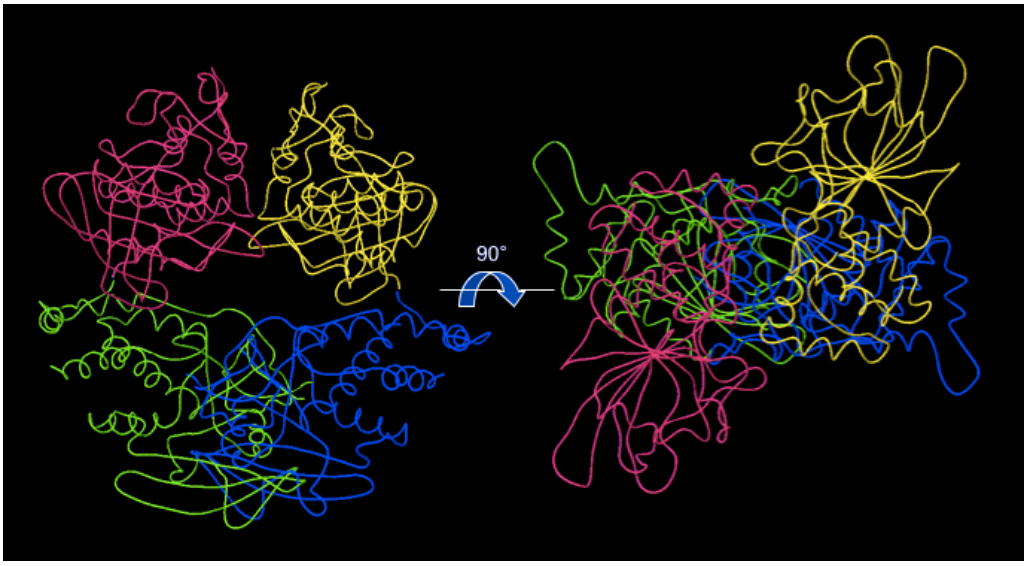
(A) Overlay of CD45 (blue) and RPTPα D1 (red) with the wedge structure indicated

(B) Steric clash in CD45 dimers. Two molecules of CD45 were oriented such that their D1s (red and yellow) formed a dimer similar to that observed for RPTPα D1. The overlap of the D2s (blue and green) can be seen looking from the side (left) or from above (right).

A.



B.



conformation that is rigid enough to crystallize, while other possible inactive conformations are too flexible. Is there any evidence for flexibility between domain 1 and domain 2?

Closer inspection of the CD45 crystal structures suggests that the domain 1/domain 2 interaction may not be completely rigid. CD45 was crystallized with with two different phosphopeptides, each with two CD45 molecules per asymmetric unit (Nam et al., 2005). Although CD45 is almost identical in the two crystals, overlaying all four molecules reveals that each one has a slightly different angle between D1 and D2. This difference is also present when comparing CD45 to the unliganded LAR structure. Evidence also exists for flexibility between the two domains of RPTPα in solution. In the structure of RPTPα D2 the N-terminal portion of D2, the segment joining it to D1, is disordered, suggesting that the link between the two domains may be disordered and mobile as well (Sonnenburg et al., 2003). In addition, limited proteolysis of the tandem D1/D2 domains revealed a number of cleavage sites in the region between D1 and D2, pointing to this region being solvent exposed and flexible.

Many other factors may influence CD45 structure and dimerization including the extracellular domain, lipid bilayer, transmembrane segment, and juxtamembrane region. The effects of the acidic loop, which did not crystallize, and large C-terminal tail, which had to be removed to produce crystals, are also unclear.

Finally, CD45 conformation and activity may also be dynamically regulated by phosphorylation or oxidation of the second phosphatase domain. Although none of the proposed sites of phosphorylation or oxidation lie immediately adjacent to the domain 1/domain 2 interface, these modifications may induce structural changes that are

transmitted to the interface, ultimately disrupting the interaction between domain 1 and domain 2 and allowing movement of domain 2. In fact the crystal structure of oxidized PTP1B revealed a dramatic conformational change with the formation of a novel sulphenyl-amide ring at the active site and large scale movement of a number of loops (Salmeen et al., 2003; van Montfort et al., 2003). Releasing domain 2 from its interaction with domain 1 may then allow CD45 to adopt a new conformation that permits dimerization. Were domain 2 to be reoriented, domain 1 could be free to form dimers similar to RPTPα. However, until more biophysical studies examining the effect of phosphorylation and oxidation on CD45 structure are performed, these conformational changes will remain pure speculation.

Domain 2

The second phosphatase domain of CD45 may have an important role in regulating the overall activity of CD45. Although it does not have any intrinsic catalytic activity, when domain 2 is deleted, domain 1 has significantly less activity than full length CD45 (Johnson et al., 1992; Streuli et al., 1990). A careful analysis of full length CD45 versus domain 1 alone revealed that full length CD45 is more stable over wider pH and temperature ranges (Felberg and Johnson, 1998). It is likely that some sort of interaction between domain 1 and domain 2 contributes to the greater stability and activity of full length CD45. Isolated domain 1 and domain 2 do associate with one another (Felberg and Johnson, 1998, 2000; Hayami-Noumi et al., 2000), and the CD45 crystal structure shows an extensive area of contact between the two domains (Nam et al.,

2005). Disruption of the association by deletion of domain 2 may alter the conformation of domain 1, directly resulting in loss of activity.

Assuming domain 2 does modulate domain 1 activity, an intriguing possibility is that it does so in a regulated manner. Specific signals leading to the covalent modification of CD45 could alter its conformation, disrupt the domain 1/domain 2 interface, and allow the cell to turn off CD45. CD45 is known to be covalently modified by phosphorylation and potentially by oxidation, both of which could influence activity.

In unstimulated cells, CD45 is constitutively phosphorylated on a number of serine residues in domain 2, possibly via casein kinase 2 (CK2) (Kang et al., 1997; Wang et al., 1999). In vitro, CK2 phosphorylates recombinant CD45 on a unique, 19 amino acid insert in domain 2. This acidic loop is phylogenetically conserved, but is not present in domain 1 or any other phosphatase. Interestingly, treatment with CK2 enhances CD45 activity three fold, but not when the acidic loop serines are mutated to alanine (Wang et al., 1999). Conversely, decreased CD45 serine phosphorylation upon ionomycin stimulation has been reported to correlate with a loss of activity (Ostergaard and Trowbridge, 1991). One interpretation of these data is that phosphorylation stabilizes the interaction between domain 1 and domain 2, maintaining higher CD45 activity, while dephosphorylation destabilizes the interaction, resulting in loss of activity. Alternatively, phosphorylation of the acidic loop may help to recruit substrates. However, the role of acidic loop phosphorylation in vivo is unclear as its deletion or mutation reduced changes in intracellular calcium and MAPK phosphorylation in one report (Greer et al., 2001), but in another resulted in only somewhat sustained intracellular calcium increase, without affecting its magnitiude or MAPK phosphosphorylation (Wang et al., 2000). Mutation or

deletion of the acidic loop also had no effect on immunoprecipitated CD45 phosphatase activity (Wang et al., 2000). Tyrosine phosphorylation of CD45 has also been observed upon stimulation with phosphatase inhibitors directly or phytohemagglutinin in the presence of phosphatase inhibitors, and has been reported to increase CD45 activity (Autero et al., 1994; Stover et al., 1991; Stover and Walsh, 1994), but has no known in vivo function.

Reactive oxygen species are also emerging as potent modifiers of phosphatases (den Hertog et al., 2005). The catalytic cysteine of phosphatases is particularly sensitive to oxidation because of its low pKa. Oxidation of this cysteine completely eliminates phosphatase activity. As with phosphorylation, oxidation is reversible, and phosphatase activity is restored by cellular reducing agents such as glutathione and thioredoxin. Surprisingly, despite their lack of activity, almost all D2s of tandem domain phosphatases, including that of CD45, have retained this cysteine (Andersen et al., 2001). They are therefore likely to be oxidized as well. Moreover, it appears that the second domain of RPTPα is actually more sensitive to oxidation than the first domain (Groen et al., 2005; Persson et al., 2004), and upon oxidation it undergoes a conformational change measured by FRET (Blanchetot et al., 2002). Such a conformational change could disrupt the domain 1/domain 2 interface, abrogating phosphatase activity. Recently, the RPTPα D2 oxidation induced conformational change was visualized by crystallography (Yang et al., 2007). These crystals confirmed the presence of the sulfenyl-amide ring observed in PTP1B, suggesting it may be a common feature of oxidized phosphatases, but the conformational change was not as significant as PTP1B and did not appear to affect other portions of the molecule. However, this observation was made by oxidizing

preformed crystals, and it is possible that the crystal constrained the conformation of the oxidized protein. Attempts to crystallize pre-oxidized protein were unsuccessful, suggesting that the oxidized protein might be more flexible. Thus, these results leave open the possibility of more significant oxidation induced conformational changes, possibly allowing dimerization.

Although the CD45 E613R wedge mutation has a robust autoimmune phenotype on a mixed C57BL/6 x 129 background, on a homogenous B6 background the phenotype is much milder. We therefore set out to determine whether or not the CD45 wedge mutation could act as a genetic modifier with other mutations predisposing to autoimmunity on the B6 background. Two strains of mice were selected, the complement *C4* knockout and the Fas mutation *lpr*. We found that CD45 wedge mutation in the context of C4 deficiency did not alter the phenotype of single mutant mice. However, combination of the CD45 wedge and lpr mutations results in dramatic autoantibody production, lymphocyte activation, and lymphoproliferation.

Chapter 2: Results

Introduction

The adaptive immune system must be strictly regulated to prevent it from responding to self-antigens. A number of mechanisms have evolved to prevent the inappropriate generation, activation, or survival of auto-reactive cells. Disruption of any of these mechanisms can result in autoimmunity. For most adult autoimmune diseases, these disruptions take the form of polymorphisms in immunoregulatory genes that in and of themselves have very subtle effects. Only in the right context or combination will these polymorphisms synergize to overcome the protective mechanisms in place.

A similar phenomenon occurs in mice, where autoimmune phenotypes are highly background dependent. This is true for the CD45 E613R wedge mutation. On a mixed C57BL/6 x 129 background, CD45 wedge mutant mice develop a lupus-like syndrome characterized by lymphoproliferation, autoantibody production, and immune complex-mediated glomerulonephritis. However, when the CD45 wedge mutation is backcrossed on to a homogenous B6 background, much of the phenotype resolves. Therefore, the CD45 wedge mutation must require other modifiers on the B6 background, or may act as a modifier itself for other autoimmune prone mice.

To test this hypothesis, we selected two other mutations that predispose mice to autoimmunity, the complement *C4* knockout and the Fas mutation *lpr*, and studied how the phenotypes of these mice changed when combined with the CD45 wedge mutation. We found that combination of the CD45 wedge mutation and C4 deficiency had no effect, while CD45 wedge and lpr double mutant mice showed dramatically accelerated autoantibody production, lymphocyte activation, and lymphoproliferation. Thus, the CD45 wedge mutation acts as a potent modifier with the lpr mutation, but not C4

deficiency. In addressing the mechanisms behind the CD45 wedge/lpr phenotype, we further demonstrate that T cells, and specifically signaling through the TCR, as well as B cells are required for lymphoproliferation, suggesting the presence of a positive feedback loop between these two cell types. Moreover, B cells appear to be intrinsically activated, and therefore their activation may be the primary event driving this feedback loop.

Abstract

CD45 and Fas regulate lymphocyte signaling and death, respectively. Mutation of an inhibitory wedge motif in CD45 results in hyper-responsive T and B cells, while deletion of Fas is responsible for a mild autoimmune disease on the C57BL/6 background. Here we show that when combined in mice, these two mutations synergize, causing early lethality, autoantibody production, and significant lymphoproliferation. In double mutant mice, signaling in response to endogenous antigens is required for T cell activation and expansion, as is the presence of B cells. Likewise, T cells are necessary for B cell expansion and autoantibody production. However, B cells appear to be intrinsically activated even in the absence of T cells, suggesting that they may be responsible for driving the phenotype of these mice. These results reveal a requirement for careful control of B cell signaling and cell death in maintaining and preventing inappropriate lymphocyte activation and autoimmunity.

Background

In the adaptive immune system, multiple barriers minimize the likelihood that a randomly generated antigen receptor will recognize and initiate a response to self-peptide. These barriers are at work throughout the lifetime of antigen receptor bearing cells, starting with the elimination of a majority of self-reactive cells during their development, and followed by mechanisms that induce anergy, suppression, or apoptosis of any self-reactive cells escaping to the periphery (Bidere et al., 2006; Fathman and Lineberry, 2007; Goldrath and Bevan, 1999; Zheng and Rudensky, 2007). In some rare, inherited autoimmune syndromes, single gene defects trigger a failure of one of these processes. For example,

mutations in AIRE disrupt aspects of negative selection, while FOXP3 deficiency prevents appropriate generation of regulatory T cells, both resulting in autoimmune polyendocrinopathies (Mathis and Benoist, 2007; Zheng and Rudensky, 2007). However, for the pathogenesis of most autoimmune diseases, such as lupus and arthritis, rarely has one genetic lesion proven to be sufficient (Wakeland et al., 2001). Instead, multiple interacting loci, each with a relatively small effect of its own, are likely to be necessary for disease. Studying how these genetic modifiers cooperate is therefore critical to our understanding of autoimmunity.

CD45 plays an essential role in the immune system, regulating lymphocyte development and activation through the Src family kinases (SFKs) (Hermiston et al., 2003). CD45 maintains SFKs in a primed, quiescent state by dephosphorylating both inhibitory and activating tyrosines. These unphosphorylated SFKs assume an open conformation that can be rapidly activated by phosphorylation of the activation loop tyrosine. (Palacios and Weiss, 2004). In the absence of CD45 activity, the inhibitory tyrosine is phosphorylated by the kinase Csk and the SFK adopts a closed, inactive conformation. One means of regulating CD45 is by spontaneous homodimerization, which inhibits its activity, preventing SFK activation and antigen receptor signaling (Desai et al., 1993; Majeti et al., 1998). The crystal structure of a related phosphatase, RPTPα, revealed a possible mechanism for this inhibition by dimerization. RPTPα crystallized as a symmetric dimer in which a helix-turn-helix motif, termed the "wedge", from one molecule was inserted into the catalytic site of the opposing molecule (Bilwes et al., 1996). Mutation of an acidic amino acid at the tip of the CD45 wedge prevented dimerization mediated inhibition in vitro, and moreover, led to a lupus like syndrome in

mice when knocked into the endogenous CD45 locus (E613R) (Majeti et al., 1998; Majeti et al., 2000). In a mixed C57BL/6 (B6) and 129 genetic background, the E613R wedge mutant mice develop a pronounced lymphoproliferative disease, autoantibodies, and immune complex-mediated glomerulonephritis (Majeti et al., 2000). However, as is the case with many autoimmune phenotypes, when the wedge mutation was backcrossed onto a homeogenous B6 background, much of the disease resolved (Hermiston et al., 2005). The CD45 wedge mutation still results in a much milder lymphoproliferation and elevated IgM levels, but autoantibodies and glomerulonephritis are absent. B cells from these backcrossed wedge mutant mice are also hyper-responsive to various stimuli, suggesting that the CD45 wedge mutant behaves much like a hypermorphic allele, but is not sufficient to cause autoimmunity on the B6 background.

We therefore asked whether or not the wedge mutation could act as a genetic modifier with other mutations predisposing humans and mice to autoimmunity. The criteria for selecting such mutations to study were their ability to induce a mild, slowly progressing autoimmunity on the B6 background. The complement *C4* knockout and a naturally occurring Fas mutation called *lpr* met these requirements. Complement is a component of the innate immune system involved in the opsonization and clearance of pathogens and possibly apoptotic bodies (Carroll, 1998; Manderson et al., 2004; Taylor et al., 2000). Fas, when bound by Fas-ligand (FasL), triggers apoptosis, frequently on activated cells, and hence is part of the activation induced cell death pathway. Fas is expressed in numerous tissues, while FasL is thought to be restricted to activated T cells and certain "immune privileged" tissues such as the eye and testis (Ferguson and Griffith, 2006; Nagata and Golstein, 1995). In mice, the phenotypes of the *C4* knockout and *lpr*

mutation are highly dependent on the strain background. Both mixed B6 x 129 C4 knockout and MRL/lpr mice develop very robust autoimmune lupus-like disease, characterized by lymphocyte activation, autoantibodies against nuclear components, immune complex deposition, and glomerulonephritis (Andrews et al., 1978; Chen et al., 2000; Cohen and Eisenberg, 1991; Einav et al., 2002; Manderson et al., 2004; Nagata and Golstein, 1995; Prodeus et al., 1998). However, backcrossing both *C4* and *lpr* to the B6 background mitigates most of their phenotype. In these mice, low levels of autoantibody are detected only in older mice, without evidence of overt organ damage (Izui et al., 1984; Paul et al., 2002; Pisetsky et al., 1982). Here, we find that combination of C4 deficiency and the CD45 wedge mutation does not alter disease compared to single mutant mice. However, the wedge mutation and lpr act synergistically, dramatically accelerating autoantibody production, lymphocyte activation and lymphoproliferation. Thus, the wedge mutation acts as a potent modifier with the lpr mutation, but not C4 deficiency. In addressing the mechanisms behind the wedge/lpr phenotype, we further demonstrate that T cells, and specifically signaling through the TCR, as well as B cells are required for lymphoproliferation, suggesting the presence of a positive feedback loop between these two cell types. Moreover, B cells appear to be intrinsically activated, and therefore their activation may be the primary event driving lymphocyte activation and expansion.

Results

CD45 wedge/lpr mice die at an accelerated rate with autoantibody production

We generated wedge/lpr and wedge/C4 homozygous double mutant mice and followed cohorts consisting of 10 mice, 5 male and 5 female, from wild-type (WT), single, and double mutant backgrounds over the course of a year. Of these cohorts, wedge/lpr double mutant mice showed significantly accelerated mortality, with 100% of mice dying by 9 months (Figure 2-1A). Lpr mice also died at an accelerate rate, but not to the same extent as wedge/lpr, while the other cohorts, wedge, C4, and C4/wedge showed no difference in survival compared to WT. To understand the increased mortality, we analyzed serum collected from these mice for the presence of anti-nuclear antibodies (ANA) by Hep2 nuclei staining and dsDNA antibodies by ELISA. Only wedge/lpr mice consistently and rapidly developed ANA and dsDNA antibodies (Figure 2-1B, C). ANAs appeared as early as 2 months of age in wedge/lpr mice, whereas dsDNA antibodies of the IgG subtype peaked at approximately 4 months. Only serum from older single mutant lpr mice contained low titer autoantibodies, as has been previously reported for the B6 background (Izui et al., 1984). Six month old lpr mice possessed ANAs, while dsDNA antibodies were present by 9 months (data not shown). C4 and wedge/C4 mice, however, did not develop any detectable autoantibodies during the course of the experiment. Other studies have found dsDNA antibodies in C4 knockouts on a mixed B6 x129 genetic background, but mice backcrossed five generations to B6 produced only low levels of IgG autoantibody (Chen et al., 2000; Paul et al., 2002). The mice used in these experiments were backcrossed eight generations, which may explain the absence of autoantibodies. Serum levels of total IgG were also significantly higher in CD45 wedge/lpr double mutant mice, and total IgM levels were elevated to a similar extent in both wedge and wedge/lpr (data not shown).

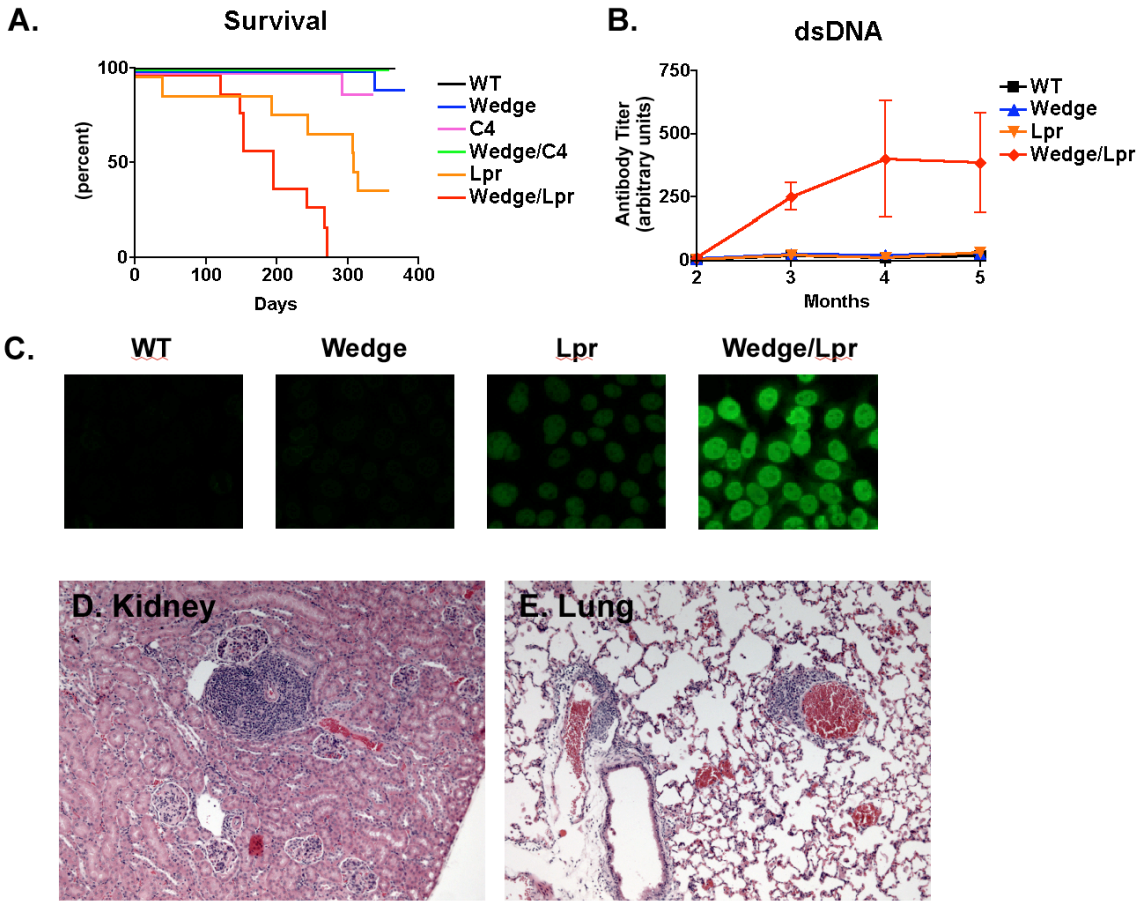
Figure 2-1: Early lethality and autoantibody production in CD45 wedge/lpr double mutant mice.

(A) Kaplan Meier survival curve from aging cohorts. N=10 mice per group, 5 males and 5 females.

(B) anti-dsDNA ELISA of serum collected at monthly intervals from aging mice, 6-10 mice per group. Error bars represent SEM.

(C) Staining of Hep2 nuclei with serum diluted 1:40 from 2 month old animals. Images are representative of 3 animals per group

Perivascular lymphocyte accumulation in kidney (D) and lungs (E) of CD45 wedge/lpr mice.


A.
Survival
100
50
0
(percent)
0
100
200
300
400
Days
WT
Wedge
C4
Wedge/C4
Lpr
Wedge/Lpr
B.
dsDNA
750
500
250
0
Antibody Titer
(arbitrary units)
2
3
4
5
Months
WT
Wedge
Lpr
Wedge/Lpr
C.
WT
Wedge
Lpr
Wedge/Lpr
D. Kidney
E. Lung

In MRL/lpr mice, autoantibodies and early death are often linked to antibody deposition in the kidneys and glomerulonephritis. Proteinuria is commonly used as a marker for kidney damage in these mice, but surprisingly neither immune complex deposition nor proteinuria were detected in CD45 wedge/lpr mice as late as nine months of age (data not shown). Histological examination of kidneys from six to nine month old wedge/lpr mice did, however, reveal increasing amounts of perivascular lymphocyte accumulations with age. The extent and organs affected by these perivascular infiltrates varied from mouse to mouse, but the accumulations were most prominent in kidney and lung and to a lesser extent the liver (Figure 2-1D, E). The lymphocytes were typically well circumscribed and did not infiltrate into the surrounding tissue, suggesting they might be contained within dilated lymphatic vessels. Immunohistochemical analysis revealed the cells to consist almost exclusively of CD3+ and CD4+ T cells (Figure 2-2A-D). Mice with defects in Fas also possess a unique population of CD3+, B220+, CD4/CD8 double negative (DN) cells (Cohen and Eisenberg, 1991; Morse et al., 1982), but these cells were not observed in kidney sections by B220 immunohistochemistry, nor were B cells detected, which express the same marker (data not shown). In addition to the perivascular infiltrates, kidneys from older wedge/lpr mice appeared pale and contained enlarged, hypercellular glomeruli, which could represent early, subclinical renal ischemia (Figure 2-2E-I).

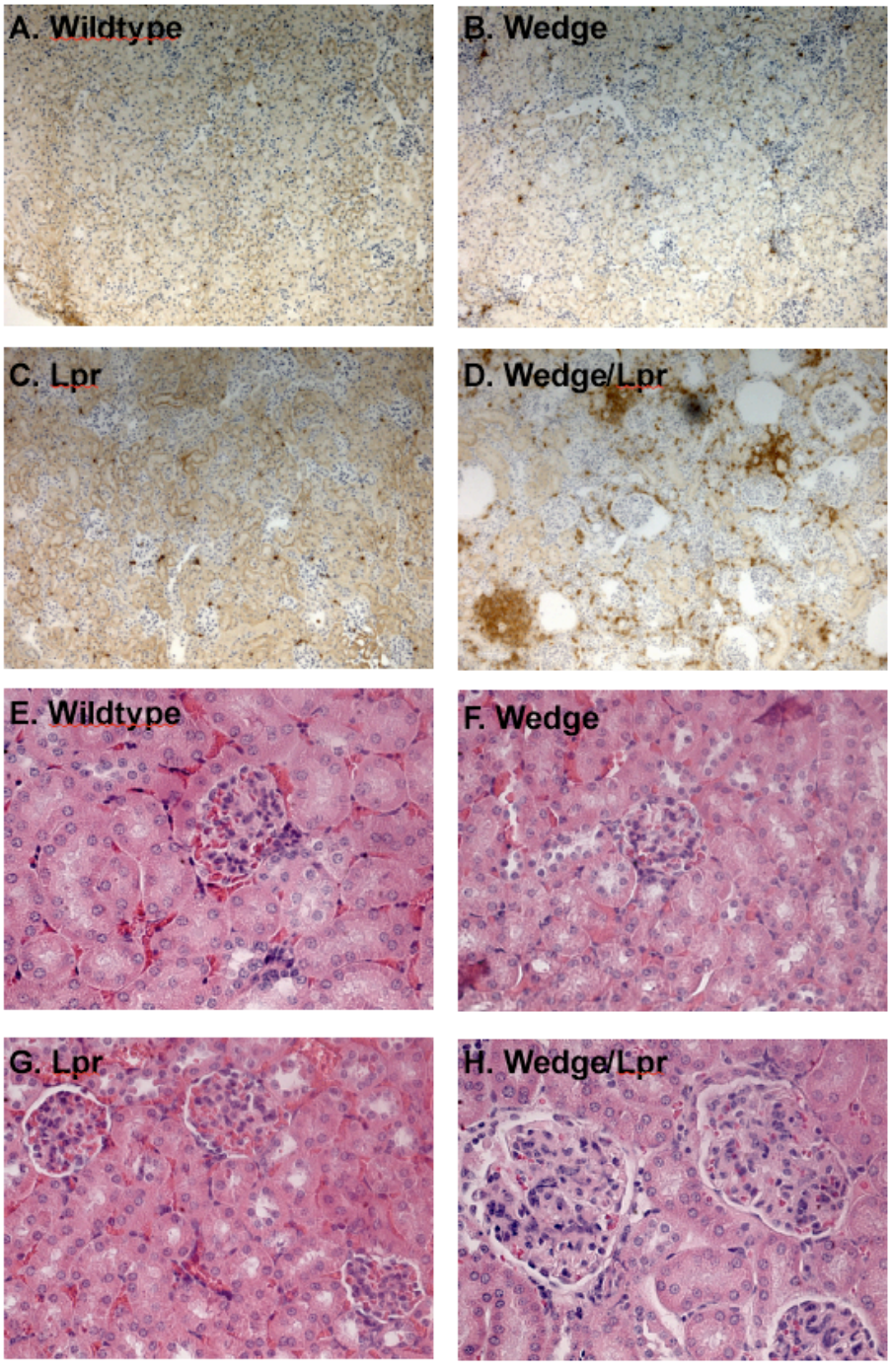
Wedge and lpr mutations cooperate to induce lymphocyte activation and expansion

On gross examination, the most striking feature of wedge/lpr mice is dramatic lymphadenopathy and splenomegaly, which progresses with age. Splenic weight in the

Figure 2-2: Kidney pathology in Wedge/Lpr mice

Representative fields of CD4 immunohistochemistry from (A) WT, (B) Wedge, (C) Lpr, and (D) Wedge/Lpr mice. Representative fields of H&E stained sections from (E) WT, (F) Wedge, (G) Lpr, and (H) Wedge/Lpr mice.

(I) Gross kidney appearance.


A. Wildtype
B. Wedge
C. Lpr
D. Wedge/Lpr
E. Wildtype
F. Wedge
G. Lpr
H. Wedge/Lpr
I.
Wildtype
Wedge
Lpr
Wedge/Lpr

wedge/lpr double mutant mice was significantly higher than either single mutant or wildtype spleens at 2, 4, and 6 months (Figure 2-3A). At 2 months, lymph node cellularity was already increased almost 10 fold in the double mutant mice (Figure 2-3B). Lymphocyte subsets in young wedge/lpr mice mirrored that of CD45 wedge single mutant mice, which we found to have a consistently higher proportion of CD3+ T cells versus CD19+ B cells and a slightly skewed CD4 to CD8 ratio (M. Hermiston manuscript in preparation; Figure 2-3C). However, as a result of the overall increased cellularity, the absolute numbers of both T and B cells were much higher in wedge/lpr mice (Figure 2-3D). Of note, wedge/lpr double mutant mice had a marked increase in the percentage and number of CD4/CD8 double negative peripheral T cells, which came at the expense of CD4 and CD8 single positive cells. The origin of these cells is still somewhat controversial, but they are believed to represent a population of activated T cells that fail to undergo activation induced cell death as a result of the Fas mutation (Laouar and Ezine, 1994; Pestano et al., 1999). Instead, the cells downregulate their CD4 or CD8 coreceptor and become refractory to stimulation (Giese and Davidson, 1992).

To determine whether the lymphocyte expansion was a consequence of increased proliferation in vivo, we measured BrdU incorporation in all four strains of mice. Mice 7-8 weeks old were given BrdU in their drinking water for 10 days and then analyzed. In wedge/lpr mice, approximately 60% of CD4 and CD8 T cells as well as B cells had divided and incorporated BrdU, a significantly higher percentage than wedge, lpr, and wildtype cells (Figure 2-3E). Wedge and lpr T cells also reproducibly incorporated BrdU at a rate faster than wildtype, although the increased incorporation in wedge mice was not statistically significant. Surprisingly, B cells were turning over much more rapidly in

Figure 2-3: Lymphocyte expansion in CD45 wedge/lpr mice.

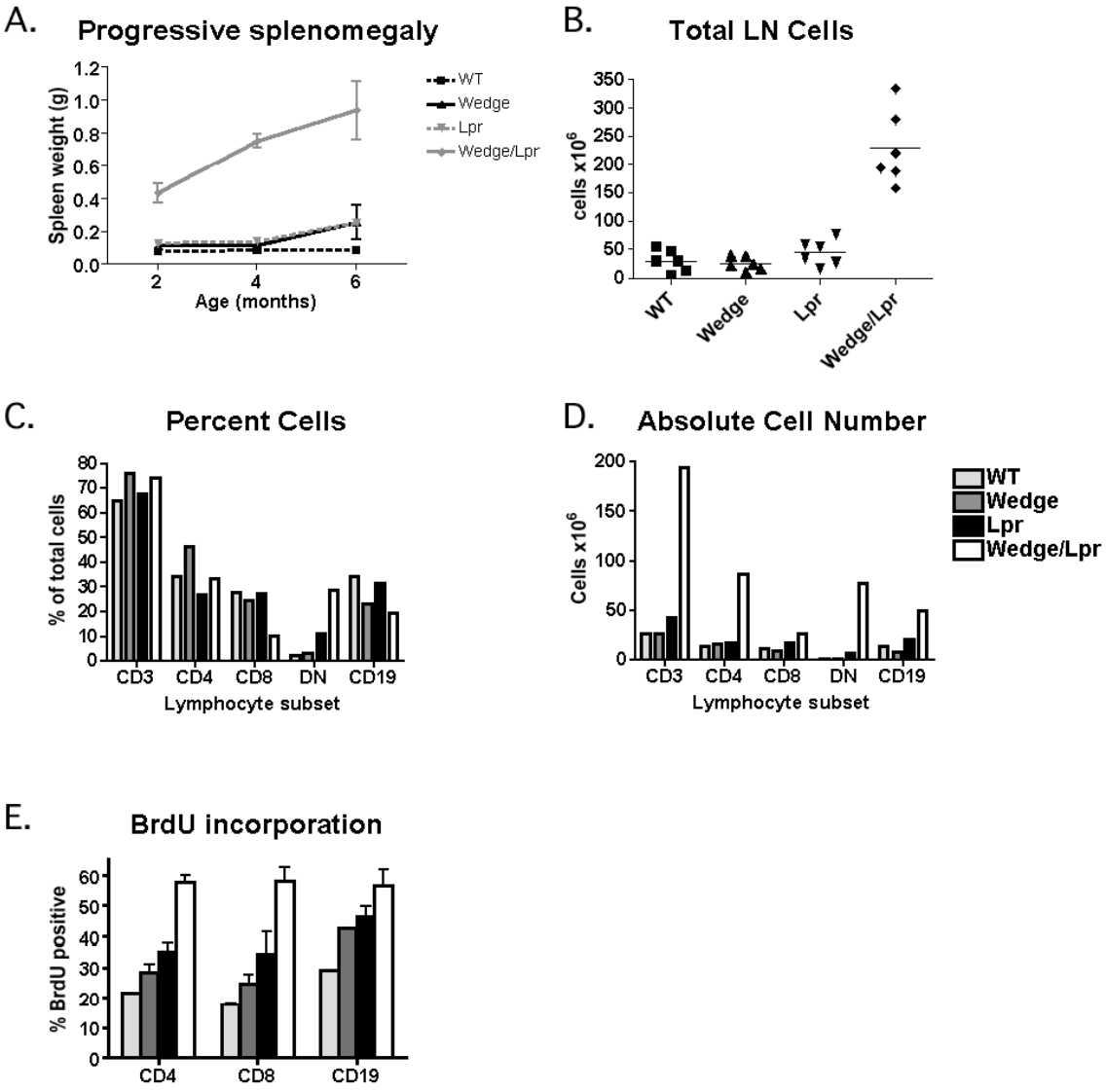
(A) Splenic weight of 2, 4, and 6 month old mice. 3-6 mice per time point.

(B) Total number of cells pooled from inguinal, axillary, brachial, cervical, and mesenteric lymph nodes of 2 month old mice.

(C) Lymphocyte subset analysis of lymph nodes from 2 month old mice determined by flow cytometry. CD3+ cells are broken down into CD4+, CD8+, and double negative (CD3+, B220+, CD4-/CD8- (DN)) cells

(D) Absolute number of T cell subsets and B cells in lymph nodes from 2 month old mice determined by multiplying the percent of each subset with the total number of lymph node cells.

(E) BrdU incorporation in CD4 and CD8 T cells and B cells. Mice were given BrdU in their drinking water for 10 days before analyzing


A.
Progressive splenomegaly
Spleen weight (g)
Age (months)
WT
Wedge
Lpr
Wedge/Lpr
B.
Total LN Cells
cells x10^6
WT
Wedge
Lpr
Wedge/Lpr
C.
Percent Cells
% of total cells
CD3
CD4
CD8
DN
CD19
Lymphocyte subset
D.
Absolute Cell Number
Cells x10^6
WT
Wedge
Lpr
Wedge/Lpr
Lymphocyte subset
E.
BrdU incorporation
% BrdU positive
CD4
CD8
CD19

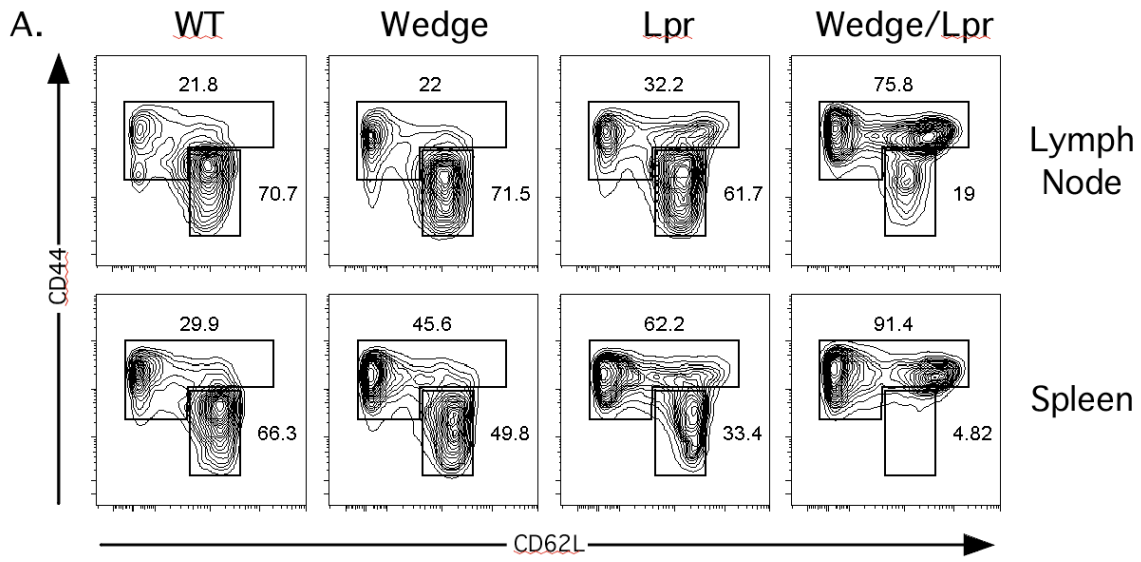
both wedge and lpr mice. Since these mice do not display any lymphadenopathy or splenomegaly at this age, other homeostatic mechanisms must be controlling total cell number. These data strongly suggest that wedge/lpr T and B cells are in fact actively proliferating in vivo. However, we cannot exclude the possibility that wedge/lpr T cells are being produced and emigrating from the thymus at an accelerated pace, but the percentage and absolute number of wedge/lpr thymic subsets are indistinguishable from normal, arguing against any large changes in T cell development (data not shown).

Fitting with this observed lymphoproliferation, both peripheral T and B cells in wedge/lpr mice also appeared to be activated at a higher frequency. In 2 month old mice, a majority of CD4 and CD8 T cells had already acquired a memory phenotype, defined by upregulation of CD44. This was most impressive in the spleen, where the fraction of memory CD4 and CD8 cells reached 90% and 75%, respectively. (Figure 2-4A and Figure 2-5A). Lpr single mutant mice possess an unusual population of CD4 cells that have upregulated CD44 but failed to downregulate CD62L, as is typical of normal memory cells, and this population was likewise expanded in wedge/lpr double mutants. The wedge/lpr memory phenotype cells also showed a strong Th1 polarization, with a large fraction of the CD4+ CD44 high cells producing IFNγ, but no IL-4 or IL-17, upon direct ex vivo stimulation with PMA and ionomycin (Figure 2-5B and data not shown). The IFNγ production is only seen in a much smaller fraction of cells from the other genotypes, and not in CD44 low cells. These results suggest that the memory cells in wedge/lpr mice have in fact been primed and are capable of secreting IFNγ in vivo. In addition to the increase in memory cell markers, wedge/lpr mice have a higher fraction of cells that express the immediate activation marker CD69, which suggests that an ongoing

Figure 2-4: Enhanced lymphocyte activation

(A) Representative flow cytometric analysis of CD4 memory phenotype cells. CD3+ CD4+ gated cells from lymph node and spleen were costained for CD44 and CD62L expression. Memory phenotype cells are CD44 high, while naïve cells are CD44 low, CD62L high. Numbers represent percent of CD4+ cells.

(B) Graphic representation of MHC class II expression on CD19+ B cells and CD86 expression on CD11b+ CD11c+ dendritic cells.


A.
WT
Wedge
Lpr
Wedge/Lpr
21.8
70.7
22
71.5
32.2
61.7
75.8
19
Lymph Node
29.9
66.3
45.6
49.8
62.2
33.4
91.4
4.82
Spleen
CD44
CD62L

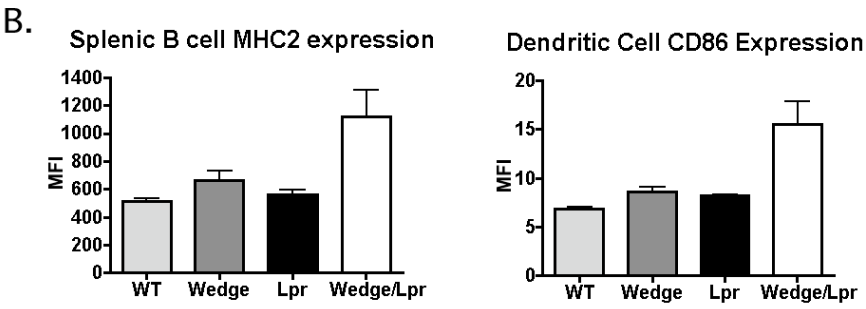
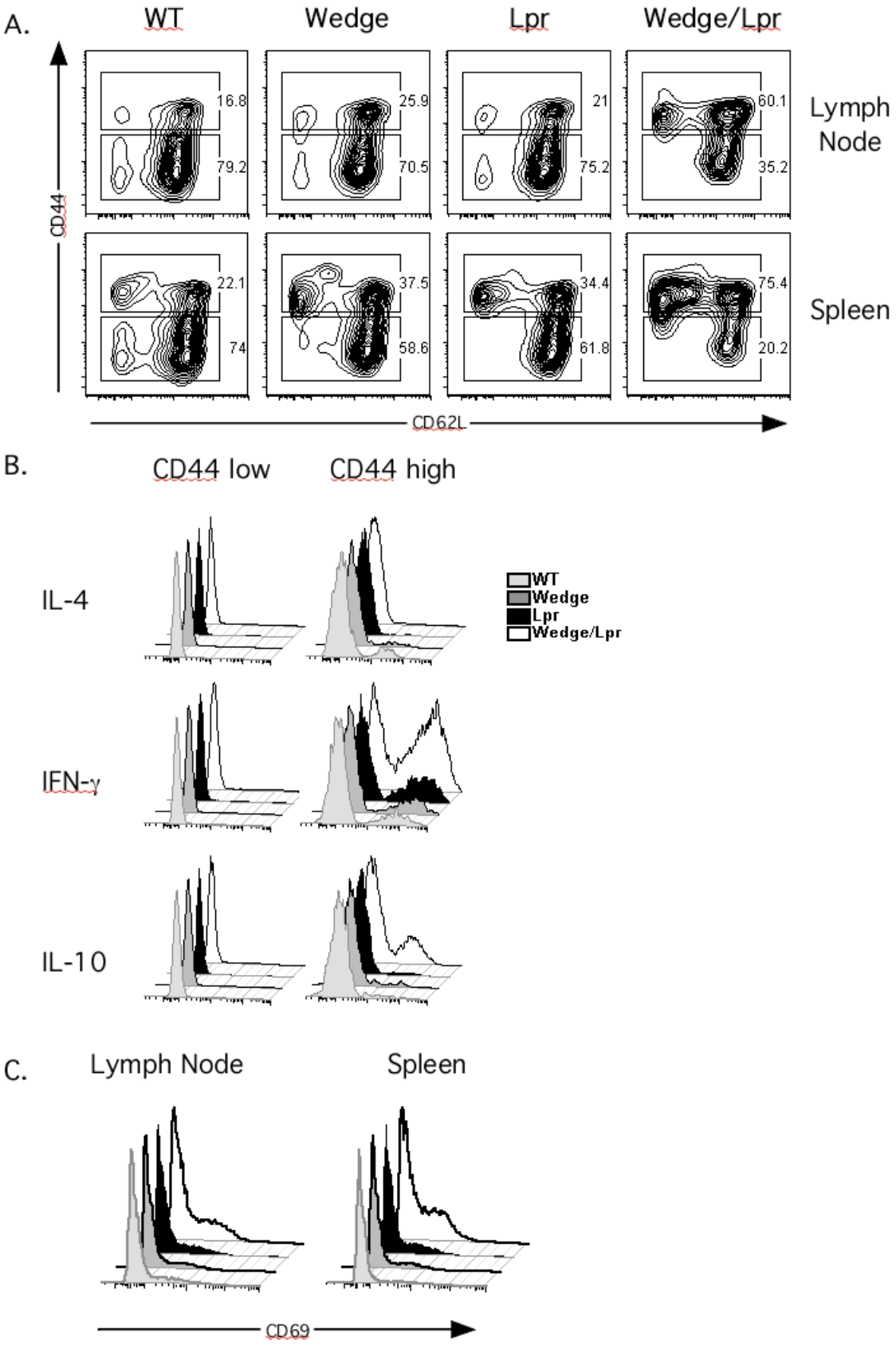

B.
Splenic B cell MHC2 expression
MFI
1400
1200
1000
800
600
400
200
0
WT
Wedge
Lpr
Wedge/Lpr
Dendritic Cell CD86 Expression
MFI
20
15
10
5
0
WT
Wedge
Lpr
Wedge/Lpr

Figure 2-5: T cell activation and cytokine production

(A) Representative flow cytometric analysis of CD8 memory phenotype cells. CD3+ CD8+ gated cells from lymph node and spleen were costained for CD44 and CD62L expression. Memory phenotype cells are CD44 high, while naïve cells are CD44 low. Numbers represent percent of CD8+ cells.

(B) Representative overlays showing intracellular cytokine staining for IL-4, IFNγ, and IL-10. Isolated lymph node cells were stimulated with PMA and ionomycin in the presence of Brefeldin A for 5 hours. Cells were gated as CD3+ CD4+ and then separated based on high and low CD44 expression.

(C) Representative overlays of CD69 expression on CD3+ CD4+ cells from lymph node and spleen

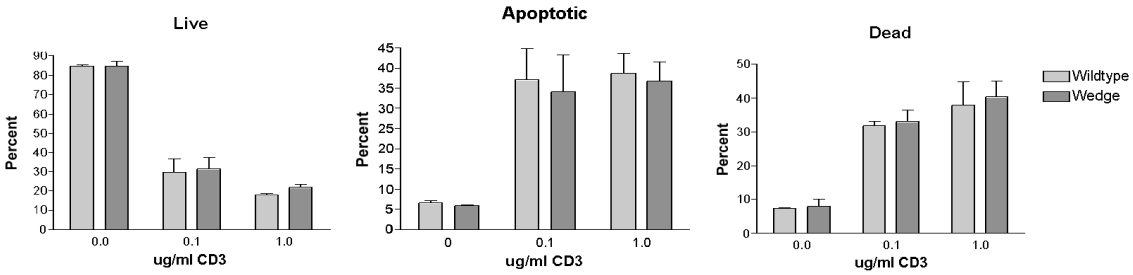

A.
WT
Wedge
Lpr
Wedge/Lpr
16.8
79.2
25.9
70.5
21
75.2
60.1
35.2
Lymph Node
22.1
74
37.5
58.6
34.4
61.8
75.4
20.2
Spleen
CD44
CD62L
B.
CD44 low
CD44 high
IL-4
IFN-γ
IL-10
WT
Wedge
Lpr
Wedge/Lpr
C.
Lymph Node
Spleen
CD69

process of T cell activation is occurring (Figure 2-5C). Analysis of B and myeloid cells suggests that these lineages were also activated in the double mutant mice. MHC class II molecules and the costimulator CD86 are upregulated on activated cells, and these markers were approximately two fold higher on wedge/lpr B cells and dendritic cells compared to wildtype cells (Figure 2-4B-D).

The wedge mutation does not enhance activation induced cell death or proliferation in vitro

We next set out to understand the mechanism driving the T cell activation and expansion in the CD45 wedge/lpr double mutant mice. Based on our observations that wedge mutant B cells are hyper-responsive to stimulation, we hypothesized that wedge T cells would also have a reduced threshold for activation and thus be more susceptible to activation induced cell death (AICD) mediated by Fas. We further reasoned that removal of Fas by addition of the lpr mutation to wedge mice would prevent AICD, allowing activated cells to survive and accumulate, and accounting for the dramatic phenotype of wedge/lpr mice. To our surprise, standard in vitro AICD assays did not reveal any differences in the apoptosis of wedge versus wildtype T cells (Figure 2-6). Lpr and wedge/lpr T cells were resistant to death in these experiments, indicating that induction of death was Fas dependent (data not shown). Although Fas is not believed to play a role in controlling lymphocyte proliferation (Van Parijs et al., 1996), we had reasoned that the wedge mutation may unmask an effect of Fas, but there was also no difference in CD3 plus CD28 induced in vitro proliferation of CD4 or CD8 T cells amongst the four genotypes at multiple doses and time points (data not shown). These data raise the

Figure 2-6: Wedge T cells are not more sensitive to activation induced cell death. WT and Wedge cells were stimulated in vitro for 3 days with 5 μg /ml plate bound anti-CD3 and 1μg/ml soluble anti-CD28, rested for 1 day with IL-2, and restimulated with 0, 0.1, and 1.0 μg /ml plate bound anti-CD3 for 6 hours before analysis. Cells were stained for Annexin V and 7-AAD. Live cells are defined as Annexin V-, 7-AAD-. Apoptotic cells are Annexin V+, 7-AAD-. Dead cells are Annexin V+, 7-AAD+


Live
Percent
ug/ml CD3
0.0
0.1
1.0
Apoptotic
Percent
ug/ml CD3
0
0.1
1.0
Dead
Percent
ug/ml CD3
0.0
0.1
1.0
Wildtype
Wedge

possibility that the wedge mutation has different effects on responses to strong in vitro stimulation versus in vivo endogenous signals.

TCR signaling and B cells are required for lymphoproliferation

Having not gained any insight into the cause of the increased T cell activation using in vitro experiments, we undertook a genetic approach. To examine the role of signaling through the T cell receptor, we restricted T cells to a single TCR specificity by crossing wild-type, single mutant, and double mutant mice to the ovalbumin-peptide reactive OT2 TCR transgene, limiting the potential antigens to which T cells can respond. The OT2 TCR transgene completely eliminated the presence of memory phenotype T cells in wedge/lpr mice, arguing that a subset of wedge/lpr T cells are in fact being activated through the TCR in response to certain endogenous antigens, possibly self-peptide or commensal organisms (Figure 2-7B). Restricting the TCR repertoire also corrected the lymphadenopathy and splenomegaly, as did complete elimination of T cells using TCR$\alpha^{-/-}$ mice (Figure 2-7A). Furthermore, wedge/lpr TCR$\alpha^{-/-}$ mice failed to produce autoantibodies by 5 months of age (data not shown). Wedge/lpr B cells must therefore require T cell help to expand and secrete autoantibodies. Interestingly, B cells and DCs from wedge/lpr TCR$\alpha^{-/-}$ mice still expressed higher levels of MHC class II and CD86, suggesting that antigen presenting cell activation is a T cell independent effect (Figure 7C).

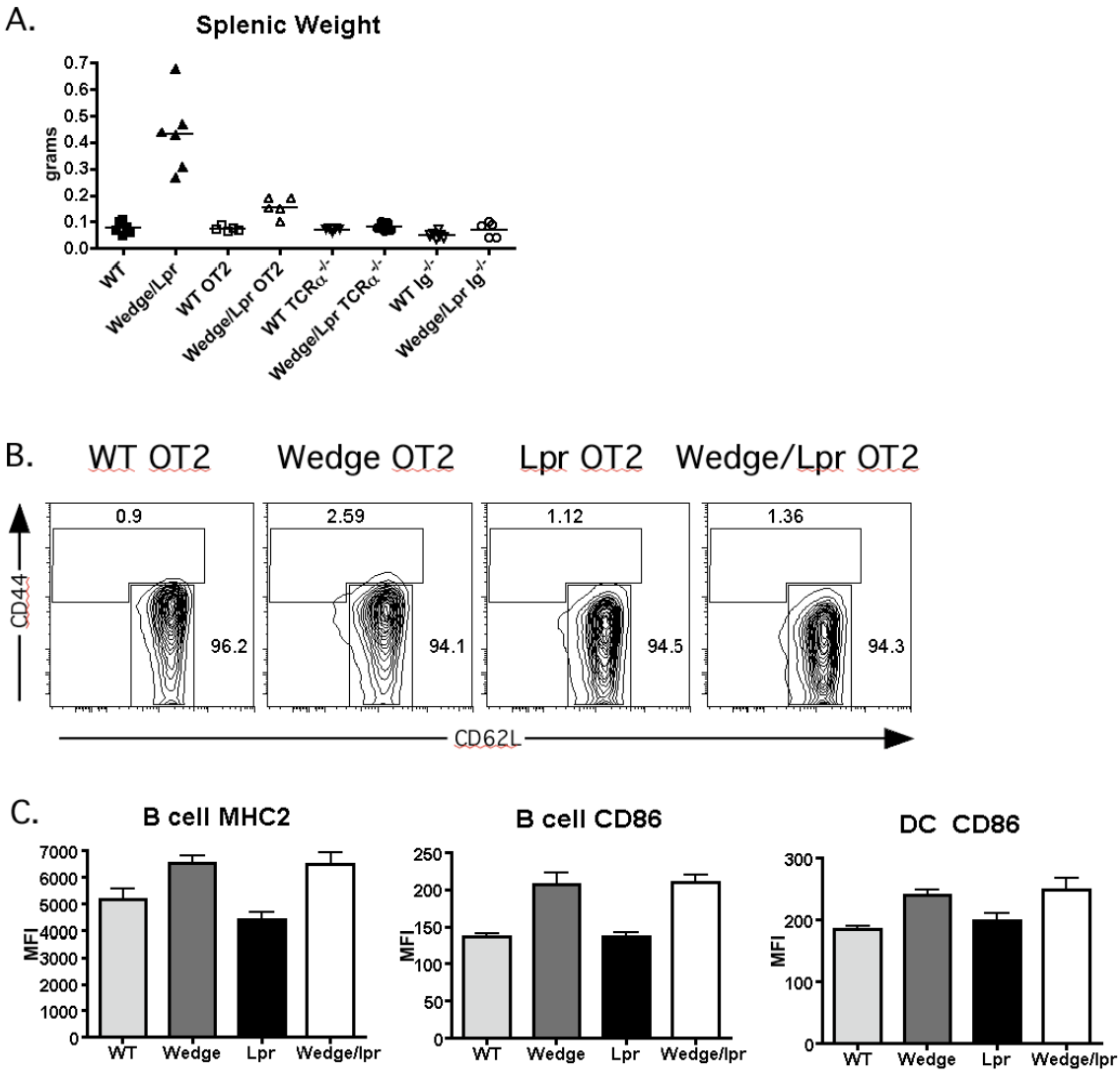
Previous work has demonstrated a role for B cells in the lymphocyte expansion of both mixed background wedge mutant and lpr mice (Akashi et al., 1998; Chan and Shlomchik, 1998; Hermiston et al., 2005). We therefore crossed the μMT mutation (Ig$^{-/-}$)

Figure 2-7. TCR specificity and T cells contribute to lymphocyte activation and expansion.

(A) Splenic weight as an indicator of lymphoproliferation in normal, OT2 TCR transgenic (OT2), T cell deficient (TCR$\alpha^{-/-}$), and B cell deficient mice ($Ig^{-/-}$) comparing WT and Wedge/Lpr. Each point represents a single mouse. 5-6 mice per group. Bars represent the mean of the group.

(B) Representative flow cytometric analysis of CD4 memory phenotype cells in OT2 TCR transgenic mice. OT2 cells were gated as CD3+ CD4+ Vα2+ Vβ5+.

(C) Graphic representation of activation markers on B cell and DCs from TCR$\alpha^{-/-}$ mice.


A.
Splenic Weight
grams
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
WT
Wedge/Lpr
WT OT2
Wedge/Lpr OT2
WT TCRα-/-
Wedge/Lpr TCRα-/-
WT Ig-/-
Wedge/Lpr Ig-/-
B.
WT OT2
Wedge OT2
Lpr OT2
Wedge/Lpr OT2
0.9
96.2
2.59
94.1
1.12
94.5
1.36
94.3
CD44
CD62L
C.
B cell MHC2
B cell CD86
DC CD86
MFI
7000
6000
5000
4000
3000
2000
1000
0
250
200
150
100
50
300
WT
Wedge
Lpr
Wedge/lpr

onto wedge/lpr mice to eliminate B cells and found that the extent of T cell activation was significantly reduced (Figure 2-8A). The percentage of memory phenotype T cells in wedge/lpr $Ig^{-/-}$ mice was similar to that of Lpr $Ig^{-/-}$ T cells, arguing that B cells are playing a role in the activation of T cells. In addition, the absence of B cells completely abrogated lymphadenopathy and splenomegaly (Figure 2-7A). Therefore, elimination of either B or T cells prevents lymphocyte expansion.

B cells could be contributing to the accumulation of activated T cells by either directly stimulating activation and proliferation of T cells, or by promoting the survival of intrinsically activated T cells. To address these two possibilities we compared the BrdU incorporation of T cells from mice with and without B cells. If B cells were contributing to T cell proliferation, we anticipated a reduced rate of BrdU incorporation in T cells when B cells are absent. Alternatively, if they were promoting only survival, we would expect an equal rate of BrdU incorporation in the presence or absence of B cells. Removal of B cells in wedge/lpr $Ig^{-/-}$ mice reduced the rate of BrdU incorporation in T cells by 50%, consistent with B cells contributing to the proliferation of T cells (Figure 2-8B). However, the residual increase in turnover implies that there is also a partial defect in T cell survival in the absence of B cells, as there is no lymphadenopathy and splenomegaly in these mice. Thus, in contrast to the intrinsic activation of wedge/lpr B cells, T cells appear to be dependent on B cells for both activation and expansion.

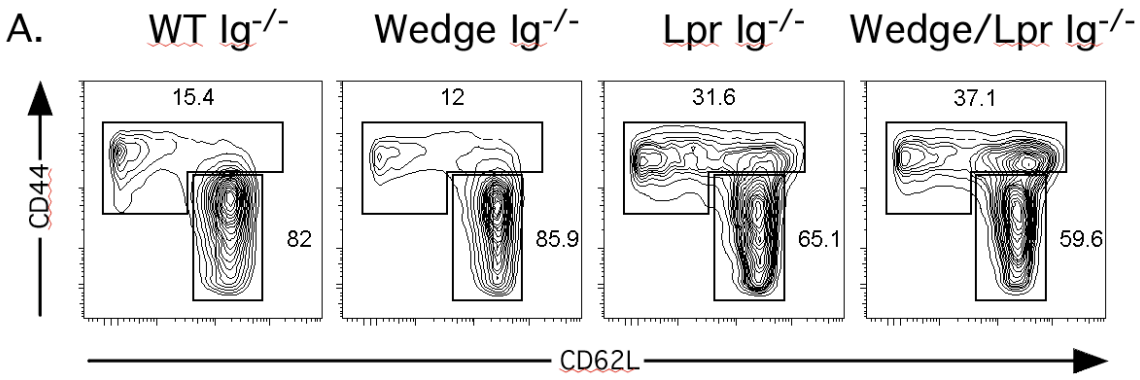
Discussion

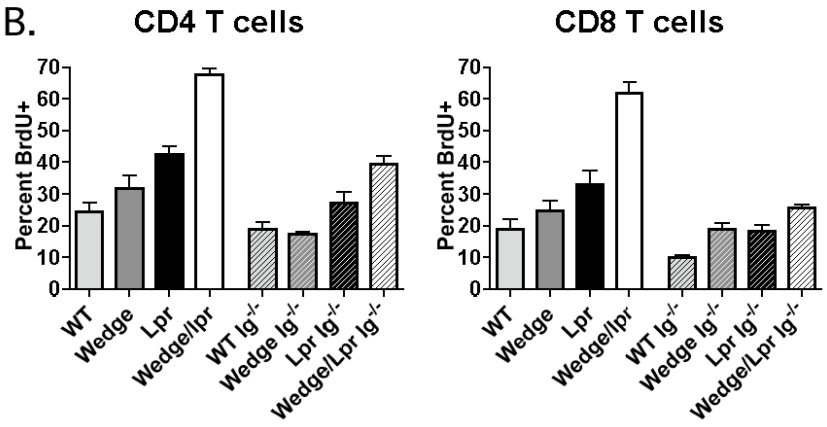
We have shown that the CD45 wedge mutation can act as a genetic modifier for the development of autoimmunity, but not in all contexts. When combined with the *lpr*

Figure 2-8. Reduced T cell activation and expansion in the absence of B cells

(A) Representative flow cytometric analysis of CD4 memory phenotype cells in B cell deficient ($Ig^{-/-}$) mice. Cells are gated as CD3+ CD4+

(B) T cell Brdu incorporation in the presence and absence of B cells. Normal (N = 2) and B cell deficient ($Ig^{-/-}$; N = 4) mice were administered BrdU in their drinking water for 10 days, and BrdU incorporation was measured in TCRβ+ CD4+ and TCRβ+ CD8+ cells


A.
WT Ig-/-
Wedge Ig-/-
Lpr Ig-/-
Wedge/Lpr Ig-/-
15.4
82
12
85.9
31.6
65.1
37.1
59.6
CD44
CD62L


B.
CD4 T cells
CD8 T cells
Percent BrdU+
0
10
20
30
40
50
60
70
WT
Wedge
Lpr
Wedge/lpr
WT Ig-/-
Wedge Ig-/-
Lpr Ig-/-
Wedge/Lpr Ig-/-

mutation in Fas, the wedge mutation dramatically accelerates and exacerbates the loss of tolerance, lymphocyte activation, and lymphoproliferation. In contrast, the wedge mutation and C4 deficiency do not cooperate despite the strong predisposition of C4 deficiency to result in a lupus-like syndrome. What does our data tell us about how the CD45 and Fas pathways interact? We propose the following model: It is likely that careful titration of antigen receptor signaling in both B and T cells is absolutely essential for keeping potentially autoreactive cells quiescent. When the balance of signaling is disrupted, as may be occurring with the wedge mutation, activation induced cell death through the Fas pathway provides a second failsafe to eliminate dangerous cells. However, failure of both these pathways then results in the autoimmune and lymphoproliferative phenotype seen in wedge/lpr mice.

T and B cells are positively selected based on the weak interactions of the their antigen receptors with different forms of endogenous self-antigens. Once the cells mature and reach the periphery, they continue to be exposed to endogenous ligands that induce tonic, low level signals, that they are critically dependent upon for survival. Ablation of either the antigen receptor or downstream signaling machinery in mature, naïve cells significantly shortens their lifespan (Kraus et al., 2004; Labrecque et al., 2001; Lam et al., 1997; Polic et al., 2001a). Signaling through the antigen receptor requires SFKs, and deletion of the T cell SFKs lck and fyn also limits T cell survival (Seddon and Zamoyska, 2002). CD45 is absolutely essential for SFK activity and normal development of T and B cells, and may therefore also be important for the tonic signal in the periphery.

Our lab has proposed a model for CD45 regulation, whereby homodimerization inhibits CD45 function via a wedge like structure (Desai et al., 1993). Mutation of the wedge in the cytoplasmic domain of CD45 relieves the inhibition, resulting in a hypermorphic CD45 allele that is more competent for signaling (Majeti et al., 1998; Majeti et al., 2000). This increased signaling is evident in both wedge mutant B cells stimulated through the BCR, and in wedge mutant T cells based on enhanced positive selection seen in the thymus (Hermiston et al., 2005) (M. Hermiston, manuscript in preparation). The same is likely to be true for the tonic signal in the periphery. Instead of the low signal required for maintaining naïve cell survival, enhanced signaling in the CD45 wedge mutant cells may be enough to activate some of them in the context of the right antigenic exposure. The slightly higher rate of BrdU incorporation in wedge T and B cells compared to wildtype cells is consistent with this idea.

Despite the increased turnover of wedge cells, they do not accumulate in young mice. The most likely explanation is that Fas keeps the activated cells in check through an inducible cell death pathway. The initial function ascribed to Fas was based on its ability to induce the death of in vitro stimulated cells (Brunner et al., 1995; Dhein et al., 1995; Ju et al., 1995). This observation led to the idea that Fas may clear activated cells at the end of an immune response (Mogil et al., 1995; Singer and Abbas, 1994; Van Parijs et al., 1998a), but a number of other studies have suggested otherwise (Hildeman et al., 2002; Scott et al., 1993). Fas deficient cells contract normally after strong antigenic stimulation, either with superantigen or pathogen, while Bim deficient cells persist (Hildeman et al., 2002; Pellegrini et al., 2003). Therefore Bim, rather than Fas, appears to be the essential molecule downregulating the immune response (Strasser and

Pellegrini, 2004). What then is the role of Fas? Fas deficient mice still accumulate activated cells in the absence of antigenic stimulation and even when kept in a germ free environment on antigen free food, arguing that Fas must be inhibiting some population of cells (Strasser and Pellegrini, 2004). Two groups have suggested that in fact, Fas may be controlling autoreactive cells activated by the tonic signal from endogenous antigen (Chen et al., 2006; Stranges et al., 2007). They further propose that in addition to killing the autoreactive T and B cells, Fas deletes cells presenting the autoantigen, such as dendritic cells. We believe our data fit this hypothesis. The wedge mutation increases the activation of antigen presenting cells as well as the proportion of cells that respond to self antigen, but Fas quickly deletes them. If Fas is removed, these cells survive. Hence the dramatic number of activated, dividing cells and the resulting lymphadenopathy and splenomegaly in wedge/lpr mice represents the failure of this peripheral checkpoint mechanism. Prevention of T cell activation and lymphoproliferation by restricting wedge/lpr T cells to the OT2 TCR supports the idea that activation of T cells with a more diverse repertoire is the result of auto-reactive TCRs responding to endogenous antigens, and that this could then lead to Fas dependent deletion of such antigen presenting cells and the responding T cells.

Although both T and B cells show increased activation and turnover in wedge/lpr mice, neither cell type alone is sufficient to cause lymphoproliferation. Neither T cell deficient nor B cell deficient wedge/lpr mice develop lymphadenopathy or splenomegaly. This phenomenon has also been previously documented for B cell deficiency in both lpr and wedge single mutant mice in appropriate genetic backgrounds. Elimination of B cells prevents the lymphadenopathy and splenomegaly that occur in mixed background

wedge mutant mice (Hermiston et al., 2005). The same is true for B cell deficient MRL/lpr and aged B6/lpr mice, which also fail to develop glomerulonephritis (Akashi et al., 1998; Chan and Shlomchik, 1998; Shlomchik et al., 1994). Together, these data suggest the presence of a positive feedback loop between B and T cells that lead to their mutual expansion (Shlomchik et al., 2001). In wedge/lpr mice activated antigen presenting cells, and more specifically B cells, may be responsible for initiating this loop. Wedge/lpr B cells express increased levels of the activation markers MHC class II and CD86, even in the absence of T cells. T cell activation, however, is significantly reduced in the absence of B cells even though dendritic cells and other antigen presenting cells might be present. Thus, the elevated levels of MHC class II and CD86 on B cells appear to be cell intrinsic and might represent the first event leading to T cell activation. Our BrdU data also suggests that B cells are contributing to T cell survival, based on the observation that wedge/lpr T cells turn over faster, but without accumulating. The enhanced activation and survival of wedge/lpr T cells may be responsible for their increased ability to produce IFNγ, which might then further promote the activation of B cells and the production of autoantibodies. Indeed, deletion of IFNγ or the IFNγ receptor in MRL/lpr mice reduced autoantibody production and nephritis, supporting a role for this cytokine in the feedback loop (Balomenos et al., 1998; Haas et al., 1997; Peng et al., 1997; Schwarting et al., 1998).

Collectively these data demonstrate the combined need for tight regulation of CD45 activity by the wedge motif and Fas mediated death, as well as the importance of B cells in promoting autoimmunity. Disrupting the CD45 wedge by substitution of a single amino acid may be amplifying the tonic signals from endogenous antigens that cells

constantly receive, pushing auto-reactive cells over the threshold for activation. Fas is likely to be an important checkpoint designed to control the activation of these auto-reactive cells. Mutation of both the wedge and Fas then allows B cells to become activated and survive, driving a positive feedback loop resulting in the activation of auto-reactive T cells, autoantibody production, and lymphoproliferation.

More broadly, this work highlights the ability of polymorphisms with subtle effects to act as significant genetic modifiers in autoimmunity. Interestingly, genetic modifiers may be an important factor in a human disease caused by Fas mutations called autoimmune lymphoproliferative syndrome (ALPS). Even though Fas mutations are autosomal dominant, parents of ALPS patients may carry the same mutation but not display any symptoms (Straus et al., 1999). Subtle differences in the signaling threshold of lymphocytes from these individuals may account for this variable penetrance. Wedge and wedge/lpr mice may therefore serve as a useful tool to begin understanding not only the pathogenesis of ALPS, but also the roles of B cells and tonic signaling thresholds in other autoimmune conditions.

Materials and Methods

Mice

CD45 E613R mice backcrossed at least nine generations to C57BL/6 were bred to C57BL/6 lpr and C4 knockout mice (Jackson Laboratories, Bar Harbor, Maine), respectively and maintained at homozygousity. OT2 mice were a generous gift from M. Peters. C57BL/6 TCR$\alpha^{-/-}$ and μMT mice were obtained from Jackson Laboratories. Mice were bled monthly from the tail vein for serum antibody analysis. All animals were

housed in a specific pathogen-free barrier facility at UCSF according to University and National Institutes of Health guidelines.

Autoantibody Assays

For anti-nuclear antibody analysis, serum was diluted 1:40 in PBS/1% FBS and applied to Hep2 ANA slides (iNOVA) for 30 minutes at room temperature. Slides were washed three times in PBS, incubated with FITC anti-mouse IgG for an additional 30 minutes at room temperature, and finally washed again. Images were captured using a Marianis system with a Sensicam cooled CCD camera (Cooke) attached to an Axiovert microscope. Anti-dsDNA antibody titers were determined by ELISA. Flat bottom 96 well polyvinyl chloride plates (Becton Dickinson) were first coated with poly-L-lysine (100μg/ml in 0.1M Tris HCl, pH 7.3) overnight at 4°C. Following three washes with water, poly dA-dT (20μg/ml in 0.1M Tris; Sigma) was added to each well and incubated overnight at 4°C. Plates were washed six times with water and then blocked with ELISA blocking buffer (1% BSA, 0.05% Tween 20 in PBS) for 1 hour at room temperature. Serum was applied to the plate at a starting dilution of 1:100 in blocking buffer, and then diluted in 2-fold steps up to seven times. Pooled MRL/lpr serum was used as a positive control. Plates were incubated with serum overnight at 4°C and then washed 6 times with water. The secondary antibody, HRP-conjugated goat anti-mouse (Southern Biotechnology) diluted 1:10,000 in blocking buffer, was added to the wells and incubated at room temperature for 1 hour. Plates were washed six more times and then developed with 3,3',5,5' Tetramethylbenzidine (Sigma) liquid substrate. Reactions were stopped by the addition of HCl and absorbance read at 450nm on a Molecular Devices plate reader.

Histology

Tissue was fixed in 10% formalin (Fisher) for at least 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For immunohistochemistry, tissue was immersed in OCT and snap frozen in 1,1,1,2 tetrafluoroethane (Fisher).

Flow Cytometry and antibodies

Pooled lymph nodes (inguinal, brachial, axillary, cervical, and mesenteric) and spleen were homogenized by grinding between frosted slides and passing through a 30μm filter. Red blood cells were removed from spleen by ACK treatment. Typically, $2x10^6$ cells were stained and analyzed immediately or fixed in 1% paraformaldehyde (Electron Microscopy) for analysis the following day. Data were collected on either a FACSCalibur (Becton Dickinson) or CyAn ADP (Dako). Antibodies used were conjugated to FITC/Alexa 488, PE, PE-Texas Red, PerCP-Cy5.5, PE-Cy7, Pacific Blue, Pacific Orange, or APC/Alexa 647. CD3, CD11b, CD11c, CD19, CD45R (B220), CD62L, CD69, TCR Vα2 and Vβ5 antibodies came from BD Biosciences; CD86 from Biolegend; CD4, CD44, and MHC class II from eBiosciences; and CD8 from Invitrogen.

Intracellular cytokine staining

Lymph node cells were stimulated for 5 hours with 20ng/ml PMA and 1μM ionomycin in the presence of 10μg/ml Brefeldin A. They were then washed in cold FACS buffer (2% FCS, 2mM EDTA in PBS), stained for CD3, CD4, CD8, and CD44, and fixed in 2% paraformaldehyde for 20 minutes at room temperature. Following three washes in PBS,

cells were washed one more time in permeabilization buffer (0.5% saponin, 2% FCS in PBS). Staining for cytokine was done in super permeablization buffer (3 parts permeablization buffer, 1 part FCS) using PE-conjugated anti-IL-4, anti-IFNγ, anti-IL-17, and anti-IL-10 (BD Biosciences) for 60 minutes at room temperature. Cells were washed twice in permeablization buffer, once in PBS, and once in FACS buffer before analyzing by flow cytometry.

Activation induced cell death assay

Lymph node CD4 T cells were purified by MACS negative selection (Miltenyi Biotec) and stimulated for 3 days in plates coated with 5μg/ml anti-CD3 (2C11, Harlan) and with 1 μg/ml soluble anti-CD28. At the end of 3 days, dead cells were removed with Lympholyte (Cedarlane Laboratories) according to manufacturer's instructions. Live cells were resuspended at 0.5 x 10^6/ml with 50 units/ml IL-2 and incubated for 24 hours. At the same time, 96 well plates were coated with 0, 0.1, and 1.0 μg/ml anti-CD3 overnight at 4°C. After 24 hours, dead cells were again removed with Lympholyte and live cells were added to the anti-CD3 coated plate at 0.2 x 10^6/well. Cells were harvested 6-24 hours later, stained with Annexin V and 7-AAD (BD Biosciences) according to manufacturer's instructions, and analyzed by flow cytometry.

Proliferation

Lymph node T cells were purified by MACS negative selection (Miltenyi Biotec) and labeled with 2.5μM CFSE. 96 well plates were coated with 0, 0.1, 1.0, and 10 μg/ml of anti-CD3 (2C11) overnight at 4°C. 0.5 x 10^6 cells were added to each well with or

without 1 μg/ml of soluble anti-CD28. Cells were harvested 48-120 hours later, stained for CD4 and CD8, and analyzed by flow cytometry.

In vivo BrdU incorporation

Experiments were performed essentially as described (Tough 1994). Briefly, mice were given water containing 0.8 mg/ml BrdU and 2% glucose for 10 days. Single cell suspensions were made from lymph node and spleen (ACK treated) and then stained for extracellular markers with antibodies conjugated to FITC/Alexa 488, Pacific Blue, and Pacific Orange. Cells were then fixed in 70% ethanol for 30 minutes on ice, washed, and resuspended in 1% paraformaldehyde/PBS for 30 minutes at room temperature. Intracellular DNA was digested using DNase solution (50 Kunitz U/ml , 4.2 mM $MgCl_2$, 0.15 M NaCl). After washing away the DNase solution, cells were stained with anti-BrdU (PRD-1) conjugated to Alexa 647 (Invitrogen), washed again, and analyzed by flow cytometry.

Chapter 3: Discussion

These experiments raise a number of questions about the interaction of genetic modifiers. Despite the pronounced autoimmunity induced individually by C4 deficiency and the CD45 E613R wedge mutation on a mixed C57BL/6 (B6) x 129 background, the two mutations together on a homogenous B6 background are not sufficient to cause disease. On the other hand, combination of the lpr and wedge mutations results in dramatic lymphoproliferation and loss of tolerance, but little to no end organ damage. Why do C4 deficiency and the wedge mutation not cooperate, while lpr and the wedge mutation do? How are the lpr and wedge mutations cooperating? Finally, although wedge/lpr double mutant mice show a loss of tolerance to auto-antigens, why is there no end organ damage?

CD45 wedge mutation and C4 knockout

C4 and C1q deficiencies are strong predisposing factors for lupus in humans, and the initial descriptions of mouse knockouts for these molecules seemed to corroborate these observations (Botto et al., 1998; Chen et al., 2000; Manderson et al., 2004). However, the findings in mice were made on mixed backgrounds, and when the C4 was backcrossed to B6, or the C1q knockout to either 129 or B6, the phenotypes all but disappeared (Botto et al., 1998; Mitchell et al., 2002; Paul et al., 2002). One explanation for the lack of cooperation between C4 deficiency and the wedge mutation might simply be the ability of other complement factors to compensate for C4 deficiency. Alternatively, complement deficiencies may not be significant contributors to autoimmunity on the B6 background and may have different roles in the pathogenesis of autoimmune disease in mice and humans. Although macrophages from complement

deficient humans and mice showed similar defects in uptake of apoptotic bodies, the mice used were from a mixed background, and the connection between reduced uptake of apoptotic bodies and autoimmune disease is still speculative (Manderson et al., 2004; Taylor et al., 2000).

Another possibility for the lack of cooperation may be that instead of cooperating, the two mutations may have opposing effects. Macrophage phagocytosis mediated by Fcγ receptors proceeds through a SFK and ITAM dependent pathway, and may therefore be regulated by CD45 (Berton et al., 2005). Although we have not investigated phagocytosis in CD45 wedge mutant macrophages, it is not unreasonable to expect phagocytosis to be enhanced in the context of the wedge mutation, which could compensate for the reduced immune complex clearance in C4 deficiency. Similarly, in B cells, the complement receptor CD21/35 (Cr2) exists in a complex with CD19, which enhances BCR signaling through SFK recruitment (Rickert, 2005). Signaling through this complex may play a role in B cell anergy induction. Knocking out either *Cr2* or *C4* has been reported to inhibit anergy, while the CD45 wedge mutation promotes anergy, and thus the *C4* and wedge mutations may have opposing effects that together cancel out (Hermiston et al., 2005; Prodeus et al., 1998). This hypothesis could be tested using the same transgenic Ig-HEL/sHEL system used in the single mutant mice.

Finally, on the B6 background C4 deficiency may require types of genetic modifiers other than the wedge mutation. The CD45 signaling pathway and complement deficiency may not overlap sufficiently to cause disease when altered. A recent study has identified loci from the 129 and B6 backgrounds that contribute to the C1q knockout phenotype on the mixed background (Heidari et al., 2006). None of the loci overlapped

with CD45, but whether the loci contain genes involved in signaling or other immune modulators may indicate what other pathways are necessary for disease in complement deficiency. Similar loci might cooperate with C4 deficiency.

CD45 wedge and lpr mutations

CD45 wedge/lpr double mutant mice develop pronounced activation and expansion of lymphocytes as well as auto-antibody production. The two mutations by themselves have relatively mild phenotypes on the B6 background, arguing that the double mutant phenotype is truly a synergistic effect of the two mutations. However, *lpr* has been used to accelerate autoimmunity in a number of contexts, and therefore the possibility that lpr non-specifically enhances autoimmunity when crossed to any immune dysregulating mutation always exists. The *lpr* mutation has been bred to knockouts for *C1q, C4, Cr2, PD-1*, and *FcγRIIb* among others, each with different outcomes. *C4* and *PD-1* knockouts combined with lpr accelerated disease in both double mutants, although the C4/lpr double mutants were on a mixed B6 x 129 background, and the *PD-1* knockout by itself developed significant autoimmunity (Nishimura et al., 1999; Prodeus et al., 1998). The *Cr2* knockout and lpr mutation interacted in a more synergistic manner because neither mutation by itself had much of a phenotype (Wu et al., 2002). The phenotype of C1q/lpr double mutant mice was no different from that of single mutant controls (Mitchell et al., 2002). Finally, in the case of FcγRIIb, two groups published directly contrasting results, with one showing that *lpr* actually rescued the FcγRIIb phenotype, while the other showing that lpr exacerbated it (Bolland et al., 2002; Yajima et al., 2003). Collectively, these finding suggest that lpr does not necessarily interact

with any mutation that predisposes to autoimmunity, but instead has specific requirements for cooperating.

Origin of lymphoproliferation in wedge/lpr mice

Wedge/lpr mice have a massive expansion of the lymphoid compartment, and BrdU incoporation shows that the rate of T and B cell turn over is dramatically accelerated. However, the increased turnover could be a consequence of a number of processes, including increased production, proliferation, or survival, and distinguishing amongst these possibilities will be important for understanding the source of the lymphoproliferation. Cells are rapidly dividing and incorporating BrdU in the thymus, and thus the increased percentage of BrdU positive cells could be a result of greater thymic output. Increased production of T cells in wedge/lpr mice seems unlikely, though, based on the similarity in thymocyte subset percentages and absolute numbers compared to WT mice. A shorter 2 day BrdU pulse would more specifically rule out the contribution of production because 2 days is not long enough for T cells to transit through thymic development. To more directly assess the rate of proliferation, a cell cycle analysis could be performed using a DNA binding dye such as DAPI to determine the percentage of dividing cells. If, as we believe, the lymphocyte expansion is a result of mature peripheral cell proliferation, we would expect to see a greater percentage of dividing cells. Even in wedge/lpr mice, however, this percentage is likely to be very small, and therefore might be difficult to differentiate from WT. Finally, the increase in BrdU positive cells could represent enhanced survival of labeled cells rather than enhanced proliferation. In other words, the same number of cells could be dividing in

wedge/lpr mice compared to other genotypes, but if these cells had a survival advantage, they would accumulate more, thus appearing to be incorporating BrdU faster. Survival might be measured one of two ways. Mice could be given BrdU until 100% of their cells were labeled with BrdU, followed by a chase period to determine how quickly they are replaced by unlabeled cells. This approach might be confounded by dilution of the BrdU in dividing cells. Alternatively, cells could be labeled and adoptively transferred into mice and their half-life determined. This experiment might be best performed as a competition with two cell types, distinguished by congenic markers or labeled with CFSE and CMTMR, mixed at a 50:50 ratio to determine survival of one genotype relative to another.

T cell activation in wedge/lpr mice

The data presented here only begin to address how the lpr and wedge mutations cooperate. Based on the presumed roles of CD45 in lymphocyte activation and lpr in activation induced cell death (AICD), the most parsimonious hypothesis would have the wedge mutation increasing the activation of lymphocytes and the lpr mutation allowing these cells to survive and accumulate by disrupting AICD. However we failed to demonstrate either increased anti-CD3 induced activation of wedge mutant T cells or increased sensitivity to AICD. There are a number of explanations for these results.

In terms of AICD, the assays used are extremely artificial. The sequence of stimulating cells, resting them in IL-2, and then restimulating them, is not likely to mimic any process occurring in vivo. In vivo, cells are not being stimulated so strongly, but rather are only receiving the low-level survival signals induced by self-peptide/MHC

complexes. Another possibility is that in mature wedge mutant lymph nodes and spleen, the cells isolated for in vitro experiments have already been selected to be less sensitive to AICD. If fas mediated death is controlling wedge mutant cells, it may be eliminating cells very quickly after they mature into the periphery, and thus the sensitive cells are not represented in the assays. One way of getting around this problem may be to use CD4 single positive thymocytes. Assuming AICD is occurring in the periphery, this population may still contain the cells destined to undergo apoptosis. Thymocytes, however, are not fully mature, and may behave differently from peripheral cells.

Wedge mutant cells also failed to show an increased response to anti-CD3 stimulation as measured by proliferation. This result does not fit with previous results from wedge mutant B cells, which had an augmented response to anti-IgM stimulation (Hermiston et al., 2005). These observations suggest that CD45 plays a very different role in B versus T cells. In B cells, CD45 activity determines the output of BCR stimulation. T cell signaling, however, seems to be set independently of CD45 activity. It is possible that B and T cell signaling pathways are set up differently such that B cells generate a graded response, whereas T cells have more of an all-or-nothing response. However, given the homology between T and B cell signaling components, and the teleologic argument that both cell types would want to be designed to respond maximally to very limiting amounts of antigen, it seems unlikely there would be significant differences between the sensitivity of T and B cells. Moreover, T cells do not show a dose response that is so abrupt, except in the thymus where the difference between positive and negative selection is rather acute.

A second explanation for the difference between T and B cells may be the CD45 isoform usage. B cells predominantly express the high molecular weight B220 isoform, while T cells express lower molecular weight isoforms that are developmentally regulated (Hermiston et al., 2003). Although CD45 dimerization seems to occur less efficiently in higher molecular weight isoforms compared to lower molecular weight, the regulation may be more complicated (Xu and Weiss, 2002). In T cells it is also possible the wedge mutation may play a more important role in memory cells than naïve cells, as has been reported for the phosphatase PEP (Hasegawa et al., 2004). Isoform usage changes as cells differentiate into memory cells, and could thus affect CD45 dimerization. This could be tested by generating memory cells in vivo using TCR transgenic cells and immunization with their cognate antigen. The response of wildtype and wedge mutant memory cells to stimulation could then be compared.

The explanation I favor for the lack of difference in wedge and wildtype T cell stimulation involves differential roles for CD45 in strong and weak signaling. Although based on our results CD45 may not determine signal strength in response to strong TCR stimulation, it may determine the signal strength from the weak TCR stimulation cells receive in response to endogenous self-peptide/MHC complexes. Such signaling is required for naïve T cell survival. Experiments transferring cells into MHC deficient mice or deleting the TCR or Lck resulted in reduced T cell survival and demonstrated the importance of these weak, tonic signals (Labrecque et al., 2001; Polic et al., 2001b; Seddon and Zamoyska, 2002; Takeda et al., 1996; Tanchot et al., 1997). B cells have a similar requirement for signaling from the BCR and Igαβ (Kraus et al., 2004; Lam et al., 1997). In addition to its function in priming cells for maximum activation, CD45 may be

necessary for allowing tonic signaling. Moreover, the wedge mutation may turn the tonic signal into an activating signal. Unfortunately the most direct test to determine whether or not CD45 regulates tonic signaling would be inducible deletion of CD45 in mature peripheral cells, which would require a new transgenic mouse model. Nor has a quantitative analysis of the signal strength required for homeostasis been performed, making it difficult to judge how increased signaling from the wedge mutation might affect homeostasis.

Testing the role of tonic signaling in vitro is difficult because of the need for proper T cell/APC interactions and the lack of applied stimulus. In addition, differences in phosphorylation induced by tonic signals are likely to be very subtle and thus unlikely to be detectable by western blot. We do, however, provide some in vivo support for this hypothesis. Correction of the lymphoproliferation and T cell activation by the OT2 TCR transgene suggests that signaling through the TCR is required for lymphoproliferation and that restricting the repertoire eliminates the subset of T cell receptors that are hyperreponsive. This conclusion assumes that the TCR transgene, which must also interact with self-peptide/MHC, transmits a tonic signal that falls below the threshold for activation even with the wedge mutation. The multitude of TCRs generated randomly likely have a broad spectrum of responses to self-peptide/MHC. The wedge mutation may increase slightly the response of all TCRs. The increased positive selection of wedge mutant DO11.10 TCR transgenic thymocytes provides evidence of this enhanced signaling (M. Hermiston, manuscript in preparation). In the periphery, however, wedge mutant DO11.10 TCR transgenic do not appear to be more activated, probably because the increased activation is not enough to induce them to adopt a memory phenotype.

Presumably, there is a population of TCRs that are at the upper end of the spectrum of responses, but just below the threshold for activation to memory phenotype cells. It is in these cells that the wedge mutation might have the most dramatic effect, pushing them across the activation threshold. At the same time, though, in wedge mice these activated cells are deleted by Fas, preventing them from appearing in the periphery. In the absence of Fas, the cells survive and expand. One way of identifying such a TCR might be to clone the TCR from an activated or double negative cell from wedge/lpr mice. If our hypothesis is correct, cells with this TCR might survive as naive in wildtype and possibly lpr mice, be activated but immediately deleted by Fas in wedge mutant mice, and accumulate as activated cells in wedge/lpr mice. This experiment, of course, is not trivial.

A simpler, indirect experiment may be to assess the amount of primed Lck not phosphorylated on tyrosine Y505. If the wedge mutant CD45 is more active, the increased activity might be reflected in more primed Lck, which might contribute to increased tonic signaling. A system previously used to detect the reduced amount of unphosphorylated Y505 in CD45 knockout mice could be applied for this purpose (Sieh et al., 1993). These experiments used a phospho-peptide to bind and pulldown the Lck SH2 domain. Phosphorylated Y505 binds the SH2 domain to inhibit Lck activity, but in Lck not phosphorylated on Y505, the SH2 domain is free and can thus bind phospho-peptide. This system has the advantage of measuring an increase in the relatively small amount of unphosphorylated Lck as opposed to detecting a small decrease in the amount of abundant Y505 phosphorylated Lck by phospho-specific western blot. An alternative approach, albeit one more technically challenging and possibly less sensitive, would be to

use a FRET based reporter for Lck activity, similar to those created by Roger Tsien (Wang et al., 2005). Increased Lck activity would alter the amount of FRET, which could be detected by microscopy or FACS. This reporter could be retrovirally transduced into wildtype and wedge mutant BM and used to reconstitute irradiated animals. FRET could then be measured in peripheral T cells.

Another factor that may account for T cell activation in wedge/lpr double mutant mice is a shift in TCR repertoire. To compensate for increased signaling in wedge mutant cells, a repertoire with decreased affinity for the same self-peptides may be selected. However, analyzing the TCR repertoire is extremely difficult and is currently only done by brute force sequencing of hundreds of T cell receptors. Furthermore, how an altered repertoire might translate into the wedge/lpr phenotype is also unclear.

B cell role in T cell activation

This work also demonstrates a key role for B cells in the activation and expansion of T cells. In the absence of B cells, splenomegaly and lymphadenopathy are abrogated and T cell activation, turn over, and survival are diminished. Similar observations have been made in MRL/lpr, aged B6/lpr, and mixed B6 x 129 background CD45 wedge mutant mice (Akashi et al., 1998; Chan and Shlomchik, 1998; Hermiston et al., 2005). In all of these models, B cell deficiency corrects lymphoproliferation, and furthermore blocks glomerulonephritis in MRL/lpr mice. The first question is how specific is the prevention of autoimmunity in B cell deficient wedge/lpr mice?

Few reports on the effect of B cell deficiency on autoimmunity have been published. One study examining the role of T and B cells in Sle1 mediated autoimmunity

found that T cells from μMT Sle1 mice were activated to the same extent as cells from Sle1 mice with B cells (Sobel et al., 2002). These results contrast with our findings that T cell activation is reduced in the absence of B cells, suggesting that the requirement for B cells is not universal, but context dependent. Further studies of B cell deficiency in other models of autoimmunity such as CTLA-4 and FoxP3 knockouts might address the settings in which B cells are necessary.

The mechanism behind B cell driven activation of T cells is also unclear. One strong possibility is that activated B cells may be directly enhancing T cell activation by acting as antigen presenting cells. Indeed, wedge/lpr B cells appear to have increased expression of MHC class II and CD86, both of which are upregulated upon B cell activation and are involved in stimulation of T cells. The reduced fraction of memory phenotype T cells in the absence of B cells is consistent with a direct role for B cells in activating T cells. The increased expression occurs even in the absence of T cells and lymphoproliferation, suggesting that B cell activation may be a primary cause of T cell activation rather than a consequence of general inflammation. The wedge mutation clearly sensitizes B cells to anti-IgM stimulation (Hermiston et al., 2005), and may also increase the activation induced by tonic signals from endogenous antigens. The influence of tonic signaling could be tested as it was for T cells by introducing a transgenic BCR such as Ig-HEL. Lymphadenopathy and splenomegaly have been reported to be delayed in Ig-HEL/lpr mice, and it is therefore likely this transgene would delay lymphoproliferation in wedge/lpr mice as well (Rathmell and Goodnow, 1994).

In addition to directly simulating T cells, B cells could also be enhancing T cell activation and survival indirectly a number of ways. One non-specific explanation might

be disruption of lymphoid architecture by B cell deficiency. B cells are an important source of lymphotoxin $\alpha_1\beta_2$ (LTβ) during lymphoid organogenesis (Mebius, 2003). Lymphotoxin helps establish T and B cell zones and promote homing chemokine secretion (Ngo et al., 2001; Ngo et al., 1999). Absence of B cells and the lymphotoxin they produce could thus prevent T cells from migrating properly to areas where they might interact with APCs. However, in Sle1mice, B cell deficiency did not reduce T cell activation, suggesting T cells were homing to and interacting with APCs (Sobel et al., 2002). To separate the developmental role of B cells from their antigen presenting function, B cells could be depleted from mature adult mice and the effect on T cells monitored. Reduction of T cell activation or resolution of the lymphoproliferation would argue that any developmental defects are not responsible for the phenotype of wedge/lpr/μMT mice.

In addition to producing LTβ during development, activated B cells also upregulate LTβ expression (Browning et al., 1993). This increased LTβ could be a homeostatic factor for stromal cells and dendritic cells (Kabashima et al., 2005; Mackay and Browning, 1998; McCarthy et al., 2006). Stimulation of stromal cells by LTβ may induce them to secrete other T cell survival cytokines such as IL-7 and IL-15 (Husson et al., 2000), which may allow the increased T cell accumulation in wedge/lpr mice. LTβ stimulation of DCs is also essential for their survival, as LTβ deficient mice have reduced numbers of DCs, and for maximal expression of CD86 and efficient priming of T cells (Summers-DeLuca et al., 2007). By promoting DC survival and activation, B cells could be indirectly contributing to T cell activation. Increased expression of LTβ could be

detected by quantitative PCR. If an increase is found, the role of LTβ may be tested by blocking it in vivo with the available LTβR-Ig fusion protein.

A B cell and T cell feedback loop

Despite what appears to be increased activation of B cells even in the absence of T cells, B cells are not sufficient to cause lymphoproliferation since T cell deficient wedge/lpr mice do not have lymphadenopathy or splenomegaly. Furthermore, wedge/lpr B cells do not produce autoantibodies in the absence of T cells. Together with the lack of lymphoproliferation in B cell deficient mice, there appears to be a positive feedback loop between B and T cells that lead to their mutual expansion (Shlomchik et al., 2001). Such a feedback loop might be initiated by the increased activation of antigen presenting cells such as DCs and B cells. Elevated levels of MHC class II and costimulatory molecules might break the tolerance of quiescent auto-reactive T cells. Activation of T cells might lead to their expression of molecules such as CD40L that would further promote the activation of antigen presenting cells.

Fas may be an important checkpoint designed to interrupt this loop. Inhibiting DC apoptosis by overexpression of an anti-apoptotic molecule or targeted deletion of Fas in DCs resulted in autoantibody production, arguing that apoptosis of DCs may be an important mechanism for maintaining tolerance (Chen et al., 2006; Stranges et al., 2007). Thus the wedge mutation may be important for activating APCs, and the Fas mutation prevents their destruction by responding T cells. It is likely that both the fas and wedge mutations are operating in multiple cell types. Unfortunately, the widespread expression of both Fas and CD45 in the immune system makes it difficult to separate their

contribution to the wedge/lpr phenotype. One approach to address this issue is use of bone marrow chimeras that express different combinations of the mutations in T and B cells.

Lack of end organ damage

Like MRL/lpr mice, wedge/lpr mice produce significant quantities of autoantibodies at an early age and develop lymphadenopathy and splenomegaly consisting of activated lymphocytes. On the other hand, wedge/lpr mice do not have any signs of glomerulonephritis, arthritis, or other autoimmune manifestations seen in the MRL/lpr strain. So while the wedge mutation and lpr seem to cooperate quite readily in loss of tolerance and lymphocyte activation, the two mutations are not sufficient to cause end organ damage. Despite the high levels of anti-nuclear and dsDNA antibodies, wedge/lpr mice have no signs of the immune complex deposition or proteinuria typically associated with these antibodies. Other groups have made similar observations, showing that auto-antibodies and kidney damage are not strictly correlated (Deshmukh et al., 2006). Auto-antibodies can be present without kidney damage, as seen here, or on the other hand, may be absent in animals that have glomerulonephritis (Clynes et al., 1998; Waters et al., 2004). Therefore, in these models additional loci must be required to make these antibodies pathogenic.

A genome wide screen of F2 MRL x B6 lpr mice identified four loci that contributed to autoantibodies, lymphoproliferation, and glomerulonephritis (Vidal et al., 1998). The locus linked to glomerulonephritis was located on chromosome 10 and came from the MRL background. It is possible that a wedge/lpr congenic containing this MRL

locus may develop renal disease. A locus from the NZM2410 strain that may also contribute to nephritis has been postulated to be Sle1d on chromsome 1 (Morel et al., 2001). Mice that were congenic for Sle1a, Sle1b, and Sle1c developed autoantibodies, but no renal damage. Analyses of other autoimmune models have identified additional loci for different pathology. A number of genes, including FcγRIII, have also been found to influence a serum transfer model of arthritis (Ji et al., 2002). It is possible that transfer of serum from wedge/lpr mice may induce nephritis in a susceptible strain.

Alternatively, wedge/lpr mice may be succumbing to some other pathologic process before overt autoimmune kidney damage develops. Indeed, there is evidence of histologic alterations in the glomeruli of wedge/lpr mice, but the etiology of these changes is unclear. In MRL/lpr mice, deletion of IFNγ or the IFNγ receptor reduces autoantibody production and nephritis (Balomenos et al., 1998; Haas et al., 1997; Peng et al., 1997; Schwarting et al., 1998). Given the pronounced ability of wedge/lpr T cells to produce IFNγ in vitro, this cytokine may be partially responsible for the enlarged glomeruli we observe. Blocking or deleting IFNγ genetically may alleviate the autoantibody production and hypercellular glomeruli in wedge/lpr mice as well.

Conclusions

Based on our results, the CD45 E613R wedge mutation can act as genetic modifier for autoimmune disease in some, but not all, contexts. Combination of the wedge mutation with the complement C4 knockout failed to alter the disease course compared to single mutant mice. Autoimmunity in wedge/lpr double mutant mice, however, was significantly accelerated and exacerbated. Both B and T cells contributed

to the lymphoproliferation. Further study of these mice may provide valuable insight into the feedback driving expansion of these cells as well as the individual function of Fas and CD45 in normal immune homeostasis.

Appendix

Summary

One of the central ideas in our model for CD45 regulation is wedge mediated inhibition of phosphatase activity by dimerization. Unfortunately, previous studies in the lab have failed to demonstrate any differences in phosphatase activity of the CD45 wedge mutant in vitro or in the phosphorylation of its substrate Lck in vivo. Highly purified recombinant protein may be a useful tool for further biochemical and biophysical analysis of CD45. Here I describe a protocol for CD45 purification.

Introduction

Two critical aspects of the wedge model that remain to be demonstrated are CD45 dimerization in vitro and wedge mediated inhibition of phosphatase activity. The current evidence for dimerization comes from crosslinking of cell surface CD45 (Majeti et al., 2000; Xu and Weiss, 2002). Although dimerization of purified domain 1 has been observed in vitro, there is no evidence for dimerization of full length CD45 (Felberg and Johnson, 1998). Furthermore, CD45 crystallized in a conformation that would preclude wedge mediated dimerization (Nam et al., 2005). For measuring phosphatase activity, previous experiments have used CD45 immunoprecipitated from EGFR-CD45 chimera expressing cell lysate. However, immunoprecipitation of CD45 may disrupt the dimerization in membranes. Another in vitro approach used a recombinant Gyrase B-CD45 that could be induced to dimerize with coumermycin (Maxwell, 1993). No difference was seen with coumermycin, but dimerization was never formally shown.

The failure to detect CD45 dimers may reflect the need for specific conditions. Recently oxidation has emerged as potent regulator of phosphatase activity (Tonks,

2005). Oxidation has been shown to alter the structure of phosphatases as well as stabilize dimers of a related phosphatase RPTPα (den Hertog et al., 2005). Oxidation could therefore play a role in CD45 dimerization and activity and warrants further study.

Given the caveats to the previously used approaches, other techniques are worth considering. However, many of the biophysical techniques for measuring dimerization require highly purified recombinant protein. We generated recombinant protein that was of sufficient quality for analysis using analytical ultracentrifugation, small angle x-ray scattering, and mass spectrometry. These techniques also failed to show any dimerization of CD45 under normal conditions, but some evidence for dimerization was seen with oxidation.

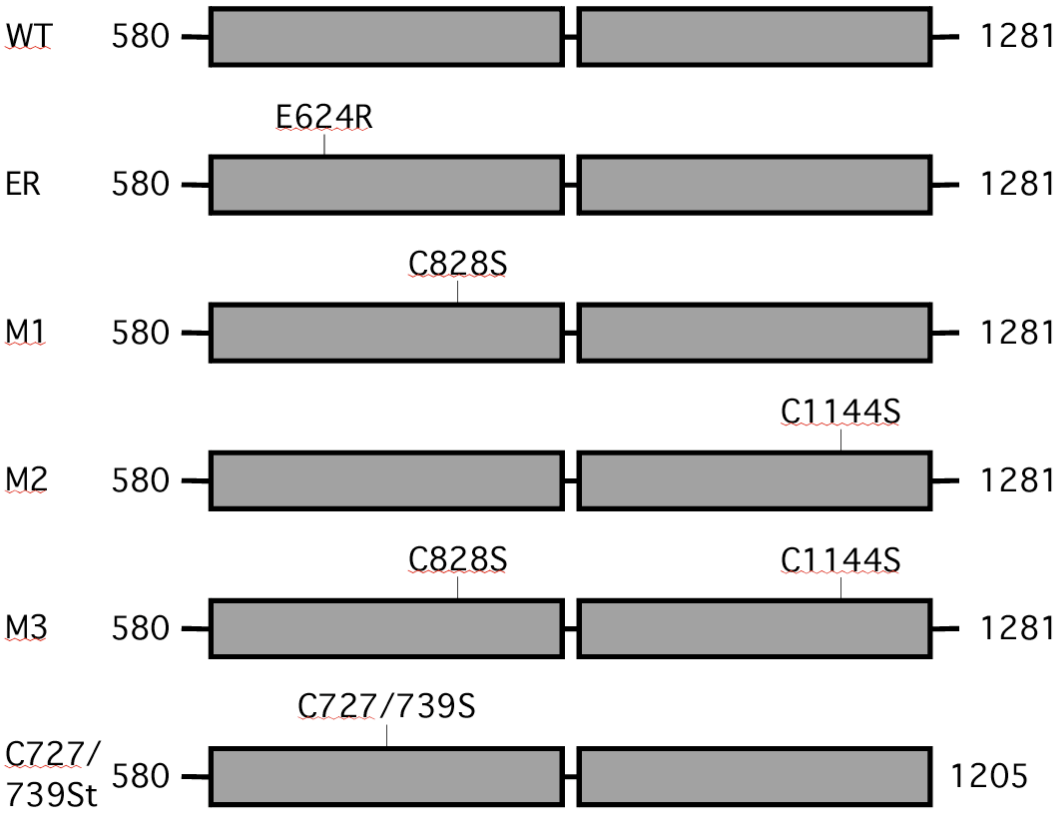
Results and Discussion

We designed expression plasmids containing an N-terminal 6xHis tag and thrombin cleavage site followed by the cytoplasmic domain of CD45 and various mutants (Figure A-1). Protein was expressed in bacteria and extracted from lysate using Co^{++} beads. Following cleavage of the 6xHis tag, the protein was run sequentially over ion exchange (Q), hydrophobic (phenyl), and size exclusion (superdex 75) columns, and finally concentrated using Amicon Ultra centrifugal filtration devices.

An unexpected problem I was unable to correct was poor binding of 6xHis tagged CD45 to Co^{++} beads. A majority of protein, in fact, was left in the flow through after incubating with the beads for one hour. Ni^{++} beads fare just as poorly if not worse. As a result, yield from the purification was inconsistent and never exceeded approximately one milligram for every liter of bacteria. I compensated by simply growing more bacteria.

Figure A-1: Constructs

Constructs used for recombinant protein production shown with introduced mutations


WT
580
1281
E624R
ER
580
1281
C828S
M1
580
1281
C1144S
M2
580
1281
C828S
C1144S
M3
580
1281
C727/739S
C727/
739St
580
1205

Use of different induction conditions did not seem to make a difference in the binding to beads. Both 0.1mM IPTG for 24 hours at 15-25°C and 0.02mM IPTG for 40 hours at 22°C produced similar results. I did not encounter this problem with the original 6xHis tagged construct I used, but the disadvantage of the original construct was the use of an Xpress cleavage site. Unfortunately, I found that CD45 seemed to contain a cryptic Xpress site because use of the Xpress protease produced a smaller than expected protein. It is possible something about the thrombin cleavage site I introduced disrupted binding to the beads. If large-scale purification is necessary in the future, subcloning the constructs into a different expression plasmid may be worthwhile.

Following elution from Co^{++} column, thrombin was added and the protein was dialyzed into the starting buffer for the Q column. The Q column seemed to give slightly better results than DEAE. A problem with the ion exchange column was the difficulty separating the CD45 peak from a closely eluting second peak. My solution was to use a long, slow gradient, which worked to some extent, but resulted in CD45 eluting as a broad peak in a large volume (Figure A-2A).

Ammonium sulfate was added to the eluted protein to bring it up to the starting concentration for the phenyl column, 900mM. At this ammonium sulfate concentration, CD45 came out in the flow through, which is less than ideal. However, at 1M ammonium sulfate some protein bound, but a large proportion still came through in the flow through, splitting it into two fractions. At 1.2M, the protein bound efficiently to the column, but when eluted was not sufficiently separated from a second contaminating peak (Figure A-2B).

Figure A-2: Chromatograms of recombinant CD45 purification

(A) Chromatogram from the Q ion exchange column run with wildtype CD45 protein. P1 indicates the collected CD45 peak.

(B) Chromatogram from the Phenyl hydrophobic column with wildtype CD45 protein. FT indicates the collected CD45 fraction.

(C) Chromatogram from the Superdex size exclusion column with wildtype CD45 protein. P1 indicates the collected CD45 peak.

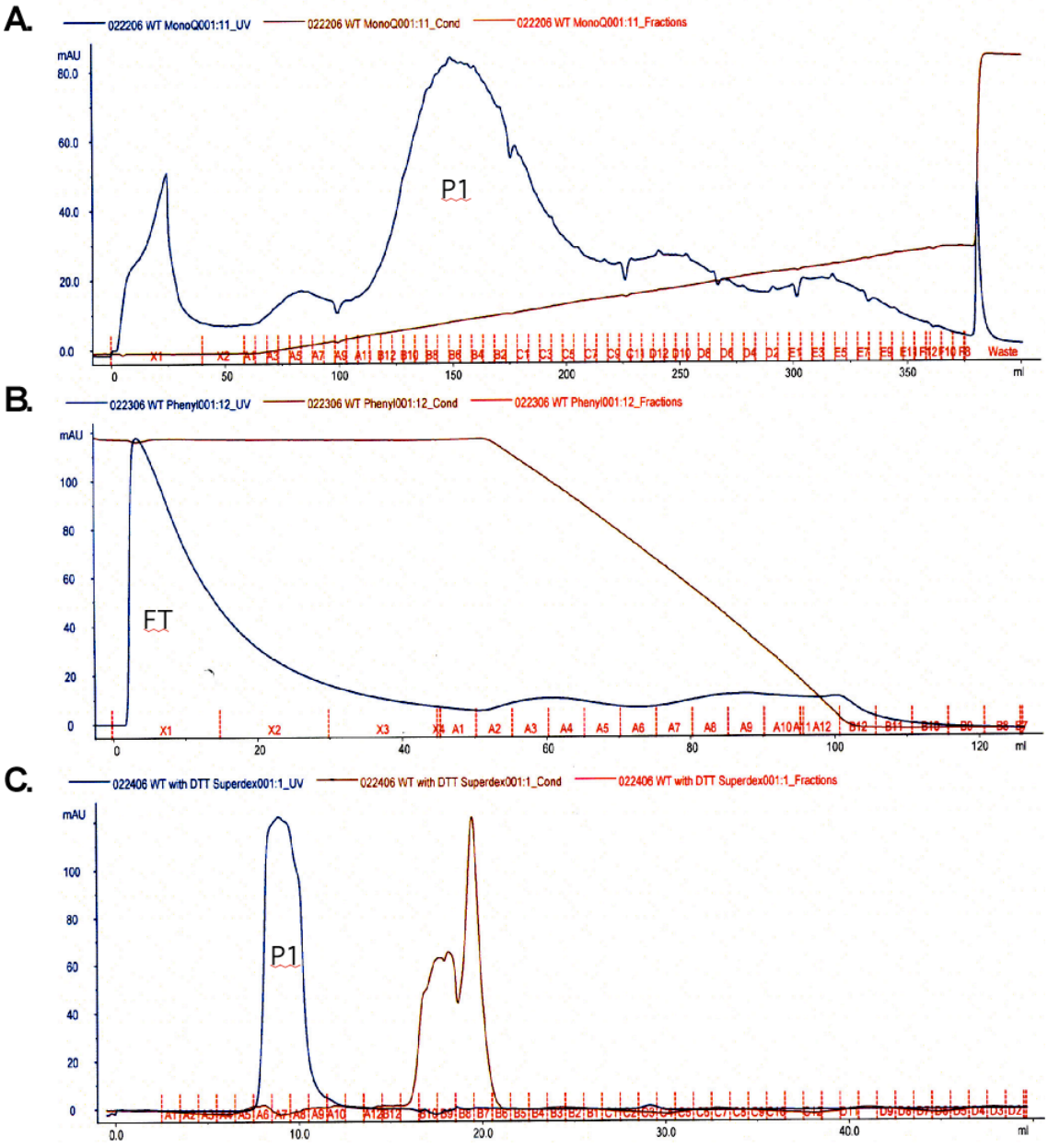
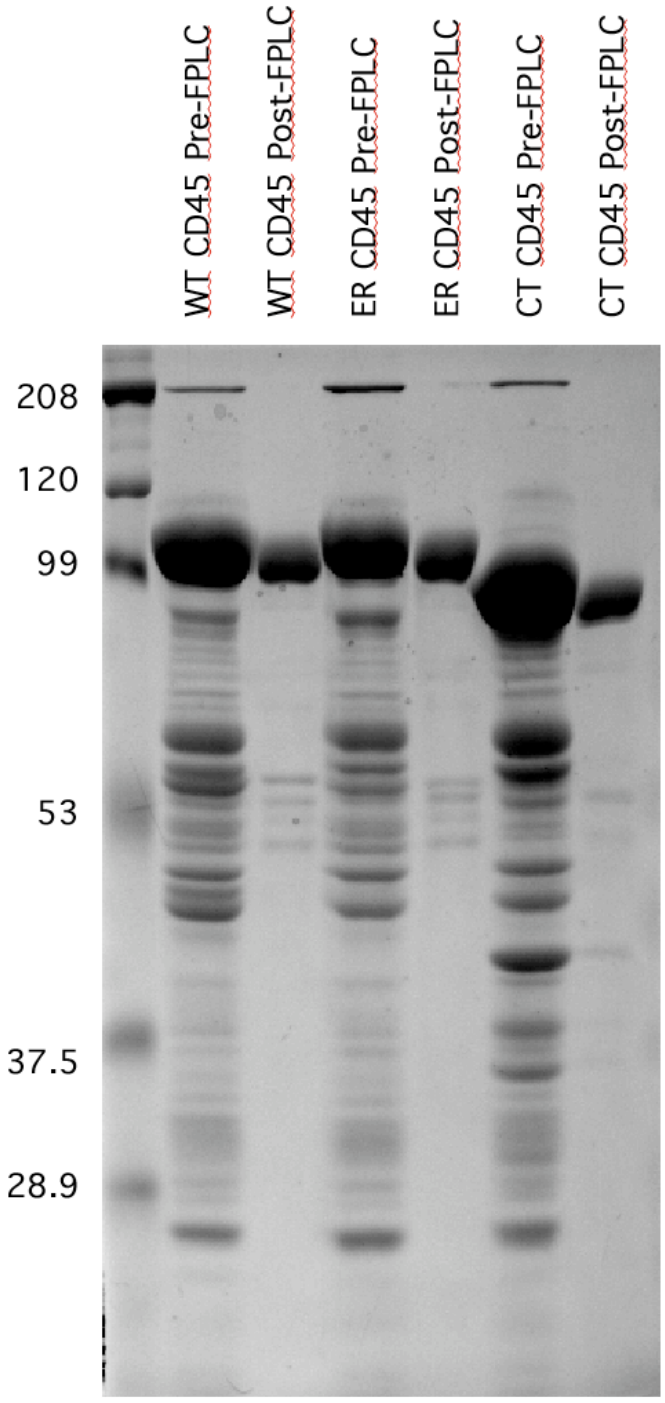

A.
022206 WT MonoQ001:11_UV
022206 WT MonoQ001:11_Cond
022206 WT MonoQ001:11_Fractions
mAU
P1
Waste
ml
B.
022306 WT Phenyl001:12_UV
022306 WT Phenyl001:12_Cond
022306 WT Phenyl001:12_Fractions
mAU
FT
ml
C.
022406 WT with DTT Superdex001:1_UV
022406 WT with DTT Superdex001:1_Cond
022406 WT with DTT Superdex001:1_Fractions
mAU
P1
ml

Figure A-3: Recombinant CD45 purity

Coommassie stained SDS-PAGE gel of recombinant CD45.


WT CD45 Pre-FPLC
WT CD45 Post-FPLC
ER CD45 Pre-FPLC
ER CD45 Post-FPLC
CT CD45 Pre-FPLC
CT CD45 Post-FPLC
208
120
99
53
37.5
28.9

After collecting the phenyl column flow through, the solution was concentrated to less than 200μl for the size exclusion column. The final protein came off as a single peak and looked extremely clean by coomassie staining of SDS-PAGE gels (Figure A-2C, A-3). The purified CD45 was very stable under different conditions. It could be concentrated up to 60 mg/ml and did not precipitate out in buffered water without salt. The protein was enzymatically active against pNPP and phospho-peptide.

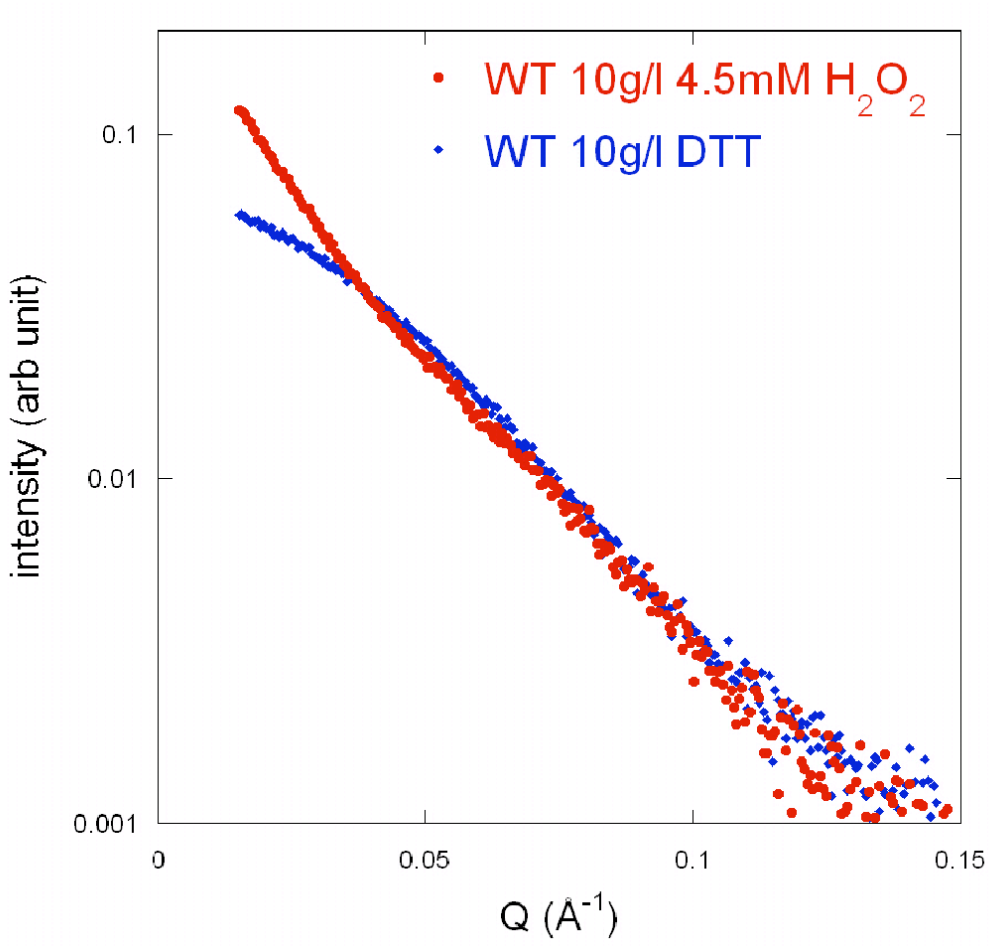
Our primary hypothesis was that CD45 activity and dimerization was regulated by oxidation of the domain 2 cysteine. This hypothesis was tested by mutating the domain 2 cysteine to serine (M2), and comparing the behavior of this mutant to wildtype protein in response to oxidation. If our hypothesis was correct, we expected M2 to be resistant to the effects of oxidation. A number of approaches were taken, both enzymatic and biophysical.

Our first system measured phosphatase activity in vitro in the presence or absence of the oxidizing agent hydrogen peroxide. The results of these experiments were inconclusive for the most part. Various concentrations of phosphatase, substrate, and hydrogen peroxide were used, but overall there was no reproducible difference between wildtype and M2 activity. It is possible a more rigorous analysis of Km and Vmax for these enzymes will reveal a difference.

We also used analytical ultracentrifugation (AUC) and small angle x-ray scattering (SAXS) to assess dimerization in response to oxidation. AUC did not reveal any effect of oxidation on dimerization. With SAXS we saw a reproducible difference in scattering before and after oxidation (Figure A-4). This difference was in the form of an approximately two fold increase in the radius of gyration which could represent the

Figure A-4: Detection of dimers upon oxidation using small angle X-ray scattering

Change in radius of gyration upon addition of hydrogen peroxide to recombinant wildtype CD45 protein.


WT 10g/l 4.5mM H2O2
WT 10g/l DTT
intensity (arb unit)
0.1
0.01
0.001
0
0.05
0.1
0.15
Q (Å-1)

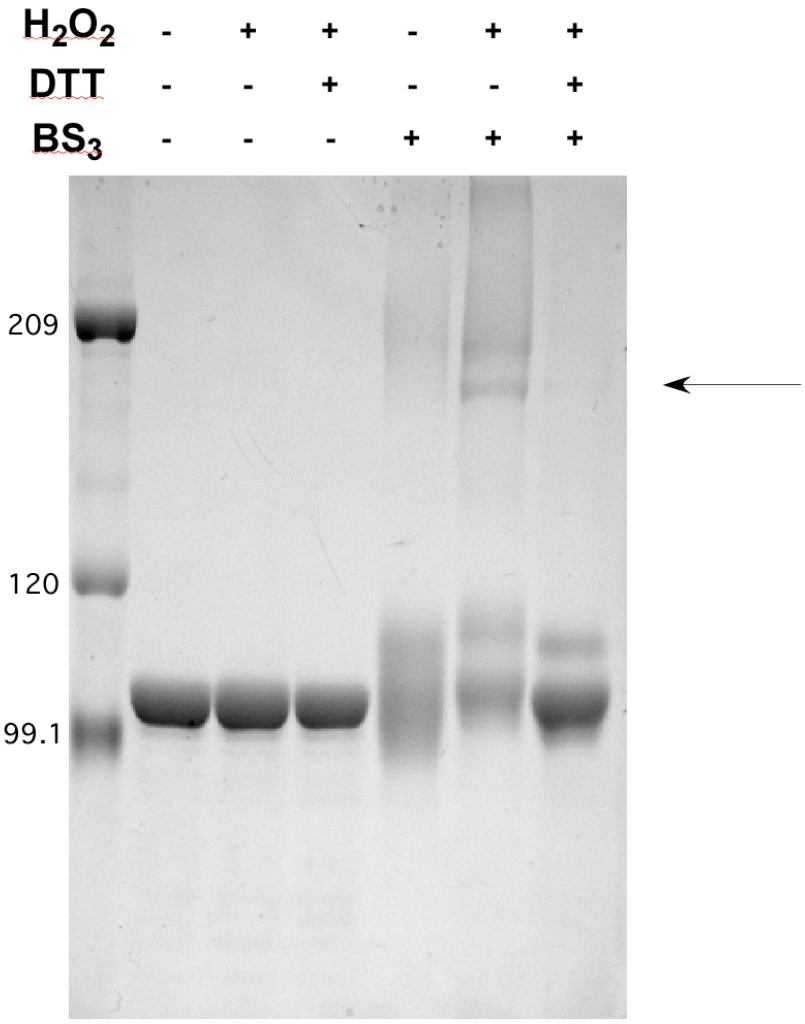
presence of dimers. Unfortunately, the same effect was seen with a number of mutants, including M2 and E624R, which we would have predicted not to be oxidized or dimerize. In addition, one limitation of this technique is that scattering reflects the average of all the molecular species, and thus a two fold change could represent a large fraction of dimers or a small fraction of higher molecular weight aggregates. We attempted to overcome this problem by filtration of the samples through a 1MD cutoff filter. Although filtration had only a minimal effect on the scattering profile, we had no way of confirming the effectiveness of the filter. Reduction of the treated samples with DTT did, however, reverse the increase in radius of gyration, suggesting that the oxidation and/or aggregation was reversible.

In order to independently validate the presence of dimers, we used non-reducing gels and crosslinking. Oxidation of CD45 was done using conditions similar to the SAXS experiments, and then run on a non-reducing gel. No dimers were seen, arguing that any dimer formation is not the result of an intermolecular disulfide bridge. The second approach was to crosslink dimers to allow visualization on a gel. Oxidized and untreated protein were crosslinked with BS_3 and subject to SDS-PAGE. Oxidized and crosslinked CD45 showed a band at approximately the size of the expected dimer (Figure A-5). Reduction of the oxidized protein before crosslinking eliminated this band.

Demonstrating dimerization is a critical aspect of our model for CD45 regulation. Once a simple, reproducible method for measuring CD45 dimerization is identified, the molecular basis for this dimerization can be dissected. The domain 2 cysteine mutant or wedge mutation can be tested for their role in dimerization, or mass spectrometry can be used to identify other residues that may be oxidized in a non-biased manner.

Figure A-5. Detection of dimers upon oxidation by crosslinking

Wildtype recombinant CD45 was treated with an approximately equimolar concentration of hydrogen peroxide. A portion of this protein was then reduced using DTT. Finally the untreated and treated CD45 was either crosslinked with BS_3 or left untreated. Samples were then separated by SDS-PAGE and visualized by coomassie staining. Arrow indicates dimer species.


H2O2 - + + - + +
DTT - - + - - +
BS3 - - - + + +
209
120
99.1

Materials and Methods

Protein Purification

1. Grow 5 ml overnight culture of expression plasmid in BL21 codon plus bacteria.
2. Inoculate 1 L TB-Amp with 5 ml culture.
3. Grow at 37°C until OD600~0.8-1.0.
4. Cool at 4°C for 10 minutes.
5. Induce with 1ml 100 mM IPTG (final 0.1 mM IPTG) and shake for 24 hours at 15°C
6. Pellet cells 30 minutes at 3000 rpm, 4°C.
7. Freeze pellet at -80°C for later use or resuspend in lysis buffer for immediate lysis.
8. Resuspend pellet in 30 ml lysis buffer with protease inhibitors
9. Lyse with french press (Stroud Lab, Mission Bay; Oakes lab may now have one).
10. Spin lysate at 15,000 rpm for 60 minutes.
11. Incubate supernatant with 5 ml prewashed Co^{++} beads for 1 hour, tumbling at 4°C.
12. Pour beads into column and wash with 20 bed volumes wash buffer (no PMSF).
13. Elute with 2 bed volumes elution buffer (no PMSF).
14. Add 500 U thrombin.
15. Inject into slide-a-lyzer dialysis cassette.

16. Dialyze overnight in dialysis buffer with one buffer change.
17. Prepare Q column by washing with 10 volumes 100% Q buffer B followed by 10 volumes 100% Q buffer A.
18. Equilibrate Q column in 5% Q buffer B.
19. Load Superloop with dialyzed protein.
20. Run Q column from 5% to 40% Q buffer B (50-400 mM NaCl) over 20 column volumes.
21. Replace Q column with Phenyl column.
22. Wash Phenyl column with 100% Phenyl buffer B followed by 100% Phenyl buffer A.
23. Meanwhile, pool fractions containing peak 1 (Figure A-2A), add 3.6 M ammonium sulfate to 900mM final concentration. Filter with Steriflip to remove any precipitate. Concentrate using Amicon Ultra to less than 1.5ml.
24. Load concentrated protein into 1.5ml loop.
25. Run Phenyl column with 25ml column wash and collect flow through (Figure A-2B). Continue with 100% - 0% B gradient to elute contaminating proteins.
26. Equilibrate size exclusion column with 2 volumes final buffer.
27. Concentrate phenyl column flow through to less than 200µl using amicon ultra for size exclusion column.
28. Load concentrated protein into 0.5ml loop.
29. Run size exclusion column with 2 volumes final buffer.
30. Collect peak (Figure A-2C), concentrate, and store at 4°C or flash freeze and place at -80°C.

Buffers

Lysis/Wash Buffer

20 mM Tris pH 7.6

150 mM NaCl

5 mM β-mercaptoethanol

Elution Buffer

20 mM Tris pH 7.6

150 mM NaCl

5 mM β-mercaptoethanol

200 mM imidazole

Dialysis Buffer

20 mM Tris pH 7.6

50 mM NaCl

1 mM EDTA

1 mM DTT

Q Buffer A

20 mM Tris pH 7.6

1 mM EDTA

Q buffer B

20 mM Tris pH 7.6 1M NaCl

1 M NaCl

1 mM DTT

1 mM EDTA

1 mM DTT

Phenyl Buffer A

50 mM Tris pH 7.6

900 mM ammonium sulfate

0.1 mM EDTA

1 mM DTT

Phenyl Buffer B

50 mM Tris pH 7.6

0.1 mM EDTA

1 mM DTT

Gel Filtration/Final buffer

50 mM Tris pH 7.4

150 mM NaCl

1 mM EDTA

1 mM DTT

Crosslinking

1. Use gel filtration to exchange buffer into crosslinking buffer

   20 mM HEPES pH 7.4 (no primary amines - Tris)

   150 mM NaCl,

   no reducing agent – use immediately, or low concentration, 50 μM DTT, to use soon.

2. Concentrate with amicon ultra to 20 mg/ml (~ 245 μM)

3. Dilute hydrogen peroxide into crosslinking buffer, final concentration 300 μM

4. Make 10 mM BS^3 in crosslinking buffer

5. Mix 5 μl protein + 5 μl buffer ± hydrogen peroxide

6. Incubate 5 minutes, room temperature

7. To reverse oxidation, add DTT to 10 mM

8. Add 1 μl BS^3

9. Incubate 30 minutes room temperature

10. Add Tris to 50 mM to quench crosslinker

11. Run on SDS-PAGE

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