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Destabilization of peptide: MHC interaction induces IL-2 resistant anergy in diabetogenic T cells

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Abstract

Autoreactive T cells are responsible for inducing several autoimmune diseases, including type 1 diabetes. We have developed a strategy to induce unresponsiveness in these cells by destabilizing the peptide:MHC ligand recognized by the T cell receptor. By introducing amino acid substitutions into the immunogenic peptide at residues that bind to the MHC, the half life of the peptide:MHC complex is severely reduced, thereby resulting in abortive T cell activation and anergy. By treating a monoclonal diabetogenic T cell population with an MHC variant peptide, the cells are rendered unresponsive to the wild type ligand, as measured by both proliferation and IL-2 production. Stimulation of T cells with MHC variant peptides results in minimal Erk1/2 phosphorylation or cell division. Variant peptide stimulation effectively initiates a signaling program dominated by sustained tyrosine phosphatase activity, including elevated SHP-1 activity. These negative signaling events result in an anergic phenotype in which the T cells are not competent to signal through the IL-2 receptor, as evidenced by a lack of phospho-Stat5 upregulation and proliferation, despite high expression of the IL-2 receptor. This unique negative signaling profile provides a novel means to shut down the anti-self response.

Keywords

T cell anergy; type 1 diabetes; I-A^{g7}; signaling

1. Introduction

Antigen specific targeting of self-reactive CD4⁺ T cells to induce an unresponsive state would provide a powerful intervention for autoimmune disease. In the NOD mouse model of type 1 diabetes, an autoimmune disease driven largely by T cells, various strategies have been used to induce tolerance, the majority of which drive deviation from a Th1 to a Th2 type response or induce regulatory T cells [1–7]. Skewing to a Th2 phenotype may be a less than ideal approach, as hypersensitivity may occur, a risk that has been observed in diabetic

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Conflicts of interest

The authors report no conflicts of interest.

mice treated with peptide [8–10], mice with experimental autoimmune encephalomyelitis [11, 12] and clinical trials of altered peptide ligands as a treatment for multiple sclerosis [13].

One factor complicating the induction of antigen specific tolerance in the NOD mouse model is the unique features of its class II MHC molecule, I-A^{g7}. This MHC is a major factor in diabetes development [14–16], and shares several characteristics with the human diabetes-associated allele HLA-DQ. The I-A^{g7} and DQ β -chains contain a common polymorphism in the peptide binding pocket, a non-aspartic acid residue at position 57 [17]. This position contains an Asp residue in all other mouse class II molecules. Another key feature of I-A^{g7} is its promiscuous ability to bind peptides [18]. It has been demonstrated that this MHC binds a broad range of peptides, favoring small, hydrophobic amino acids at P4 and P6; the P9 pocket is relatively larger, and this is able to accommodate larger and/or charged residues at that position [19–22]. In addition to being a promiscuous peptide binder, I-A^{g7} is a less stable MHC, having a shorter half-life on the cell surface than both I-A^b and I-A^d. However, the position 57 mutation does not appear to be the cause of this instability, as mutating the residue back to Asp did not alter the stability of I-A^{g7} [19].

Studies from our lab have demonstrated that destabilization of the antigenic peptide: MHC complex is a safe and effective means of inducing T cell anergy in another autoimmune model, experimental autoimmune encephalomyelitis [12, 23]. As in other forms of T cell anergy, T cells treated with MHC variant peptides, which are defined by their short half life of binding to MHC, exhibit a hyporesponsive phenotype in which the cells fail to proliferate or produce IL-2 in response to normally agonistic stimuli [24]. To determine if the unique features of the diabetes-associated MHC alleles may hamper this immunotherapeutic approach, we have examined BDC-2.5 TCR transgenic cells to demonstrate that an MHC variant peptide with a short half life can be utilized to induce anergy in I-A^{g7} restricted T cells. Stimulation with the variant peptide results in undetectable early phosphorylation events, with a concomitant increase in total tyrosine phosphatase activity. Specifically, activity of the phosphatase SHP-1 is increased following stimulation with an anergy-inducing peptide. Unlike other forms of anergy, MHC variant peptide-induced anergy is not overcome by the addition of exogenous IL-2, and we demonstrate a lack of Stat5 phosphorylation following IL-2 stimulation of anergic cells. Thus we have characterized several novel aspects of the molecular mechanisms regulating the CD4⁺ T cell response to MHC variant peptides, in addition to successfully inducing anergy in an autoreactive T cell population restricted by a diabetes-associated MHC Class II allele.

2. Materials and Methods

2.1 Mice

BDC-2.5 TCR transgenic [25] breeders were a kind gift from Mark Rigby. NOD mice were purchased from either Jackson Laboratory (Bar Harbor, Maine) or Taconic Farms (Germantown, New York). All mice were housed by the Emory University Division of Animal Resources. Breeding and experiments were carried out in accordance with protocols approved by the Emory University Institutional Animal Care and Use Committee and other applicable regulations.

2.2 Peptides and reagents

Peptides were synthesized in house using Fluorenylmethyloxycarbonyl chloride chemistry on a Peptide Technologies Incorporated Prelude synthesizer. The specific pancreas-derived epitope recognized by BDC-2.5 T cells was previously unknown [26, 27], but several synthetic mimotopes have been identified that mimic the initiating antigen sufficiently to stimulate the T cells [28, 29]. The mimotope sequence utilized in these studies was AHHPIWARMDA [29], and the sequence of the YPDV MHC variant was AHYIPADMDV; additional variant sequences can be found in Fig. 1.

2.3 HPLC and half life determination

To determine the half life of the peptide: MHC interaction, high performance size exclusion chromatography was performed as previously described [30, 31]. I-A^{g7} monomers were acquired from the NIH tetramer core facility at Emory University. Monomers were incubated with the peptide of interest containing a C-terminal fluorescein for 24–72h at 37°C in a buffer containing 100mM citrate phosphate buffer, pH 5, 0.2% NP-40, 0.02% sodium azide, 1 μ M I-A^{g7} monomer, 10 μ M fluorescently labeled peptide, 10mM EDTA and a protease inhibitor cocktail (Calbiochem). Unbound peptide was removed by passing the sample through a Sephadex G-50 spin column (GE) that had been blocked with 10mg/ml BSA in buffer containing PBS plus 25.5mM citric acid and 53.4mM sodium phosphate dibasic anhydrous. Unlabeled competitor peptide AVRPLWVRME [28] was added to a final concentration of 2mM and samples were allowed to dissociate at 37°C for the indicated time points. To determine dissociation, samples were resolved using a 300 $\times$ 7.80mm Phenomenex BioSep-SEC-S 3000 size exclusion column with a Phenomenex guard cartridge kit in buffer containing 19.5mM sodium phosphate monobasic monohydrate, 30.5mM sodium phosphate dibasic heptahydrate, and 0.05% sodium azide. Samples were visualized with an SPD-10AVP UV-Vis detector and a RF-10AXL fluorescence detector on a Shimadzu HPLC system. Data were analyzed on LC Solution software (version 1.25, Shimadzu Corporation). Decay curves were generated by normalizing the height of the fluorescence peak to the height of the UV peak and plotting these natural log of the peak height at each time point (B) divided by the baseline peak height (B₀) versus time. Half life was calculated by natural log 2 divided by the slope of the decay curve.

2.4 Cell culture

BDC-2.5 spleen cells were cultured *ex vivo* with 1 μ M mimotope peptide for 14 days in 24-well plates. Live cells were purified over a Ficoll gradient and restimulated for 14 days with irradiated syngeneic splenocytes (3000 rad) and either 1 μ M mimotope peptide or 10 μ M variant peptide.

For proliferation assays, naïve or previously activated T cells and irradiated syngeneic splenocytes (3000 rad) were cultured in 96-well plates with the indicated concentration of peptide at 37°C. After 48h in culture, 0.4 μ Ci/well of [³H] thymidine was added. After an additional 18h, cells were harvested on a FilterMate harvester (Packard Instrument) and [³H] thymidine incorporation was assessed on a 1450 LSC Microbeta TriLux counter (PerkinElmer). Where indicated, recombinant mouse IL-2 was added to a final concentration

of 3.5ng/well. Stimulation indices were calculated as stimulated CPM divided by CPM of unstimulated samples.

Culture media consisted of RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 0.01M HEPES buffer, 100µg/mL gentamicin (Mediatech, Herndon, VA) and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO).

2.5 Cytokine ELISA

After induction of anergy, T cells were stimulated with irradiated syngeneic splenocytes (3000 rad) and 10µM mimotope peptide for 24h. Supernatants were incubated in triplicate on microtiter plates coated with purified anti-IL-2 (5 µg/ml clone JES6-1A12; BD Pharmingen). Recombinant IL-2 was used as a standard. Captured cytokines were detected using biotinylated anti-IL-2 (100 µg/ml JES6-5H4, 100 µl per well; BD Pharmingen) followed by alkaline phosphatase-conjugated avidin and p-nitrophenylphosphate substrate (Sigma). Colorimetric change was measured at dual wavelengths of 405 and 630 nm on a Microplate Autoreader (Biotek Synergy HT).

2.6 Flow cytometry

Cells were stimulated with either 10µM peptide presented by C3.G7 hybridomas [19] or 100µg recombinant mouse IL-2 for the indicated periods of time. 3×10^5 cells were fixed in a final concentration of 1.5% formaldehyde (Polysciences) for 30min-18h. Cells were then permeabilized in 100% ice cold methanol for 10 minutes. Cells were stained for 30 min. on ice with antibodies to CD4 (RM4-5, BD Biosciences), CD25 (clone PC61, BD Biosciences), p-p44/42 (D13.14.4E, Cell Signaling) and/or pStat5 (Y694, BD Biosciences). Staining buffer consisted of phosphate buffered saline containing 0.1% BSA and 0.05% sodium azide. Data was collected on a BD FACSCalibur and analyzed using FlowJo software (TreeStar).

2.7 Phosphatase assays

For whole cell lysate phosphatase activity, cell lysates were prepared at various times after stimulation by lysing cells with a buffer containing 20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 0.5% Igepal and protease inhibitor cocktail (Calbiochem). p-nitrophenylphosphate substrate solution was added to lysates, incubated overnight at 37°C and colorimetric change was read at dual wavelengths of 405 and 630 nm on a Microplate Autoreader (Biotek Synergy HT). For specific SHP-1 activity, SHP-1 was immunoprecipitated from whole cell lysates prepared without the addition of sodium orthovanadate. Immunoprecipitation was performed with 2µg of anti-SHP1 antibody (Santa Cruz) and a Pierce Protein A/G plate based immunoprecipitation kit. The immunoprecipitated protein was reduced using p-nitrophenylphosphate Tyr Assay Buffer (Millipore) and incubated for 1h at 37°C with a pTyr containing substrate peptide (AEEELpYGEFEA). Malachite green was added to assess the amount of free phosphate in the solution, and colorimetric change was assessed at 620nm on a BioTek microplate reader.

2.8 Statistical analysis

Data were analyzed using GraphPad Prism. Statistical significance was determined by Student's *t* test or ANOVA, as indicated in the figure legends. For figures in which percent maximum is presented, data were normalized using GraphPad Prism and appropriate minimum and maximum values for each experiment.

3. Results

3.1 Design of MHC variant peptides for I-A^{g7} with minimal activation of BDC-2.5

Peptide substitutions were designed based on existing studies of peptide binding to I-A^{g7} class II MHC molecules and our previous work through introducing non-favored amino acids [12, 20, 23, 32]. The parent peptide sequence and the variants utilized in this study are aligned in Figure 1a. The binding groove of I-A^{g7} exhibits a preference for hydrophobic residues at p4 and p6 and larger and/or positively charged amino acids at p9 [19, 20, 22, 33]. In systems with other MHC alleles including I-A^b and I-A^s, we have utilized a strategy of substituting an aspartic acid at p6 to successfully reduce peptide MHC half life and induce T cell anergy [12, 23]. In the case of I-A^{g7}, an aspartic acid substitution at p6 was not sufficient to induce anergy in BDC-2.5 T cells (data not shown). Instead, we introduced amino acid changes at all 4 anchor residues. To determine the immunogenicity of our panel of 7 variant peptides, we performed a proliferation assay. Naïve BDC-2.5 splenocytes were stimulated with various doses of each peptide (Fig. 1b), and variants that exhibited minimal proliferation above background were further tested for their ability to induce anergy.

3.2 MHC variant peptide treatment induces anergy

CD4 T cell anergy has been defined as an unresponsive state in which T cells do not proliferate or secrete IL-2 upon challenge with agonist peptide. To induce anergy with our variant peptide, we first activate cells for two weeks with 1 μ M of mimotope (agonist) peptide. Live cells are then restimulated with additional antigen presenting cells and either wild type or variant peptide. Cells that were passed for two cycles on the control peptide exhibit typical dose dependent proliferation to the activating peptide (Fig. 2A). Conversely, cells cultured on the variant peptide and then rechallenged with the agonist peptide show no proliferation above background (Fig. 2A). The variant selected for these studies was YPDV, which contains the following substitutions: H $\rightarrow$ Y, W $\rightarrow$ P, R $\rightarrow$ D and A $\rightarrow$ V at positions 1, 4, 6 and 9 respectively.

Similar to the phenotype observed for proliferation, cells passed on the agonist peptide secrete significant amounts of IL-2, while those cells cultured on variant peptide secrete very limited amounts of IL-2 when re-challenged with agonist peptide (Fig. 2B). These data indicate that the YPDV variant peptide is similar to the MHC variant peptides we have previously described for myelin antigens in experimental autoimmune encephalomyelitis [12, 23], i.e. it is minimally stimulatory, and stimulation with variant is sufficient to induce an anergic phenotype characterized by minimal proliferation and IL-2 production in response to stimulation with the agonist ligand.

3.3 Determination of peptide: MHC Half life

We used high performance size exclusion chromatography (HPSEC) to determine the half life of both the parent mimotope sequence as well as the MHC variant peptide. As shown in Fig. 3a, the mimotope peptide forms an extremely stable complex with I-A^{g7}, with a half life of greater than 36h. The exact value of the mimotope half life cannot be determined in this time frame due to the minimal decay observed. The variant peptide containing the YPDV substitutions formed a much less stable complex with MHC, resulting in an average half life of 4h.

3.4 Variant stimulation induces no detectable Erk1/2 or c-Jun phosphorylation

To characterize the mechanism by which MHC variant peptides induce anergy, we examined positive signaling events following initial encounter of activated T cells with the variant peptide. We assessed phosphorylation of the MAP kinase Erk1/2 following stimulation of activated BDC-2.5 T cells with either the agonist or variant peptide. Upon agonist peptide stimulation, we observed robust phosphorylation of Erk1/2 that persisted up to an hour after stimulation and waned by 3–5 hours after stimulation (Fig. 4A). Treatment with the variant peptide YDPV resulted in no detectable Erk1/2 phosphorylation at any time point tested (Fig. 4A).

Previous studies have demonstrated that p-c-Jun can accumulate even in the absence of detectable Erk1/2 phosphorylation [34, 35]. Despite robust accumulation of p-c-Jun in agonist stimulated cells, no substantial accumulation of c-Jun phosphorylation was observed in variant stimulated cells (Fig. 4B).

3.5 Variant stimulated cells exhibit increased total tyrosine phosphatase and specific SHP-1 phosphatase activity

In T cell signaling, with a few notable exceptions (e.g., CD45), kinases drive the positive signaling events while phosphatases are responsible for negative regulation. To determine if treatment with our variant peptide upregulated a generally negative signaling profile, we assessed bulk T cell lysates for their phosphatase activity. Activated BDC-2.5 T cells were stimulated with antigen presenting cells and either mimotope or variant peptide. At various times following stimulation, cells were harvested and lysed, and phosphatase activity was assessed by incubating the lysates with p-nitrophenylphosphate as a substrate. Agonist stimulation resulted in a low level of tyrosine phosphatase activity (Fig. 5A). Variant stimulated cells, however, exhibited a significantly different pattern of phosphatase activity. Activated BDC-2.5 T cells restimulated with variant peptide exhibited an early peak of phosphatase activity that was approximately three times greater in magnitude than that produced by wild type stimulated cells. Additionally, phosphatase activity was sustained in variant stimulated cells throughout the 30 minute time course assayed (Fig. 5A).

To clarify the specific negative signaling events occurring in this system, we next assayed the activity of the phosphatase SHP-1. This tyrosine phosphatase has been shown to be necessary for both T cell antagonism by altered peptide ligands [36, 37] as well as the induction of anergy by MHC variant peptides [38]. To determine the SHP-1 activity in this system, cells were stimulated as for the total phosphatase assay and lysed. SHP-1 was

immunoprecipitated from the cell lysates, and its activity was assessed by incubating the immunoprecipitated protein with a phospho-tyrosine containing target peptide. Phosphate release was measured by malachite green, which exhibits a colorimetric change in the presence of free phosphate. Similar to the bulk phosphatase activity, SHP-1 activity in agonist stimulated cells peaked and then waned over time (Fig. 5B). Variant stimulated cells peaked at the same time point as agonist treated cells, but then maintained high levels of phosphatase activity for the duration of the assay (Fig. 5B).

3.6 Anergic T cells are unresponsive to IL-2 stimulation

In many forms of anergy, the provision of exogenous IL-2 is sufficient to restore the cells' ability to proliferate [39]; on the other hand, MHC variant peptide induced anergy is not readily overcome by the addition of IL-2. To determine whether the anergized BDC-2.5 T cells were resistant to IL-2 induced proliferation, we stimulated anergic cells with agonist ligand in the presence of 3.5ng/well recombinant IL-2. Agonist activated cells cultured without the addition of IL-2 exhibit a typical proliferation profile, while variant cultured cells have little proliferation above background (Fig. 6). Mimotope cultured cells rechallenged with mimotope plus IL-2 exhibited increased background proliferation in cells not stimulated with peptide, but maintained a normal dose response to agonist stimulation. However, variant cultured cells challenged with agonist peptide plus IL-2 did not exhibit proliferation above background. This is consistent with the inability of cells anergized by MHC variant peptides to be rescued by the addition of IL-2 (Fig. 6).

To characterize the mechanisms mediating the IL-2 resistant phenotype of the anergized BDC-2.5 cells, we first assessed their expression of the high affinity IL-2 receptor by staining for CD25. Both groups of cells expressed high levels of CD25 (Fig. 7A). Interestingly, the anergic cells maintained levels of CD25 that were slightly elevated relative to the control cells. Thus, a lack of high affinity receptor expression does not explain the inability of the anergized cells to be rescued by the addition of exogenous IL-2. Ligation of the IL-2 receptor drives both proliferative and survival signals [40], and the proliferative program is mediated at least in part by signaling through Stat5. To determine if this aspect of signaling via the IL-2 receptor was disrupted in MHC variant peptide anergized cells, we stimulated the cells with IL-2 and assessed Stat5 phosphorylation by flow cytometry. BDC-2.5 T cells stimulated with IL-2 exhibited robust phosphorylation of Stat5 by 30 minutes (Fig. 7B and C). In contrast, anergized cells displayed minimal Stat5 phosphorylation (Fig. 7B and C).

4. Discussion and Conclusions

Type 1 diabetes is caused in large part by autoreactive CD4⁺ T cells; therefore we sought to develop an antigen specific means of inducing tolerance in diabetogenic T cells by destabilizing the peptide:MHC interaction. The unusual binding characteristics of the diabetes-associated class II MHC I-A^{g7} expressed by NOD mice pose some challenges to the induction of anergy by MHC variant peptides. Although the I-A^{g7} molecule is relatively unstable as compared to some MHC molecules, we have demonstrated herein that peptide:I-A^{g7} complexes possess sufficient stability such that an MHC variant peptide that effectively

anergizes T cells may be generated. As we and others have demonstrated, agonistic epitopes typically exhibit a half life on MHC on the order of days [30, 31, 38, 41–49]. By modifying all four MHC binding residues of the mimotope peptide, we have generated an epitope with a half life of approximately 4 hours, which can induce anergy in BDC-2.5 T cells. Of interest, this finding is in accordance with our previous data suggesting that peptides with half lives of less than 8 hours are sufficient to induce anergy [23, 38].

Historically, the BDC-2.5 T cells autoantigen was unknown, but recent data suggest that these T cells may recognize an epitope derived from the protein Chromogranin A, although there is not yet a consensus regarding the exact epitope [50, 51]. There is also some evidence suggesting that a Chromogranin A-derived epitope may bind the MHC molecule in an unusual fashion [50]. Regardless of the antigen specificity, our method for creating MHC variant peptides generated a peptide that induced T cell anergy, suggesting that a single set of unfavorable MHC anchors may be inserted into any antigenic peptide that binds to a given MHC molecule and destabilize the pMHC sufficiently to render the reactive T cells anergic.

Our data demonstrate that an MHC variant peptide with a short half life induces undetectable levels of Erk1/2 phosphorylation. This may be due to Erk1/2 phosphorylation being completely absent, rapidly reversed, or a positive signal through this pathway may be delivered via a “trickle through” type effect as we and others have previously described [34, 52]. Furthermore, we observed a concomitant increase in total and specific SHP-1 tyrosine phosphatase activity upon stimulation with the anergizing peptide. This increased activity of SHP-1 following stimulation with an anergizing peptide would likely have far-reaching effects in the cell, due to the potential of this phosphatase to interact with a plethora of signaling cascades, including those downstream of the T cell receptor, CD28 and various cytokine receptors, perhaps pointing to SHP-1 as a global regulator of T cell activation.

How signals are transduced through the TCR and subsequently trigger intracellular events is an area of intense investigation, and our knowledge of these mechanisms are continually evolving. Both conformational and thermodynamic parameters determine the outcome of the interaction between a TCR and a pMHC ligand. We postulate that the ability of our MHC variant peptide to induce anergy hinges primarily on thermodynamic alterations, although structural changes could also have an impact. We have demonstrated that the anergy-inducing variant has a shortened half life of interaction with the MHC, but as these changes are on the order of hours and differential signaling occurs within minutes of encounter, we hypothesize that the interaction between a weak ligand and the MHC may be more dynamic than that of an agonist, i.e. a weak ligand may disassociate and rebind rapidly whereas an agonist may bind strongly and rarely dissociate. These differences in the dynamics of the pMHC interaction would not be detectable by the approaches we have utilized, but represent an interesting question regarding how pMHC interaction kinetics are translated into intracellular signaling events via the TCR.

An anergic phenotype can be induced in CD4⁺ T cells in a variety of ways, including provision of a strong signal through the TCR in the absence of sufficient costimulation, by means of chemically fixed antigen presenting cells, antibody cross-linking or antibody

blockade, low or high doses of antigen, altered peptide ligands and MHC variant peptides [12, 23, 53–59]. Anergy induced by each of these methods results in a stable phenotypic shift that can frequently be overcome by the provision of IL-2, although this is not the case for anergy induced by MHC variant peptides [39, 60]. The variety of ways to induce anergy and the subtle differences in the phenotypes generated by each model imply that the molecular mechanisms likely vary. For example, cells anergized by provision of TCR signal alone exhibit a blockade in the activation of the kinases Erk and Jnk and the GTP-ase Ras following CD3/CD28 cross-linking [61, 62], while cells anergized by treatment with an altered peptide ligand exhibit alterations in TCR zeta chain phosphorylation and a failure to recruit ZAP-70 [63]. For the MHC variant peptides, increased phosphatase levels appear to mediate the anergic phenotype. The mechanistic differences observed in different forms of anergy necessitate the further study of those approaches if they are to be applied therapeutically.

Importantly, anergy induced by MHC variant peptides cannot be overcome by the addition of exogenous IL-2 (Fig. 6). This failure to respond to IL-2 stimulation is not due to differences in expression of the high affinity IL-2 receptor, as expression levels are similar in agonist treated and anergized populations. Binding of IL-2 to its receptor activates several signaling pathways in T cells, including Jak/Stat, MAPK, PI3K/Akt, by which it drives both T cell proliferation and survival [64]. Despite their high levels of CD25 expression, anergized cells fail to upregulate Stat5 phosphorylation upon treatment with IL-2 (Fig. 7), similar to what has been previously described for anergy induced by superantigen treatment, which is also not overcome by exogenous IL-2 [65]. Interestingly, T cells anergized by superantigen are also unable to phosphorylate Stat5 in response to IL-7 or IL-15, suggesting there may be a broad blockade of positive signaling events in these cells. It is possible that this resistance to IL-2 stimulation is mediated by the activity of SHP-1, as some evidence suggests that this phosphatase can bind directly to the IL-2 receptor beta chain [66], although these pathways have not been directly linked in anergy. Furthermore, SHP-1 has been shown to associate with many of the downstream mediators of IL-2 signaling including Stat5 [67–71]. It is possible this blockade in IL-2 signaling represents a key mechanism for limiting proliferation and IL-2 production by anergic cells.

In addition to its role in maintaining tolerance, anergy can also be a consequence of various pathological states including infection and cancer [72, 73]. Viral escape mutants or tumor antigens that have decreased affinity of peptide: MHC interaction can anergize reactive T cells [72–74]. Better understanding the mechanisms regulating the induction and maintenance of the anergic state may allow for therapeutic utilization of anergy as a means to treat autoimmune diseases or prevent graft rejection. Furthermore, these mechanistic insights may also elucidate ways to reverse anergy, allowing for the revitalization of a previously dysfunctional T cell response. Because T cells anergized by MHC variant peptides are resistant to stimulation via the IL-2 receptor, knowledge of the signaling events downstream of IL-2 stimulation of anergized cells can provide unique approaches to inhibit or reinvigorate the T cell response.

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A.

Parent

		P1			P4		P6			P9
A	H	H	P	I	W	A	R	M	D	A
		Y			P		D			V
		F			Q		A			N
					P		D			V
					P					V
										V
		Y					D			V
		Y			P					V

B

Figure 1. BDC2.5 T cells do not proliferate in response to variant peptide

A. Sequence of the mimotope (parent) and variant peptides. **B.** BDC2.5 splenocytes were stimulated with a range of concentrations of both parent and variant peptides. Proliferation was assessed by [^3H] thymidine incorporation as described in the methods. Data are representative of at least three independent experiments. Points represent means $\pm$ SEM. All variant peptides differ significantly from the parent mimotope ($p < 0.05$) as determined by One-way ANOVA with Dunnett post test.

Figure 2. Variant peptide treatment induces anergy

A. BDC2.5 cells were activated for 14d with 1 μ M mimotope, restimulated with either mimotope or variant (YPDV) for an additional 14d and subsequently challenged with the indicated concentrations of mimotope peptide. Proliferation was assessed by [3 H] thymidine incorporation. Data represents average of four independent experiments performed in duplicate. Points indicate mean values $\pm$ SEM. $p=0.0015$ as determined by Student's t test.

B. Anergy was induced as in part A. Mimotope or variant treated BDC2.5 T cells were restimulated with 10 μ M mimotope peptide. Supernatants were harvested after 24h, and IL-2

production was determined by ELISA. Samples were assessed in triplicate and data are representative of three independent experiments. Error bars indicate SEM. $P=0.0310$ by Student's t test.

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Figure 3. MHC anchor substitutions decrease half life of peptide: MHC complexes
Decay curves (A) and half life (B) of mimotope and variant peptide as determined by HPSEC. Decay curves are representative of five independent repeats. Half life values represent the average of five replicates. Y-axis values represent the natural log of the normalized peak height at each time point divided by the baseline peak height. Half life values are calculated as the natural log 2 of the slope of the decay curve. $p=0.0067$ for differences in half life as determined by Student's t test.

Figure 4. Variant stimulation induces no detectable Erk1/2 phosphorylation

A. BDC2.5 splenocytes were stimulated with mimotope peptide for 14d. Live cells were stimulated with an I-A^{g7} expressing cell line and either 10 μ M mimotope (black bars, top row) or 10 μ M variant (YPDV, white bars, bottom row) for the indicated times. Cells were fixed, permeabilized and Erk1/2 phosphorylation was determined by flow cytometry. Y-axis represents percent of CD4⁺ T cells that are pErk1/2⁺. Right side displays representative plots of pErk1/2 vs. SSC at time=0 (no stimulation), 30 min (peak), and 5h (post-peak). Flow plots are gated on CD4⁺ lymphocytes. Data compiled from three independent experiments, $p=0.0031$ by Student's t test, error bars represent SEM. **B.** c-Jun phosphorylation. Cells were stimulated as in A. Y-axis represents percent of CD4⁺ T cells that are p-c-Jun⁺. Right panel displays representative histograms of c-Jun phosphorylation, with shaded histograms representing mimotope stimulated cells and open histograms representing variant stimulation for the indicated time points. Data compiled from two independent experiments, $p=0.0007$ by Student's t test, error bars represent SEM.

Figure 5. Variant stimulated cells exhibit increased total tyrosine phosphatase and specific SHP-1 phosphatase activity

A. BDC2.5 T cells were stimulated as in Figure 4. *p*-nitrophenylphosphate solution was added to whole lysates and allowed to develop overnight at 37°C. Colorimetric change was assessed at 405nm. Data graphed are the averages of three independent experiments and represent mean $\pm$ SEM. $p=0.0086$ by Student's *t* test. **B.** Cells were stimulated as in Figure 4. After cell lysis, SHP-1 was immunoprecipitated and incubated with a pTyr containing substrate peptide. Malachite green was added and amount of free phosphate was determined

by colorimetric change at 620nm. Data graphed are representative of three independent experiments.

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Figure 6. Addition of exogenous IL-2 does not rescue proliferation of anergized T cells
 BDC2.5 T cells were stimulated for 14d with 1 μ M mimotope peptide. Live cells were restimulated for an additional 14d with either 1 μ M mimotope or 10 μ M variant peptide plus irradiated antigen presenting cells. Live cells were restimulated with irradiated antigen presenting cells and indicated doses of mimotope without the addition of IL-2 (Mimotope vs. variant, $p=0.05$) or with IL-2 (mimotope+IL-2 vs. variant+IL-2 $p=0.001$). Data represent mean $\pm$ SEM, and p values were calculated by a one way ANOVA with Bonferroni post test.

Figure 7. MHC variant peptide anergized cells are resistant to signaling through the IL-2 receptor

A. Expression of CD25 is slightly elevated in variant anergized cells (open histogram) as compared to mimotope-cultured cells (filled, dark grey histogram). Light grey tinted histogram represents no stain control. **B.** 30 minutes following stimulation with 100ng/ml recombinant IL-2 mimotope cultured cells exhibit significant Stat5 phosphorylation (open histogram) compared with unstimulated cells (filled histogram). Anergized cells stimulated under the same conditions exhibit no Stat5 phosphorylation above background (open histogram: IL-2 stimulated; filled histogram: no stimulation). Data are representative of two independent experiments. **C.** Average of percent maximum pStat5 geometric mean fluorescence intensity at various times following IL-2 stimulation. Data represents average of two independent experiments. Points indicate averages of percent maximum fluorescence intensity $\pm$ SEM. * $p < 0.02$.