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# γδ T cells recognize the insulin B:9–23 peptide antigen when it is dimerized through thiol oxidation☆

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ABSTRACT

The insulin peptide B:9–23 is a natural antigen in the non-obese diabetic (NOD) mouse model of type 1 diabetes (T1D). In addition to αβ T cells and B cells, γδ T cells recognize the peptide and infiltrate the pancreatic islets where the peptide is produced within β cells. The peptide contains a cysteine in position 19 (Cys19), which is required for the γδ but not the αβ T cell response, and a tyrosine in position 16 (Tyr16), which is required for both. A peptide-specific mAb, tested along with the T cells, required neither of the two amino acids to bind the B:9–23 peptide. We found that γδ T cells require Cys19 because they recognize the peptide antigen in an oxidized state, in which the Cys19 thiols of two peptide molecules form a disulfide bond, creating a soluble homo-dimer. In contrast, αβ T cells recognize the peptide antigen as a reduced monomer, in complex with the MHCII molecule I-$A^{g7}$. Unlike the unstructured monomeric B:9–23 peptide, the γδ-stimulatory homo-dimer adopts a distinct secondary structure in solution, which differs from the secondary structure of the corresponding portion of the native insulin molecule. Tyr16 is required for this adopted structure of the dimerized insulin peptide as well as for the γδ response to it. This observation is consistent with the notion that γδ T cell recognition depends on the secondary structure of the dimerized insulin B:9–23 antigen.



## 1. Introduction

The adaptive immune system consists of three lymphocyte-types, B cells, αβ T cells and γδ T cells, which share the ability to express diverse antigen receptors encoded by genes that undergo somatic gene rearrangement. Widely distributed in present day vertebrates, all three cell types can be traced back ~500 million years through their antigen receptor genes (Rast et al., 1997), suggesting that they each have essential functions in the survival of the species (Bonneville et al., 2010; Vantourout and Hayday, 2013).

Like the other lymphocyte-types, γδ T cells take part in immune responses (Bonneville et al., 2010; Vantourout and Hayday, 2013), become mobilized during sterile or infectious inflammation (Mukasa et al., 1997; Simonian et al., 2010), and contribute to host protection, especially early in life (Ramsburg et al., 2003). They have been implicated in tissue repair (Jameson and Havran, 2007), antigen presentation to T cells (Brandes et al., 2005), B cell help

*Abbreviations:* T1D, type 1 diabetes; B:9–23, insulin2 B chain peptide (amino acids 9–23); TCR, T cell receptor for antigen; APC, antigen presenting cell; mAb, monoclonal antibody; MHC, major histocompatibility complex.

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(Wen et al., 1996), the elaboration and control of certain cytokines (*e.g.* IFN-γ, IL-4, IL-17, IL-22) (Simonian et al., 2010; Roark et al., 2008; Yin et al., 2002; Ferrick et al., 1995), and much evidence suggests that γδ T cells monitor and respond to stressed cells and tissues (Janeway et al., 1988; Hayday, 2009). Many of these functions are overlapping with those of other lymphocyte populations, which besides conventional T and B cells include innate-like NKT cells, MAIT cells and B1 B cells. However, γδ T cells express antigen receptors that are distinct from the BCRs and αβ TCRs (Brenner et al., 1986; Chien et al., 1987; Born et al., 1987), suggesting that they recognize antigens differently. Differences in TCR triggering (Zeng et al., 2012) and responsiveness also set γδ T cells apart. Thus, comparison of TCR-mediated signaling showed that γδ TCRs signal more robustly when compared to αβ TCRs (Hayes and Love, 2002). Second, studies of the antigen binding site revealed that the γδ TCR differs from the αβ TCR in that the complementarity-determining regions 3 (CDR3) of the γ and δ chains are more variable in length (Rock et al., 1994), and from immunoglobulins (Igs) by lesser diversity of CDR1 and 2 (Davis and Bjorkman, 1988). Finally, the antigens recognized, and the mode of antigen recognition, indicate that antigens for γδ T cells are structurally far more diverse than those of αβ T cells (Vantourout and Hayday, 2013; Born et al., 2013), and γδ T cells tend to recognize antigens directly, without requirement for processing and presentation (Chien et al., 1996). The broad range of molecular moieties stimulating TCR-dependent γδ T cell responses includes intact proteins (cell surface expressed or soluble), protein fragments (peptides), phosphoantigens (*e.g.* isoprenyl-pyrophosphate), phospholipids and/or complexes between phospholipids or sulfatides and proteins (Vantourout and Hayday, 2013; Born et al., 2013). Furthermore, individual γδ TCRs can mediate responses to several structurally unrelated molecules (Born et al., 2003), so that the mode of recognition might change depending on the antigen involved. As a caveat, many of the presumed γδ antigens have not yet been directly shown to bind to the γδ TCR, or to elicit physiological γδ responses *in vivo*.

Early reports that small synthetic peptides elicit TCR-dependent responses of γδ T cells (Born et al., 1990; Fu et al., 1993) left unanswered how peptides might be recognized, and whether natural peptides can be antigens for γδ T cells. However, these studies, which involved γδ T hybridomas, already indicated that such responses do not require antigen-presenting cells (APCs) (O'Brien et al., 1992), in marked contrast to the peptide antigen-specific, MHC-restricted responses of αβ T cells. This suggested that γδ T cells and αβ T cells recognize peptides by different mechanisms. More recently, we reported a TCR-dependent γδ response to the insulin B chain-derived peptide B:9–23 (Zhang et al., 2010), which is a natural auto-antigen in non-obese diabetic (NOD) mice (Mohan et al., 2010). NOD mice spontaneously develop a type-1 diabetes (T1D)-like autoimmune disease, which unfolds in several stages, including the early appearance of autoantibodies directed against pancreatic islet antigens, insulitis, and finally β cell destruction and diabetes (Bluestone et al., 2010). γδ T cells appear to play both pathogenic and regulatory roles in this autoimmune disease (Markle et al., 2013; Harrison et al., 1996; Han et al., 2010), but what triggers their engagement remains unclear. Insulin is an early, prominent and essential auto-antigen in this disease (Nakayama et al., 2005; Zhang et al., 2008). The naturally occurring insulin B chain-derived peptide B:9–23 is recognized by B cells (Mohan et al., 2010; Liu et al., 2002) and $CD4^+$ αβ T cells (Simone et al., 1997), and by γδ T cells (Zhang et al., 2010). NOD-derived αβ T cells "see" this peptide antigen in the context of the MHCII molecule I-$A^{g7}$, and the molecular parameters of this recognition have been studied in much detail (Mohan et al., 2011; Crawford et al., 2011). Here, we provide an initial account of requirements for the recognition of the insulin peptide B:9–23 by γδ T cells, which is not restricted by I-$A^{g7}$ but might depend on a distinct secondary structure associated with dimerization of the oxidized peptide.

## 2. Results

### 2.1. Oxidizing the insulin peptide B:9–23 improves its capability of stimulating γδ T cell hybridomas

We previously found that a γδ T cell hybridoma derived from a mouse of the non-obese diabetic (NOD) genetic background (hybridoma SP9D11 expressing Vγ4 and Vδ10 TCR-genes) responded specifically to the insulin peptide B:9–23. The response was TCR-dependent. SP9D11 cells also responded specifically to pancreatic islet cells but not to the intact insulin molecule (Zhang et al., 2010). Specific reactivity to the B:9–23 peptide was also seen with several other NOD-derived γδ TCR-expressing hybridomas, revealing considerable diversity among γδ TCRs capable of supporting this response. Unlike αβ T cells, the γδ hybridomas responding to the insulin peptide did not require APCs, in this regard reminiscent of previously reported peptide responses by γδ hybridomas (Born et al., 1990; O'Brien et al., 1992), and even isolated single SP9D11 cells were activated by this soluble peptide (Zhang et al., 2010). Interestingly, a B:9–23 peptide in which the cysteine in position 19 (Cys19) was replaced with alanine (B:19A) was not stimulatory, suggesting that the cysteine might be required for the γδ response, in marked contrast to B:9–23-reactive αβ T cells, which respond well to the B:19A peptide (Alleva et al., 2002; Stadinski et al., 2010). In addition, we noted variations between batches of untreated synthetic B:9–23 peptide in terms of their stimulatory capacity. Because cysteine contains a thiol group, which can be oxidized to form disulfides and higher oxidized states (Go and Jones, 2013), we considered the possibility that the peptide must be oxidized to be stimulatory. We therefore compared the stimulatory capacity of fresh B:9–23 peptide preparations that were untreated *vs.* those that were intentionally oxidized, either by prolonged exposure to ambient air, or by adding copper chloride, which accelerates the oxidative process (Pecci et al., 1997). We also tested in this manner other peptides, including the previously identified non-stimulatory B:19A peptide (Zhang et al., 2010), and another non-stimulatory B:9–23 peptide in which Tyr16 was replaced with alanine (B:16A). The experiments assembled in Fig. 1, panel A show that oxidation substantially enhanced the ability of the wild-type B:9–23 peptide to stimulate SP9D11 cells, but failed to produce responses to the two alanine-substituted non-stimulatory peptides. In contrast, a B:9–23-reactive αβ T cell hybridoma (I.29; Levisetti et al. (2007)) did not respond to the oxidized peptide (Fig. 1, panel B). These findings suggested that while oxidation of Cys19 is not required for the αβ response, it might be critical for the γδ response.

### 2.2. The oxidized insulin B:9–23 peptide stimulates γδ T cells as a dimer, and without requirement for MHCII

The thiol group in cysteine is readily oxidized to form disulfides, whereas higher oxidized forms require stronger oxidants (Pecci et al., 1997). Hence, the oxidized B:9–23 peptide might form a dimer and stimulate γδ T cells in this configuration. To examine this possibility, and to exclude other oxidized forms, we again employed the B:9–23 peptide, oxidized it with the comparatively weak oxidant DMSO (Liu et al., 2009), separated monomers from dimers by HPLC, and finally analyzed the purified fractions for their monomer and dimer content using EMS. Monomeric and dimeric peptide preparations with a purity of >95% were then tested for their ability to stimulate SP9D11 cells (Fig. 2). SP9D11 hybridoma cells readily responded to the dimer fraction, even at