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Antibodies that react with bovine T lymphocytes expressing the T cell receptor β chain subgroup BV20 inhibit antigen recognition

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Abstract

In recent years, molecular studies have provided detailed information on the bovine T cell receptor (TCR) variable gene repertoire, both in resting T cells and during T cell responses. However, studies of the biological function of the receptor have been hampered by a lack of reagents that recognise the protein. Herein, we describe the characterisation of two antibodies (IL-A47 and IL-A98) that recognise T cells expressing the TCR VB20 subfamily of BV genes. These antibodies each recognise a small subset of αβ T cells in PBMC, including subsets of both CD4 and CD8 T cells. One of the antibodies (IL-A98) recognises a smaller subset of cells within the IL-A47+ population. When tested on a panel of T cell clones expressing different αβ TCR subfamilies of B chain genes, IL-A47 was found to react only with clones expressing the BV20 subfamily, which in cattle has undergone expansion due to gene duplication; IL-A98 reacted with a subset of the BV20 subfamily members. IL-A47 was shown to profoundly inhibit recognition of target cells by cytotoxic T cell clones, an effect that was mediated via the effector T cell rather than the target cells.

Introduction

The capacity to generate T cell-mediated immune responses to pathogens relies on a repertoire of T lymphocytes that can recognise and respond to a diverse array of antigens. This ability is determined by expression of an antigen-specific receptor that recognises antigenic peptides presented in association with major histocompatibility complex (MHC) proteins on the surface of pathogen-infected cells or antigen-presenting cells. This T cell receptor (the α/β TCR) is a heterodimeric protein, the two chains of which (α and β) are generated during T cell development in the thymus by somatic recombination of genes (V and J for the alpha chain and V, D and J for the beta chain) from a germline repertoire of genes. Random addition or deletion of nucleotides at the junctions of the V, D and J genes further enhances sequence diversity of the α/β TCR chains. These processes result in an extensive repertoire of α/β T cells expressing unique receptors capable of recognising diverse foreign antigens. Antigen recognition by the TCR is determined predominantly by three hypervariable peptide regions in each chain, known as complementarity determining regions (CDRs). CDR1 and CDR2, encoded within the variable gene segments, interact with the presenting MHC protein, whereas CDR3 encoded by the VDJ junctional region interacts predominantly with the MHC-presented antigenic peptide.

Humans possess 60 distinct TCR β variable (BV) genes, of which 40-42 genes are functional, belonging to 23 subfamilies, defined as those with > 75% nucleotide identity (Folch and Lefranc 2000). The identification of orthologous BV subfamilies in other mammalian species indicates that they evolved prior to speciation. However, inter-species differences in the complement of BV genes have arisen as a result of gene deletion and duplication events, the latter resulting in multiple members of some subfamilies. Analyses of the bovine T cell receptor have revealed a markedly expanded repertoire of BV genes, due predominantly to expansion of 4 subfamilies BV6, BV9, BV20 and BV21, which account for over 50% of the expressed BV genes (Connelley et al. 2009). Over 130 BV genes have been identified in the bovine genome and 89 genes belonging to 22 BV subfamilies have

been identified as transcripts in cDNA from circulating T cells. Many of the members of each of the expanded subfamilies show a high level of sequence identity (Connelley et al. 2009), indicating that gene duplication in cattle has occurred relatively recently.

Although there has been excellent progress in defining the bovine T cell repertoire at the molecular level, the ability to study the biological function of the receptor has been constrained by a lack of reagents that allow identification of the protein. Herein, we describe the characterisation of two antibodies that recognise T cells expressing the TCR BV20 subfamily of V genes and demonstrate that one of these antibodies inhibits antigen recognition.

Methods

Cell phenotyping

Analyses of antibody specificity used peripheral blood mononuclear cells (PBMC) harvested by density gradient centrifugation from blood of healthy 6-12 month old Holstein or Boran cattle and CD8 T cell clones specific for either the protozoan parasite *Theileria parva* or allogeneic MHC antigens. T cell clones were generated *in vitro* as previously described (Goddeeris and Morrison 1988; Toye et al. 1990). Some experiments utilised T cell lines infected with *T. parva*, established by infection of PBMC *in vitro* with sporozoites as previously described (Brown et al. 1973). Cells subjected to single-colour and two-colour staining by indirect immunofluorescence used monoclonal antibodies (MAb) specific for CD3 (MM1A), CD4 (CC30), CD8 (IL-A63) and the γδ TCR (GB21A) and secondary mouse isotype-specific antibodies labelled with Alexa Fluor 488 or Alexa Fluor 647. Stained cells were analysed on a FACScalibur cell analyser (Becton-Dickenson Biosciences, Oxford, UK).

Production of monoclonal antibodies

Murine MAb were produced by conventional procedures from two cell fusions each using spleen cells from a Balb/c mouse that had received two doses of 10^7 CD8 T cells from a

single T cell clone, administered intraperitoneally at an interval of 3 weeks, and spleen cells harvested 4 days later.

Identification of expressed VB genes

The sequences of BV genes expressed by cloned T cells were determined by Sanger sequencing of PCR products obtained using PCR primers that detect all BV genes, as described previously (Connelley et al, 2008a).

Inhibition of antigen recognition

Experiments to examine antigen recognition used allo-reactive cytotoxic CD8 T cell clones generated *in vitro* from an animal that had been immunised with allogeneic PBMC as previously described (Toye et al 1990). The T cell clones were maintained *in vitro* by weekly stimulation with gamma-irradiated cells from a *T. parva*-infected cell line of the appropriate MHC type, with added recombinant human IL-2 (Chiron, Emryville, CA, USA). Antigen recognition was determined by measuring cytotoxicity employing a conventional 4-hour Chromium-51 (^{51}Cr) release assay conducted in 96-well plates as described previously (Goddeeris and Morrison 1988). Effector T cells were incubated with ^{51}Cr-labelled *T. parva*-infected target cells (5 x 10^4 cells/well) at a range of effector to target cell ratios. The ability of the MAb to inhibit antigen recognition was tested by measuring cytotoxicity in the presence of MAb. MAb was added to the effector T cells or target cells at a concentration 10 times that giving maximal binding, as assessed by staining by flow cytometry, followed by addition of target cells or effector T cells respectively after 30 minutes and incubation at 37^{o} C for 4 hours.

T cell receptor nomenclature

In initial publications of bovine TCR BV genes (Tanaka et al. 1990, Houston and Morrison 1999), the identified genes were allocated subfamily numbers based on orthology with human BV subfamilies (Arden et al. 1995). Following complete sequencing of the human TCR BV locus (Rowen et al. 1996), human BV gene subfamilies were allocated new

numbers according to their order within the genome (Folch and Lefranc, 2000; Lefranc et al. 2003). Bovine subfamilies were then re-numbered, retaining orthologies with the new human BV subfamilies (Houston et al. 2005; Connelley et al. 2009); one gene referred to as BVX does not have an obvious human orthologue.

Approval of animal studies

Studies in Kenya using mice and cattle were approved by the ILRI Institutional Animal Care and Use Committee. Experiments using cattle in Edinburgh were completed under Licence from the UK Home Office in accordance with the UK Animals (Scientific Procedures) Act 1986 and were also subject to approval by the University of Edinburgh ethical review process.

Results and Discussion

Antibodies that detect a small subset of T cells

Two separate experiments, in which mice were immunised with cloned CD8 T cells for monoclonal antibody production, each yielded a hybridoma producing antibodies (IL-A47 and IL-A198, IgA and IgG1 respectively) that reacted with 100% of the CD8 T cells used to generate the antibodies and a small population of cells in PBMC. In PBMC from twelve 6-12-month old calves, the percentage of cells in PBMC stained for IL-A47 ranged from 4% to 7% in different animals, while IL-A198 consistently reacted with a smaller population. The IL-A47$^+$ cells represented 12-19% (mean 15.1%) of the T cells in these animals. The results of two colour staining (shown in Figure 1) demonstrated that the positive cells were CD3+ and that the smaller population of IL-A198+ cells represented a subset of the IL-A47+ cells. Co-staining for T cell subset markers revealed that both CD4 and CD8 subsets contained small populations of IL-A47$^+$ cells, whereas γδ T cells were negative for IL-A47.

Association with T cell receptor BV gene expression

Attempts to immunoprecipitate the IL-A47 and IL-A198 target proteins proved inconclusive. However, the pattern of staining with the antibodies suggested a possible reaction with the αβ T cell receptor. The reactivity of IL-A47 with a panel of CD8 T cell clones that had been typed for BV gene subfamily expression was therefore examined. Fourteen of the 22 functional subfamilies of BV genes identified in cattle (Connelley et al. 2009b) were represented in this panel of cloned T cells. As shown in Figure 2, only the clone that expressed BV20 reacted with IL-A47. This is one of the VB subfamilies that has undergone expansion in cattle, with 9 functional subfamily members identified (Connelley et al 2009b). Over 20 T cell clones typed as BV20 have now been phenotyped with IL-A47 and all found to be positive, whereas a similar number of clones expressing the other BV subfamilies shown in figure 2 were all negative (data not shown). T cell clones expressing 6 of the BV20 subfamily members were available to examine reactivity with IL-A47 and IL-A198. IL-A47 reacted with representatives of all 6 subfamily members, whereas IL-A198 recognised only 2 of the 5 subfamily members examined. These results indicate that reactivity of IL-A47 is restricted to T cells expressing the BV20 subfamily of TCR β chains and that IL-A198 recognises a subset of the BV20 subfamily members.

IL-A47 blocks antigen recognition by CD8 T cells

The ability of IL-A47 to inhibit antigen recognition by CD8 T cells was tested using alloreactive cytotoxic CD8 T cell clones specific for the class I MHC allele 2*00102, which is expressed by *Bos indicus* animals with the A10 class I serotype. The T cell lines included IL-A47^{+} and IL-A47^{-} clones. The availability of target cells that were also either IL-A47^{+} or IL-A47^{-} allowed the influence BV20 expression on the effector or target cell to be examined. Addition of IL-A47 antibody to IL-A47^{+} CD8 T cells incubated with IL-A47^{-} target cells resulted in profound inhibition of cytotoxicity whereas no effect was observed when the antibody was incubated with IL-A47^{-} T cells and IL-A47^{+} target cells (Figure 3). Incubation of the cells with an isotype control MAb had no inhibitory effect on cytotoxicity. These results clearly demonstrate that interference in T cell recognition by the antibody acts at the level of

the T cell rather than the target cell, a finding that is consistent with recognition of the T cell receptor.

Conclusions:

Collectively, the results of this study suggest that IL-A47 and IL-A98 are specific for BV20. However, further experiments will be required to confirm their specificity. Generation of recombinant TCR proteins that retain native conformation, as described for human TCR (Card et al. 2004), would allow studies to formally confirm reactivity of the antibodies with the α/β TCR. Moreover, further investigation of reactivity with T cells expressing different BV subfamilies is required to ascertain whether or not the MAb recognise other BV subfamilies and other BV20 subfamily members not represented in the T cell clones examined in this study. While this information could be obtained by examining further T cell clones, deep sequencing of BV cDNA from purified IL-A47+ and IL-A98+ cells would provide a more direct and sensitive approach to addressing this question.

These antibodies represent the first reagents available for studying the biology of the bovine αβ TCR. In some instances, particularly involving CD8 T cells, which frequently utilise a restricted set of BV genes (Connelley et al. 2008a, 2016), they will have applications for monitoring their responses. Moreover, their ability to inhibit T cell recognition will be of value for functional studies of bovine T cells *in vitro*.

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Table 1. Reactivity of monoclonal antibodies with T cell clones expressing different members of the T cell receptor VB 20 subfamily

BV subfamily member	T cell clones[1]	Monoclonal antibody	
		IL-A47	IL-A198
20.2	T29.10	+	-
20.3	485.53, 485.130	+	-
20.4	592.15	+	+
20.5	T28.1	+	-
20.6	592.27	+	Nt
20.7	T.40.58, T54.18,	+	+

[1] T cell clone T28.1 is an alloreactive CD8 T cell. All other clones are CD8 T cells specific for cells infected with *T. parva*

Figure 1

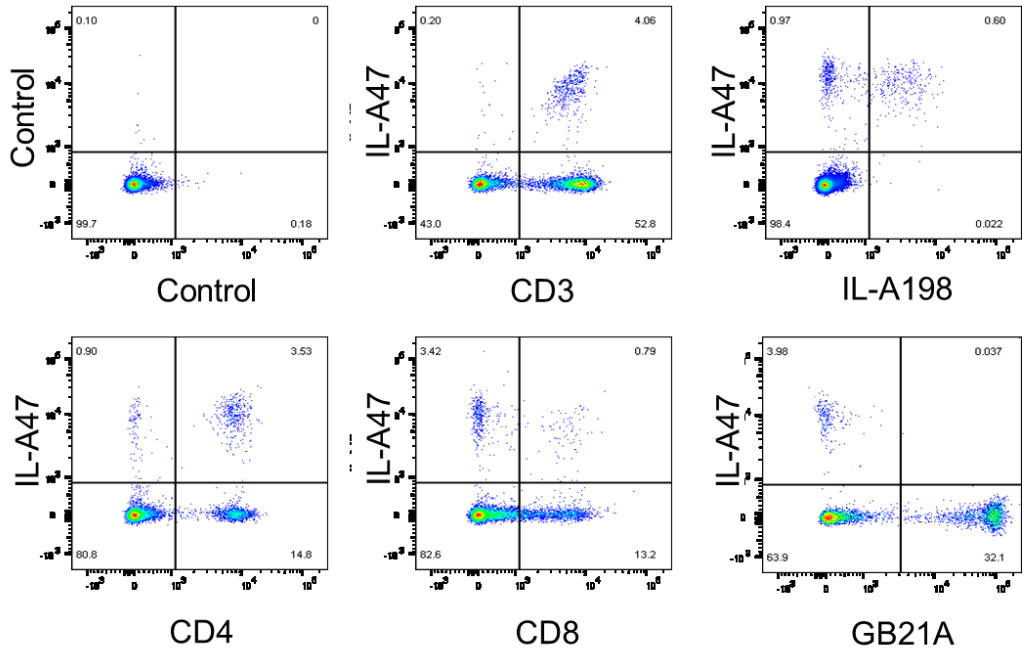

Figure 1: Two-colour immunofluorescence staining of PBMC with monoclonal antibodies IL-A47 (IgA) and IL-A98 (IgG1) and antibodies specific for CD3 and T cell subpopulation markers CD4 (CC30, IgG1), CD8 (IL-A63, IgG1) and γδ TCR (GB21A, IgG1). Cells were stained by indirect immunofluorescence using secondary IgG1-specific and IgA-specific antibodies labelled with Alexa Fluor 488 and Alexa Fluor 647 respectively.

Figure 2

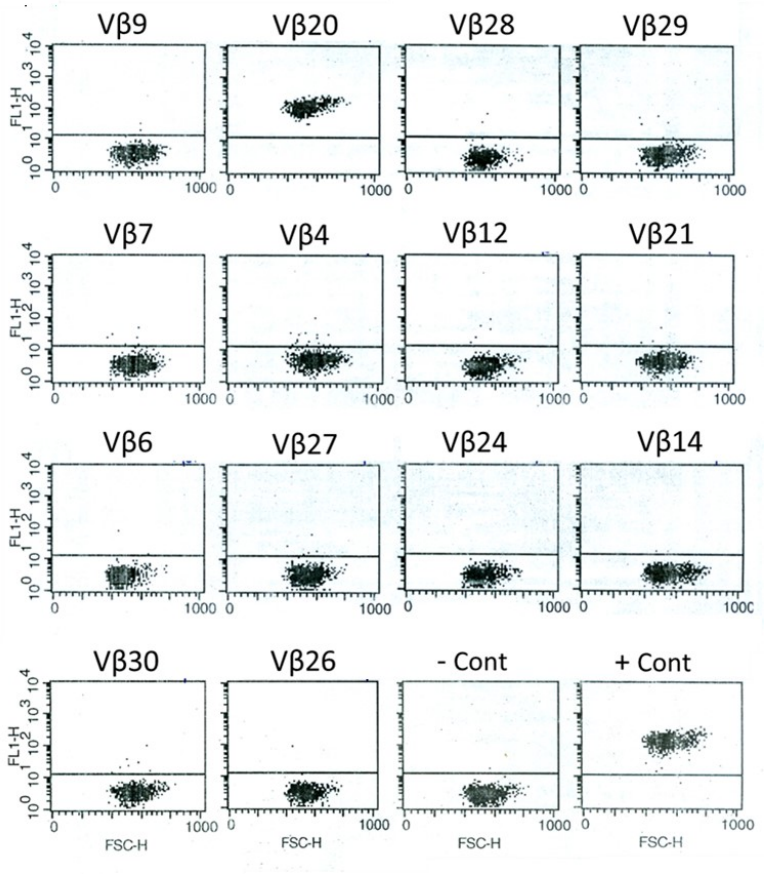

Figure 2: Reactivity of IL-A47 with CD8 T cell clones expressing different TCR BV20 subfamily genes. Cells from each clone were also incubated with secondary antibody alone as a negative control (-Cont) and stained for CD3 (antibody MM1A) as a positive control (+Cont): Results obtained for positive and negative controls are shown for cells expressing Vβ20.

Figure 3

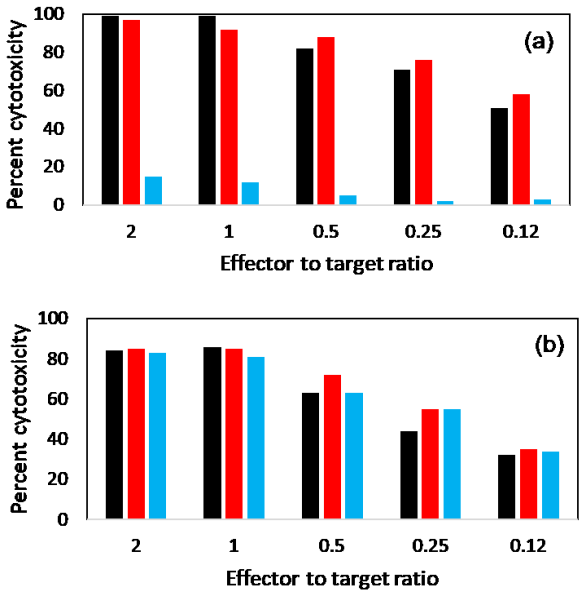

Figure 3: Inhibition of CD8 T cell recognition of target cells by IL-A47: Results are shown for alloreactive cytotoxic CD8 T cells specific for the class I MHC allele 2*00102. Panel (a) shows profound inhibition of cytotoxicity by IL-A47 is observed with IL-A47+ T cells (T28.16) incubated with IL-A47- target cells (E49); panel (b) shows lack of inhibition cytotoxicity of IL-A47- T cells (T28-38) incubated with IL-A47+ target cells (F104). Black = results obtained without added antibody; Red = results with addition of a control IgA antibody; Blue = results with addition of IL-A47.