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NKp46+CD3+ cells - a novel non-conventional T-cell subset in cattle exhibiting both NK cell and T-cell features

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Abstract

The NKp46 receptor demonstrates a high degree of lineage-specificity, being expressed almost exclusively in natural killer cells. Previous studies have demonstrated NKp46 expression by T-cells, but NKp46+CD3+ cells are rare and almost universally associated with NKp46 acquisition by T-cells following stimulation. In this study we demonstrate the existence of a population of NKp46+CD3+ cells resident in normal bovine PBMC which include cells of both the αβ TCR+ and γδ TCR+ lineages and is present at a frequency of 0.1-1.7%. NKp46+CD3+ cells express transcripts for a broad repertoire of both natural killer (NKR) and T-cell receptors (TCR) and also the CD3ζ, DAP10 and FcεR1γ but not DAP12 adaptor proteins. In vitro functional analysis of NKp46+CD3+ cells confirm that NKp46, CD16 and CD3 signalling pathways are all functionally competent and capable of mediating-re-direct cytolysis. However, only CD3 cross-ligation elicits IFN-γ release. NKp46+CD3+ cells exhibit cytotoxic activity against autologous Theileria parva infected cells in vitro and during in vivo challenge with this parasite an expansion of NKp46+CD3+ cells was observed in some animals, indicating the cells have the potential to act as an anti-pathogen effector population. The results presented herein identifies and describes a novel non-conventional NKp46+CD3+ T-cell subset that is phenotypically and functionally distinct from conventional NK and T-cells. The ability to exploit both NKR and TCR suggests these cells may fill a functional niche at the interface of innate and adaptive immune responses.

Introduction

The immune system is classically segregated into innate and adaptive components which operate in an integrated fashion to recognise and respond to pathogens. Natural Killer (NK) and T-cells are lymphocyte subsets that show some similarities in function, development and

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transcriptional profile but sit at opposite ends of the spectrum of innate and adaptive immunity (1, 2). As part of the adaptive immune system, conventional T-cells require priming before attaining full functional competency and their activation is predominantly achieved through somatically rearranged and clonotypically distributed antigen-specific receptors – the T cell receptor (TCR). Conversely NK cells, as part of the innate immune system, are capable of rapidly mounting effector responses and their activation is dependent on the balance of signals received from a set of germline encoded activatory and inhibitory NK receptors (NKR). NKRs are heterogeneous and include members of the KIR, Ly49, CD161 and NKG2D families as well as 2B4 (CD244), CD16 and the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46 (3).

Most NKR are not lineage-restricted but can be expressed on other cell types including CD3+ T-cell subsets. Conventional T-cells may acquire expression of a broad range of NKRs following activation, which can serve as co-stimulatory molecules modulating TCR signalling thresholds (4-9) or occasionally provide an alternative TCR-independent activation pathway (10, 11). In addition, small subsets of non-conventional T-cells, such as Natural Killer T-cells (NKT) and Mucosal Associated Invariant T-cells (MAIT), constitutively co-express CD3 and NKR. These non-conventional T-cell subsets appear to have a phenotype intermediate between NK and T-cells, with the ability to function as innate effectors and there is accumulating evidence that they may play important roles in providing early responses against pathogens by bridging innate and adaptive immune responses (12, 13).

In contrast to other NKR, expression of NKp46 is highly specific to NK cells (14) and is widely regarded as the most reliable phenotypic marker for this population (15, 16). Although initial characterisation of NKp46 suggested it was NK cell-specific (17, 18) recent work has identified rare human and murine NKp46+CD3+ T-cell subsets (reviewed in (19)) including i) chronically activated intra-epithelial cytotoxic T cells (CTL) in celiac disease, where NKp46 up-regulation is a component of a general and profound dysregulation of NKR expression associated with a ‘re-programming’ of CTL to become NK-like cells (20), ii) subpopulations of γδ+ and cord blood T-cells stimulated with IL-15 (21, 22), iii) a minor population of aberrant murine CD3loγδ T-cells termed ‘NK-like γδ T-cells’ (23) and iv) a minute fraction of NKT cells (24). Notably, with the exception of NKT cells, expression of NKp46 by CD3+ cells appears to be a consequence of induced NKp46 acquisition following some form of T-cell stimulation. Following identification of these populations it has been proposed that mammalian NK cells could be phenotypically defined as NKp46−CD3+ (16).

Initial characterisation of bovine NKp46+ cells suggested they were uniformly CD3−, although the presence of a rare NKp46+CD3+ population could not be excluded (25, 26). As in humans and mice, subsequent studies have reported that activated γδ T-cells can acquire NKp46 expression following activation (27, 28). Herein, we report that a small population of NKp46+CD3+ lymphocytes, incorporating both αβ and γδ T-cell subsets, is constitutively present in the blood of healthy cattle. Functionally, these NKp46+CD3+ cells can be activated via either NKR or TCR dependent pathways, but they exhibit a number of phenotypic and functional differences that distinguish them from either conventional T-cells or NK cells. NKp46+CD3+ cells are capable of lysing autologous Theileria parva-infected
cells and responding to infection with this parasite in vivo. Our results indicate that these cells represent a novel subset of lymphocytes that, like other non-conventional T-cell populations, may occupy a unique functional niche at the interface between the innate and adaptive immune responses.

**Materials and Methods**

**Animals**

Animals used in the study were Holstein-Friesian cattle obtained from the University of Edinburgh Farm. Immunisation of animals was achieved by infection and treatment as described previously (29) using a cryopreserved sporozoite stock of *T. parva* Muguga. In vivo challenges using lethal doses of either Muguga or the antigenically heterologous Marikebuni stocks were clinically monitored based on a standard protocol (30) and buparvaquone (a theileriacidal drug - Parvexon, Bimeda, Ireland) administered accordingly. All animal experimental work was completed under license and in accordance with the Animal (Scientific Procedures) Act 1986.

**Cell isolation and culture of NKp46+ cells**

PBMC were isolated from peripheral blood collected into 2mM EDTA by density gradient centrifugation (Ficoll-Paque Plus - Amersham Biosciences, Chalford St. Giles, Beds., UK). For isolation of NKp46+ populations cells were re-suspended at 5 × 10^7 cells/ml in PBS/2mM EDTA/0.5% BSA and incubated with 3μg/ml anti-ovine NKp46 monoclonal antibody EC1.1 (31) which cross-reacts with bovine NKp46 (unpublished data) for 30 min at 4°C. After two washes in PBS/2mM EDTA/0.5% BSA, PBMC were incubated with immunomagnetic anti-mouse Ig beads (Dynabeads Pan Mouse IgG, Life Technologies, Paisley, UK) for 30 min at 4°C on a rotator, placed in a magnet, washed three times and positively selected cells collected.

Where specified, ex vivo NKp46+ and NKp46+CD3+ populations were isolated by sorting PBMC stained with AKS6 (murine IgG2b anti-bovine NKp46) and MM1A (murine IgG1 anti-bovine CD3) monoclonal antibodies (each at 1μg/ml) and then Alexa Fluor 488 conjugated anti-murine IgG2b and Alexa Fluor 647 conjugated anti-murine IgG1 antibodies by flow cytometry using a FACSaria III (BD Biosciences, Oxford, UK). Isolated NKp46+ cells were co-cultured with irradiated autologous *T. parva*-infected cells at a ratio of 1:4 in RPMI1640 supplemented with 10% FCS, 25mM HEPES buffer, 50 μM 2-mercaptoethanol, 100u/ml penicillin, 10μg/ml streptomycin, 0.29mg/ml L-glutamine and 100U recombinant human IL-2 (Chiron, Emeryville, CA) for 9-10 days. NKp46+ cells were then harvested, labelled with MM1A (at 1μg/ml) and purified CD3+ and CD3− populations isolated by flow cytometry sorting. NKp46+CD3+ and NKp46+CD3− cells were then counted and co-cultured with irradiated autologous *T. parva*-infected cells under the conditions described above, assessed for purity by flow cytometry and re-passaged every 9-10 days. If required, cell-lines were subjected to additional purification by flow cytometry; all cell-lines were >97% pure at the time of functional and phenotypic analysis. Using this method, NKp46+CD3+ lines could be maintained for up to 6 weeks during which a 2-5 ×10^3 fold expansion was achieved.
**Theileria-infected and uninfected lymphoblast cell lines**

Cell lines infected with *T. parva* or *T. annulata* were established by *in vitro* infection of PBMC using sporozoites from cryopreserved stabilates as described previously (32) and then maintained by passage every 2-3 days. Cultured lymphoblasts were established from PBMC stimulated with 2.5μg/ml of Concaivalin A (Sigma-Aldrich, Dorset, UK) and then passaged in medium supplemented with 100U/ml recombinant human IL-2.

**Cell phenotyping by flow cytometry**

Multi-colour flow cytometric analysis of PBMC and cell-lines was conducted using a panel of primary monoclonal antibodies including anti-CD3 (MM1A; IgG1; VMRD, WA, USA), anti-NKp46 (AKS1, AKS6, AKS8; IgG1, IgG2b, IgG2a; (25, 33) and unpublished data), anti-CD2, (IL-A43 (34); IgG2a), anti-CD4 (IL-A12 (35); IgG2a), anti-CD5 (CC17 (36); IgG1), anti-CD6 (IL-A57 (37); IgG2a), anti-CD8 (IL-A105; (38); IgG2a; a gift from Dr. N. MacHugh, The Roslin Institute, Edinburgh, UK), anti-CD16, (KD1 (26); IgG2a; a gift from Daniela Pende, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy), anti-γδ TCR (GB21A; IgG2b; VMRD), anti-WC1 (CC15 (39); IgG2a) and anti-Vδ20 (IL-A47; IgA; Prof. W.I. Morrison and Dr. N. MacHugh, unpublished data) antibodies. Following secondary staining with Alexa Fluor 488 conjugated anti-IgG2b, Alexa Fluor 647 conjugated anti-IgG1 and PE-conjugated anti-IgA secondary antibodies (Life Technologies, Paisley, UK), samples were analysed on a FACS Calibur using CellQuest (BD Biosciences, Oxford, UK) and FloJo software (Treestar, OR, USA); for PBMC and cell-lines, a minimum of $5 \times 10^5$ and $1 \times 10^4$ events were acquired respectively and in each analysis controls using isotype-matched primary antibodies (ABDserotec, Oxford, UK) were included. For all data an initial gate for live cells based on FSC and SSC parameters was used.

**Analysis of TCR and NKR transcription**

Total RNA was isolated from cell-lines using Tri-reagent (Sigma-Aldrich, Dorset, UK) and cDNA subsequently synthesized using the Reverse Transcription System (Promega, Madison, WI, USA), with priming by the Oligo(dT)15 primer, according to the manufacturer’s instructions. TCRβ chains were PCR amplified using Vβ subgroup-specific primers as described previously (40) and TCRα chains were amplified using an equivalent panel of Vα subgroup specific primers (Connelley *et al.*, manuscript in preparation). TCR genes were named using a nomenclature based on that of the IMGT system (41). Gene-specific primer pairs for NKR and adaptor protein genes (Supplementary Material 1) were designed using Primer3.0 software (http://bioinfo.ut.ee/primer3-0.4.0) based on genomic sequence data from the UMD 3.1 assembly accessed through Ensembl (www.ensembl.org). These PCRs were conducted using 1μl cDNA template, 10 pmol each of the relevant forward and reverse primers, 0.5 units of Biotaq (Bioline, London, UK) and 2 μl SM-0005 10x buffer (ABgene, Epsom, Surrey, UK) per 20 μl reaction. Cycling conditions were 5 min at 95°C, 5 cycles of (1 min at 94°C, 1 min at 55°C, 1 min at 72°C), 25 cycles of (30 s at 94°C, 1 min at 60°C, 1 min at 72°C) and a final extension period of 5 min at 72°C. 10μl of the products were resolved on 1.5% agarose gels and visualised with SafeView stain (Gentaur, Belgium).
Cytotoxicity assays

A 4-h $^{111}$In-release assay was used to examine the cytotoxic activity of cell-lines. All assays were conducted in duplicate. Percentage specific lysis was calculated as \[ \frac{\text{sample release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100\% \] and expressed as a mean of the duplicated assays. Maximal and spontaneous release were derived from triplicates of target cells incubated with 0.2% Tween 20 and RPMI 1640/5% FCS, respectively. In re-directed cell lysis assays and NKp46-blocking assays, target or effector cells were pre-incubated with anti-CD3, -CD16 or anti-NKp46 (AKS1) monoclonal antibodies (all at 2μg/ml) for 30 min prior to completion of the cytotoxicity assays. Anti-CD8 (IL-A51 (38); IgG1) and anti-MHCI (IL-A88 (42); IgG2a) monoclonal antibodies were used for isotype controls during these assays.

IFN-γ assays

To assess the ability of ligation of CD3, CD16 and NKp46 receptors to elicit IFN-γ production, cells were cultured in 96-well MaxiSorp plates (NUNC, Denmark) pre-coated overnight in 0.05 M carbonate buffer (pH 9.6) with either MM1A, KD1 or AKS1 and with ILA-51 and ILA-105 as isotype controls, at a concentration of 10μg/ml. To assess the ability of recombinant bovine IL-12 to induce IFN-γ expression cells were cultured with or without rbIL-12 at 10U/ml. In all assays individual wells contained 1×10^5 cells and the media was supplemented with 100U/ml rhIL-2 and supernatant was harvested after 20 hours of culture at 37°C in 5% CO\textsubscript{2}. Triplicate wells were prepared for each condition examined. The IFN-γ content in supernatants was measured using a sandwich ELISA, using non-competing monoclonal antibodies specific for bovine IFN-γ (clones CC330 and CC302 both from AbD Serotec). The latter was conjugated with biotin and binding detected with horseradish peroxidase-conjugated streptavidin (AbD Serotec) followed by addition of TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution (BD Biosciences). A standard curve for IFN-γ determination was generated using a series of doubling dilutions of bovine IFN-γ (AbD Serotec). The mean IFN-γ concentration for each culture condition was then calculated by correlation to the standard curve generated by the IFN-γ standards.

Results

Identification of an in vivo NKp46+CD3+ subset

During studies of cellular responses of naïve cattle against *Theileria parva*, CD8+ cell lines generated from PBMC stimulated with irradiated autologous *T. parva*-infected cells were found to contain a variable proportion of NKp46+ CD3+ cells (data not shown). In previous studies bovine NKp46+ CD3+ cells have been reported to originate from T-cells which acquired NKp46+ following activation as they were not identified pre-stimulation (27, 28). However, analysis of PBMC from a panel of naïve animals (n=23) by 2-colour flow cytometry identified a small NKp46+ CD3+ population (0.1-1.7% of total PBMC) \textit{ex vivo} in all animals examined. In the majority of animals NKp46+CD3+ cells formed only a minor subset (<10%) of the total NKp46+ population although in 4 animals it represented between 21-44% of the NKp46+ population. The inclusion in our panel of 3 neonates (<96 hours old) suggests that NKp46+ CD3+ cells are constitutively present from birth and do not represent a subset of \textit{in vivo} activated T-cells accumulated post-natally.
Three-colour phenotypic analysis of PBMC from a cohort of 3-8 month old animals (n=13) showed that in vivo NKp46+CD3+ populations included CD8+ , CD4+ and γδ TCR+ fractions (Figure 1). Although most animals had a dominant CD8+ subset with relatively minor CD4+ and γδ TCR+ components (including some expressing WC1), there was substantial inter-animal variation and in some individuals CD4+ or γδ TCR+ populations constituted a third or more of the NKp46+CD3+ population (Table I). Repeat analyses on a subset of animals conducted a month apart showed that within individuals the frequency and relative proportions of the CD8+, CD4+ and γδ TCR+ components were stable. Consistent with the phenotype of the total NKp46+ population, NKp46+CD3+ cells were predominantly CD16+ (81.0-97.9%) and included both CD2+ and CD2− subsets (39.0-87.9% CD2+). However, CD8 staining of NKp46+CD3+ cells (MFI = 473.6 ± 266.4) was approximately one log higher than that seen on NKp46+CD3− cells (MFI = 45.1 ± 6.4) and equivalent to that seen on T-cells (MFI = 396.9 ± 96.7, no significant difference, paired T-test p=0.477). Notably, expression of CD3 on NKp46+CD3+ cells (MFI = 190.7 ± 51.8) was approximately 70% that seen on T-cells (MFI = 272.5 ± 73.8, paired T-test p<0.05).

To determine if the NKp46+CD3+ cells observed in T. parva-stimulated cultures were derived from the in vivo NKp46+CD3+ population, we established parallel T. parva-stimulated cultures from highly purified ex vivo NKp46+CD3− and NKp46+ populations. After 9-11 days in culture, a distinct NKp46+CD3+ population was observed in the NKp46+ but not the NKp46+CD3− derived cell lines (n=4), confirming that the NKp46+CD3+ cells observed in the in vitro cultures originated from the in vivo NKp46+CD3+ population (Figure 2).

**NKp46+CD3+ cell-surface phenotype is distinct from conventional T-cells**

To enable characterisation of their functional capacity we sought to establish a method to purify and expand NKp46+CD3+ cells. In vitro maintenance and proliferation of NKp46+CD3+ cells using recombinant bovine IL-2 alone was limited and insufficient to generate enough NKp46+CD3+ cells for analysis (data not shown). We therefore optimised a system exploiting co-culture with irradiated autologous T. parva-infected cells in the presence of recombinant IL-2, to facilitate the in vitro expansion of NKp46+CD3+ cells (see Materials and Methods). NKp46+CD3+ cell lines were generated in vitro from 3 Theileria-naive animals. NKp46+CD3− cell lines from the same animals and a T. parva-specific CD3−NKp46−CD8+ T-cell line from a Theileria-immune animal were also established to facilitate comparison with conventional NK cells and T-cells.

The 3 NKp46+CD3+ cell lines had similar phenotypes (Figure 3). Compared to the ex vivo populations, NKp46+CD3+ cell lines had an increased proportion of CD8+ cells (>97%) and a concomitantly reduced proportion of CD4+ cells (<4%), indicating preferential expansion of the CD8+ subset in this culture system. The effect of culture on the γδ TCR+ fraction was variable; in two animals the proportion of γδ TCR+ cells decreased (from 21.0% and 31.9% respectively to approximately 10%) whilst in the third animal γδ TCR+ T-cells increased from 19% to 33%.

NKp46+ CD3+ cell lines were predominantly CD5−, CD6−, CD16+ and had defined CD2− and CD2+ fractions – a phenotype similar to that of the conventional NK (NKp46+CD3+)
NKp46+CD3+ cells and NK cells express a similar repertoire of NKR but not adaptor proteins

Although other cell lineages may express selected NKR, expression of a comprehensive NKR repertoire is generally restricted to NK-cells. To investigate the NKR expressed by NKp46+CD3+ cells, we performed RT-PCR to detect mRNA transcripts for 2B4 (CD244), NKp30 (NCR3), NKp80 (KLRF1), NKG2A/C/D (KLRC1/2 and KLRK1), CD94 (KLRD1) and several KIR. Both NKp46+CD3+ and NKp46+CD3− cell lines expressed transcripts for all of the NKR examined (Table II and Supplementary Material 2). The conventional CD8+ T-cell line expressed transcripts for 2B4, CD94, NKG2A, NKG2D and KIR, all of which are commonly found on activated T-cells in other species (43, 44). Surprisingly, transcripts for NKp30, which has not been reported previously to be expressed on αβ T-cells, were also detected in the CD8+ T-cell line. In contrast, the CD8+ T-cell line did not have transcripts for either NKp80 or NKG2C, which in other species both show a high level of NK-cell restriction and are only rarely expressed on T-cells (10, 20, 45).

Adaptor proteins containing ITAMs are required for the initiation of intra-cellular signalling following ligation of TCRs and many activatory NKR. As with the cell surface receptors with which they associate, adaptor proteins often exhibit a lineage-restricted expression. We analysed mRNA transcript expression of 4 adaptor proteins – CD3ζ, DAP10, DAP12, and FceR1γ. As anticipated, the conventional CD8+ T-cell line expressed transcripts for DAP10 and CD3ζ and the NKp46+CD3− cells expressed transcripts for all 4 proteins (Table II). In contrast, NKp46+CD3+ cell lines expressed DAP10, FceR1γ and CD3ζ but not DAP12. Consequently, although they express similar NKR repertoires the ability of conventional NK cells and NKp46+CD3+ cells to be activated by some NKR (e.g. NKG2C) may differ.

NKp46+CD3+ cells express a diverse TCR repertoire

Several non-conventional T-cell subsets such as invariant NKTs (iNKTs) and MAIT cells express semi-conserved TCR repertoires (46, 47). To determine if NKp46+CD3+ cells also express a restricted TCR repertoire we used panels of Vα and Vβ subgroup-specific primers to analyse TCRα and TCRβ mRNA transcripts by RT-PCR. Each of the NKp46+CD3+ cell lines expressed a highly diverse αβ TCR repertoire with all 18 Vβ subgroups and most of the Vα subgroups represented (Figure 4 panels A and B upper row). Sequencing from a limited number of NKp46+CD3+ cell clones (n=5), generated by limiting dilution from 2 additional animals, corroborated a diversity within the TCRβ repertoire (Supplementary Material 3). The CD8+ T-cell line expressed TCR utilising a broad but incomplete repertoire of Vα and Vβ subgroups, consistent with a population that has undergone antigen-driven clonal selection (Figure 4 panels A and B lower rows), whilst the NKp46+CD3− cell lines expressed neither TCRα nor TCRβ mRNA transcripts (Figure 4 panels A and B middle row).
NKp46+CD3+ cells exhibit NK-like cytotoxicity but not IFN-γ production

Bovine NK cells exhibit a range of characteristic functions including lysis of the target cell line MDBK, re-directed cytotoxicity through ligation of both CD16 and NKp46 and production of IFN-γ in response to stimulation with IL-2 and IL-12 as well as cross-linking of CD16 or NKp46 (25, 26, 48). We conducted a series of assays to determine if NKp46+ CD3+ cells retained these NK-like functions.

In cytotoxicity assays, NKp46+ CD3+ cells demonstrated strong killing of MDBK cells, giving 63-94% lysis at an effector to target ratio (E:T) of 10:1, comparable to the levels demonstrated by NKp46+ CD3− cells (Figure 5). In contrast, the NKp46− CD3+CD8+ T-cell line did not kill the MDBK target (data not shown). Blocking NKp46 by pre-incubation of effector cells with anti-NKp46 monoclonal antibody reduced lytic activity of NKp46+ CD3+ cells (range 14-54% lysis at E:T of 10:1), demonstrating that cytotoxic activity against MDBK, as with conventional NKp46+ CD3− cells is partially NKp46 dependent. In re-directed lysis assays using the FcR+ mouse p815 mastocytoma cell line, cytolytic function of NKp46+ CD3+ cells could be substantially increased by pre-incubation with either anti-CD16 or anti-NKp46 antibodies, but not by isotype-matched control antibodies (Figure 6). Notably, as with conventional NK cells (NKp46+ CD3−), the degree to which anti-NKp46 and anti-CD16 antibodies blocked killing of MDBK (Figure 5) and elevated cytotoxicity against p815 (Figure 6) exhibited considerable variation between different NKp46+ CD3+ cell lines. Together these results indicate that NKp46+ CD3+ cells have NK-like cytotoxic function and that both NKp46 and CD16 are functionally competent activating receptors.

The ability of NKp46+ CD3+ cells to produce IFN-γ in response to activation was examined by measuring IFN-γ in the supernatants of cells cultured in cytokine-supplemented medium or in wells pre-coated with monoclonal antibodies. NKp46+ CD3+ cells failed to produce IFN-γ in response to IL-2/IL-12 or following cross-linking NKp46 or CD16 (Figure 7, grey bars). In contrast, NKp46+ CD3− cells (Figure 7, black bars) produced IFN-γ in response to these stimuli although the quantity produced was highly variable depending on the combination of the cell line and stimulus (3-35ng/ml). Thus, with respect to IFN-γ production NKp46+ CD3+ cells are distinct from conventional NK-cells.

NKp46+CD3+ retain a functional CD3 signalling pathway

In the absence of defined antigens recognised by TCR expressed by NKp46+CD3+ cells, we used CD3 cross-linking as a proxy for TCR ligation to examine if NKp46+CD3+ cells retained a functional TCR signalling pathway. In re-directed cytotoxicity assays pre-incubation of p815 cells with anti-CD3 antibody resulted in increased lysis by NKp46+CD3+ cells and the conventional CD8+ T-cells but not the line NKp46+CD3− cell lines (Figure 6). The enhancement of cytotoxic activity was notably more pronounced in the NKp46+CD3+ cell lines than in the conventional CD8+ T-cell line. Ligation with CD3-specific antibody induced IFN-γ production in NKp46+CD3+ cells (Figure 7), demonstrating these cells are competent at producing IFN-γ and indicating that failure of cross-ligation of CD16 or NKp46 to induce IFN-γ is a receptor-dependent phenomenon. The levels of IFN-γ produced by the 3 different NKp46+CD3+ cell lines were similar (16-22ng/ml) but markedly less than that produced by the conventional CD8+ T-cell line (70ng/ml).
NKp46*CD3+ cells demonstrate effector function against Theileria-infected cells

To examine the effector activity of NKp46*CD3+ cells against pathogens, we assayed their ability to lyse Theileria-infected cells. NKp46*CD3+ cell lines efficiently lysed the autologous T. parva-lines with which they had been stimulated during *in vitro* co-culture (45-58% at E:T ratio of 10:1), exhibited variable efficacy in lysing autologous T. annulata-infected cells and MHC-I-mismatched T. parva-infected cells, but did not kill autologous uninfected T-cell lymphoblasts (Figure 8). The restriction of killing to parasitized cells, which was neither MHC-I-restricted nor parasite species-specific, was similar to that observed with NKp46*CD3− cells and in marked contrast to the MHC-restricted, highly specific cytotoxicity of the CD8+ T-cell line from the T. parva-immunised animal. Although NKp46*CD3+ cells exhibited ‘NK-like’ lytic activity against Theileria-infected cells, the relative susceptibilities of target cell lines to lysis by NKp46*CD3− and NKp46*CD3+ cell lines from two of the animals differed, suggesting some disparity in the receptor/ligand interactions involved in recognition of the pathogen-infected cells.

Pre-incubation of NKp46*CD3+ cells with NKp46-specific antibody resulted in inhibition of Theileria-infected cell lysis (Figure 9a and b), demonstrating the involvement of this receptor in recognition and killing of Theileria-infected cells. However, the level of inhibition of lysis observed with different effector/target combinations varied, and in some cases was minimal (Figure 9c), indicating that other receptors expressed by NKp46*CD3+ cells have a role in eliciting effector function.

Expansion of the NKp46*CD3+ subset during Theileria infection in vivo

To determine if NKp46*CD3+ populations could respond to *T. parva* infection *in vivo*, we monitored the frequency of this population in PBMC of animals during experimental parasite challenge. Five *T. parva* Muguga-immunised cattle were challenged by infection with a lethal dose of either the homologous *T. parva* stock (Muguga, n=2) or a heterologous stock (Marikebuni, n=3). Two naïve animals were similarly infected with the Marikebuni stock. Both immunised animals challenged with the homologous Muguga stock were solidly immune (102121 and 402082), 2 of the immunised animals challenged with the Marikebuni stock exhibited partial immunity with delayed parasite clearance (102170 and 402145), whilst the third immunised animal (702162) and the 2 naïve animals (102107 and 202192) challenged with Marikebuni stock were unprotected, developing severe clinical disease that required anti-parasite therapy 15-17 days after infection.

During the first 11 days following challenge the frequency of NKp46*CD3+ cells in the PBMC of all the animals remained relatively unaltered (Figure 10a). However, from day 13 the frequency of the NKp46*CD3+ populations in the 2 Marikebuni-challenged animals that exhibited partial protection was markedly increased (from 0.3% on day 11 to 3% and 2% on day 13), whereas in all other animals it remained low or decreased. Total NKp46+ frequency showed a general decrease in all 7 animals during the first week of challenge but in the 2nd week the frequency was elevated in the 2 partially-protected Marikebuni-challenged and one of the immunised animals challenged with Muguga (102121), whilst in the other animals it remained low (Figure 10b). Together the results demonstrate an ability of NKp46*CD3+ cells to respond to *T. parva* infection *in vivo*, however in naïve and completely immune
animals this response may either be absent or below the threshold for detection by direct quantification in PBMC.

**Discussion**

In resting lymphocytic populations the cell surface proteins NKp46 and CD3 have been considered to be lineage specific markers of NK cells and T cells respectively. In this study we report the identification and initial characterisation of a novel subset of NKp46+CD3+ lymphocytes in the PBMC of healthy cattle. These cells were present in all individuals examined, including new-born animals. Functionally and phenotypically this population was distinct from both conventional NK cells and T-cells. NKp46+CD3+ cells have the capacity to lyse autologous *Theileria parva*-infected cells and participate in *in vivo* responses against this parasite, demonstrating their potential to contribute to immunity against pathogens.

Rare subsets of NKp46+CD3+ cells have been described previously in cattle as well as in mice and humans (15, 20, 23, 24, 27, 28); however, the subset we describe here appears to be distinct from these populations. Previously reported populations of bovine NKp46+γδ TCR+ cells were derived from γδ T-cells that acquired expression of NKp46 following *in vitro* or *in vivo* activation (27, 28) and similarly, with the exception of a minute fraction of NKp46+ NKT cells (24), human NKp46+CD3+ cells originate from activated T-cell populations that acquire NKp46 following activation (20-22). Analysis of the TCR repertoire expressed by NKp46+CD3+ cells in one of these studies demonstrated a high degree of oligoclonality (20), consistent with their derivation from a small number highly activated T-cells that had undergone marked clonal expansion. In direct contrast, we have demonstrated that bovine NKp46+CD3+ cells exist as a population in healthy animals and that cultured lines derived from this population express diverse TCR repertoires, indicating a polyclonal origin rather than derivation from a highly activated, clonally restricted T-cell population. Although murine NK-like γδ T-cells are also normally resident in PBMC they, unlike the bovine NKp46+CD3+ cells we describe, express exceptionally low levels of surface CD3 (~10-15% of that on conventional T-cells), such that by surface staining for CD3 they are difficult to distinguish from conventional NKp46+CD3− NK cells. The CD3lo phenotype of murine NK-like γδ T-cells suggests they represent a chronically stimulated T-cell population that have acquired NKR as a consequence of activation (15, 23). Thus, we propose that the bovine NKp46+CD3+ cells we describe herein represent a previously undescribed non-conventional T-cell subset.

Cell surface phenotype and gene expression analysis revealed additional features that distinguished these NKp46+CD3+ cells from both conventional NK cells and T-cells. Unlike conventional T-cells, NKp46+CD3+ cells lack cell-surface expression of CD5 and CD6 and upon *in vitro* stimulation down-regulate expression of CD2 to become predominantly CD2−. Additionally, NKp46+CD3+ cells express a full repertoire of NKR including CD16, NKp80 and NKG2C, which like NKp46, are rarely expressed on T-cells (10, 45, 49, 50). Similarly, NKp46+CD3+ populations could be differentiated from conventional NK cells by several criteria including the higher level of CD8 expression *ex vivo* and absence of mRNA transcripts for the DAP12 adaptor protein. This adaptor is universally expressed by NK cells and is a key component in the signalling pathways of a variety of activatory NK receptors.
including NKG2C/CD94, NKp44 and KIR2DS (51). Furthermore, NKp46^CD3+ cells were functionally distinct from NKp46^CD3^- cells, with cross-linking of NKp46 or CD16 inducing IFN-γ production in the latter but not the former. Together, these characteristics confirm that NKp46^CD3+ cells have a complex phenotype and cannot be considered simply as either NKp46+ T-cells or TCR+ NK-cells.

Functional analyses verified the ability of NKp46^CD3+ cells to exploit both innate and adaptive (TCR) signalling pathways, implying that, as with other non-conventional T-cell subsets, NKp46^CD3+ cells can be activated by integrating TCR-dependent and innate signals (52, 53). In our *in vitro* studies cross-linking of NKp46 or CD16 was capable of eliciting cytotoxicity but not IFN-γ production from NKp46^CD3+ cells. Such disparity in the ability to induce cytotoxicity and cytokine production by cross-linking NK receptors including NKp46, NKG2D and KIRs has been reported previously (23, 54, 55). It has been suggested that in these situations NKR are serving as co-stimulatory molecules, lowering the threshold for cell activation by other receptors; the obvious candidate in NKp46^CD3+ cells being the TCR. However, the ability of a mono-clonal antibody blocking NKp46 ligation to reduce the lysis of autologous *Theileria*-infected cells in 2 out of 3 animals demonstrates that NKp46 can be, but is not always, a major factor in eliciting a response from NKp46^CD3+ cells. With the variety of innate and TCR-dependent signalling pathways at the disposal of NKp46^CD3+ cells, the interplay of different receptors in achieving activation are likely to be complex and variable. Recent work has demonstrated that the relative contribution of TCR-dependent and -independent signalling to activation of NKT cells can depend on the biological context (56). Unfortunately, due to the absence of the relevant antibodies, it is not currently possible to perform the assays that would enable the roles of other NKRs and TCR in enabling NKp46^CD3+ cell recognition and lysis of *Theileria*-infected cells to be examined.

Vα- and Vβ-subgroup analysis demonstrated that bovine NKp46^CD3+ cells expressed a highly diverse TCR repertoire. This is in stark contrast to 2 of the best characterised non-conventional T-cell subsets – iNKT and MAIT cells – which are defined by the expression of a highly restricted TCR. The semi-invariant TRCs of iNKT and MAITs act effectively as innate-like pattern-recognition receptors (57), enabling recognition of a conserved lipid moiety presented by CD1d and MR1 respectively (53, 58). This suggests that the function of TCR in NKp46^CD3+ cells may be more similar to that of non-invariant NKT cells in which a diverse but oligoclonal TCR (59-61) repertoire permits recognition of a series of heterogeneous, but limited, ligands or conventional T-cells where expression of a genuinely polyclonal TCR repertoire enables recognition of highly diverse peptide-MHC ligands. Large-scale sequencing of TCR chains expressed by NKp46^CD3+ cells will be required to provide the higher resolution data needed to fully characterise the TCR repertoire expressed by NKp46^CD3+ cells. Conducting such analysis serially during active NKp46^CD3+ cell responses could demonstrate TCR selection that would provide insights into the functional role of, and possibly the nature of the ligands recognised by, NKp46^CD3+ cell TCR.

The ability of NKp46^CD3+ cells from PBMC of *Theileria*-naïve animals to expand during *in vitro* co-culture with, and efficiently lyse, autologous *T. parva*-infected cells indicated their capacity to function as an innate-like anti-pathogen effector population. However,
during *in vivo* experiments naïve animals failed to generate a detectable NKp46<sup>+</sup>CD3<sup>+</sup> cell expansion following *T. parva* infection. The absence of a conventional NK cell response in these naïve animals suggests that the immune dysregulation seen in primary *T. parva* infection (62) may extend to suppression of innate immune responses or that there is sequestration of NKp46<sup>+</sup>CD3<sup>+</sup> and NKp46<sup>+</sup>CD3<sup>-</sup> cells to lymphoid tissues during active infection. *In vivo* NKp46<sup>+</sup>CD3<sup>+</sup> cell expansions were observed in the 2 immunised animals that exhibited partial protection against challenge with a heterologous parasite stock. In these animals NKp46<sup>+</sup>CD3<sup>+</sup> cells expanded from 0.4% of PBMC pre-challenge to a maximum of 2 and 3% by day 13 post-challenge – co-incident with the onset of clinical resolution of disease.

Efficient lysis by NKp46<sup>+</sup>CD3<sup>+</sup> cells of autologous *T. parva*-infected cells *in vitro* suggests that the expanded NKp46<sup>+</sup>CD3<sup>+</sup> populations have the potential to make a substantial contribution to pathogen control. *Ex vivo* pMHCI-tetramer data from fully immunised animals undergoing challenge shows the peak frequency of CD8<sup>+</sup> T-cells specific for immunodominant epitopes was similar, ranging from 1.5-4.4% (Xiaoying Li, unpublished data). Similarity in the kinetics of the NKp46<sup>+</sup>CD3<sup>+</sup> and NKp46<sup>+</sup>CD3<sup>-</sup> cell expansions in these 2 animals is consistent with the response of the NKp46<sup>+</sup>CD3<sup>+</sup> cells against the antigenically novel pathogen being innate-like, suggesting a function of this subset may be to exploit NKR co-expression to facilitate the recruitment of a population of innate-like T-cells to augment conventional NK responses. Although there is a clear association with partial protection seen in the immunised animals subjected to a heterologous parasite challenge the role, if any, of NKp46<sup>+</sup>CD3<sup>+</sup> (and NKp46<sup>+</sup>CD3<sup>-</sup>) responses in mediating immunological protection is yet to be defined. No NKp46<sup>+</sup>CD3<sup>+</sup> cell responses were detected in the 2 immunised animals challenged with a homologous parasite, suggesting that the presence of an established and highly protective adaptive memory CD8<sup>+</sup> T-cell population (63) may restrict NKp46<sup>+</sup>CD3<sup>+</sup> cell responses due to competition or functional redundancy. Together, our data demonstrates that NKp46<sup>+</sup>CD3<sup>+</sup> cells have the capacity for anti-*Theilerial* effector function and are able to respond to pathogen challenge *in vivo*.

However, cytotoxicity is only one of many functions attributed to NK cells and conventional and non-conventional T-cells (53, 64-66). Analyses of the ability of NKp46<sup>+</sup>CD3<sup>+</sup> cells to form memory, interact with and regulate other components of innate immunity and direct adaptive immune responses were out-with the scope of the present study but will be required to fully understand the biological role of NKp46<sup>+</sup>CD3<sup>+</sup> cells and their function in immunity against *T. parva*.

In addition to our work here demonstrating a response against *T. parva*, bovine NKp46<sup>+</sup> cells have been shown to respond to a variety of pathogens that cause economically important cattle diseases including *M. bovis* (67-70), *Neospora* (48, 71, 72) and *Babesia* (73). Determining if NKp46<sup>+</sup>CD3<sup>+</sup> cells form a component of these NKp46<sup>+</sup> cell responses could have implications for our understanding of innate immune responses against these as well as other important bovine pathogens. Recent observations of small CD3<sup>+</sup> subsets within NKp46<sup>+</sup> cells in the pig (74) and possibly horse (75), suggest that NKp46<sup>+</sup>CD3<sup>+</sup> cells may be common to ungulates and possibly other mammalian species. Therefore, improved
knowledge of the function of these cells in the bovine system may have wider implications for our understanding of immune responses to pathogens in other species.

In conclusion, the NKp46\(^+\)CD3\(^+\) cells identified in this study represent a novel non-conventional T-cell population with features of both innate and adaptive immune cell types. The ability to utilise both NKR and TCR signalling pathways indicates a cell that may be specialised, like other unconventional T-cells, to combine facets of innate and adaptive immunity. Such cells can have significant roles in the induction or provision of protective immunological memory and represent potential new targets in vaccination strategies (12, 64, 76). Our data provide evidence that NKp46\(^+\)CD3\(^+\) can respond to \textit{T. parva}. Inevitably there are fundamental questions that need to be addressed following the identification of a novel lymphoid subset. The availability of \textit{in vivo} models for immunisation and challenge with \textit{T. parva}, coupled with the ability to grow parasitized cells \textit{in vitro} provides a useful system in which to conduct further studies into the biological function and ligand specificities of NKp46\(^+\)CD3\(^+\) cells. Such studies will help to determine the role of NKp46\(^+\)CD3\(^+\) cells in immunity and consequently if there is potential for their exploitation more widely in immunisation strategies.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**References**


27. Johnson WC, Bastos RG, Davis WC, Goff WL. Bovine WC1(−) gammadelta T cells incubated with IL-15 express the natural cytotoxicity receptor CD335 (NKp46) and produce IFN-gamma in response to exogenous IL-12 and IL-18. Dev Comp Immunol. 2008; 32:1002–1010. [PubMed: 18329100]


Figure 1. Flow cytometric analysis of *ex vivo* NKp46<sup>+</sup>CD3<sup>+</sup> cell phenotype
Freshly isolated PBMC were analysed by 3-colour flow cytometry using antibodies against NKp46, CD3 and the markers indicated in the panels. Plots show the staining observed within the NKp46<sup>+</sup> gated population. The percentages of cells within each quadrant are shown. Results presented are from 1 individual representative of those obtained from 13 animals.
Figure 2. NKp46+CD3+ cells present after \textit{in vitro} culture are derived from an \textit{in vivo} population

Highly purified NKp46+ (upper panels) and NKp46+CD3− (lower panels) populations obtained from PBMC were co-cultured with autologous \textit{T. parva}-infected cells and after 9-11 days analysed for CD3 and NKp46 expression by 2-colour flow cytometry. A NKp46+CD3+ subset was present within cultures established from \textit{ex vivo} NKp46+ but not NKp46+CD3− purified populations (right hand panels) demonstrating \textit{in vitro} NKp46+CD3+ populations are derived from a pre-existing \textit{in vivo} NKp46+CD3+ population. The percentages of cells within each quadrant are shown. Results presented are from 1 individual representative of those obtained from 4 animals.
Figure 3. Comparison of the phenotype of in vitro NKp46⁺CD3⁺ cell, NK cell (NKp46⁺CD3⁻), and CD8⁺ T-cell lines

NKp46⁺CD3⁺ cell (upper panels), NK cell (NKp46⁺CD3⁻ - middle panels) and CD8⁺ T-cell (lower panels) lines were subjected to 2-colour flow cytometric analysis. The percentages of cells expressing the markers indicated on the horizontal axis of each plot are shown in the upper right quadrant. Data for NKp46⁺CD3⁺ and NKp46⁺CD3⁻ cell lines are from 1 animal representative of 3.
Figure 4. TCR repertoire of NKp46$^+$CD3$^+$ cell, NK cell (NKp46$^+$CD3$^-$), and CD8$^+$ T-cell lines RT-PCR analysis of the repertoire of rearranged TCR$\alpha$ (panel A) and TCR$\beta$ (panel B) chain transcripts expressed in NKp46$^+$CD3$^+$ cell (upper rows), NKp46$^+$CD3$^-$ cell (middle rows) and CD8$^+$ T-cell (lower rows) lines using V-gene subgroup specific primers. V-gene subgroups have been named in accordance to the IMGT nomenclature system. Indented white arrows show the position of the 300bp MW marker. Data for NKp46$^+$CD3$^+$ and NKp46$^+$CD3$^-$ cell lines are from 1 animal representative of 3.
Figure 5. Cytotoxic activity of NKp46\(^+\)CD3\(^+\) and NKp46\(^+\)CD3\(^-\) cell lines against MDBK

Cytotoxicity of NKp46\(^+\)CD3\(^-\) (A-C) and NKp46\(^+\)CD3\(^+\) (D-F) cell lines against MDBK targets was measured in 4hr \(^{111}\)In-release assays. Prior to the assay effector cells were pre-incubated with either no antibody, anti-NKp46 antibody or an isotype control (IgG1 anti-CD8) antibody. MDBK were not lysed by a conventional CD8\(^+\) T-cell line (data not shown).
Figure 6. Re-directed cytotoxic activity of NKp46+CD3−, NKp46+CD3+ and CD8+ T-cell lines mediated by cross-linking NKp46, CD16 and CD3

Cytotoxic activity of NKp46+CD3− cell (A-C), NKp46+CD3+ cell (D-F) and CD8+ T-cell (G) lines against p815 which had been pre-incubated with antibodies as indicated in the legend was assessed in 4hr $^{111}$In-release assays.
Figure 7. Production of IFNγ by NKp46+CD3−, NKp46+CD3+ and CD8+ T-cell lines following stimulation

IFNγ release by NKp46+CD3− cells (black bars), NKp46+CD3+ cells (grey bars) and CD8+ T-cell (diagonal pattern bars) lines in response to stimulation with recombinant IL-2 and IL-12 or cross-linking of NKp46, CD16 and CD3 receptors by plate bound antibodies was measured by ELISA of supernatant harvested after 20 hours of stimulation.
Figure 8. Cytotoxic activity of NKp46+CD3−, NKp46+CD3+ and CD8+ T-cell lines against *Theileria*-infected cells

Cytotoxicity of NKp46+CD3− (A-C), NKp46+CD3+ (D-F) and CD8+ T-cell (G) lines against autologous *T. parva*-, autologous *T. annulata*- and MHCI mis-matched *T. parva*-infected cells and autologous Concanavalin-A blasted cells was measured in 4hr 111In-release assays.
Figure 9. Cytotoxic activity of NKp46+CD3+ cells against Theileria-infected cells shows variable dependency on NKp46

Blocking NKp46 had a variable efficacy in reducing lysis of autologous *T. parva*-infected cells by 201961 (A), 202094 (B) and 102107 (C) NKp46+CD3+ cell lines as measured in 4hr $^{111}$In-release assays. NKp46+CD3+ cells were pre-incubated with either no antibody, anti-NKp46 antibody or an isotype control (anti-CD8) antibody. In parallel assays NKp46+CD3+ lysis of autologous *T. annulata* and MHCI-mismatched *T. parva*-infected cells exhibited a similarly variable dependency on NKp46 as did lysis by NKp46+CD3− cell lines (data not shown). Notably, although 102107 NKp46+CD3+ lysis of autologous *T. parva*-infected cells was not reduced by blocking NKp46, lysis of autologous *T. annulata* and MHCI-mismatched *T. parva*-infected cells was.
The frequencies of NKp46+CD3+ (A) and NKp46 (B) cells in Muguga-immunised animals receiving either a Muguga (black lines) or Marikebuni (dashed lines) challenge and naïve animals receiving a Marikebuni challenge (grey lines) were monitored by flow cytometric analysis during in vivo challenge. Substantial increase in the frequency of NKp46+CD3+ was detected from day 13 in the 2 Muguga-immunised animals that exhibited partial immunity following challenge with the antigenically heterogenous Marikebuni strain of the parasite (102170 and 402145).
Table I

Phenotype of *ex vivo* NKp46⁺CD3⁺ cell populations

Results of 3-colour flow cytometric analysis of PBMC from 13 animals. The percentage of i) NKp46⁺ cells in PBMC, ii) NKp46⁺CD3⁺ cells in PBMC subset and iii) CD3⁺ cells in the NKp46⁺ are detailed (columns 1, 2 and 3 respectively) as are the percentage of NKp46⁺CD3⁺ cells that express CD2, CD4, CD8, CD16, WC1 and γδ TCR (columns 4-9). The arithmetic mean and standard deviation (S.D. – in parentheses) for these percentages across the cohort are given in the bottom row. For 4 individuals (marked with *) 2 samples taken at least 1 month apart were analysed and showed the frequency of NKp46⁺CD3⁺ cells and the subsets expressing the different markers examined remained consistent.

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Table II
Expression of NKR and adaptor protein transcripts by NKp46^+CD3^+ cell, NK cell (NKp46^+CD3^-), and CD8^+ T-cell lines

RT-PCR analysis was performed for a range of NKRs including 2B4 (CD244), NKp30 (NCR3), NKp80 (KLRF1), NKG2A/C/D (KLRC1/2 and KLRK1) and CD94 (KLRD1) and the adaptor proteins DAP10, DAP12, FcεRIγ and CD3ζ on cDNA derived from NKp46^+CD3^+ cell, NK cell (NKp46^+CD3^-) and CD8^+ T-cell lines. Presence of mRNA transcripts is denoted by grey shading in the table.

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