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This information is current as of April 20, 2017.


*J Immunol* 2014; 192:3868-3880; Prepublished online 17 March 2014;
doi: 10.4049/jimmunol.1302464
http://www.jimmunol.org/content/192/8/3868

Supplementary Material
http://www.jimmunol.org/content/suppl/2014/03/17/jimmunol.1302464.DCSupplemental

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NKp46⁺CD3⁺ Cells: A Novel Nonconventional T Cell Subset in Cattle Exhibiting Both NK Cell and T Cell Features

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The NKp46 receptor demonstrates a high degree of lineage specificity, being expressed almost exclusively in NK 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 PBMCs that includes 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 NKRs and TCRs and also the CD3ζ, DAP10, and FcεRIγ but not DAP12 adaptor proteins. In vitro functional analysis of NKp46⁺CD3⁺ cells confirm that NKp46, CD16, and CD3 signaling pathways are all functionally competent and capable of mediating/redirecting 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 in this study identify and describe a novel nonconventional NKp46⁺CD3⁺ T cell subset that is phenotypically and functionally distinct from conventional NK and T cells. The ability to exploit both NKRs and TCRs suggests these cells may fill a functional niche at the interface of innate and adaptive immune responses. The Journal of Immunology, 2014, 192: 3868–3880.

The immune system is classically segregated into innate and adaptive components that operate in an integrated fashion to recognize and respond to pathogens. NK and T cells are lymphocyte subsets that show some similarities in function, development, and 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 Ag-specific receptors, the TCRs. 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 NKRs. NKRs are heterogeneous and include members of the killer Ig-related receptor (KIR), Ly49, CD161, and NKG2D families as well as 2B4 (CD244), CD16, and the natural cytotoxicity receptors (NCRs) Nkp30, Nkp44, and Nkp46 (3).

Most NKRs 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 costimulatory molecules modulating TCR signaling thresholds (4–9) or occasionally provide an alternative TCR-independent activation pathway (10, 11). Additionally, small subsets of nonconventional T cells, such as NKT cells and mucosal-associated invariant T cells (MAITs), constitutively coexpress CD3 and NKRs. These nonconventional 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 NKRs, 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 characterization 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 Ref. 19), including 1) chronically activated intraepithelial CTLs in celiac disease, where NKp46 upregulation is a component of a general and profound dysregulation of NKR expression associated with a “reprogramming” of CTL to become NK-like cells (20); 2) subpopulations of γδ⁺ and cord blood T cells stimulated with IL-15 (21, 22); 3) a minor population of aberrant murine CD3⁺γδ⁺ T cells termed “NK-like γδ T cells” (23); and 4) 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.

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Received for publication September 12, 2013. Accepted for publication February 7, 2014.

This work was supported by Combating Infectious Diseases of Livestock for International Development Grant BB/H009515/1 from the Department for International Development and the Biotechnology and Biological Sciences Research Council (BBSRC), U.K. government, as well as BBSRC Institute Strategic Grants on Innate Immunity and Endemic Disease awarded to The Roslin Institute and on Livestock Viral Diseases awarded to The Pirbright Institute.

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The online version of this article contains supplemental material.

Abbreviations used in this article: iNKT, invariant NKT; KIR, killer Ig-related receptor; MAIT, mucosal-associated invariant T cell; MDBK, Madin–Darby bovine kidney; MFI, mean fluorescence intensity; NCR, natural cytotoxicity receptor.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1302464
Following identification of these populations, it has been proposed that mammalian NK cells could be phenotypically defined as NKp46+CD3− (16).

Initial characterization 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, similar to other nonconventional 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. Immunization 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 Marikubuni stocks were clinically monitored based on a standard protocol (30) and buparvaqone (a thieniraidic drug; Parvexon from Bimeda, Dublin, 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

PBMCs were isolated from peripheral blood collected into 2 mL EDTA by density gradient centrifugation (Ficoll-Paque Plus from Amersham Biosciences, Chalford St. Giles, U.K.). For isolation of NKp46+ populations, cells were resuspended at 5 × 107 cells/ml in PBS/2 mM EDTA/0.5% BSA and incubated with 3 μg/ml anti-ovine NKp46 mAb EC1.1 (31), which cross-reacts with bovine NKp46 (T. Connelley, unpublished observations) for 30 min at 4°C. After two washes in PBS/2 mM EDTA/0.5% BSA, PBMCs were incubated with immunomagnetic anti-mouse Ig beads (Dynabeads pan mouse IgG, Life Technologies, Paisley, U.K.) for 30 min at 4°C. Ten microliters of the products was amplified using V beta subgroup-specific primers as described previously (27, 28) and PCR-MarkR-chains were amplified using an equivalent panel of V β subgroup–specific primers (T. Connelley, K. Degnan, and W.I. Morrison, submitted for publication). 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 (Supplemental Table I) were designed based on that of the IMGT system (41). Gene–specific primer pairs for NKp46+ cells

PBMCs were isolated from peripheral blood collected into 2 mL EDTA by density gradient centrifugation (Ficoll-Paque Plus from Amersham Biosciences, Chalford St. Giles, U.K.). For isolation of NKp46+ populations, cells were resuspended at 5 × 107 cells/ml in PBS/2 mM EDTA/0.5% BSA and incubated with 3 μg/ml anti-ovine NKp46 mAb EC1.1 (31), which cross-reacts with bovine NKp46 (T. Connelley, unpublished observations) for 30 min at 4°C. After two washes in PBS/2 mM EDTA/0.5% BSA, PBMCs were incubated with immunomagnetic anti-mouse Ig beads (Dynabeads pan mouse IgG, Life Technologies, Paisley, U.K.) for 30 min at 4°C on a rotator, placed in a magnet, and washed three times, and positively selected cells were collected.

Where specified, ex vivo NKp46+ and NKp46+CD3+ populations were isolated by sorting PBMCs stained with AKS6 (murine IgG2b anti-bovine NKp46) and MM1A (murine IgG1 anti-bovine CD3) mAbs (each at 1 μg/ml) and then Alexa Fluor 488–conjugated anti-murine IgG2b and Alexa Fluor 647–conjugated anti-murine IgG1 Abs (Dynabeads pan mouse IgG, Life Technologies, Paisley, U.K.) for 30 min at 4°C on a rotator, placed in a magnet, and washed three times, and positively selected cells were collected.

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Analysis of TCR and NKR transcription

Total RNA was isolated from cell lines using TRI Reagent (Sigma-Aldrich), and cDNA was subsequently synthesized using a reverse transcription system (Promega, Madison, WI), with priming by the oligo(dT)15 primer, according to the manufacturer’s instructions. TCRβ-chains were PCR amplified using V β subgroup–specific primers as described previously (41) and TCR-γ chains were amplified using an equivalent panel of V γ subgroup–specific primers (T. Connelley, K. Degnan, and W.I. Morrison, submitted for publication). TCR genes were named using a nomenclature based on that of the IMGT system (41). Gene–specific primer pairs for NKp46+ and adaptor protein genes (Supplemental Table I) were designed based on that of the IMGT system (41). Gene–specific primer pairs for NKp46 and adaptor protein genes (Supplemental Table I) were designed based on that of the IMGT system (41). Gene–specific primer pairs for NKp46+ and adaptor protein genes (Supplemental Table I) were designed based on that of the IMGT system (41).

Cytotoxicity assays

A 4 h [3H]Thymidine release assay was used to examine the cytotoxic activity of cell lines. All assays were conducted in duplicate. Percentage specific lysis was calculated as [(sample release − spontaneous release) × 100%] / [(maximal release − spontaneous release) and expressed as a means 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 redirected cell line assays and NKp46-blocking assays, target or effector cells were preincubated with anti-CD3, anti-CD16, or anti-NKp46 (AKS1) mAbs (all at 2 μg/ml) for 30 min prior to completion of the cytotoxicity assays. Anti-CD8 (IL-A51 (38); IgG1) and anti-MHC-1 (IL-A88 (42); IgG2a) mAbs 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, Roskilde, Denmark) precoated overnight in 0.05 M carbonate buffer (pH 9.6) with either MM1A, KD1, or AKS1 and with IL-2 as a source of recombinant bovine IL-12 to induce IFN-γ expression, cells were cultured with or without recombinant bovine IL-12 at 10 U/ml. In all assays individual wells contained 1 × 105 cells and the media were supplemented with 100 U/ml recombinant human IL-2 and supernatant was harvested after 20 h of culture at 37°C in 5% CO2. Triplicate wells were prepared for each condition examined. The IFN-γ content in supernatants was measured.
using a sandwich ELISA, using noncompeting mAbs specific for bovine IFN-γ (clones CC330 and CC302, both from AbD Serotec). The latter was conjugated with biotin and binding was detected with HRP-conjugated streptavidin (AbD Serotec) followed by addition of 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 naive cattle against *T. parva*, CD8+ cell lines generated from PBMCs 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 before stimulation (27, 28). However, analysis of PBMCs from a panel of naive animals (n = 23) by two-color flow cytometry identified a small NKp46+CD3+ population (0.1–1.7% of total PBMCs) ex vivo in all animals examined. In most animals NKp46+CD3+ cells formed only a minor subset (<10%) of the total NKp46+ population, although in four animals it represented between 21 and 44% of the NKp46+ population. The inclusion in our panel of three neonates (<96 h old) suggests that NKp46+CD3+ cells are constitutively present from birth and do not represent a subset of in vivo–activated T cells accumulated postnatally.

Three-color phenotypic analysis of PBMCs from a cohort of 3- to 8-mo-old animals (n = 13) showed that in vivo NKp46+CD3+ populations included CD8+, CD4+, and γδ TCR+ fractions (Fig. 1). Although most animals had a dominant CD8+ subset with relatively minor CD4+ and γδ TCR+ components (including some expressing WC1), there was substantial interanimal 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 (mean fluorescence intensity [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 ∼70% that seen on T cells (MFI = 272.5 ± 73.8, paired t test p < 0.05).

To determine whether 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 d 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 (Fig. 2).

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

To enable characterization 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 optimized a system exploiting coculture with irradiated autologous *T. parva*-infected cells in the presence of recombinant bovine 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 three *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.

**FIGURE 1.** Flow cytometric analysis of ex vivo NKp46+CD3+ cells. Freshly isolated PBMCs were analyzed by three-color flow cytometry using Abs against NKp46, CD3, and the markers indicated in the panels. Plots show the staining observed within the NKp46+ gated population. The percentages of cells within each quadrant are shown. Results presented are from 1 individual representative of those obtained from 13 animals.
The three NKp46+CD3+ cell lines had similar phenotypes (Fig. 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 ~10%) whereas 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−) cell cultures. Conversely, the conventional CD8+ T cell line was CD5+, CD6−, CD16−, CD2+, and thus had a phenotype very different from that of the NKp46+CD3+ cells. In addition to being CD3+, both the conventional CD8+ T cell line and the NKp46+CD3+ lines included a subset that stained with a mAb (IL-A47) specific for bovine Vβ20, providing confirmation that NKp46+CD3+ cells express αβ TCR on their cell surface.

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Table I. Phenotype of ex vivo NKp46+CD3+ cell populations

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<th>Animal</th>
<th>% PBMCs NKp46+</th>
<th>% NKp46+CD3+ CD2+</th>
<th>% NKp46+CD3+ CD4+</th>
<th>% NKp46+CD3+ CD8+</th>
<th>% NKp46+CD3+ CD16+</th>
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Results of three-color flow cytometric analysis of PBMCs from 13 animals. The percentages of NKp46+ cells in PBMCs, NKp46+CD3+ cells in a PBMC subset, and CD3+ cells in the NKp46+ are detailed (columns 2, 3, and 4, respectively), as are the percentages of NKp46+CD3+ cells that express CD2, CD4, CD8, CD16, WC1, and γδ TCR (columns 5–10). The arithmetic mean and SD (in parentheses) for these percentages across the cohort are given in the bottom row. For four individuals (marked with *), two samples taken at least 1 mo apart were analyzed and showed the frequency of NKp46+CD3+ cells and the subsets expressing the different markers examined remained consistent.

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 KIRs. Both NKp46+CD3+ and NKp46+CD3− cell lines expressed transcripts for all of the NKR examined (Table II, Supplemental Table II). 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).

FIGURE 2. NKp46+CD3+ cells present after in vitro culture are derived from an in vivo population. Highly purified NKp46+ (upper panels) and NKp46+CD3− (lower panels) populations obtained from PBMCs were cocultured with autologous T. parva–infected cells and after 9–11 d analyzed for CD3 and NKp46 expression by two-color flow cytometry. An NKp46+CD3+ subset was present within cultures established from ex vivo NKp46+ but not NKp46+CD3− purified populations (right panels) demonstrating that in vitro NKp46+CD3+ populations are derived from a pre-existing in vivo NKp46+CD3+ population. The percentages of cells within each quadrant are shown. Results presented are from one individual representative of those obtained from four animals.
Adaptor proteins containing ITAMs are required for the initiation of intracellular signaling following ligation of TCRs and many activatory NKRs. As with the cell surface receptors with which they associate, adaptor proteins often exhibit a lineage-restricted expression. We analyzed mRNA transcript expression of four adaptor proteins: CD3z, DAP10, DAP12, and FcεR1g. As anticipated, the conventional CD8+ T cell line expressed transcripts for DAP10 and CD3z, and the NKp46+CD32 cells expressed transcripts for all four proteins (Table II). In contrast, NKp46+CD3+ cell lines expressed DAP10, FcεR1g, and CD3z 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 NKRs (e.g., NKG2C) may differ.

**NKp46+CD3+ cells express a diverse TCR repertoire**

Several nonconventional T cell subsets such as invariant NKTs (iNKTs) and MAITs express semiconserved TCR repertoires (46, 47). To determine whether NKp46+CD3+ cells also express a restricted TCR repertoire, we used panels of Vα and Vβ subgroup-specific primers to analyze 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 (Fig. 4, upper rows). Sequencing from a limited number of NKp46+CD3+ cell clones (n = 5), generated by limiting dilution from two additional animals, corroborated a diversity within the TCRβ repertoire (Supplemental Table III). The CD8+ T cell line expressed TCR utilizing a broad but incomplete repertoire of Vα and Vβ subgroups, consistent with a population that has undergone Ag-driven clonal selection (Fig. 4, lower rows), whereas the NKp46+CD3+ cell lines expressed neither TCRα nor TCRβ mRNA transcripts (Fig. 4, middle rows).

**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 Madin–Darby bovine kidney (MDBK), redirected 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.

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**Table II. Expression of NKRs and adaptor protein transcripts by NKp46+CD3+, NK (NKp46+CD3−), and CD8+ T cell lines**

<table>
<thead>
<tr>
<th>NKR</th>
<th>NKp46+CD3+</th>
<th>NKp46+CD3−</th>
<th>CD8+ T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>2B4</td>
<td>201961</td>
<td>202094</td>
</tr>
<tr>
<td>NKR</td>
<td></td>
<td></td>
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<tr>
<td>NKp30</td>
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<tr>
<td>NKG2D</td>
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<tr>
<td>CD94</td>
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<tr>
<td>NKG2A</td>
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<td>NKG2C</td>
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<td>NKp80</td>
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<tr>
<td>Adaptor protein</td>
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<tr>
<td>FCER1G</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CD3ZETA</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DAP10</td>
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<td></td>
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<tr>
<td>DAP12</td>
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</table>

RT-PCR analysis was performed for a range of NKRs, including 2B4 (CD244), NKp30 (NCR3), NKp80 (KLRF1), NKG2A/D (KLRCl/2 and KLRK1), and CD94 (KLRD1) and the adaptor proteins DAP10, DAP12, FcεR1γ, and CD3ζ on cDNA derived from NKp46+CD3+, NK (NKp46+CD3−), and CD8+ T cell lines. Presence of mRNA transcripts is denoted by gray shading in the table.
We conducted a series of assays to determine whether 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 E:T ratio of 10:1, comparable to the levels demonstrated by NKp46+CD3+ cells (Fig. 5). In contrast, the NKp46−CD3+CD8+ T cell line did not kill the MDBK target (data not shown). Blocking NKp46 by preincubation of effector cells with anti-NKp46 mAb 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 redirected lysis assays using the FcR+ mouse p815 mastocytoma cell line, cytolytic function of NKp46+CD3+ cells could be substantially increased by preincubation with either anti-CD16 or anti-NKp46 Abs, but not by isotype-matched control Abs (Fig. 6). Notably, as with conventional NK cells (NKp46+CD3+), the degree to which anti-NKp46 and anti-CD16 Abs blocked killing of MDBK (Fig. 5) and elevated cytotoxicity against p815 (Fig. 6) exhibited considerable variation between different NKp46+CD3+ cell lines. Taken 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

![FIGURE 4. TCR repertoire of NKp46+CD3+, NK (NKp46+CD3−), and CD8+ T cell lines. RT-PCR analysis of the repertoire of rearranged TCRα (A) and TCRβ (B) chain transcripts expressed in NKp46+CD3+ (upper rows), NKp46+CD3− (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 300-bp MW marker. Data for NKp46+CD3+ and NKp46+CD3− cell lines are from one animal representative of three.](http://www.jimmunol.org/)

![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 4-h [111In]-release assays. Prior to the assay, effector cells were preincubated with either no Ab, anti-NKp46 Ab, or an isotype control (IgG1 anti-CD8) Ab. MDBK cells were not lysed by a conventional CD8+ T cell line (data not shown).](http://www.jimmunol.org/)
wells precoated with mAbs. NKp46+CD3+ cells failed to produce IFN-γ in response to IL-2/IL-12 or following cross-linking NKp46 or CD16 (Fig. 7, gray bars). In contrast, NKp46+CD3+ cells (Fig. 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 (3–35 ng/ml). Thus, with respect to IFN-γ production, NKp46+CD3+ cells are distinct from conventional NK cells.

NKp46+CD3+ cells retain a functional CD3 signaling pathway

In the absence of defined Ags recognized by TCR expressed by NKp46+CD3+ cells, we used CD3 cross-linking as a proxy for TCR ligation to examine whether NKp46+CD3+ cells retained a functional TCR signaling pathway. In redirected cytotoxicity assays preincubation of p815 cells with anti-CD3 Ab resulted in increased lysis by NKp46+CD3+ cells and the conventional CD8+ T cells but not the line NKp46+CD3− cell lines (Fig. 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 Ab induced IFN-γ production in NKp46+CD3+ cells (Fig. 7), demonstrating that 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 three different NKp46+CD3+ cell lines were similar (16–22 ng/ml) but markedly less than that produced by the conventional CD8+ T cell line (70 ng/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 culture (45–58% at E:T ratio of 10:1), exhibited variable efficacy in lysing autologous T. annulata–infected cells and MHC class I–mismatched T. parva–infected cells, but did not kill autologous uninfected T cell lymphoblasts (Fig. 8). The restriction of killing to parasitized cells, which was neither MHC class 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–immunized animal. Although NKp46+CD3+ cells exhibited “NK-like” lytic activity against Theileria-infected cells, the rela-

**FIGURE 6.** Redirected 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− (A–C), NKp46+CD3+ (D–F), and CD8+ T cell (G) lines against p815, which had been preincubated with Abs as indicated in the legend, was assessed in 4-h $^{111}$In-release assays.
tive 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.

Preincubation of NKp46+CD3+ cells with NKp46-specific Ab resulted in inhibition of Theileria-infected cell lysis (Fig. 9A, 9B), 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 (Fig. 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 whether NKp46+CD3+ populations could respond to T. parva infection in vivo, we monitored the frequency of this population in PBMCs of animals during experimental parasite challenge. Five T. parva Muguga–immunized 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 naive animals were similarly infected with the Marikebuni stock. Both immunized animals challenged with the homologous Muguga stock were solidly immune (1021121 and 402082), two of the immunized animals challenged with the Marikebuni stock exhibited partial immunity with delayed parasite clearance (102170 and 402145), whereas the third immunized animal (702162) and the two naive animals (102107 and 202192) challenged with Marikebuni stock were unprotected, developing severe clinical disease that required anti-parasite therapy 15–17 d postinfection.

During the first 11 d following challenge the frequency of NKp46+CD3+ cells in the PBMCs of all the animals remained relatively unaltered (Fig. 10A). However, from day 13 the frequency of the NKp46+CD3+ populations in the two 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 seven animals during the first week of challenge but in the second week the frequency was elevated in the two partially protected Marikebuni-challenged and one of the immunized animals challenged with Muguga (102121), whereas in the other animals it remained low (Fig. 10B). Taken together, the results demonstrate an ability of NKp46+CD3+ cells to respond to T. parva infection in vivo; however, in naive and completely immune animals this response may either be absent or below the threshold for detection by direct quantification in PBMCs.

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 characterization of a novel subset of NKp46+CD3+ lymphocytes in the PBMCs of healthy cattle. These cells were present in all individuals examined, including newborn 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 T. 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 in the present study 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 of 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 PBMCs, 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 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 in the present study represent a previously undescribed nonconventional 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 downregulate expression of CD2 to become predominantly CD2+. Additionally, NKp46+CD3+ cells express a full repertoire of NKRs, including CD16, NKp80, and NKG2C, which, similar to 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 signaling 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. Taken 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) signaling pathways, implying that, as with other nonconventional 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 NKRs are serving as costimulatory molecules, lowering the threshold for cell activation by other receptors, with the obvious candidate in NKp46+CD3+ cells being the TCR. However, the ability of a mAb blocking NKp46 ligation to reduce the lysis of autologous *Theileria*-infected cells in two of three 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 signaling 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 signaling to activation of NK cells can depend on the biological context (56). Unfortunately, owing to the absence of the relevant Abs, it is not currently possible to perform the assays that would enable the roles of other NKRs and TCRs in enabling NKp46+CD3+ cell recognition and lysis of *Theileria*-infected cells to be examined.

**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 4-h [111In]-release assays. NKp46+CD3+ cells were preincubated with either no Ab, anti-NKp46 Ab, or an isotype control (anti-CD8) Ab. In parallel assays NKp46+CD3+ lysis of autologous *T. annulata* and MHC class I-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 MHC class I-mismatched *T. parva*-infected cells was.

**FIGURE 10.** Frequency of NKp46+ and NKp46+CD3+ cells in PBMCs during in vivo challenge with *T. parva*. The frequencies of NKp46+CD3+ (A) and NKp46+ (B) cells in Muguga-immunized animals receiving either a Muguga (black lines) or Marikebuni (dashed lines) challenge and naïve animals receiving a Marikebuni challenge (gray lines) were monitored by flow cytometric analysis during in vivo challenge. Substantial increase in the frequency of NKp46+CD3+ cells was detected from day 13 in the two Muguga-immunized animals that exhibited partial immunity following challenge with the antigenically heterogeneous Marikebuni strain of the parasite (102170 and 402145).
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 characterize 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 recognized by NKp46+CD3+ cell TCRs.

The ability of NKp46+CD3+ cells from PBMCs of Theileria parva-infected animals to expand during in vitro coculture 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 naive animals failed to generate a detectable NKp46+CD3+ cell expansion following T. parva infection. The absence of a conventional NK cell response in these naive 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+CD3+ and NKp46+CD3− cells to lymphoid tissues during active infection. In vivo NKp46+CD3+ cell expansions were observed in the two immunized animals that exhibited partial protection against challenge with a heterologous parasitic stock. In these animals NKp46+CD3+ cells expanded from 0.4% of PBMCs prechallenge to a maximum of 2 and 3% by day 13 postchallenge, coincident with the onset of clinical resolution of disease.

Efficient lysis by NKp46+CD3+ cells of autologous T. parva-infected cells in vitro suggests that the expanded NKp46+CD3+ populations have the potential to make a substantial contribution to pathogen control. Ex vivo pMHC class I-tetramer data from fully immunized animals undergoing challenge show the peak frequency of CD8+ T cells specific for immunodominant epitopes was similar, ranging from 1.5 to 4.4% (X. Li, unpublished data). Similarity in the kinetics of the NKp46+CD3+ and NKp46+CD3− cell expansions in these two animals is consistent with the response of the NKp46+CD3− cells against the antigeneically novel pathogen being innate-like, suggesting that a function of this subset may be to exploit NKR coexpression 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 immunized animals subjected to a heterologous parasite challenge, the role, if any, of NKp46+CD3+ (and NKp46+CD3−) responses in mediating immunological protection is yet to be defined. No NKp46+CD3+ cell responses were detected in the two immunized animals challenged with a homologous parasite, suggesting that the presence of an established and highly protective adaptive memory CD8+ T cell population (63) may restrict NKp46+CD3− cell responses due to competition or functional redundancy. Taken together, our data demonstrate that NKp46+CD3+ cells have the capacity for anti-Theileria effector function and are able to respond to pathogen challenge in vivo. However, cytotoxicity is only one of many functions attributed to NK cell functions and conventional and nonconventional T cells (53, 64–66). Analyses of the ability of NKp46+CD3+ cells to form memory, interact with and regulate other components of innate immunity, and direct adaptive immune responses were outside the scope of the present study but will be required to fully understand the biological role of NKp46+CD3+ cells and their function in immunity against T. parva.

In addition to our work in this study demonstrating a response against T. parva, bovine NKp46+ cells have been shown to respond to a variety of pathogens that cause economically important cattle diseases, including Mycobacterium bovis (67–70), Neospora (48, 71, 72), and Babesia (73). Determining whether NKp46+CD3+ cells form a component of these NKp46+ 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+ subsets within NKp46+ cells in the pig (74) and possibly horse (75) suggest that NKp46+CD3+ 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 nonconventional T cell population with features of both innate and adaptive immune cell types. The ability to use both NKR and TCR signaling pathways indicates a cell that may be specialized, similar to 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 vaccine development (12, 64, 76). Our data provide evidence that NKp46+CD3+ cells can respond to T. parva. Inevitably there are fundamental questions that need to be addressed following the identification of a novel lymphoid subset. The availability of in vivo models for immunization and challenge with T. parva, coupled with the ability to grow parasitized cells 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 whether there is potential for their exploitation more widely in immunization strategies.

Acknowledgments
We thank Bob Fleming for excellent assistance with flow cytometric cell sorting and the staff at the large animal facility at The Roslin Institute.

Disclosures
The authors have no financial conflicts of interest.

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