

NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken

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Keywords: chicken, cytotoxicity, mucosal immunity

Abstract

Non-mammalian NK cells have not been characterized in detail; however, their analysis is essential for the understanding of the NK cell receptor phylogeny. As a first step towards defining chicken NK cells, several tissues were screened for the presence of NK cells, phenotypically defined as CD8⁺ cells lacking T- or B-lineage specific markers. By this criteria, ~30% of CD8⁺ intestinal intraepithelial lymphocytes (IEL), but <1% of splenocytes or peripheral blood lymphocytes were defined as NK cells. These CD8⁺CD3⁻ IEL were used for the generation of the 28-4 mAb, immunoprecipitating a 35-kDa glycoprotein with a 28-kDa protein core. The CD3 and 28-4 mAb were used to separate IEL into CD3⁺ IEL T cells and 28-4⁺ cells, both co-expressing the CD8 antigen. During ontogeny, 28-4⁺ cells were abundant in the IEL and in the embryonic spleen, where two subsets could be distinguished according to their CD8 and c-kit expression. Most importantly, 28-4⁺ IEL lysed NK-sensitive targets, whereas intestinal T cells did not have any spontaneous cytolytic activity. These results define two major, phenotypically and functionally distinct IEL subpopulations, and imply an important role of NK cells in the mucosal immune system.

Introduction

Mammalian NK cells have been intensively characterized in the last years, providing a wealth of information regarding their receptors, tissue distribution and function (1–5). In particular, activatory and inhibitory receptors essential for NK cell function have been defined. It is now generally accepted that NK cells control the integrity of self-MHC class I molecules and target cell lysis is prevented by ligation of inhibitory NK cell receptors and self-MHC class I (6). Lack of MHC class I molecules on target cells fails to generate an inhibitory signal and NK cells can be induced to lyse targets by activatory receptors (1–5,7).

Three major groups of inhibitory receptors have been identified on NK cells. Mouse NK cells express members of the Ly-49 family, proteins with type II orientation and homology to C-type lectins (8). In contrast, human NK cells utilize type I glycoproteins with two or three Ig-like extracellular domains (4,5,9). The CD94/NKG2 heterodimer with homology to C-type lectins is present in both species (10,11).

Individual members of the three families have been functionally described to either activate or inhibit NK cells (4,5,7,9). The inhibition is mediated via the cytoplasmic immunoreceptor

tyrosine-based inhibition motif, which, upon phosphorylation, recruits the phosphatase SHP-1 (12,13). Activatory NK cell receptors within the same family consistently lack this motif, but contain a charged transmembrane residue, the putative interaction site for DAP-12. DAP-12, like the ζ chain in T cells, forms homodimers and harbors a cytoplasmic immunoreceptor tyrosine-based activation motif (14). To gain more insight into the evolution of these three distinct NK cell receptor families it would be beneficial to characterize NK cells in non-mammalian vertebrates.

NK cell-like activity has been demonstrated in numerous vertebrate phyla and is regarded as an early form of the immune defense, most likely occurring before the emergence of T and B lymphocytes. In addition, allorecognition in invertebrates shares very similar features to mammalian NK cells (15). The paucity of information regarding NK cell biology in non-mammalian species is mainly due to the lack of NK cell-specific mAb, which would allow us to identify, purify and culture NK cells. In previous studies, we have characterized an embryonic splenocyte population, which was identified as chicken NK cell homologues by morphological, phenotypical and functional criteria (16).

In order to extend these analyses to adult chicken NK cells, several tissues were screened for the presence of NK cells by phenotypical markers. In striking contrast to mammals, chicken NK cells were barely detectable in peripheral blood lymphocytes (PBL) and spleen, but they were abundant in the intestinal epithelium where they represent a distinct intestinal epithelial lymphocyte (IEL) subset besides T cells. The 28-4 mAb was generated to specifically identify this IEL subset, and was used to study their ontogeny and function.

Methods

Animals, mAb and cell lines

The partly inbred H.B19 (B19) chicken strain and white leghorn chickens were all raised and maintained in our animal facility. The mAb CT3 (17), CT4, CT8 (18) and L1 which react with the chicken CD3, CD4, CD8 α and IgL chains respectively were kindly provided by Dr C. Chen (University of Alabama at Birmingham, Birmingham, AL), the mAb against $\alpha V_{\beta}1$ TCR (TCR2) (19) by Dr J. Cihak (Institute for Animal Physiology, Munich, Germany), and the mAb against c-kit by Dr O. Vainio. The 28-4 mAb (mouse IgG-3) was generated according to standard procedures. The intestinal cell line IEL-1 was generated using the replication-defective avian reticuloendotheliosis virus strain T and the chicken syncytial virus as a helper virus. The LSCC-RP9 B lymphoblastoid cell line derived from tumor induced by Rous-associated virus 2 and is commonly used as chicken target cell line (20). All cell lines were propagated in IMDM supplemented with 5% FCS, 50 IU/ml penicillin, 50 μ g/ml streptomycin and 2 mM glutamine (complete medium; all reagents Gibco/BRL, Bethesda, MD).

Cell preparations and immunofluorescence analysis

All analyses were performed on tissues obtained from 2- to 3-month-old birds. Single cell suspensions of PBL, spleen and embryonic spleen were prepared according to standard procedures. IEL were obtained as described in detail (21). Briefly the duodenal loop was rinsed and cut longitudinally in 1 cm pieces. Following several washes in cold PBS, the intestinal pieces were stirred in prewarmed solution of PBS containing 10 mM DTT and 0.1 mM EDTA for 30 min. Cells were collected by centrifugation, passed through a nylon wool column and further enriched on a Ficoll-Paque density gradient. The interphase cells were collected and washed. Immunofluorescence staining was performed with primary unlabeled mAb followed by isotype-specific anti-mouse fluorochrome conjugates (Southern Biotechnology Associates, Birmingham, AL). For double immunofluorescence analysis the cells were first incubated with the primary mAb, followed by washing and incubation with an anti-mouse-IgG1-phycoerythrin conjugate and a 28-4-FITC conjugate. Labeled cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) or sorted on a FACS Vantage cell sorter. Analysis was performed using CellQuest software.

Immunoprecipitation and gel electrophoresis

The lactoperoxidase catalyzed reaction was used to label cell-surface proteins with 125 I. Cells were lysed with 1% NP-40 in 0.15 M NaCl/0.05 M Tris-HCl, pH 7.4, containing

various protease inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany). Insoluble matter was removed by 30 min centrifugation at 15,000 *g*. Lysates were immunoprecipitated by a solid-phase technique (22) and immunoprecipitates were separated on 4–20% gradient PAGE (23). For deglycosylation samples were treated with PNGase F prior to electrophoresis.

Cytotoxicity assay

The effector cells were obtained from freshly isolated IEL, and sorted according to their expression of CD3 and 28-4. The cells were washed, resuspended in complete medium and distributed on 96-well round-bottom plates. LSCC-RP9 cells (10^6) were labeled using 500 μ Ci of 51 Cr (sodium chromate in aqueous solution containing 0.9% NaCl, sp. act. 10 mCi/ml; Amersham, Arlington Heights, IL) for 45 min at 37°C. Cells were washed once and incubated for 45 min at 37°C. Following two more washes, 10^4 target cells were added to the effector cells and incubated for 4 h at 37°C. The supernatants were harvested using a Scatron supernatant collection system (Scatron Instruments, Norway) and specific cytotoxicity was calculated using the formula: specific release = [(test release – spontaneous release)/(total release – spontaneous release)] \times 100. Spontaneous cytotoxicity never exceeded 15%.

Results

Tissue distribution of a putative CD3⁺CD8⁺ NK cell population

Specific avian NK cell markers have not been available. Since mammalian NK cells are phenotypically defined as lymphocytes lacking surface TCR-CD3 and Ig, splenocytes and PBL were screened for a similar cell population. Virtually all embryonic chicken NK cells that were *in vitro* expanded expressed CD8, which thus could serve as an additional marker of adult NK cells (16). Only a small lymphocyte subset in the spleen fulfilled these criteria of a CD8⁺CD3⁻ population (Fig. 1A). To exclude the possibility of a CD8⁻ NK cell subpopulation, splenocytes were simultaneously analyzed for their expression of CD3, IgL and CD8. Lymphocytes lacking B and T cell-specific markers were barely detectable, some of which expressed CD8 (Fig. 1A).

In striking contrast, when IEL were analyzed for their CD3 and CD8 expression, ~30% of the CD8⁺ cells lacked CD3 (Fig. 1B). Thus, a putative NK cell subset phenotypically identified as non-T, non-B cells that co-expressed CD8 was found in substantial numbers in the IEL, while other peripheral tissues contained only low frequencies of these cells.

Two major IEL populations defined by their expression of the CD3 and 28-4 antigens

The CD3⁻CD8⁺ IEL cells were subsequently sorted for immunization of mice in order to generate mAb. The 28-4 mAb was selected for further analyses since it specifically reacted with the CD3⁻ IEL subset. Double immunofluorescence analyses using the 28-4 mAb in conjunction with various well-characterized mAb revealed that the 28-4 antigen is mainly expressed by CD3⁻CD8⁺ lymphocytes, that do not express Ig (Fig. 2). Although both CD3⁺ T cells and 28-4⁺ cells expressed the

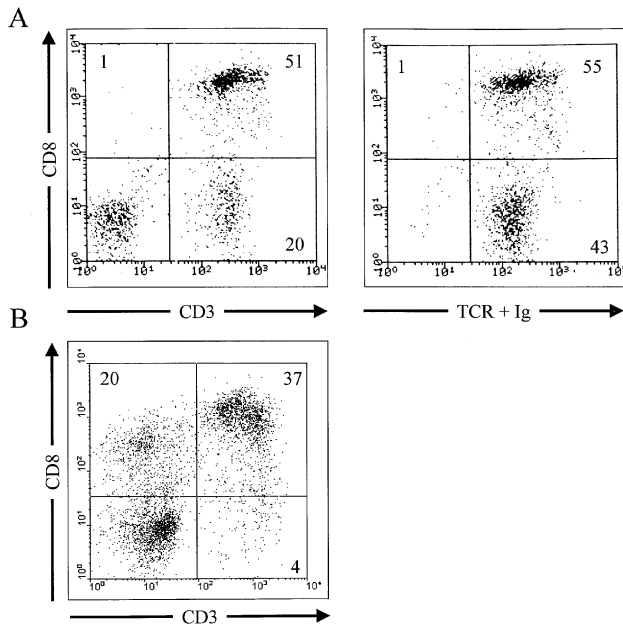


Fig. 1. Tissue distribution of CD3⁺CD8⁺ lymphocytes. Cells obtained from spleen (A) and intestine (B) were analyzed for their CD3, IgL and CD8 expression to identify a putative NK cell subset lacking TCR/CD3 and IgL.

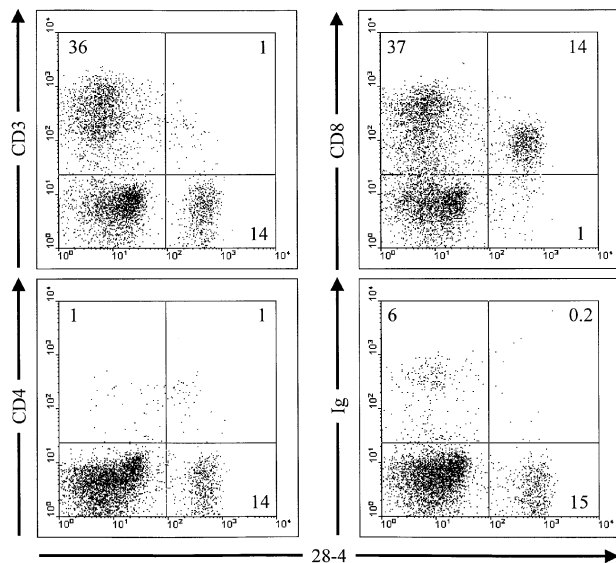


Fig. 2. The 28-4 antigen expression on IEL. Dual immunofluorescence analysis of IEL utilizing the 28-4 mAb together with mAb specific for CD3, CD4, CD8 and IgL defined two major lymphocyte subpopulations expressing the CD3 and 28-4 antigen respectively.

CD8 antigen, the CD8 expression levels on 28-4⁺ were homogenous but lower as compared to the heterogeneous and higher levels observed on CD3⁺ cells (Figs 1 and 2). These differences can be explained by the exclusive usage of the CD8 $\alpha\alpha$ homodimer on 28-4⁺ cells (data not shown) in contrast to the expression of both the CD8 $\alpha\alpha$ homodimer and the CD8 $\alpha\beta$ heterodimer on different CD3⁺ IEL subsets. Cells

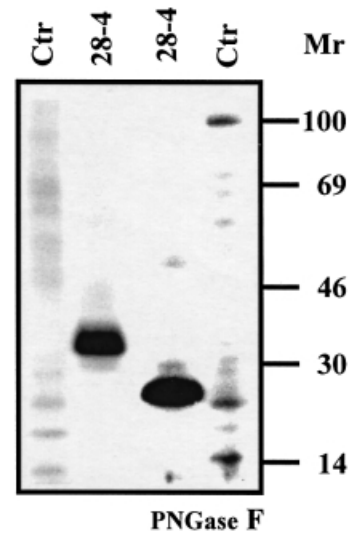


Fig. 3. The 28-4 antigen is a 35 kDa monomer. ¹²⁵I-labeled NK cell lysates were immunoprecipitated with the 28-4 mAb (28-4) or isotype-matched control mAb (Ctr) and separated on a 4–20% PAGE under non-reducing conditions. PNGase F was used to remove N-linked carbohydrates as indicated.

expressing the CD4 antigen can barely be detected in the IEL. However, some of these low-frequency cells co-expressed the 28-4 antigen (Fig. 2). Moreover, ~3% of the total CD3⁺ cells were also 28-4⁺ (Fig. 2). These CD3⁺28-4⁺ cells were also detected in embryonic IEL preparations (Fig. 3) and could resemble an NKT cell-like population.

For biochemical analysis 28-4⁺ cells were sorted and retrovirally transformed to generate a cell line. This cell line expressing high levels of the 28-4 antigen was surface iodinated, and lysates were immunoprecipitated and separated on PAGE gels. The 28-4 mAb precipitated a glycosylated monomeric 35 kDa molecule under both non-reducing (Fig. 3) and reducing conditions (data not shown). Removal of N-linked carbohydrates yielded a protein core size of 28 kDa (Fig. 3). Similar results were obtained using freshly isolated IEL (data not shown). In conclusion, the 28-4 mAb specifically reacts with a surface glycoprotein, expressed on all CD8⁺ CD3⁻ IEL.

Ontogeny of 28-4⁺ cells in the embryonic spleen and gut

We initially described chicken NK cells localized in the embryonic spleen (16). Therefore embryonic day 19 splenocytes were analyzed for the presence of the 28-4 antigen. The 28-4 antigen was expressed on most CD8 $\alpha^+\beta^-$ and CD25⁺ embryonic splenocytes, whereas Ig⁺ cells were 28-4⁻ (Fig. 4A). A similar population of 28-4⁺ cells was also detected in embryonic and adult bone marrow (data not shown). The 28-4⁺ cells were further divided into CD8⁺ and CD8⁻ subpopulations (Fig. 4B). The light scatter characteristics of the 28-4 single-positive cells were heterogeneous, whereas the 28-4⁺ CD8⁺ double-positive cells represented a homogeneous cell population. Moreover, c-kit was expressed on virtually all 28-4 single-positive cells at high levels, while c-kit was only expressed by some 28-4⁺ CD8⁺ cells (Fig. 4B).

To address the ontogeny of intestinal NK cells, IEL were

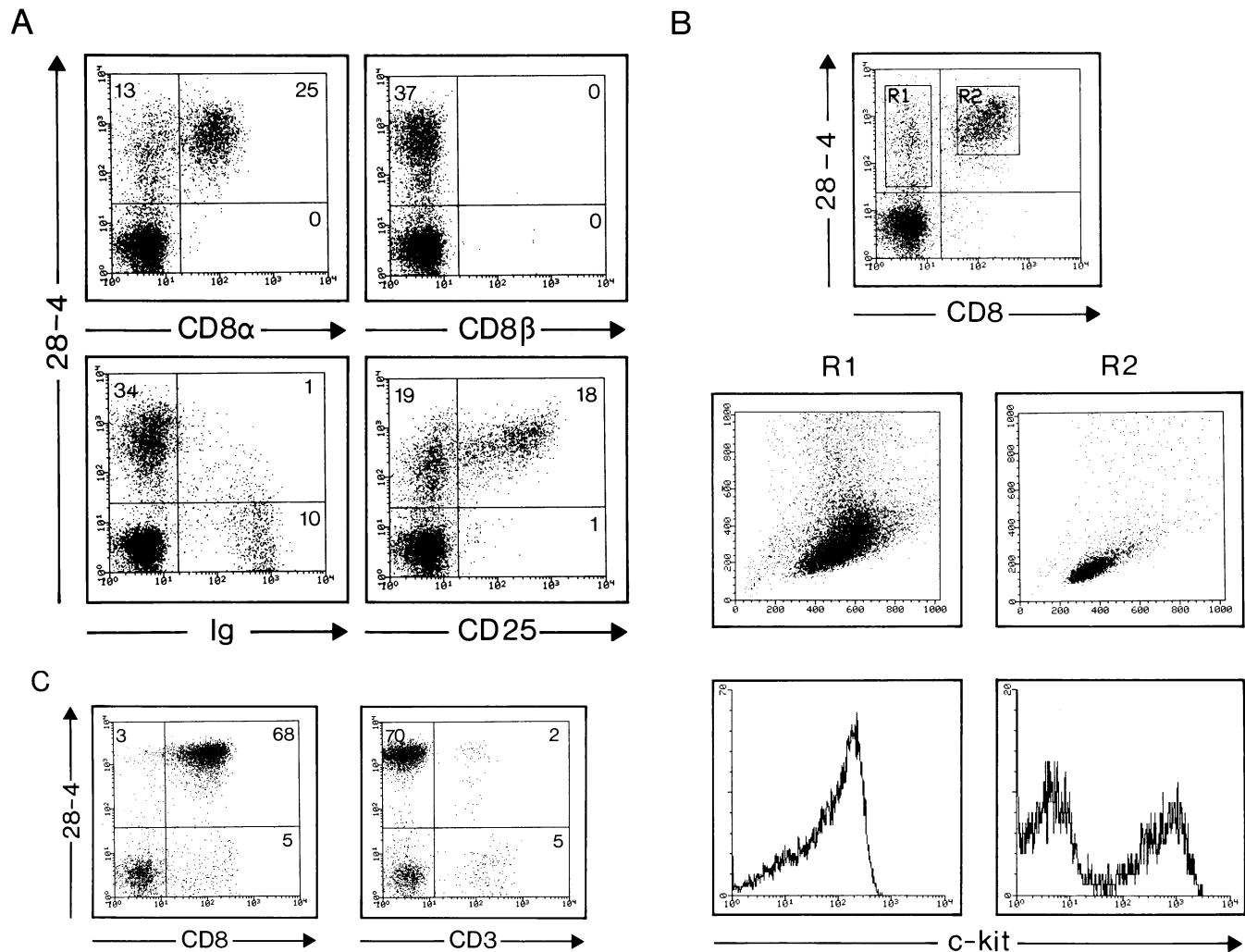


Figure 4. 28-4⁺ cells are detected during embryonic development. 28-4⁺ cells present in 19-day-old embryonic splenocytes co-express CD8 α and CD25 (A), and can be further separated into two subpopulations according to their CD8 expression (B). 28-4⁺ NK cells are the most abundant IEL subset in day 19 embryos (C).

prepared from 19-day-old chicken embryos, and analyzed for 28-4 and CD3 expression. Almost all embryonic IEL expressed the 28-4 antigen, while only few CD3⁺ T cells were present at this developmental stage (Fig. 4C). Thus, 28-4⁺ cells represent the first detectable IEL subset, before CD3⁺ T cells migrate into the intestine.

Two functionally different IEL subsets

NK cells are functionally defined by their ability to spontaneously lyse target cells. In order to test whether the 28-4⁺ IEL functionally represent NK cells, CD3⁺ and 28-4⁺ cells were sorted from IEL and tested in a 4 h Cr-release assay. The 28-4 cells lysed the target cell line in a dose-dependent fashion, whereas T cells did not kill the targets (Fig. 5). When the 28-4 hybridoma was incubated with IEL effector cells, the hybridoma was not killed, indicating that the mAb does not induce redirected lysis. (data not shown). Taken together, these data suggest that the two major IEL subpopulations differ functionally in their ability to spontaneously lyse target cells.

Discussion

This report describes a novel mAb specific for chicken intestinal NK cells, which allows the identification of two major IEL subpopulations: CD3⁺ T cells and 28-4⁺ NK cells. When chicken tissues were screened for potential NK cells by phenotypic means, it was apparent that very few NK cells reside in the spleen and PBL. It is unlikely that a distinct NK cell population in these tissues was missed, since almost all lymphocytes in the spleen and PBL express either T- or B-lineage-specific markers. Since the CD8 $\alpha\alpha$ homodimer is expressed by *in vitro* activated embryonic NK cells (16) and by 28-4⁺ IEL (Figs 1 and 2), CD8 could serve as an additional NK cell marker and it is unlikely that all splenic- or blood-derived NK cells do not express the CD8 antigen. However, virtually all CD8⁺ cells detectable in blood and spleen were characterized as CD3⁺ T cells.

This low frequency of NK cells in peripheral tissues is rather unexpected given that in human, mice and rat the frequency of NK cells in spleen and blood is higher. Chickens have a

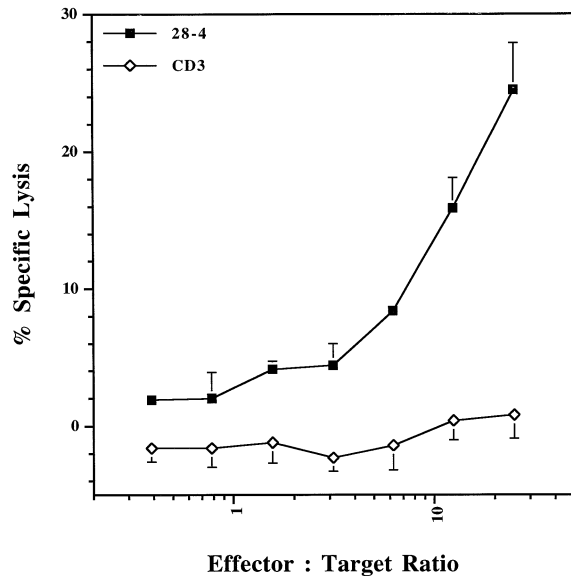


Fig. 5. Spontaneous cytolytic activity is restricted to 28-4⁺ IEL. Freshly isolated IEL were sorted into 28-4⁺ and CD3⁺ subsets, and the spontaneous cytotoxic capacity of both subsets was tested in a 4 h standard assay. Data are represented as mean \pm SD of triplicate values.

different composition of lymphocyte subsets in the periphery as compared to man and mouse. In particular, the number of $\gamma\delta$ T cells is higher with up to 50% of the total splenic or blood T cells (24). $\gamma\delta$ T cells and NK cells may have redundant functions and they may compete for the same physiological niche. It is apparent that different vertebrates need to adapt their tissue-specific lymphocyte ratios to meet specific requirements against the various pathogens they might encounter. Interestingly, a small CD3⁺28-4⁺ population was detected in adult and embryonic IEL. These cells may resemble avian homologues of the mammalian NKT cells (25).

28-4⁺ cells were also readily detected in the embryonic bone marrow and spleen. NK cells are generated in the bone marrow from pluripotent stem cells and then migrate into different sites. It is not clear if and where NK cells are selected. The presence of NK cells in the chicken embryonic spleen suggests that part of this differentiation and selection might take place in the spleen. Since two populations of splenic 28-4⁺ cells could be differentiated, it seems likely that the 28-4⁺ c-kit⁺ population is an immature NK cell precursor that differentiates into the 28-4⁺ cells which co-express high levels of CD25 and CD8 $\alpha\alpha$, and start to down-modulate c-kit levels. These cells might then migrate and home to the intestine.

In mammals, an IEL resident CD8⁺ lymphocyte sub-population has been characterized to constitute thymic-independent T cell progenitors (26). It is unlikely that the cells described here are T cell progenitors, since no extrathymic development is found in the chicken including the IEL compartment (27). Moreover, cells of similar phenotype are also detected in the embryonic spleen and bone marrow. In addition, the spontaneous cytotoxicity of these cells identifies them as fully differentiated effector cells, rather than progenitor cells.

The antigen recognized by the 28-4 mAb resembles a small glycoprotein. Cross-linking of the 28-4 effector cells during the cytotoxicity assay did not have any effect and the 28-4 mAb did not induce redirected killing. We were unable to test the possible role of the 28-4 antigen as a putative inhibitory receptor due to experimental limitations of the chicken system. Cloning of the respective antigen will clarify the exact nature of the antigen and is currently performed using expression cloning techniques.

The tissue distribution and ontogeny of NK cells suggests that these cells develop in the bone marrow, mature in the spleen and then mainly home to the intestinal epithelium. Although remarkable low, there is a small splenic population (~0.5–1%) of cells which expresses CD8 in the absence of Ig and CD3. This putative splenic NK cell population could, however, be expanded following viral infections.

The physiological role of the high number of intestinal NK cells is not known. Obviously, NK cells might constitute the first line of defense once epithelial cells get infected with pathogens. Epithelial $\gamma\delta$ T cells are critically involved in the homeostasis of epithelial cells as demonstrated in several systems. In fact activated $\gamma\delta$ T cells have been demonstrated to produce keratinocyte growth factor, a growth factor for epithelial cells (28). In parallel it may well be that NK cells would serve similar functions and that a functional redundancy between $\gamma\delta$ T cells and NK cells exists.

Acknowledgements

This work was supported by a grant from the 'Deutsche Forschungsgemeinschaft' no. GO 489/3.

Abbreviations

IEL intestinal intraepithelial lymphocyte
PBL peripheral blood lymphocyte

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