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## Characterization of avian natural killer cells and their intracellular CD3 protein complex\*

Natural killer (NK) cell activity appears to be conserved throughout vertebrate development but NK cells have only been well characterized in mammals. Candidate NK cells have been identified in the chicken as cytoplasmic CD3<sup>+</sup> and surface T cell receptor (TCR)/CD3<sup>-</sup> (TCRO) lymphocytes that often express CD8. The fact that the TCRO cells are abundant in the embryonic spleen before T cells enter this organ allowed us to cultivate the embryonic TCRO cells using growth factors derived from activated adult lymphocytes. These TCRO cells were cytotoxic for an NK target cell line. They expressed cell surface CD8, a putative interleukin-2 receptor, CD45 and a receptor for IgG, but did not express CD4, major histocompatibility complex class II or immunoglobulin. Biochemical analysis of the cytoplasmic CD3 antigen revealed two of the three CD3  $\gamma$ ,  $\delta$  and  $\epsilon$  homologues, and RNA transcripts for the third. The CD3 monoclonal antibody also precipitated a 32-kDa dimer that may represent a heterodimer of different CD3 constituents. TCR  $\alpha$  and  $\beta$  gene transcripts were not detected in the TCRO cells. These results indicate that the avian TCRO cell is the mammalian NK cell homologue. The shared evolutionary features of T cells and NK cells in birds and mammals support the idea that they derive from a common progenitor.

### 1 Introduction

A third lineage of lymphoid cells has been identified in the chicken that is developmentally independent of both the thymus and the bursa [1, 2]. These cells are identified by their intracellular expression of a CD3 antigenic determinant and absence of TCR/CD3 determinants on the cell surface. Many of these cells express surface CD8, but none express CD4. These cells, called TCRO, first appear on embryonic day 8 (E8) in the spleen, 4 days before TCR<sup>+</sup> cells appear in the thymus, and they rapidly increase in frequency to reach peak levels of approximately 10% of the splenocytes by E14. In adult birds, the TCRO cells are found mainly in the intestinal epithelium, where they comprise approximately 25% of the CD3<sup>+</sup> cells as detected in tissue sections.

The thymus-independent development of TCRO cells was established in studies of interspecies chick-quail chimeras [1], taking advantage of mAb specific for chicken lymphocyte antigens [3, 4]. Splenic transplants from E6 chick embryos generate TCRO cells in quail recipients [1], whereas precursor cells do not enter the epithelial thymus

before E6.5 [5]. TCRO cells derived from the embryonic spleen fail to develop into T or B cells, thus establishing this as a third lymphocyte lineage.

The functional capabilities of the TCRO cells have not been determined, but the possibility that they may be NK cells is suggested by their phenotypic similarity with human NK cells [6–9]. Moreover, TCRO cells are present in lymphoid tissues that have been shown to contain cells with NK activity [10, 11]. In order to characterize further the composition, nature and function of these candidate NK cells, it was necessary to isolate them in relatively large numbers. For this we have established a culture system for TCRO cells employing the E14 splenocyte population, which contains an abundance of TCRO cells but virtually no T cells [2, 12]. Functional, immunological and biochemical analyses of these TCRO cells indicate that they have NK activity and closely resemble mammalian NK cells in their phenotypic profile.

### 2 Materials and methods

#### 2.1 Animals

Fertile eggs of SC (B<sup>2</sup>B<sup>2</sup>) white leghorn chickens were obtained from Hy-Line International (Dallas Center, IA) and incubated at 41 °C. Embryonic age was estimated by the number of days of incubation. Chicks were hatched and raised in our animal facility.

#### 2.2 Monoclonal antibodies

The CT3, CT4, CT8, TCR1, and TCR3 mAb, respectively defining the chicken CD3, CD4, CD8,  $\gamma\delta$ TCR and  $V\beta 2$ - $\alpha\beta$ TCR, were produced in our laboratory [12–16]. The TCR2 mAb, specific for  $V\beta 1$ - $\alpha\beta$ TCR, and the mAb CL-1, specific for chicken CD45, were kindly provided by Drs. J. Cihak and U. Lösch (Institute for Animal Physiology,

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**Abbreviation:** E: Embryonic day

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Munich, FRG) [17–19]. The TAP-1 mAb recognizing MHC class II molecules [20] was supplied by Dr. N. LeDouarin (Institut d'Embryologie, Nogent-sur-Marne, France). The Ch T6 mAb, which reacts with the putative chicken IL-2R [19, 21], was provided by Dr. G. Wick (Institut for General and Experimental Pathology, Innsbruck, Austria). The Ep42 mAb recognizing the CD8  $\beta$  chain was provided by Michael Ratcliffe (McGill University, Montreal, Canada; [19] and Young, J. et al., unpublished).

### 2.3 Cell lines

The TCR2<sup>+</sup> Marek's disease virus-transformed cell (MDV) line UG9 was provided by Dr. L. W. Schierman (University of Georgia, Athens, GA); the TCR3<sup>+</sup> CD132A MDV line and the avian leukosis virus-induced cell line LSCC-RP9 were from Dr. K. A. Schat (Cornell University, Ithaca, New York); and BM2, an avian myeloblastosis virus-transformed macrophage cell line, was kindly provided by Dr. D. L. Ewert (Wistar Institute, Philadelphia, PA). All cell lines were grown in RPMI 1640 supplemented with 15% FCS, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), nonessential amino acids (1%), sodium pyruvate (1 mM) and 2-ME (50  $\mu$ M) (complete RPMI) at 41°C.

### 2.4 Culture of E14 spleen cells

Single-cell suspensions were obtained by homogenizing E14 spleens between two frosted-end glass slides. Viable cells were isolated using a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient and the viability (> 95%) was determined by trypan exclusion. Cells ( $1 \times 10^6$ /ml) were cultured at 41°C in 6-well plates containing complete RPMI and 10–20% of a conditioned medium, which was changed daily when the cultures were split to maintain a cell density of  $1 \times 10^6$ /ml. The conditioned medium was prepared by culturing adult splenocytes ( $1 \times 10^7$ /ml) in complete RPMI with 10  $\mu$ g/ml Con A (Sigma, St. Louis, MO) for 48 h. The cytokine-containing SN was filtered through a 30-kDa filter (Amicon, Beverly, MA), sterilized by filtration and stored at –20°C.

### 2.5 Immunofluorescence analysis

Cells were stained indirectly with the primary mAb followed by FITC-conjugated goat anti-mouse-Ig (Southern Biotechnology Associates, Birmingham, AL) before analysis on a FACScan flow cytometer as previously described [14]. For cytoplasmic staining, cells ( $1 \times 10^6$ ) were fixed for 20 min at 4°C with 10% phosphate-buffered formaldehyde. After washing, the cell membrane was permeabilized by incubation with 0.125% Triton X-100 in PBS for 3.5 min at room temperature before immunofluorescence staining [22].

### 2.6 IgG-binding assay

Cells ( $1 \times 10^6$ ) were incubated for 30 min at 4°C with 30  $\mu$ l (0.5 mg/ml) heat-aggregated chicken IgG [23]. After three washes in medium, cells were stained with FITC-conju-

gated goat anti-chicken- $\gamma$  for 30 min at 4°C and then washed three times before analysis on a flow cytometer.

### 2.7 Electron microscopic analysis

After fixation with 1.25% glutaraldehyde in cacodylate buffer for 1 h at room temperature, cells were rinsed in cacodylate buffer, post-fixed for 1 h in 1% osmium tetroxide in the same buffer, subsequently dehydrated through a graded ethanol series and embedded in Spurr's medium [24]. Thin sections were stained with uranyl acetate and lead citrate and analyzed with a Philips 301 electron microscope.

### 2.8 NK cell assay

The NK-susceptible, target cell line LSCC-RP9 was used between passages 113 to 120, and the UG9 cell line was used as a control [25]. Target cells ( $5 \times 10^6$ ) of > 95% viability were labeled with 60  $\mu$ Ci <sup>111</sup>indium oxiquinoline solution (specific activity: > 50 mCi/ $\mu$ g indium; Amersham, Arlington Heights, IL) in 0.5 ml HBSS for 10 min at room temperature. The cells were washed three times and preincubated for 1 h at 41°C to reduce spontaneous release. After washing twice, target cells ( $2 \times 10^4$ /100  $\mu$ l) were placed in U-bottom 96-well microtiter plates and effector cells (100  $\mu$ l) in various concentrations were added. Plates were spun at  $50 \times g$  for 5 min and incubated for 4 h at 41°C. Spontaneous release was measured by culturing target cells alone and the total release measured by lysing the cells with 0.5% NP40 followed by centrifugation at  $600 \times g$  for 10 min. The spontaneous release never exceeded 12% of the total <sup>111</sup>In release. Specific release was calculated by the formula: specific release = [(test release-spontaneous release)/(total release – spontaneous release)]  $\times$  100.

### 2.9 Immunoprecipitation and gel electrophoresis

Cytoplasmic proteins were labeled by incubating  $5 \times 10^6$  cells in methionine- and cysteine-free complete RPMI supplemented with dialyzed FCS, 100  $\mu$ Ci each of [<sup>35</sup>S] methionine and [<sup>35</sup>S] cysteine (Amersham, Arlington Heights, IL) for 4 h or 8 h at 41°C. Cytoplasmic iodination was performed according to Anderson et al. [26]. Briefly, cells were fixed in 0.01% formaldehyde for 20 min, permeabilized with 8  $\mu$ M digitonin and then labeled by the lactoperoxidase-catalyzed method with Na<sup>125</sup>I. The labeled cells were lysed with 1% NP40 in 0.05 M Tris-HCl pH 7.5 containing protease inhibitors at 4°C for 30 min [27]. Lysates were immunoprecipitated by a solid-phase method in 96-well microtiter plates and absorbed molecules were eluted with 1% SDS in 0.05 M Tris-HCl with or without 2-ME [27]. Samples were electrophoresed on 12% gels as described [28] and the apparent molecular weight of precipitated molecules calculated according to molecular weight standards after autoradiography.

### 2.10 Northern blot analysis

Total RNA was prepared by the method of Chomczynski et al. [29]. Denatured samples were separated by agarose

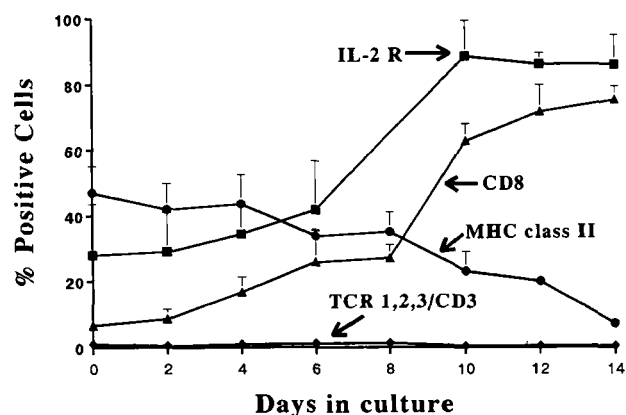
gel electrophoresis and transferred to a nylon membrane (NEN, Boston, MA). Prehybridization was carried out in 50% formamide,  $5 \times$  SSC,  $1 \times$  Denhardt's, 0.5% SDS, 10 mM EDTA, 0.1 mg/ml salmon sperm DNA at 42°C for 6 h. Blots were subsequently hybridized under identical conditions with  $10^6$  cpm random-primed DNA probe/ml. Washing was carried out in  $0.2 \times$  SSC, 0.1% SDS at 68°C. cDNA probes employed for detection included those specific for the 19-kDa CD3 protein [30], the TCR $\alpha$  C region [31], the TCR $\beta$  C region [32], and a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GPDH) as a control of RNA integrity.

### 3 Results

#### 3.1 Growth of TCRO cells from the embryonic spleen

E14 splenocytes were grown in complete RPMI with or without growth factors from Con A-stimulated adult splenocytes in the initial experiments. In both cases, the cultures could be maintained for up to 1 week but cell proliferation was not observed even when the Con A was neutralized with  $\alpha$ -MM. In contrast, the embryonic splenocyte cultures showed vigorous proliferation with a cell doubling time of approximately 36 to 48 h over the first 10 days when cultured in the presence of the conditioned medium that was filtered with a 30-kDa membrane to remove large potentially inhibitory components. When cultured with this fractionated ConA SN, the embryonic spleen cells could be maintained for 3 to 4 weeks.

The cultured E14 splenocytes were lymphoid in morphology, and the frequency of CD8<sup>+</sup> cells increased from 7% to 75% over 14 days in culture (Fig. 1). The number of IL-2R<sup>+</sup> cells similarly increased from approximately 25% to almost 90%. Two-color immunofluorescence analysis confirmed that essentially all of the IL-2R<sup>+</sup> cells coexpress CD8 and vice versa (data not shown). On the other hand, these lymphoid cells did not express HMC class II molecules, and other types of MHC class II-positive splenocytes



**Figure 1.** Cell surface antigen profile of E14 spleen cells in culture. Spleen cells from E14 embryos were cultured with low molecular weight cytokines derived from Con A-activated adult splenocytes and the expression of TCR1, TCR2, TCR3, CD3, CD8, MHC class II and IL-2R was analyzed by indirect immunofluorescence. Each point represents the mean  $\pm$  SD from three or more experiments.

decreased from approximately 50% to less than 10% over the 2-week culture interval. Virtually all of the cells expressed CD45, whereas none of the cultured cells expressed cell surface CD4, CD28, IgM, TCR1, TCR2, TCR3 or CD3 (Fig. 1, data not shown). These data indicate that the expanded population of CD8<sup>+</sup> IL-2R<sup>+</sup> lymphoid cells in the E14 splenocyte cultures were not T cells, B cells or monocytes. Furthermore, the CD8 antigen detected on these cells were CD8 $\alpha$ +CD8 $\beta$ <sup>-</sup> (Fig. 2A).

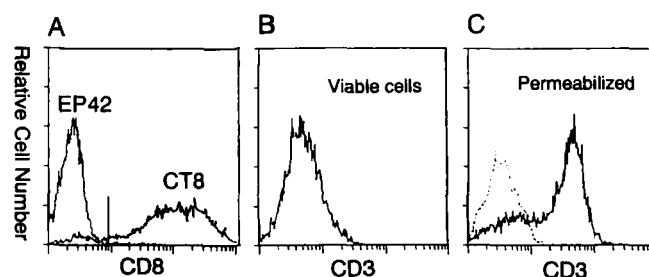
Although the CD3 antigen was not found on the cell surface (Fig. 2B), when the cells were permeabilized to allow detection of intracellular CD3, the frequency of CD3<sup>+</sup> cells was similar to that of the CD8<sup>+</sup> cells in most cultures (Fig. 2C), although the frequency of the cytoplasmic CD3<sup>+</sup> surface CD8<sup>+</sup> cells varied (data not shown). The coexpression of intracellular CD3 and CD8 on the cell surface was confirmed by microscopic examination of cells stained for surface CD8 followed by cytoplasmic staining of CD3. These results indicate that TCRO cells from the E14 spleen can be expanded in culture with lymphocyte-derived cytokines, and these TCRO cells retain most of their *in vivo* phenotypic characteristics.

#### 3.2 TCRO cells are granular lymphocytes

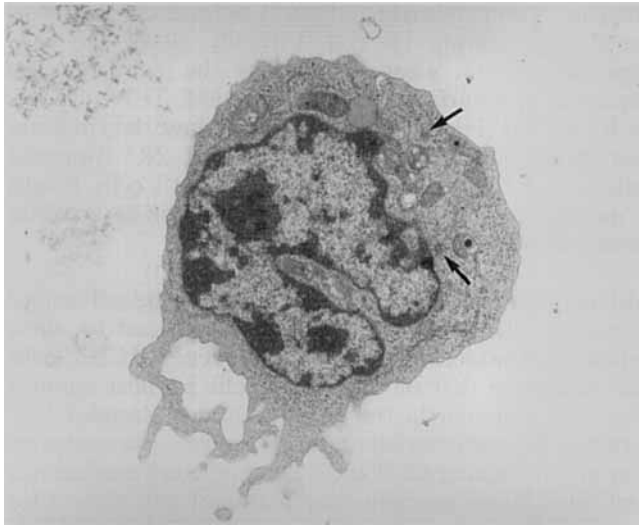
TCRO cells displayed a consistent uniformity of morphological features illustrated in Fig. 3. These lymphoid cells displayed irregular surfaces with long villi. The nuclear to cytoplasmic ratio was low and several organelles were detected in the extended cytoplasm, including mitochondria, isolated profiles of the rough endoplasmic reticulum and small stacks of Golgi cisternae. Electron-dense granules (primary lysosomes) were present, although not abundant, and were scattered in the cytoplasm. The nuclei were indented with Golgi vesicles present in the nuclear notch.

#### 3.3 TCRO cells can bind chicken IgG

A prime characteristic of mammalian NK cells, which the avian TCRO cells resemble, is the expression of Fc $\gamma$ R [33]. When the capacity to bind heat-aggregated chicken IgG was analyzed in an immunofluorescence assay, the TCRO



**Figure 2.** Cultured TCRO cells express cell surface CD8 $\alpha$  chains and intracellular CD3. Embryonic splenocytes cultured for 14 days were analyzed by indirect immunofluorescence for (A) cell surface reactivity with the CT8 mAb (anti-CD8 $\alpha$ ) or with EP42 mAb (anti-CD8 $\beta$ ), (B) cell surface expression of CD3 and (C) cytoplasmic expression of CD3 after permeabilization with detergent. Dashed line indicate background staining with an isotype-matched control mAb of irrelevant specificity.



**Figure 3.** Ultrastructure of a TCRO cell. Note the indented nucleus, the extended cytoplasm with a variety of organelles including vesicles and electron-dense granules (arrows) in this lymphoid cell. Long villi protrude at one end of the cell ( $\times 17300$ ).

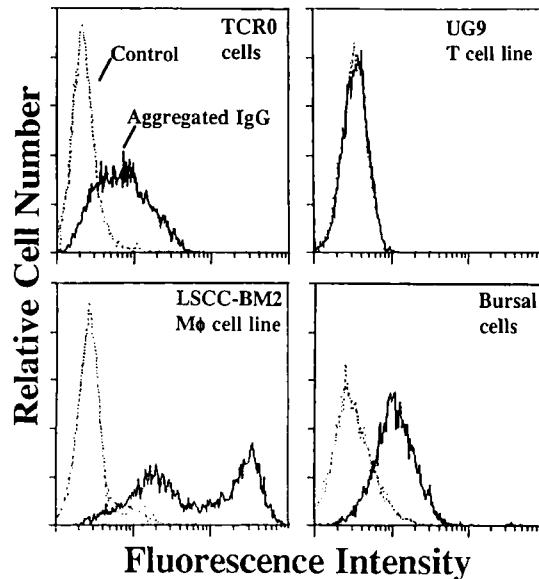
cells were able to bind chicken IgG, suggesting the presence of IgG-R on their surface. Bursal cells and a macrophage cell line also demonstrated IgG-binding capacity as expected, whereas a T cell did not (Fig. 4). The staining profiles for IgG binding were very similar for TCRO and bursal cells, whereas a characteristic bimodal staining pattern was observed for the macrophage cell line.

### 3.4 TCRO cells exhibit NK activity

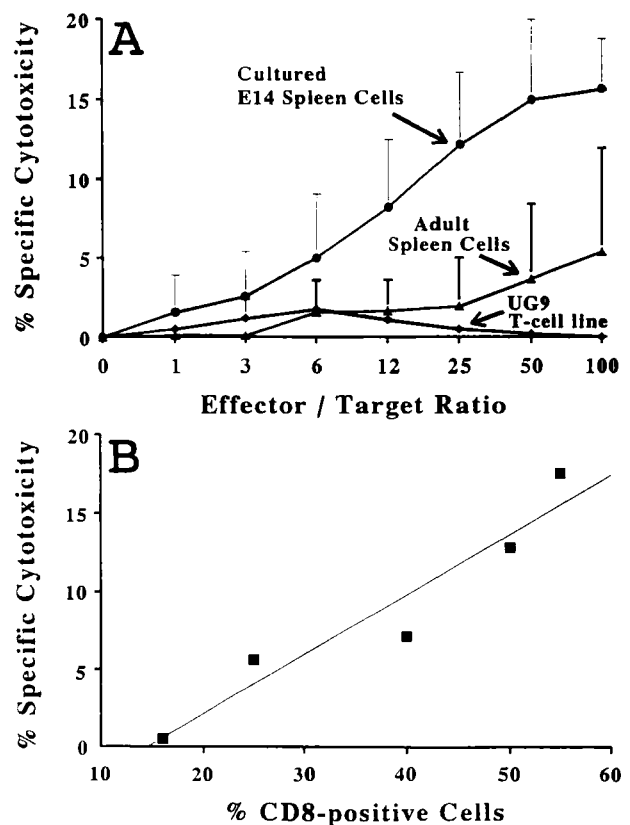
Since the TCRO cells phenotypically resemble mammalian NK cells, the E14 spleen cells cultured from SC chicks were assessed for cytotoxic capability in an  $^{111}\text{In}$  oxiquinolone release assay employing the NK-susceptible cell line, LSCC-RP9 [25]. Spontaneous cytotoxicity was observed against this NK target cell (Fig. 5A) with the highest level of cytotoxicity at an E/T ratio of 50–100:1. When adult splenocytes were analyzed for comparison, their capacity for spontaneous lysis was much lower. The UG9 T cell line lacked cytotoxicity for the LSCC-RP9 cells, and as a target cell line it was not killed by the TCRO cells (data not shown). When the level of NK activity was compared with the frequency of  $\text{CD8}^+$  cells in the TCRO cultures, a direct correlation was observed (Fig. 5B).

### 3.5 Molecular analysis of CD3 and TcR expression by TCRO cells

The gene encoding one of the three previously defined components of the chicken CD3 complex, the 19-kDa protein, has been cloned [30]. Using this cDNA probe, a 1.0-kb transcript was identified in  $\alpha\beta$  T cell lines and TCRO cells. In contrast, this CD3 transcript could not be detected in a macrophage cell line BM 2, liver or brain (Fig. 6). When the Northern blot was rehybridized with probes specific for the  $\text{TCR}\alpha$  or  $\text{TCR}\beta$  C regions, these TCR transcripts were identified in T cells, but were not present in the TCRO cells.



**Figure 4.** TCRO cells can bind chicken IgG. Cultured E14 TCRO cells, the UG9 T cell line, the BM2 macrophage line, and normal bursal cells were incubated with heat aggregated chicken IgG, washed and stained by FITC-conjugated goat antibodies to chicken IgG before analysis by FCM.



**Figure 5.** Cytotoxicity of TCRO cells. (A) TCRO cells lyse target cells in a dose-dependent fashion. The spontaneous cytotoxicity of E14 spleen cells cultured for 14 days, fresh adult spleen cells or the T cell line UG9 were analyzed in a  $^{111}\text{In}$  release assay using different E/T (LSCC-RP9) ratios as indicated. Each point represents the mean  $\pm$  SD from five different experiments. (B) NK cell activity correlates with the frequency of  $\text{CD8}^+$  TCRO cells. Cultured E14 spleen cells harvested at different intervals were assayed for  $\text{CD8}$  expression and spontaneous cytotoxicity at an E/T ratio of 100:1. Correlation coefficient: 0.96.

The protein components of the intracellular CD3 complex were analyzed by immunoprecipitation and PAGE of biosynthetically labeled proteins. After a 4-h labeling period, the same three proteins with apparent molecular weights of 32, 20 and 17 kDa were precipitated from both a T cell line and embryonic TCRO cells (Fig. 7A). When the labeling period was extended to 8 h, an additional band of 19 kDa was precipitated from T cells, whereas this band could not be demonstrated in TCRO cells even after the longer labeling interval. The protein bands of TCRO cells were consistently fainter, compared to T cells after the 8-h labeling period, probably because of rapid degradation of cytoplasmic CD3 proteins, that do not reach the cell surface. To overcome this problem, cytoplasmic proteins were labeled by iodination. When the CD3 immunoprecipitates were analyzed on a 2-D gel, 4 and 5 protein spots were detected in TCRO cells and T cells, respectively (Fig. 7B). T cells and TCRO cells contained identical CD3 proteins of approximately 20 kDa on the diagonal and 18 kDa migrating slightly above the diagonal, as well as two proteins of approximately 19 kDa and 18 kDa, with spots falling below the diagonal. An additional 17-kDa protein was observed on the diagonal in T cells but not in TCRO cells.

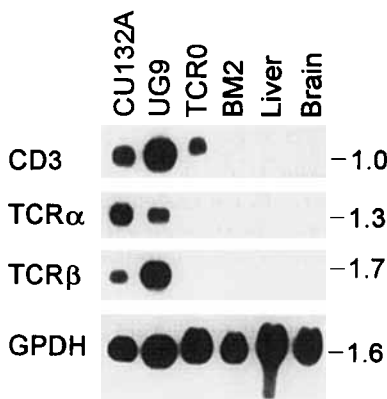


Figure 6. CD3 mRNA expression in TCRO cells. Total RNA from the TCR2<sup>+</sup> UG9 cell line, the TCR3<sup>+</sup> CU132A cell line, 14-day cultured TCRO cells, the macrophage cell line BM2, brain or liver were analyzed by Northern blot using cDNA probes as indicated.

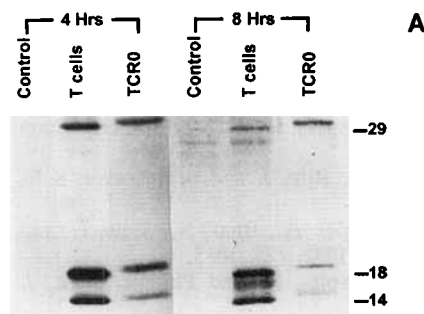


Figure 7. CD3 proteins in T cells and TCRO cells. UG9 T cells or 14 day cultured TCRO cells were metabolically labeled with [<sup>35</sup>S] methionine and [<sup>35</sup>S] cysteine (A) or intracytoplasmically iodinated (B). Cell lysates were immunoprecipitated with either an isotype control mAb or the CT3 mAb as indicated and analyzed on 12% SDS gels under (A) nonreducing conditions or (B) on a 2-D gel.

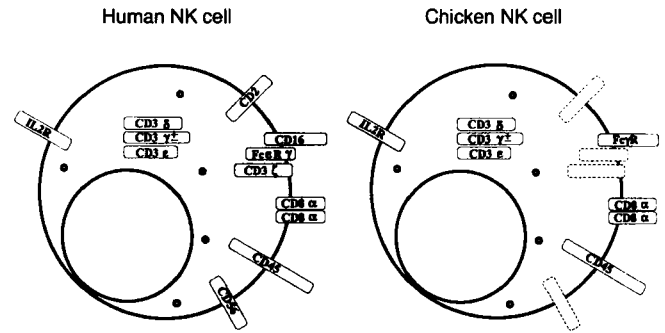


Figure 8. Schematic comparison of human and avian NK cells. Dashed lines represent molecules not yet defined.

### 4 Discussion

The comparative analysis of chicken TCRO cells suggests that they represent the avian homologue of mammalian NK cells (Fig. 8). Initially these cells were identified as a population of chick lymphocytes lacking the antigen receptors characteristic of the T and B cells, but which contain an intracellular CD3 epitope [2]. These so-called TCRO cells are generated outside of the thymus [1] and their early development in the embryonic spleen provided a tissue source virtually free of contaminating T and B cells. The acquisition of an enriched population of TCRO cells in sufficient numbers of phenotypic characterization was rendered possible by the demonstration that embryonic TCRO cells, like mammalian NK cells [34, 35], can be cultured for several weeks in the presence of low molecular weight growth factors supplied by activated T cells. The vast majority of the lymphoid cells that grew in the E14 spleen cultures expressed CD3 intracellularly, CD8α chains on their surface, a binding site for chicken IgG and the 50-kDa chain of a putative IL-2R that is found on activated T cells in the chicken [19, 21]. The putative IL-2R may be an important element in their growth, since IL-2 is one of the growth factors present in SN of Con A-activated chicken splenocytes [36, 37]. Human NK cells constitutively express an intermediate IL-2R composed of β and γ chains, and after activation they also express the 55-kDa α chain to form the high affinity IL-2R [38, 39]. Like mammalian NK cells and their *in vivo* counterparts, the cultured TCRO cells lack both CD4 and the antigen receptors characteristic of T and B cells. In contrast with human NK cells [33], the avian TCRO cells do not express MHC class II and, in this way, resemble rat NK cells [40].

Although only half of the TCRO cells express CD8 molecules in the chicken [2], a vast majority of the TCRO cells in culture were CD8<sup>+</sup>. In mammals CD8 expression has been reported on rat [40] and some human NK cells [41], but not on mouse NK cells [33]. The mammalian CD8 antigen exists in two different forms, an  $\alpha\alpha$  homodimer and an  $\alpha\beta$  heterodimer. Whereas the latter is found on most T cells, the former is expressed on many  $\gamma\delta$  T cells and on NK cells [42]. The TCRO cells react with the anti-CD8 $\alpha$  mAb, but not with the mAb EP-42, specific for the chicken CD8 $\beta$  chain [19], suggesting the presence of a CD8 $\alpha\alpha$  homodimer.

The lymphoid cells in the E14 spleen cultures displayed the typical granular lymphocyte morphology of NK cells [33] and exhibited spontaneous cytotoxicity for an established NK cell target, the LSCC-RP9 tumor cell line [25]. While the avian NK target cell appears more resistant to lysis than the classical mammalian K562 cell target, like mammalian NK cells the TCRO cells lacked cytotoxicity for T cells. The level of NK activity was found to be directly proportionate to the frequency of CD8<sup>+</sup> lymphocytes in the embryonic splenocyte cultures. These results, together with the phenotypic profile, clearly imply that the TCRO cells represent the avian NK homologue.

The intracellular presence of CD3 proteins is an intriguing feature of NK cells. Transcripts encoding the 19-kDa CD3 protein were readily detectable in TCRO cells, despite their lack of  $\alpha\beta$  TCR chain-specific transcripts (Fig. 6). However, protein analysis revealed the presence of two CD3 protein chains of 20 and 17 kDa but undetectable levels of the 19-kDa chain (Fig. 7A). 2-D gel analysis confirmed the presence of the 20-kDa protein on the diagonal and another protein above the diagonal in both T and TCRO cells. The characteristic migration pattern above the diagonal is typical of the mammalian CD3 $\epsilon$  chains because of intra-chain disulfide bonds (Fig. 7B). A third protein observed in T cells could not be detected in TCRO cells.

Adult human NK cell clones have been shown to express CD3 $\epsilon$  and CD3 $\zeta$  transcripts but no TCR proteins [6, 7]. However, NK cell clones established from human embryos have been shown to contain the CD3 $\epsilon$ , CD3 $\delta$  and CD3 $\gamma$  proteins, although the latter was found only in certain clones where the CD3 $\gamma$  protein chains were present in low levels as an immature, partially-glycosylated species [8, 9]. The chicken 19-kDa chain appears to be the CD3 $\gamma$  counterpart that is either present in very low amounts in avian NK cells or in an immature form that co-migrates with the 17-kDa band on SDS-gels. The TCRO cells grown from E14 chick embryos are thus very similar to human fetal NK cells in their intracellular expression of CD3 proteins [9].

A 32-kDa dimeric component (reduced to 19-kDa and 18-kDa proteins falling below the diagonal on the 2-D gel; Fig. 7B) of the CD3 complex was also detected in TCRO and T cells. This heterodimer may represent the association of two CD3 chains. Alternatively the heterodimer could be formed by members of the CD3  $\zeta$  family, which typically form hetero- or homodimers of similar molecular weights in CD3 complexes. However, in T cells the CD3 $\zeta$  proteins normally are associated only with TCR/CD3 complexes, which are not present in TCRO cells. It might be postulated, though, that some yet unidentified components form

complexes with the CD3 chains in NK cells. These complexes could associate with the CD3 $\zeta$  members. At present, it is impossible to identify this heterodimer, since reagents detecting each of the avian CD3 chains are not available. On mammalian NK cells the CD3  $\zeta$  chain is also associated with Fc $\gamma$ RIIIA (CD16) as a disulfide-linked  $\zeta$ - $\zeta$  homodimer or a heterodimer of  $\zeta$  and the Fc $\epsilon$ RI  $\gamma$  chain [6, 7, 43]. The binding of chicken IgG by TCRO cells suggests the existence of a Fc $\gamma$ R (CD16) homologue in the chicken, and emphasize the need to define this receptor unit.

The presence of the CD3 complex of proteins in both NK and T cells, reinforces the idea of a common developmental origin. Although TCR expression clearly distinguishes T cells from NK cells, other phenotypic similarities, such as their responsiveness to IL-2 and cytotoxic capabilities, suggest they are close relatives [44, 45] in spite of the fact that NK cells are developmentally independent of the thymus. However, NK and T cell precursors can be found in the thymus [45–48] and the Fc $\gamma$ RII/RIII<sup>+</sup> thymocyte population in fetal mice includes precursors that give rise to mature T cells in the thymus and NK cells in an extrathymic environment [49]. The identification of the CD3 protein complex in avian NK cells thus favors the speculation that T cells and NK cells may derive from a common ancestral precursor. The preservation of this intracellular complex in NK cells over more than 250 million years in vertebrate evolution could merely represent a prehistoric relic, but it seems likely that the CD3 proteins will prove to have functional significance in the NK cells.

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## 5 References

- 1 Bucy, P. R., Coltey, M., Chen, C. H., Char, D., Le Douarin, N. and Cooper, M. D., *Eur. J. Immunol.* 1989. 19: 1449.
- 2 Bucy, R. P., Chen, C. H. and Cooper, M. D., *Eur. J. Immunol.* 1990. 20: 1345.
- 3 Chen, C. H., Bucy, R. P. and Cooper, M. D., *Semin. Immunol.* 1990. 2: 79.
- 4 Cooper, M. D., Chen, C. H., Bucy, R. P. and Thompson, C. B., *Adv. Immunol.* 1991. 50: 87.
- 5 Coltey, M., Jotereau, F. V. and Le Douarin, N. M., *Cell Differ.* 1987. 22: 71.
- 6 Ciccone, E., Viale, O., Pende, D., Malnati, M., Biassoni, R., Melioli, G., Moretta, A., Long, E. O. and Moretta, L., *J. Exp. Med.* 1988. 168: 2403.
- 7 Anderson, P., Caligiuri, M., Ritz, J. and Schlossman, S. F., *Nature* 1989. 341: 159.
- 8 Phillips, J. H., Hori, T., Nagler, A., Bhat, N., Spits, H. and Lanier, L. L., *J. Exp. Med.* 1992. 175: 1055.
- 9 Lanier, L. L., Chang, C., Spits, H. and Phillips, J. H., *J. Immunol.* 1992. 149: 1876.
- 10 Lillehoj, H. S. and Chai, J. Y., *Dev. Comp. Immunol.* 1988. 12: 629.
- 11 Myers, T. J. and Schat, K. A., *Vet. Immunol. Immunopathol.* 1990. 26: 157.

- 12 Char, D., Sanchez, P., Chen, C. H., Bucy, R. P. and Cooper, M. D., *J. Immunol.* 1990. 145: 3547.
- 13 Chen, C. H., Ager, L. L., Gartland, G. L. and Cooper, M. D., *J. Exp. Med.* 1986. 164: 375.
- 14 Sowder, J. T., Chen, C. H., Ager, L. L., Chan, M. M. and Cooper, M. D., *J. Exp. Med.* 1988. 167: 315.
- 15 Chan, M. M., Chen, C. H., Ager, L. L. and Cooper, M. D., *J. Immunol.* 1988. 140: 2133.
- 16 Lahti, J. M., Chen, C. H., Tjoelker, L. W., Pickel, J. M., Schat, K. A., Calnek, B. W., Thompson, C. B. and Cooper, M. D., *Proc. Natl. Acad. Sci. USA* 1991. 88: 10956.
- 17 Cihak, J., Ziegler-Heitbrook, H. W., Schraner, I., Merkschlager, M. and Lösch, U., *Eur. J. Immunol.* 1988. 18: 533.
- 18 Chen, C. H., Cihak, J., Lösch, U. and Cooper, M. D., *Eur. J. Immunol.* 1988. 18: 539.
- 19 Ratcliffe, M. J. H., Boyd, R., Chen, C. and Vainio, O., *Vet. Immunol. Immunopathol.* 1993. 38: 375.
- 20 Guillemot, F. P., Oliver, P. D., Peault, B. M. and Le Douarin, N. M., *J. Exp. Med.* 1984. 160: 1803.
- 21 Halá, K., Schauenstein, K., Neu, N., Krömer, G., Wolf, H., Böck, G. and Wick, G., *Eur. J. Immunol.* 1986. 16: 1331.
- 22 Wilson, T. J., 1990. Ph.D. thesis, Monash University, Melbourne, Australia.
- 23 Nowak, J. S., Vainio, O., Granfors, K. and Toivanen, P., *Dev. Comp. Immunol.* 1982. 6: 329.
- 24 Spurr, A. T., *J. Ultrastruct. Res.* 1969. 26: 31.
- 25 Sharma, J. M. and Okazaki, W., *Infect. Immun.* 1981. 31: 1078.
- 26 Anderson, P., Blue, M.-L., O'Brien, C. and Schlossman, S. F., *J. Immunol.* 1989. 143: 1899.
- 27 Chen, C. H., Chanh, T. and Cooper, M. D., *Eur. J. Immunol.* 1984. 14: 385.
- 28 Laemmli, U. K., *Nature* 1970. 227: 680.
- 29 Chomczynski, P. and Sacchi, N., *Anal. Biochem.* 1987. 162: 156.
- 30 Bernot, A. and Auffray, C., *Proc. Natl. Acad. Sci. USA* 1991. 88: 2550.
- 31 Göbel, T. W. F., Chen, C. H., Lahti, J., Kubota, T., Kuo, C. L., Aebersold, R., Hood, L. and Cooper, M. D., *Proc. Natl. Acad. Sci. USA* 1994. 91: 1094.
- 32 Tjoelker, L. W., Carlson, L. M., Lee, K., Lahti, J., McCormack, W. T., Leiden, J. M., Chen, C. H., Cooper, M. D. and Thompson, C. B., *Proc. Natl. Acad. Sci. USA* 1990. 87: 7856.
- 33 Trinchieri, G., *Adv. Immunol.* 1989. 47: 187.
- 34 Suzuki, R., Handa, K., Itoh, K. and Kumagi, K., *J. Immunol.* 1983. 130: 981.
- 35 Dennert, G., *Nature* 1980. 287: 47.
- 36 Schauenstein, K., Globerson, A. and Wick, G., *Dev. Comp. Immunol.* 1982. 6: 533.
- 37 Schnetzler, M., Oommen, A., Nowak, J. S. and Franklin, R. M., *Eur. J. Immunol.* 1983. 13: 560.
- 38 Phillips, J. H., Takeshita, T., Sugarmura, K. and Lanier, L. L., *J. Exp. Med.* 1989. 170: 291.
- 39 Voss, S. D., Sondel, P. M. and Robb, R. J., *J. Exp. Med.* 1993. 176: 531.
- 40 Vujanovic, N. L., Herberman, R. B., Maghayachi, A. A. and Hiserodt, J. C., *J. Exp. Med.* 1988. 167: 15.
- 41 Ritz, J., Schmidt, R. E., Michion, J., Hercend, T. and Schlossman, S. F., *Adv. Immunol.* 1988. 42: 181.
- 42 Terry, L. A., Disanto, J. P., Small, T. N. and Flomenberg, N., in Knapp, W., Dorken, B. and Gilks, W. R. (Eds.), *Leucocyte Typing IV. White Cell Differentiation Antigens*, Oxford University Press, Oxford 1989, p. 345.
- 43 Lanier, L. L., Yu, G. and Phillips, J. H., *Nature* 1989. 342: 803.
- 44 Ortaldo, J. R. and Herberman, R. B., *Annu. Rev. Immunol.* 1984. 2: 359.
- 45 Moretta, L., Ciccone, E., Mingari, M. C., Biassoni, R. and Moretta, A., *Adv. Immunol.* 1994. 55: 341.
- 46 Poggi, A., Biassoni, R., Pella, N., Paolieri, F., Bellomo, R., Bertoni, A., Moretta, L. and Mingari, M. C., *J. Exp. Med.* 1990. 172: 1409.
- 47 Mingari, M. C., Poggi, A., Biassoni, R., Bellomo, R., Ciccone, E., Pella, N., Morelli, L., Veriani, S., Moretta, A. and Moretta, L., *J. Exp. Med.* 1991. 174: 21.
- 48 Hori, T. and Spits, H., *J. Immunol.* 1991. 146: 2116.
- 49 Rodewald, H. R., Moingeon, P., Lucich, J. L., Dosiou, C., Lopez, P. and Reinherz, E. L., *Cell* 1992. 69: 139.