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Identification of novel chicken CD4+ CD3− blood population with NK cell like features

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ABSTRACT

Chicken NK cells have been defined in embryonic spleen and intestinal epithelium as CD8+ lymphoid cells that lack BCR and TCR, whereas blood NK cells have not been phenotypically defined. Here we employed the mab, 8D12 directed against CHIR-AB1, a chicken Fc receptor, to define a previously uncharacterized lymphoid cell population in the blood. Although CHIR-AB1 expression was found on several cell populations, cells with extraordinary high CHIR-AB1 levels ranged between 0.4 and 2.8% in five different chicken lines. The widespread applicability of the CHIR-AB1 mab was unexpected, since CHIR-AB1-like genes form a polygenic and polymorphic subfamily. Surprisingly the CHIR-AB1 high cells coexpressed low MHCII, low CD4 and CD5, while other T cell markers CD3 and CD8, the B cell marker Bu1, the macrophage marker KUL01 were absent. Moreover, they stained with the mab 28-4, 20E5 and 1G7, which define chicken NK cells and they also expressed CD25, CD57, CD244 and the vitronectin receptor (αVβ3 integrin). In functional assays, PMA stimulation led to high levels of IFNγ release, while spontaneous cytotoxicity was not detectable. The expression of typical NK cell markers in the absence of characteristic B- or T-cell markers, and their IFNγ release is suggestive of a yet unidentified NK like population.

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1. Introduction

Natural killer cells are believed to represent phylogenetically ancient cells that are present in mammals and other vertebrates as part of the innate immune system (Yoder and Litman, 2011). They were initially described as cells that are cytolytic against tumor cells or virally infected cells without prior immunization. Subsequent analyses have identified NK cells as major source of interferon-γ and other cytokines (Vivier et al., 2008).

In the last decade NK cells in mammals have been intensively characterized including their functional and phenotypic properties. It is apparent from these studies that different receptor combinations can be used to phenotypically identify NK cells in various species (Walzer et al., 2007). For instance, human blood NK cells can be stained with mab against CD16 and CD56 (Lanier et al., 1983), whereas for mice the NK1.1 molecule on CD3− cells has been a useful combination. One of the markers applicable for staining NK cells in quite a lot of different species including humans, mice, cattle, and swine is NKp46 (CD335), a receptor characterized by two C2 type Ig domains and a transmembrane domain with a charged residue that allows the association to adaptor proteins (Pessino et al., 1998). It is encoded by the leukocyte receptor complex (LRC) a region on human chromosome 19q13.4 and mouse chromosome 6. This chromosomal area is of particular importance for NK cells, since it encodes distinct groups of NK cell receptors and it has highly variable gene content within and between species (Barrow and Trowsdale, 2008; Parham, 2008).

A syntenic region to the mammalian LRC is present on chicken microchromosome 31. It encodes a large family of receptors that display extracellular Ig C2 type domains. Depending on the transmembrane and cytoplasmic domains these so-called chicken Ig-like receptors (CHIR) are further grouped in activating, bifunctional and inhibitory receptors (Viertlboeck et al., 2004, 2005). CHIR are highly diversified with more than hundred receptors in a given animal (Lochner et al., 2010; Viertlboeck et al., 2010). CHIR ligands are largely unknown with the exception of CHIR-AB1, that is a high affinity ligand for IgY (Viertlboeck et al., 2007, 2009b). The single Ig domain of CHIR-AB1 shares structural homology to several mammalian LRC members such as the D1 domains of the FcαRI, KIR2DL1,

Abbreviations: CHIR, chicken Ig-like receptor; FcR, Fc receptor; LRC, leukocyte receptor complex.

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and NKp46 (Arnon et al., 2008). The identity of CHIR to several mammalian LRC encoded receptor families such as NKp46, KIR, PIR, ILT and GPVI ranges between 30 and 40%. Therefore CHIR may be functional homologs to all of these different receptor families. The CHIR expression patterns available by either RT-PCR or with the help of CHIR specific mab has revealed that individual CHIR may have either a restricted expression such as B-lymphocyte specific or they are broadly distributed on a variety of leukocyte subsets (Viertlboeck et al., 2004, 2005). Therefore, the characterization of chicken NK cells by a mab against NKp46 seems currently not straightforward since it is not possible to pick candidate CHIR genes which may functionally represent NKp46 and that are solely expressed by NK cells.

The other genomic area encoding an array of different NK cell receptors resembles the NK gene complex on human chromosome 12 and mouse chromosome 6 (Yokoyama and Plougastel, 2003). In this region, various C-type lectins are encoded that form homo- or heterodimers on the surface of NK cells and include the murine Ly-49 family and the NKG2/CD94 family. In the chicken, however, a syntenic area of this region on chromosome 1 only encodes two C-type lectin receptors that have been characterized as a CD69 and a CLEC-2 homolog with no specific expression on NK cells (Chiang et al., 2007; Neulen and Göbel, 2012). So similar to the LRC, this genomic area covered by NK cell receptor genes in mammals is also not a good source of genes to establish novel NK cell markers in the chicken. Other potential NK cell receptors such as two C-type lectins in the vicinity of the MHC locus in the chicken are reactive with T lymphocytes (Rogers et al., 2005; Viertlboeck et al., 2008b).

Up to now, chicken NK cells were mainly defined by virtue of CD8 expression in the absence of surface TCR/CD3 molecules (Rogers et al., 2008). Using this definition, we and others have characterized NK cells in the embryonic spleen and also a substantial population in intestinal epithelial lymphocytes, whereas in blood, these cells were negligible (Göbel et al., 1994, 2001). We also showed that the chicken NK cells like their mammalian counterparts have the ability to bind heat aggregated chicken IgY, thus arguing for the presence of a Fc receptor on their surface (Göbel et al., 1994). In order to track these results with novel tools, we now made use of the mab against CHIR-AB1, as a prototypic chicken Fc receptor binding to IgY. Since CHIR-AB1 has a wide tissue distribution on a variety of leukocytes we tried to utilize different markers in order to find a suitable combination staining NK cells. Here we characterize a novel blood lymphocyte subset expressing high CHIR-AB1 levels, which is lymphoid in morphology. These cells bear no markers for B-, or T-cells except CD4 and CD5. They express several NK cell markers including CD25, CD57, CD244, the vitronectin receptor and other molecularly undefined NK cell markers, and they can be induced to produce IFNγ protein.

2. Materials and methods

2.1. Animals and cell preparation

Fertilized eggs from following chicken lines were hatched at the Institute of Animal Physiology, University of Munich: M11 (RSV RES Line Mr; MHC:B2) and R11 (RSV RES Line R; MHC:B15) both kindly provided by S. Weigend, Institute for Animal Breeding, Mariensee, Germany; CB (MHC:B15) kindly provided by J. Plachy, Institute for Molecular Genetics, Prague, Czech Republic; DIAS B21-21 (MHC: H-B21) kindly provided by H. Juul-Madsen, Aarhus University, Denmark, and IAH 6_1 (MHC:B2) and IAH 7_2 (MHC:B2) kindly provided by N. Venugopal, The Pirbright Institute, Compton, Newbury, U.K. The chickens were used for the experiments at the age of 2–12 months. Heparinized blood was prepared by either density centrifugation or by slow speed centrifugation (Göbel et al., 1996b). In detail, for density centrifugation, blood was mixed with an equal volume of PBS and layered slowly above a double volume of Biocoll (with 10 mM HEPES; density 1.077 g/ml) (Biochrom AG, Berlin, Germany). After centrifugation at $600 \times g$ for 12 minutes at room temperature, the PBMC in the interphase were obtained and washed three times with cold PBS prior further analysis. For slow speed preparation blood were centrifuged at $60 \times g$ for 15 min at room temperature. Following this centrifugation, the blood was separated into a lower part containing cells and a yellowish part with plasma. The PBL in the buffy coat form a white layer, which can be collected by carefully resuspending these cells with a Pasteur pipette. The cells were washed twice with cold PBS prior further analysis.

2.2. Antibodies, flow cytometry, cell sorting and cytospin

The mab used for cell staining were: 8D12 (CHIR-AB1, mouse IgG2a) (Viertlboeck et al., 2007), CT3 (CD3, mouse IgG1, Southern Biotechnology Associates, SBA, Birmingham, USA), CT4 (CD4, mouse IgG1, SBA), 3–58 (CD5, mouse IgG1 (Koskinen et al., 1998)), CT8-PE conjugate (CD8, mouse IgG1, SBA), AV20-PE conjugate (Bu-1, mouse IgG1, SBA), 2G11 (MHCII, mouse IgM, SBA), KUL01 (mouse IgG1 (Mast et al., 1998)), 28-4 (mouse IgG3 (Göbel et al., 2001)), HNK1 (anti-human-CD57, mouse IgM, SBA), AV142 (CD25, mouse IgG1, AbD Serotec, MorphoSys GmbH, Düsseldorf, Germany), 23C6 (anti-human αVβ3 Integrin, CD51/CD61, mouse IgG1, Biolegend, San Diego, USA (Viertlboeck and Göbel, 2007)), 8C7 (CD244, mouse IgG1 (Straub et al., 2014)), 20E5 (mouse IgG1 (Jansen et al., 2010)), 1G7 (mouse IgG1 (Jansen et al., 2010)) were used. 8F2 (mouse IgG1) is a mab that putatively recognizes a CD11c homolog due to our unpublished observations regarding tissue distribution and immunoprecipitation data and it was cited by others as CD11c (de Geus et al., 2012; Wu et al., 2010). In case of double immunofluorescence analysis the cells were first incubated with a mixture of primary mab, followed by washing and incubation with a goat-anti-mouse-IgG2a-FITC conjugate (SBA) and depending on the isotype of the primary antibody with goat-anti-mouse IgG1-PE (SBA), goat-anti-mouse IgG3-PE (SBA), goat-anti-mouse IgM-PE (SBA) or the direct conjugated antibody (AV-20-PE, CD8-PE). Labeled cells were either analyzed by flow cytometry (FACS Canto II, Beckton Dickinson) using the BD FACS DIVA 6.1.3 software or cell sorting was performed (FACS Aria II, Beckton Dickinson) to isolate 8D12 high 8F2 intermediate cells and 8D12 intermediate and 8F2 high cells under liquid-cooled conditions (4 °C). Sorted cells were used for the ELISPOT assay.

2.3. ELISPOT

ELISPOT assay for IFN-γ release of CHIR-AB1 high cells was performed as described previously (Reemers et al., 2012). Briefly, 96-well Multiscreen plates (Merck MilliPore, Darmstadt, Germany) were coated with recombinant anti-chicken IFN-γ mab (2 μg/ml, Invitrogen, Karlsruhe, Germany) at 4 °C overnight. Plates were washed twice with PBS containing 0.5% (v/v) Tween 20 (Applichem, Darmstadt, Germany) and blocked with culture medium (RPMI 1640/10%FCS/P/S, Invitrogen, Karlsruhe, Germany) for 1 h at 40 °C. 5.6×10^4 cells/well were either stimulated with PMA (40 ng/ml) and Ca-Ionophore (2 μg/ml, both Sigma, Deisenhofen, Germany) or incubated with culture medium as negative control for 36 h at 40 °C, 5% CO_2. Plates were washed 5 times with PBS-T and incubated with anti-chicken IFNγ-biotin (1 μg/ml, Invitrogen, Karlsruhe, Germany) for 1 h at room temperature. After washing 5 times with PBS-T, polyHRP (0,2 μg/well, Sanquin, Amsterdam, The Netherlands) was incubated for 1 h at room temperature. Spots were developed by adding TMB substrate (Sanquin, Amsterdam, The Netherlands) until spots appear and stopped with H_2O. After drying, spot-forming cells (SFCs) were counted by an immunospot image analyzer (A.EL.VIS, Universal Plate Scanner Eli.Scan+, Hannover, Germany) using Eli.Analyse V6.1 software.

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