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CD40 ligand supports the long-term maintenance and differentiation of chicken B cells in culture

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Summary
TNF family members play crucial roles in mammalian B-cell differentiation and function, many of which have not been demonstrated in other species. To investigate the avian CD40/CD40L system, a chicken CD40 cDNA, obtained by expression screening, was used to raise monoclonal antibodies showing that CD40 was expressed on chicken B cells, monocytes and macrophages, like mammalian CD40. CD40 ligand fusion protein supported the proliferation of B cells in culture for up to 3 weeks, during which they differentiated towards a plasma cell phenotype. CD40L-activated B cells from immunised birds secreted antigen-specific IgM and IgG. These results showed important conserved functions of CD40 and its ligand in mammals and birds. CD40L provides a means for maintenance and differentiation of untransformed chicken B cells in culture, for the first time, allowing new approaches to study of post-bursal B cell biology and host–pathogen interactions with B cell tropic viruses.

Introduction

The divergence of birds from mammals, about 300 million years ago, represents an important waypoint in the evolution of the vertebrate immune system. Immunoglobulin-producing B cells, one of the hallmarks of adaptive immunity, are present in all main classes of jawed vertebrates: cartilaginous fish, bony fish, amphibians, reptiles, birds and mammals (reviewed in [1]). All jawed vertebrates generate extensive diversity of antigen-binding B cell receptors by recombination-activating gene-mediated V(D)J recombination and somatic hypermutation of immunoglobulin (Ig) genes [2], but Ig isotype class switching, attaching different constant regions to recruit effector mechanisms, is not seen before amphibians [3]. Although the generation of TCR diversity in the thymus is similar in

Abbreviations: BCM, B cell medium; GALT, gut-associated lymphoid tissue; Ig, immunoglobulin; L chain, Ig light chain.

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almost all taxa [4], BCR diversity is achieved in different ways in different vertebrate species. Reptiles, amphibians and most bony fish diversify their BCRs by gene rearrangement, like humans and mice. However, most other mammals and all bird species studied produce very limited diversity from rearrangement and rely instead on somatic hypermutation and/or gene conversion [5–10]. In these species, the extra diversification stage of B cell development takes place mainly in specialised gut-associated lymphoid tissue (GALT) such as the bursa of Fabricius in the chicken, which is the best-studied model for these so-called GALT species.

Gene conversion, as a mechanism of BCR diversification, was initially described in the chicken [5,6] and the molecular events involved are quite well understood. However, very little information exists on signals that control cell death and survival and developmental checkpoints in avian B cell compartments, either during the gene conversion process in the bursa or at later stages. In contrast to human and mouse, where B cell lymphopoesis is a lifelong process in the bone marrow [7,8], the bursa of Fabricius involutes with sexual maturity and diversification ceases. The maintenance of the peripheral antigen-responsive post-bursal B cell pool therefore requires mechanisms different from those involved in mammalian B cell homoeostasis [9].

In mammals, members of the tumour necrosis factor (TNF) family and their receptors are well known as important regulators of B cell development and function [10,11]. Although both TNFα itself and lymphotoxin-α have not been found in the chicken [12], several members of the chickens TNF/TNF-receptor family have been identified, including Fas/FasL, Ox40L, Trail/TrailL, CD30/CD30L [13] and Rank/RankL [12–15]. Recent progress has been made by the cloning and recombinant expression of chicken BAFF and chicken CD40L (CD154), two TNF family members essential for B cell survival and differentiation in mammals. Like its human orthologue, chicken BAFF was shown to function as survival factor for mature peripheral B cells [16]. Experiments with bursal B cells revealed additional functions in early B cell development, in contrast to human and mouse [17].

The importance of the CD40/CD40L system for T cell-dependent B cell responses was first recognised in patients suffering from X-linked Hyper-IgM syndrome, whose inability to form germinal centres and initiate isotype switching is caused by mutations in the CD40L gene [18,19]. Further studies have shown that the interaction between CD40 and CD40L is pivotal for the regulation of B cell proliferation, rescue of B cells from apoptosis, Ig production, differentiation into germinal centre cells, Ig class switching and maturation into memory cells (reviewed in [20,21]). Initially, CD40 expression was detected during all stages of B cell development, and the expression of its membrane-bound ligand seemed to be restricted to activated $CD4^+$ T cells. More recently a much broader expression pattern for both molecules was observed, including the major observation that activation through CD40 on dendritic cells and monocytes is critical for antigen-presenting cell maturation [22] and the fact that CD40 expression is not restricted to cells of the immune system but can be also found, for example, on endothelial cells [23,24].

Chicken orthologues of CD40 and CD40L were recently identified, and initial studies with a soluble trimeric chicken CD40L fusion protein showed that CD40 activation with this protein led to the induction of NO synthesis in a macrophage cell line and enhancement of splenic B cell survival [25].

In the present study, we used this fusion protein to further characterise the CD40/CD40L system in the chicken. Using soluble CD40L we could demonstrate the effects of CD40 activation on B cell proliferation and antibody production, and we were able to establish long-term cultures of primary chicken B cells which, for the first time, enable functional studies of cultured primary non-transformed chicken B cells.

Materials and methods

Animals

Fertilised eggs of M11 (B^2/B^2) chickens were obtained from the Institute for Animal Science, Mariensee [26], and hatched and raised at the Institute of Animal Physiology, Munich. Organ samples were taken from 6 to 8-week-old birds.

Cells

Chicken splenocytes, cells of the bursa of Fabricius and the caecal tonsils were separated by density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Freiburg, Germany). PBL were prepared by density gradient centrifugation on Ficoll-Paque or by slow speed centrifugation as described previously [27]. Cells were maintained in standard medium (RPMI 1640 medium with glutamax (Gibco, UK) supplemented with 10% FCS, 100 IE/ml Penicillin and 100 μg/ml Streptomycin) or B cell medium (IMDM with glutamax, 8% FBS, 2% chicken serum, 50 mM β-mercaptoethanol, insulin–transferrin–sodium selenite supplement (Roche Applied Science, Mannheim, Germany, 100 μl/l), 1% penicillin/streptomycin) at 40 °C in a 5% CO_2 humidified incubator.

Splenic B cell preparations of $>93\%$ purity were obtained by magnetic cell sorting on MACS separation columns LS (Miltenyi Biotec GmbH, Germany) using a chicken Ig light chain (L chain)-specific monoclonal antibody (Southern Biotechnology Associates, Birmingham, AL), anti-mouse-Ig-FITC (Sigma, Taufkirchen, Germany) and anti-FITC Micro Beads (Miltenyi Biotec GmbH, Germany).

Macrophages were isolated from PBL preparations by adherence to tissue culture dishes (Nunc, Wiesbaden, Germany) essentially as described [28]. Briefly, 1×10^7 PBL/ml were separated on Ficoll-Paque and cultured in standard medium for 72 h. Subsequently, non-adherent cells were removed by intensive washing with PBS. For flow cytometry macrophages were detached with a cell scraper (Nunc, Wiesbaden, Germany).

Recombinant fusion proteins and antibodies

The mouse CD8-chicken CD40L (m8c154) fusion protein and a control fusion protein in which a single substitution abrogated CD40 binding (m8c154 $\times$ 1) were produced in CHO cells exactly as described [25]. Each protein was purified by affinity chromatography on a separate column