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The chicken signaling lymphocytic activation molecule (SLAM) family

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Dedicated to my parents in love and gratitude

“You`ve gotta dance like there`s nobody watching,
Love like you`ll never be hurt,
Sing like there`s nobody listening,
And live like it`s heaven on earth.”

- William W. Purkey

CONTENTS

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
BLAME	B lymphocyte activator macrophage expressed
CD300L	CD300 antigen like
CD200R	CD200 receptor
CHIR	Chicken Ig-like receptor
CRACC	CD2-like receptor-activating cytotoxic cells
DAP	DNAX-activating protein
DC	Dentritic cell
EAE	Experimental autoimmune encephalomyelitis
EAT-2	Ewing`s sarcoma-associated transcript-2
EBV	Epstein-Barr virus
ERT	EAT-2 related transducer
EST	Expressed Sequence Tag
FcRγ	Fc receptor common gamma chain
GPI	Glycosyl-phosphatidylinositol
GPVI	Platelet collagen receptor glycoprotein VI
HPC	Hematopoietic progenitor cells
HSC	Hematopoietic stem cell
IgSF	Immunoglobulin superfamily
IFN-γ	Interferon-gamma
IL	Interleukin
iNKT cell	invariant natural killer T cell
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
KARAP	Killer cell-activating receptor-associated protein
KIR	Killer cell immunoglobulin-like receptor
LAIR	Leukocyte associated Ig-like receptor
LILR	Leukocyte Ig-like receptor
LLT1	Lectin-like transcript 1
LRC	Leukocyte receptor complex
LSK	$Lineage^{-/low}$ $Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$
mab	monoclonal antibody
MHC	Major histocompatibility complex
MPP	Multipotential progenitor
MM	Multiple myeloma
NCR	Natural cytotoxicity receptor
NKC	Natural killer complex
NK cell	Natural killer cell
NO	Nitric oxide
NTB-A	NK-, T- and B-cell antigen
OSCAR	Osteoclast-associated receptor
PAK	P21 activated kinase
PIR	Paired immunoglobulin-like receptor
PIX	Pak-interacting exchange factor
PKC-θ	Protein kinase C-θ
PTK	Protein tyrosine kinase

SAP	SLAM-associated adaptor protein
SIRP	Signal-regulatory protein
SH2	Src homology 2
SH3	Src homology 3
SHIP-1	SH2 domain-containing inositol-5-phosphatase
SHP-1	SH2 domain-containing protein tyrosine phosphatase-1
SHP-2	SH2 domain-containing protein tyrosine phosphatase-2
SLAM	Signaling lymphocytic activation molecule
TCR	T cell receptor
T_H1	T helper 1
T_H2	T helper 2
TNF	Tumor necrosis factor
TREM	Triggering receptor expressed on myeloid cells
XLP	X-linked lymphoproliferative disease

1. INTRODUCTION

Vertebrates possess an immune system that ensures the recognition of a multitude of exogenous and endogenous pathogens. In order to battle pathogens without causing severe immune-mediated tissue damage, sophisticated mechanisms have evolved to regulate immune cell functions. Depending on the kind of immune reaction, leukocytes can either be activated to eliminate infected or transformed cells, or be suppressed to terminate an immune response, to prevent autoreactivity and unrestrained host tissue damage. These two mechanisms, namely activation and inhibition, are achieved by an arsenal of activating and inhibitory receptors which can be mostly assigned to two groups of immunoregulatory receptor families, the C-type lectin superfamily and the immunoglobulin superfamily (IgSF). Many genes encoding C-type lectin receptors are clustered in the "natural killer gene complex" (NKC), a genomic region found on human chromosome 12p13.1 and murine chromosome 6, respectively [1, 2]. This locus harbors firstly genes found in both species, such as the receptors NKG2D and CD94/NKG2A, and secondly the Ly49 family which is abundant in mice but a non-functional gene complex in humans [3]. In contrast, the immunoglobulin superfamily comprises over 850 genes found throughout the genome. The "leukocyte receptor complex" (LRC) on human chromosome 19q13.4 constitutes a major locus of IgSF-related genes [4]. It harbors several multigene families including the killer cell Ig-like receptors (KIR), the leukocyte immunoglobulin-like receptors (LILR) as well as the leukocyte associated Ig-like receptors (LAIR). Other genes affiliated to the so called extended LRC include the osteoclast-associated receptor (OSCAR), the platelet collagen receptor glycoprotein VI (GPVI) and the natural cytotoxicity receptor 1 (NCR1, also known as NKp46). Synteny to human LRC is mapped to mouse chromosome 7, albeit structural orthologues to the KIR genes are missing. Instead, the murine LRC harbors genes encoding paired Ig-like receptors (PIR) which are closely related to the human KIRs, LILRs and LAIRs and thus might share common ancestors [4, 5].

In general, activating receptors classically possess a basic amino acid in the transmembrane region that interacts with immunoreceptor tyrosine-based activating motif (ITAM)-containing adaptors such as the DNAX-activating protein of 12 kDa (DAP12, also named killer cell-activating receptor-associated protein (KARAP)) and 10 kDa (DAP10), respectively, and the Fc receptor common γ chain (FcRγ) [6-11]. This interaction initiates an intracellular signaling cascade resulting in cellular activation.

In contrast, inhibitory receptors contain a similar motif in their long cytoplasmic domain, an immunoreceptor tyrosine-based inhibitory sequence (ITIM) [12]. Here, ligand engagement results in the phosphorylation of motif-based tyrosine residues, which impede cellular

activation by recruiting inhibitory Src homology 2 (SH2)-domain containing phosphatases [13-15].

Besides these two major genomic regions, several other though smaller gene families involved in immune regulation are found throughout the genome, such as the natural cytotoxicity receptors (NCR), the signal-regulatory protein (SIRP) family, the triggering receptor expressed on myeloid cells (TREM) family, the CD300 receptor family or genes related to the signaling lymphocytic activation molecule (SLAM) family. In mouse and man, the SLAM locus has been mapped to chromosome 1 and currently harbors nine family members, SLAMF1 to SLAMF9 [16-20].

2. OBJECTIVE

Mammals and aves have a similar immune system, albeit both diverged from a common ancestor about 300 million years ago, and comparative studies provide new insights into the evolution and development of the vertebrate immune system. Over the past years, several immunoregulatory receptor families have been described in the chicken. The syntenic locus to the human NKC has been mapped to chicken chromosome 1, although this region comprises only two C-type lectin-like genes, namely orthologues to mammalian CD69 and CLEC-2 [21, 22]. Two additional C-type lectin-like genes, chicken orthologues to mammalian NKR-P1 and lectin-like transcript 1 (LLT1), B-NK and B-Lec, respectively, are mapped to the chicken major histocompatibility complex (MHC) on chromosome 16 [23, 24]. In contrast, immunoregulatory receptors assigned to the immunoglobulin superfamily (IgSF) are found throughout the chicken genome. The chicken orthologue to the human LRC, for instance, is broadly expanded and located on microchromosome 31. Only one multigene family encoding chicken Ig-like receptors (CHIR) has been mapped to this chromosomal site [25, 26]. The CHIR locus contains over 100 genes and functional homology to KIRs, LILRs and LAIRs, gene families clustered on the human LRC, has been proposed [27-30]. Genes assigned to the extended human LRC, however, such as OSCAR, NCR1 (NKp46) or GPVI, are missing or have not been identified in chickens yet. In addition, several other immunoregulatory receptor families have been documented in the chicken, in particular the SIRP family, the TREM family, the CD200 receptor (CD200R) family and the CD300 antigen like (CD300L) family, respectively [31-33].

So far, the SLAM family of membrane receptors has been only documented in mammals and in *Xenopus tropicalis*, recently [18, 34, 35]. The present study had two objectives. The first part was conducted to clarify if SLAM- and SLAM-associated adaptor protein (SAP)-related genes exist in the chicken genome, which would provide new insights in the phylogeny of this immunoregulatory receptor family. Therefore, the chicken genome was searched for the presence of SLAM- and SAP-related genes, which were subsequently cloned, sequenced, analyzed and compared to putative murine and human homologues. The second part included the generation of a specific monoclonal antibody (mab) against SLAMF4 (CD244, 2B4) with the aim to study the expression pattern of SLAMF4 on chicken leukocytes, to determine presumed ligands as well as to clarify functional aspects on distinct immune cells.

3. LITERATURE

3.1 The SLAM family

The signaling lymphocytic activation molecule (SLAM) family of membrane receptors constitutes a subset of the greater CD2 family within the immunoglobulin superfamily (IgSF). The SLAM family currently includes nine members: SLAMF1 (CD150, IPO-3), SLAMF2 (CD48), SLAMF3 (CD229, Ly9), SLAMF4 (CD244, 2B4), SLAMF5 (CD84), SLAMF6 (CD352, NTB-A, Ly108, SF2000), SLAMF7 (CD319, CRACC, CS1), SLAMF8 (CD353, BLAME) and SLAMF9 (CD84-H1, SF2001) [18, 34, 36]. Most receptors are homophilic adhesion molecules, whereas SLAMF4 engages SLAMF2 in a heterophilic manner [37]. Affiliation to the SLAM family is primarily based on a similar structure of the extracellular domain, in particular an amino-terminal Ig variable (V)-like domain and a membrane-proximal Ig constant 2 (C2)-like domain. In addition, a key feature of most SLAM family members except SLAMF2 (CD48), SLAMF8 (CD353, BLAME) and SLAMF9 (CD84-H1, SF2001) is the presence of one or more copies of immunoreceptor tyrosine-based switch motifs (ITSM) T-I/V-Y-x-x-V/I in the cytoplasmic tail [where T represents threonine, I isoleucine, V valine, Y tyrosine and x any amino acid] (**Fig. 1**) [18, 34, 36]. Depending on the physiological condition, these motifs serve as docking sites (or switch) for both activatory and inhibitory intracellular SH2 domain-containing adaptor molecules [38-41]. Adaptor proteins involved in SLAM receptor signaling include the SLAM-associated adaptor protein (SAP, also named SH2D1A), Ewing`s sarcoma-associated transcript-2 (EAT-2, also named SH2D1B1) and EAT-2 related transducer (ERT, also named SH2D1B2), respectively. [18, 34, 36]. All SLAM receptors bind SAP and/or EAT-2/ERT, except SLAMF8 and SLAMF9, which lack cytoplasmic motifs, as well as SLAMF2, which represents a glycosyl-phosphatidylinositol (GPI)-anchored protein. Genes encoding SLAM family receptors are clustered in the SLAM locus on human and murine chromosome 1. In addition, genes encoding EAT-2 and ERT, the latter a pseudogene in humans, are located in the vicinity of the SLAM locus on chromosome 1 while the SAP gene has been mapped to human and murine chromosome X (**Fig. 2**) [36, 42].

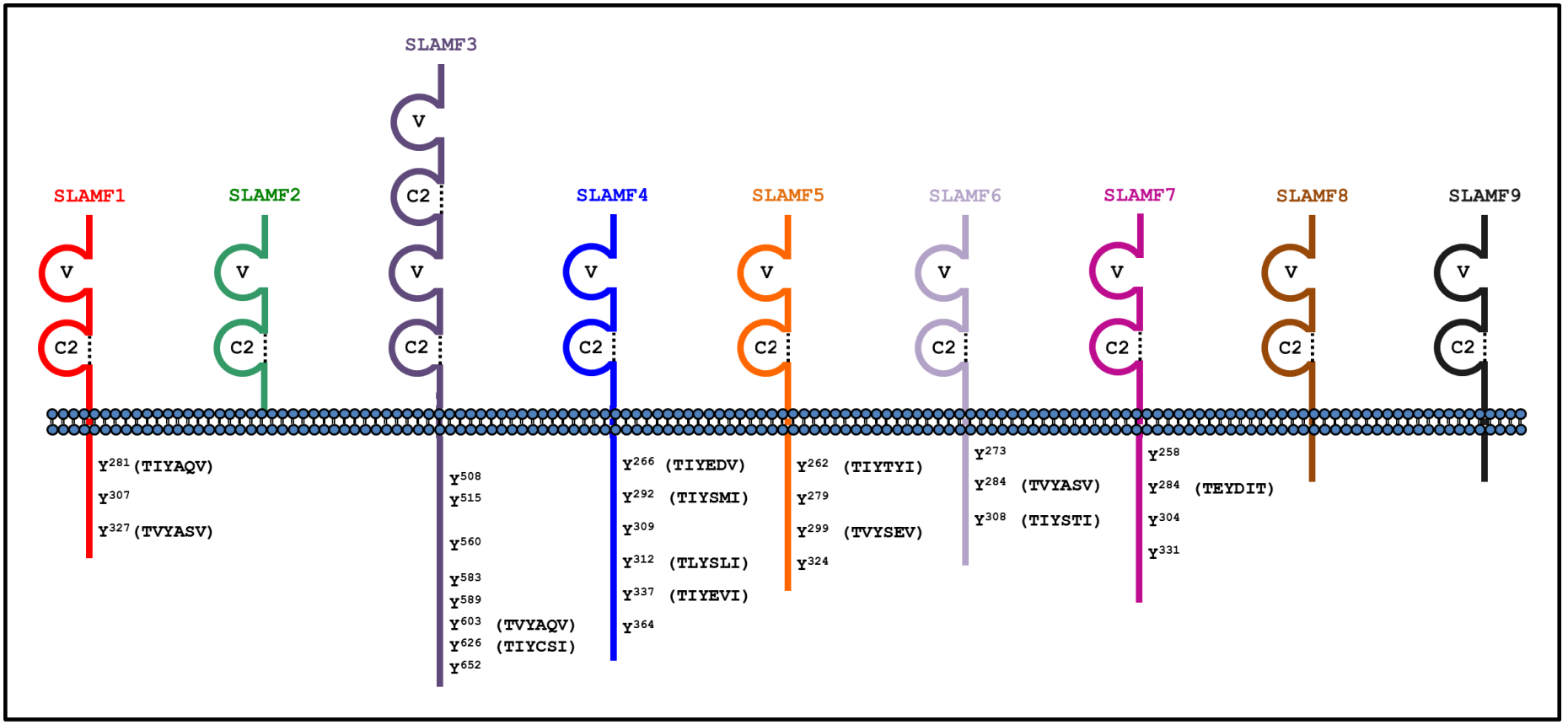

Figure 1 Schematic representation of mammalian SLAM receptors.

Family affiliation is based on homologies of the extracellular domain, each consisting of an N-terminal Ig variable (IgV)-like domain and a membrane-proximal constant 2 (C2)-like domain. Other structural elements include a single transmembrane region and a cytoplasmic tail containing the consensus motif TxYxxV/I, the latter refered to as immunoreceptor tyrosine-based switch motif (ITSM) (where T represents threonine, Y tyrosine, V valine, I isoleucine and x represents any amino acid). Modified according to [18].

The importance of the SLAM-SAP interaction is best illustrated by a genetic disorder called X-linked lymphoproliferative disease (XLP, also known as Duncan`s disease) [43]. The underlying mechanism is a mutation in the SAP gene, resulting in an increased susceptibility to Epstein-Barr virus (EBV) infection [44]. Patients suffering from XLP exhibit an uncontrolled B cell proliferation, leading to malignant B cell lymphomas as well as infiltration and failure of multiple organs. The syndrome is further characterized by both T and Natural Killer (NK) cell dysfunction to clear EBV-infected B cells. Since SAP is expressed by T and NK cells, defective SAP affects T and NK cell functions to curtail EBV infection [42, 45]. SLAM family receptors are generally involved in cytokine production, costimulation, cytotoxicity, cell development, differentiation and proliferation. XLP is therefore a good example for the important role of the SLAM family and SAP adaptors in the regulation of immune responses. Furthermore, it demonstrates how an impairment of this fine-tuned system may result in severe immunodeficiencies and autoimmune diseases, for example rheumatoid arthritis or multiple sclerosis [46-48]. Individual receptors are subsequently discussed in detail. An overview of SLAM family members, including the number of ITSM motifs, expression pattern, function, ligand(s) as well as intracellular adaptor molecules is given in **table 1**.

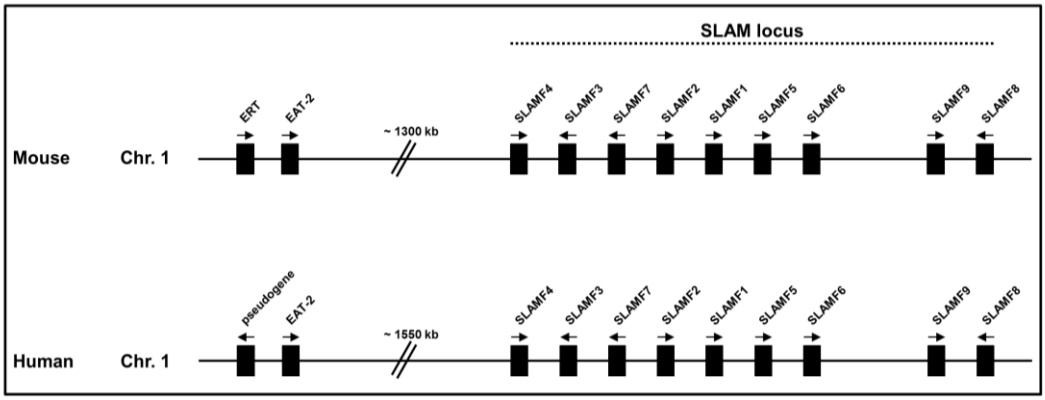

Figure 2 Schematic representation of the mammalian SLAM locus.

SLAM genes are clustered in syntenic regions on human and mouse chromosome 1. Genes encoding EAT-2 and ERT, the latter representing a pseudogene in humans, are located in the vicinity of the SLAM locus. Note that the scheme is not drawn to scale. Modified according to [18].

						Interaction with	
Family name	**Alternative name**	**ITSMs**	**Expression**	**Function**	**Ligand**	**SAP**	**EAT-2/ (ERT)**
SLAMF1	SLAM, CD150	2	B, T, Mo, Mϕ, DC, thymocytes, platelets, HSC, HPC	T: IFN-γ + proliferation↑ B: proliferation + differentiation↑ Mϕ: IL-12, TNF + NO↑ DC: IL-12 + IL-8↑ Platelets: aggregation↑ HSC: development + differentiation	Self, Measles virus, Gram-negative OmpC + OmpF	+	+
SLAMF2	CD48	None	Hematopoietic cells, HSC, MPP, HPC	NK: cytotoxicity↑; IFN-γ↑ CD4 T: proliferation ↑ HSC: development + differentiation	SLAMF4, CD2	+	+
SLAMF3	Ly9, CD229	H: 2 M:1	Thymocytes, T, B, NK, Mo, HSC, MPP	T: IFN-γ, IL-2, IL-4, IL-6, IL-10 + TNF↓ iNKT: proliferation↓; IL-4↓ HSC: development + differentiation	Self	+	+
SLAMF4	2B4, CD244	4	NK, γδ T, CD8 T, Mo, basophils, eosinophils, MPP	NK: IFN-γ↑; cytotoxicity↑ Eosinophils: cytotoxicity↑; IFN-γ, IL-4 + EPO↑ γδ T: cytotoxicity↑, IFN-γ + IL-2↑ $CD8^+$ T: $SLAMF4^{low}/SAP^{high}$: proliferation↑; cytotoxicity↑ $SLAMF4^{high}/SAP^{low}$: proliferation↓; cytotoxicity↓ MPP: development + differentiation	SLAMF2	+	+
SLAMF5	CD84	2	T, B, Mo, DCs, thymocytes, platelets	T: proliferation↑; IFN-γ↑ Platelets: aggregation↑	Self	+	+
SLAMF6	CD352, SF200 human: NTB-A, mouse: Ly108	2	T, B, NK	NK: cytotoxicity↑; proliferation↑; IFN-γ + TNF-α↑ T: proliferation↑; IFN-γ↑ B: isotype switching to IgG2a + IgG3↑	Self	+	+
SLAMF7	CD319, CRACC, CS1,	H:1 M:0	T, NK, B, plasma cells, mature DCs, malignant hematopoietic cells	NK cell: dependent on the co-expression of EAT-2 $EAT\text{-}2^+$ → cytotoxicity↑ $EAT\text{-}2^-$ → cytotoxicity↓ $CD4^+$T: proliferation + cytokines production↓ B: proliferation↑	Self	-	+
SLAMF8	BLAME, CD353	None	DC, Mϕ	Mϕ: Nox-2 activity↓	Unknown	-	-
SLAMF9	CD84-H1, SF2001	None	T, B, DCs, Mo	Unknown	Unknown	-	-

Table 1 The Signaling Lymphocytic Activation Molecule (SLAM) Family.

Abbreviations:
B: B lymphocyte; BLAME: B lymphocyte activator macrophage expressed; CRACC: CD2-like receptor-activating cytotoxic cells; DC: Dentritic cell; EAT-2: Ewing`s sarcoma-associated transcript-2; EPO: eosinophil peroxidase; ERT: EAT-2 related transducer; H: Human; HSC: Hematopoietic stem cell; HPC: Hematopoietic progenitor cells; IFN-γ: Interferon-gamma; IL: Interleukin; iNKT: invariant Natural killer T cell; ITSM: immunoreceptor-tyrosine based switch motif; T: T lymphocyte; M: Mouse; Mϕ: Macrophage; Mo: Monocyte; MPP: Multipotential progenitor NK: Natural killer cell; NO: Nitric oxide; NTB-A: NK-, T- and B-cell antigen; SAP: SLAM-associated adaptor protein; SLAM: Signaling lymphocytic activation molecule; TNF: Tumor necrosis factor. Modified according [18, 36].

3.1.1 SLAMF1 (SLAM, CD150, IPO-3)

In 1993, a novel cell surface receptor was identified on B and T cells and associated with the regulation of activated T cells [49]. The receptor was named SLAM to emphasize its role as a costimulatory activation molecule [50]. In the meantime, the SLAM family has broadly expanded and the nomenclature had to be adopted, hence the name SLAMF1 to distinguish from other SLAM family receptors.

SLAMF1 is expressed on distinct immune cells, including immature thymocytes, $CD45RO^{high}$ memory T cells, subsets of $CD4^+$ T cells, peripheral B cells and murine hematopoietic precursors, but also on B and T cells following activation. Moreover, cell surface expression has been documented in several non-lymphoid cells, in particular monocytes, platelets and dentritic cells (DCs) [49-54].

SLAMF1 is a homophilic adhesion molecule and the cellular entry-receptor for measles virus [55, 56]. Furthermore, SLAMF1 on macrophages is important for the recognition of the outer membrane components OmpC and OmpF of gram-negative bacteria. Upon contact, the SLAMF1-bacteria complex is transfered to the phagosome and degraded enzymatically, which contributes to the clearance of bacterial infections [57]. Macrophages also seem to regulate the secretion of interleukin-12 (IL-12), tumor necrosis factor (TNF) and nitric oxide (NO) through SLAMF1. This has been demonstrated in an infection model where $SLAMF1^{-/-}$ macrophages failed to clear an infection with the parasite *Leishmania major* due to an impaired production of IL-12, TNF, and NO, respectively [51].

SLAMF1 was initially described to function as costimulatory receptor in T cell receptor (TCR)-induced proliferation and interferon-gamma (IFN-γ) secretion [49, 50]. In contrast, $SLAMF1^{-/-}$ T cells produce normal levels of IFN-γ while the release of the T_H2-associated cytokines IL-4 and IL-13 is affected [51]. These phenotypic alterations are explained by **(a)** the inability of antibody-mediated ligation to reflect the physiological condition of SLAMF1 engagement or **(b)** the capability of other SLAM family receptors to replace SLAMF1 receptor function, at least partially [58]. In B lymphocytes, mab cross-linking amplifies both B cell proliferation and differentiation [49].

Moreover, SLAMF1 also regulates the cytokine profile of DCs and monocytes. SLAMF1 receptor engagement on dentritic cells intensifies the production of IL-12 and IL-8 [53]. The comprehensive function of SLAMF1 in immune regulation is completed by its role in promoting platelet aggregation stability [52].

Beyond that, SLAMF1 is involved in the development and differentiation of hematopoietic cells. Murine multipotential stem cells are generally defined as $Lineage^{-/low}$ $Sca\text{-}1^+$ $c\text{-}Kit^+$

(LSK) cells and several SLAM family members, including SLAMF1, are suitable markers to divide LSK cells into several subpopulations [59-64].

Initial studies demonstrated that murine hematopoietic stem cells (HSCs) coexpress SLAMF1 during early hematopoiesis while lacking other SLAM-related receptors [59, 64]. Subsequent development stages, in particular multipotential progenitors (MPPs) and restricted hematopoietic progenitor cells (HPCs) become SLAMF1 negative while mounting other SLAM receptors at the cell surface. This observation prompted Kiel and colleagues to propose the "SLAM code", in order to specify distinct subpopulations during hematopoiesis [59]. Recent studies allow the classification of LSK cells on the basis of the expression of SLAMF1, SLAMF2, SLAMF3 and SLAMF4, respectively, into functionally distinct fractions [60].

In conclusion, SLAMF1 functions as an important co-stimulator for cells of both innate and adaptive immunity involved in host defense against pathogens by contributing to their recognition and elimination.

3.1.2 SLAMF2 (CD48)

SLAMF2 represents a classical glycosyl-phosphatidylinositol (GPI)-anchored cell surface molecule lacking a cytoplasmic domain and the consensus motif ITSM present in most SLAM members (**Fig. 1**). SLAMF2 is broadly expressed in the hematopoietic system and binds the adhesion molecules CD2 and SLAMF4 [18, 34, 37, 59, 65-68].

The identification of SLAMF4 (CD244, 2B4) as high-affinity ligand has shifted the primary research focus towards the role of SLAMF4 in immune regulation. Yet, data exist which delineate a role not only as ligand of SLAMF4, but also as cellular activator. In this context, proliferative responses to different stimuli are impaired in SLAMF2$^{-/-}$ CD4^{+} T cells [69].

In NK cells, SLAMF2 also functions as costimulatory receptor by promoting target cell lysis as demonstrated in a redirected killing assay. Furthermore, SLAMF2 engagement on NK cells enhances IFN-γ production, a finding that corresponds to data of a SLAMF2 involvement in the IL-18 signaling pathway [70, 71]. IL-18 stimulates NK cells and T cells to produce IFN-γ, which in turn functions as potent activator of macrophages (thus originally named macrophage-activating factor), inhibitor of viral replication and promotor of hematopoiesis [72-75].

Based on its capacity to modulate IFN-γ secretion, its expression on hematopoietic progenitor cells and the role of IFN-γ in hematopoiesis, SLAMF2 consequently plays an important role in stem cell development and differentiation. This was confirmed in knockout studies where proliferation and IFN-γ secretion of SLAMF2$^{-/-}$ hematopoietic stem cells (HSC) are impaired.

At the same time, SLAMF2$^{-/-}$ mice are more likely to develop malignancies, a phenotype that has been associated with an overexpression of the p21 activated kinase (PAK) [76]. PAK is known to be involved in cancer development and the occurrence of these malignancies is explained by PAK overexpression to compensate for the missing SLAMF2-SLAMF4 signaling pathway [76-78].

3.1.3 SLAMF3 (CD229, Ly9)

SLAMF3 is an exception within the SLAM family by displaying a duplication of the V-C2-like domain in its extracellular domain (**Fig. 2**) [18, 34]. Like most SLAM receptors, SLAMF3 is a self-ligand and broadly expressed on hematopoietic cells, including thymocytes, T and B cells, NK cells, HSCs, MPPs and monocytes, respectively [79, 80].

SLAMF3 is a predominantly negative regulator of activated T cells since mab cross-linking inhibits cytokine secretion and results in the downregulation of recognized activation markers including CD25 (IL-2 receptor α-chain) and CD69 [79]. Besides its involvement in hematopoiesis (see 3.1.1), SLAMF3 plays a role in thymus development, in particular in the homeostasis of invariant Natural killer T (iNKT) cells. This has been shown in SLAMF3$^{-/-}$ mice, where the number of iNKT cells increases significantly. In addition, these cells also display elevated IL-4 levels upon activation. Both effects are explained by the loss of SLAMF3 to negatively regulate development and function of iNKT cells [81].

In conclusion, SLAMF3 receptor is mainly a cellular inhibitor, particularly in thymocytes and T cells.

3.1.4 SLAMF4 (CD244, 2B4)

SLAMF4 is one of the best characterized receptors within the SLAM family and functional aspects have been best illustrated in NK cells. Initial studies suggested SLAMF4 to be an activator of human and murine NK cells when encountering its ligand SLAMF2 [37]. This has been primarily based on its potency to promote granule exocytosis and IFN-γ release [82-84].

In contrast, new reports indicate a dichotomous role of SLAMF4 to function as either activating or inhibitory receptor, determined by the availability of the intracellular adaptor molecules SAP and EAT-2 as well as the expression levels of surface SLAMF4 [39, 85]. In the presence of SAP, SLAMF4 enhances NK cell-mediated killing whereas in SAP$^{-/-}$ NK cells, SLAMF4 engagement fails to induce a potent cytotoxic reaction. In this respect, these observations indicate an inhibitory role of SLAMF4 in the absence of SAP [85]. A similar situation could be observed in activated CD8^{+} T cells where both the amount of surface

SLAMF4 and intracellular SAP determine cellular activation or inhibition [86].

Like most SLAM family members, SLAMF4 is also present on other immune cells including γδ T cells, monocytes, basophils and eosinophils, respectively [87-89]. In contrast to NK and T cells however, no data exist indicating a similar bifunctional role in these cells, which is explained by the inability of these cells to express intracellular SAP adaptor molecules [42, 90]. The expression of SLAMF4 on γδ T cells has been mainly studied in murine epithelial tissue-type γδ T cells where antibody cross-linking promotes cytokine production and killing of certain target cells [87, 91]. Similar functions are attributed to SLAMF4 in eosinophils where mab cross-linking amplifies both cytotoxicity and cytokine production, in particular the release of eosinophil peroxidase, a recognized marker of eosinophil activation [89]. Furthermore, SLAMF4 is involved in the development and differentiation of hematopoietic precursors as discussed earlier (see 3.1.1) [59]. Due to its bifunctional character, SLAMF4 is a good example for the "switching function" of SLAM family receptors as positive and negative immune cell regulators.

3.1.5 SLAMF5 (CD84)

SLAMF5 is a self-ligand expressed on thymocytes, T and B cells, platelets, DCs and monocytes [92, 93]. In activated T cells, SLAMF5 is a co-stimulator of proliferation and IFN-γ secretion as demonstrated by *in vitro* experiments with SLAMF5 specific mab [92]. Like other SLAM family receptors, SLAMF5 associates with SAP and EAT-2, respectively [94, 95]. Interestingly, cellular activation seems to be additionally initiated by a SAP-independent mechanism since similar effects are observed in SAP-defective T cells obtained from X-linked lymphoproliferative disease (XLP) patients [96].

Furthermore, SLAMF5 is part of a group of adhesion molecules involved in the stabilization of platelet aggregation, an effect similar to the one described for SLAMF1 [52]. In contrast, further functional aspects regarding thymocytes, B cells, monocytes and dentritic cells have not been defined so far.

3.1.6 SLAMF6 (CD352, NTB-A, Ly108, SF2000)

SLAMF6 was first described as a receptor expressed on all NK, T and B cells and thus termed NK-, T- and B-cell antigen (NTB-A) [97, 98]. SLAMF6 is a homophilic molecule and considered a costimulatory molecule in NK cells important for proliferation, cytokine production and granule exocytosis [99, 100]. Similar effects are observed in $CD4^+$ T helper cells where a combinatory stimulation of SLAMF6 and CD3 increases both proliferation and

IFN-γ release.

IFN-γ is a classical T_H1-associated cytokine important for B cell isotype switching and blocking of SLAMF6 affected T_H1-mediated isotype switching significantly [48]. A relationship between SLAMF6 and the T_H1 phenotype was additionally proven in a murine model for multiple sclerosis, experimental autoimmune encephalomyelitis (EAE). EAE is a T_H1-mediated autoimmune disease and the onset of EAE is delayed when SLAMF6 is blocked with a fusion protein [48].

In summary, SLAMF6 is a costimulatory molecule and regulator of T_H1-related cytokine production.

3.1.7 SLAMF7 (CD319, CRACC, CS1)

SLAMF7 was described in 2001 as an activating receptor for NK cells, hence the initial name CD2-like receptor–activating cytotoxic cells (CRACC) [101]. It represents a membrane receptor which differs from other SLAM-related receptors in several ways. Firstly, SLAMF7 contains only one ITSM motif in its cytoplasmic tail which enables binding to EAT-2, but not SAP (**Fig. 1**). Secondly, its function is strictly dependent on the availability of the intracellular adaptor protein EAT-2 [102]. And thirdly, SLAMF7 is frequently upregulated on malignant hematopoietic cells [103].

Like most SLAM family receptors, SLAMF7 is a self-ligand and expressed on a variety of immune cells, in particular NK cells, NK-T cells, cytotoxic $CD8^+$ T cells, $CD4^+$ T cells, plasma cells, B cells and mature DCs [101, 103-105].

The bifunctionality of SLAMF7 has been investigated in NK cells carrying mutations in the EAT-2 gene. Accordingly, in the absence of EAT-2, SLAMF7 engagement inhibits target cell killing whereas in the presence of EAT-2, NK cell-mediated cytotoxicity is significantly increased [102].

In contrast, little is known about the role of SLAMF7 in T cell regulation. One study documented an inhibitory effect in $CD4^+$ T cells since mab cross-linking reduces both T cell proliferation and cytokine production, a notion supported by the inability of human $CD4^+$ T cells to express EAT-2 [102].

Human B cells also lack SAP. In this respect, these results are inconsistent with observations made in activated B cells where SLAMF7 engagement promotes cellular expansion [106].

An idiosyncrasy of SLAMF7 is its expression on malignant cells that has not been reported for other SLAM family members. Particularly its expression on multiple myeloma (MM) cells makes SLAMF7 an interesting target to an antibody-based cancer therapy. There are phase III

clinical studies in progress to investigate the potential of Elotuzumab®, a humanized anti-SLAMF7 mab as new tool in cancer therapy. The idea is that Elotuzumab® binds SLAMF7 on MM cells via its Fab fragment while simultaneously cross-linking the Fc receptor CD16 on NK cells via its Fc portion. The resulting effect is refered to as antibody-dependent cellular cytotoxicity (ADCC) where CD16 engagement activates NK cells to release cytotoxic granules and kill MM cells [103].

In contrast to this promising approach, SLAMF7 functions in DCs, NK-T cells and $CD8^+$ T cells have not been evaluated yet.

In conclusion, SLAMF7 is involved in positive and negative immune cell regulation, albeit bifunctionality is restricted to certain cell types, e.g. NK cells.

3.1.8 SLAMF8 (CD353, BLAME)

SLAMF8, also named B lymphocyte activator macrophage expressed (BLAME), was assigned to the SLAM family due to its CD2-like extracellular domain and its genomic location on human chromosome 1q21 (the genomic region encoding the SLAM genes) albeit distinctive intracellular motifs are not apparent (**Fig. 1, 2**) [107]. Potential ligands are currently unknown, and likewise few data exist regarding functional properties.

In contrast, expression on DCs and macrophages has been established. One report even implies a role of SLAMF8 as negative regulator of Nox2 activity in macrophages, an enzyme involved in the oxygen-dependent degradation and destruction of pathogens. Moreover, macrophages seem to upregulate SLAMF8 in response to IFN-γ exposure and it is being discussed whether this upregulation is mandatory to attenuate or even terminate innate immune responses [108].

In summary, SLAMF8 seems to negatively regulate macrophage function by inhibiting Nox-2 activity.

3.1.9 SLAMF9 (CD84-H1, SF2001)

SLAMF9 was initially named CD84-H1 due to its close homology to SLAMF5 (CD84) and combines features characteristic for SLAM family members, in particular an extracellular region containing two Ig-like domains and a single transmembrane region. The short cytoplasmic tail however lacks ITSM motifs (**Fig. 1**). Potential ligands of SLAMF9 as well as functional aspects are currently unknown, likewise its expression pattern on leukocytes, albeit preliminary RNA analyses indicate an expression on monocytes, DCs, T cells, and B cells, respectively [109].

3.2 The SLAM-associated protein (SAP) family

The SAP family of adaptor molecules includes SAP (SH2D1A), Ewing`s sarcoma-associated transcript-2 (EAT-2, SH2D1B1) and EAT-2-related transducer (ERT; SH2D1B2). They are composed of a SH2 domain and a short carboxy-terminal tail. The SAP gene is located on human and murine chromosome X, whereas genes encoding EAT-2 and ERT, the latter representing a nonfunctional pseudogene in humans, are clustered in the vicinity of SLAM-related genes on chromosome 1 in both species (**Fig. 2**) [36, 110, 111]. Six SLAM receptors are known to associate with either SAP or EAT-2/ERT. In contrast, this interaction has not been confirmed for SLAMF8 and SLAMF9, respectively, while SLAMF2 represents a GPI-anchored protein [36, 110]. Therefore, signal transduction mediated by intracellular SAP adaptor molecules is essential for the functionality of SLAM receptors and thus immune regulation.

3.2.1 SAP (SH2D1A)

Human and murine SAP are expressed in T cells, B cells, eosinophils, NK cells and platelets [44, 52, 89, 112-115]. An initial report, published in 1998, defined SAP adaptors as "natural blockers" of other SH2 domain-containing molecules. Accordingly, SAP adaptors block the association of SLAM receptors to inhibitory transduction molecules, in particular SH2 domain-containing protein tyrosine phosphatase-1 (SHP-1), SH2 domain-containing protein tyrosine phosphatase-2 (SHP-2), SH2 domain-containing inositol-5-phosphatase (SHIP-1) and the inhibitory kinase Csk, respectively [44]. Subsequent intracellular inhibitory signals are consequently prevented and cellular activation sustained.

Another report challenged this notion by demonstrating the potential of SAP to directly couple SLAM receptors to the protein tyrosine kinase (PTK) Fyn. This is achieved by a motif centered around arginine 78 (R78), which has been shown to serve as docking site for other Src homology 3 (SH3) domain containing molecules, including Pak-interacting exchange factor (PIX), the adaptor Nck, and the protein kinase C-θ (PKC-θ) [116-119]. These interactions initiate a cascade of protein tyrosine phosphorylation events which directly correlate with functional activation.

In the meantime, a seminal study investigating the role of SAP in NK cell activation confirmed the accuracy of both theories. Accordingly, SAP promotes NK cell activation via a dual mechanism of action. Firstly, SAP mediates the association of Fyn to SLAM receptors which in turn links SAP to Vav-1, an exchange factor essential for T cell development and activation. Secondly, SAP prevents the inhibition of SLAM family receptors by detaching the cellular

suppressor SHIP-1 [38].

These results correspond to data obtained from NK cells of genetically modified mice. Here, the recruitment site of Fyn in SAP, R78, is mutated to alanine (SAP R78A) and NK cells carrying this point mutation consequently fail to exhibit sufficient levels of granule exocytosis and IFN-γ secretion, respectively [38].

In conclusion, SAP acts as positive regulator, at least in NK cells, albeit biochemical signaling pathways downstream of the SAP-Fyn-Vav-1 cascade are not completely resolved.

3.2.2 EAT-2 (SH2D1B1)/ERT (SH2D1B2)

EAT-2 expression has been reported in NK cells, DCs and macrophages, while ERT expression is restricted to murine NK cells and nonfunctional in humans [111, 120]. The biology of both molecules has been studied in EAT-2 and ERT deficient mice displaying a SAP^+ phenotype.

In response to several stimuli, including SLAMF4, $EAT^{-/-}$ NK cells become activated as measured by the secretion of IFN-γ and the ability to kill certain target cells [111]. In addition, both effects are also observed in $ERT^{-/-}$ NK cells. In contrast to SAP, EAT-2 and presumably ERT seem to transduce intracellular signals through tyrosine residues located in the C-terminal tail, albeit the nature of this interaction is not fully resolved [111].

Given that both $EAT\text{-}2^{-/-}$ and $ERT^{-/-}$ NK cells exhibit increased cytotoxicity and IFN-γ production, a role as negative regulators of NK cell function has been proposed.

However, studies investigating the role of SLAMF7 (CD319, CRACC) in NK cells challenge this notion. Here, the cytotoxicity of $EAT\text{-}2^{-/-}$ NK cells is impaired whereas NK cell cytotoxicity is significantly increased in the $EAT\text{-}2^+$ phenotype [102].

In general, genetically modified mice offer many new insights although they do not reflect physiological conditions. Cellular responses to a variety of stimuli are orchestrated by a multitude of receptors and intracellular signaling molecules. Consequently, one cell type may harbor several SLAM receptors and SAP adaptors at the same time. NK cells, for instance, simultaneously express SLAMF2, SLAMF3, SLAMF4, SLAMF6 and SLAMF7 as well as the adaptors SAP and EAT-2/ERT [68, 71, 79, 82-84, 97, 101, 111, 121]. It is therefore conceivable that the complexity of these interactions is not completely understood and has yet to be investigated.

4. PUBLICATIONS

1) **The chicken SLAM family**
Straub C, Viertlboeck B.C., Göbel TW.
Immunogenetics 2013 Jan 65, 63-73

2) **Chicken SLAMF4 (CD244, 2B4), a receptor expressed on thrombocytes, monocytes, NK cells, and subsets of αβ-, γδ- T cells and B cells binds to SLAMF2**
Christian Straub, Marie-Luise Neulen, Birgit C. Viertlboeck, Thomas W. Göbel
Developmental and Comparative Immunology, 2014 Feb; 42(2): 159-68

3) **Chicken NK cell receptors**
Straub C, Neulen ML, Sperling B, Windau K, Zechmann M, Jansen CA, Viertlboeck BC, Göbel TW., 2013 Nov.
Developmental and Comparative Immunology 41 (3): 324-33

The chicken SLAM family

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Abstract

The signalling lymphocytic activation molecule (SLAM) family of receptors is critically involved in the immune regulation of lymphocytes, but has been only detected in mammals with one member being present in Xenopus. Here we describe the identification, cloning and analysis of the chicken homologues to the mammalian SLAMF1 (CD150), SLAMF2 (CD48), and SLAMF4 (CD244, 2B4). Two additional chicken SLAM genes were identified and designated SLAMF3like and SLAM5like in order to stress that those two receptors have no clear mammalian counterpart, but share some features with mammalian SLAMF3 and SLAMF5, respectively. Three of the chicken SLAM genes are located on chromosome 25, whereas two are currently not yet assigned. The mammalian and chicken receptors share a common structure with a V-like domain that lacks conserved cysteine residues and a C2-type Ig domain with four cysteines forming two disulfide bonds. Chicken SLAMF2 lacks like its mammalian counterpart a transmembrane and cytoplasmic domain and thus represents a glycosyl-phosphatidyl-inositol (GPI)-anchored protein. The cytoplasmic tails of SLAMF1 and SLAMF4 display two and four conserved immunoreceptor tyrosine-based switch motifs (ITSM), respectively, whereas chicken SLAMF3like and SLAMF5like both have only a single ITSM. We have also identified the chicken homologues of the SLAM associated protein-family of adaptors (SAP), SAP and EAT-2. Chicken SAP shares about 70 % identity with mammalian SAP and chicken EAT-2 is homologous to mouse EAT-2, whereas human EAT-2 is much shorter. The characterization of the chicken SLAM family of receptors and the SAP adaptors demonstrates the phylogenetic conservation of this family, in particular its signalling capacities.

Keywords

SLAM family, chicken, EAT-2, SAP

Abbreviations

GPI, glycosyl-phosphatidyl-inositol

SLAM, signalling lymphocytic activation molecule

SAP, SLAM associated protein-family of adaptors

ITSM, immunoreceptor tyrosine-based switch motif

Introduction

Immune cell responses are fine tuned by a plethora of receptors that modulate the activity of the cells. The primary signal stems either from the antigen receptors in T- and B-cells or via NK cell receptors in NK cells. These initial activation events are subsequently modulated by a number of activating and inhibitory receptors, which play an important role in determining the immune response (Ravetch and Lanier 2000; Zhu et al. 2011). Immunoreceptors with activating or inhibitory function are frequently found within the same receptor family. A typical feature of inhibitory receptors is the immunoreceptor tyrosine-based inhibition motif (ITIM), a six amino acid sequence whose conserved tyrosine is phosphorylated upon ligand binding and thereby recruits tyrosine phosphatases (Daeron et al. 2008; Rhee and Veillette 2012). Activating forms on the contrary may have a homologous extracellular domain, but they usually lack cytoplasmic ITIM but contain immunoreceptor tyrosine-based activation motifs (ITAM), where the tyrosine phosphorylation forms a docking site for kinases (Reth 1989). Alternatively, the activating receptors display only a short cytoplasmic tail and have a positively charged transmembrane residue that allows the association with adaptor molecules containing ITAM. Immunreceptor families with inhibitory and activating members include Fc receptors, receptor families encoded by the leukocyte receptor cluster, and many others (Colonna 1997; Long 1999; Nimmerjahn and Ravetch 2008).

A third group of receptors can transmit either positive or negative signals. This can be accomplished by binding to different ligands as is the case for CD28 (Odorizzi and Wherry 2012). Alternatively, the presence or absence of intracellular adaptor proteins can be critical in either the activation or inhibition of cellular immune responses. This is the case for the signalling lymphocytic activation molecule (SLAM) family of receptors, a subgroup of the CD2 receptor family (Boles et al. 2001; Cannons et al. 2011; Sidorenko and Clark 2003; Veillette 2010).

The SLAM family of receptors is composed of a group of type I transmembrane receptors, including SLAMF1 (SLAM; CD150), SLAMF3 (Ly9; CD229), SLAMF4 (2B4; CD244), SLAMF5 (CD84), SLAMF6 (NTB-A, Ly108-1), and SLAMF7 (CRACC). In man, the receptors are located on the long arm of chromosome 1, whereas the related receptors CD2 and CD58 are located on the short arm of chromosome 1. An additional receptor, SLAMF2 (CD48) is also present in the genomic cluster; however, it is a glycosyl-phosphatidyl-inositol (GPI)-anchored protein. Several features are common to the SLAM family of receptors, such as the two Ig domains composed of a V-like Ig domain lacking disulfide bonds and a membrane proximal C2-type domain, with an additional pair of disulfide bonds. All SLAM

family receptors except SLAMF2 display cytoplasmic immunoreceptor tyrosine-based switch motif (ITSM) with the consensus motif T-I/V-Y-x-x-V/I (Shlapatska et al. 2001; Sidorenko and Clark 2003). This motif is essential for SLAM to associate with the SAP (SLAM associated proteins)-family of adaptors. The SAP-family of adaptors includes three members designated SAP, EAT-2 and ERT, the latter being a pseudogene in man (Cannons et al. 2011; Veillette 2010). They are composed of a Src homology 2 (SH2) domains and a short carboxy-terminal region. It has been recently demonstrated that SLAM receptors can mediate positive as well as negative signalling which is dependent on the presence or absence of SAP adaptors (Dong et al. 2012; Kageyama et al. 2012; Zhao et al. 2012). A particular feature of all SLAM family receptors, which are found only on leukocytes is that they show homophilic interactions with the exception of SLAMF4 binding to SLAMF2.

NK cells have been mainly characterized in mammals and it would be of importance to identify NK cells in non-mammalian vertebrates as well. In the chicken, the two genomic regions encoding most of the inhibitory NK cell receptor genes have been identified (Göbel et al. 1996a; Rogers et al. 2008). Whereas the NK gene complex only encodes a small number of C-type lectins (Neulen and Göbel 2012), the synthetic locus of the mammalian leukocyte receptor complex has been massively expanded with a huge family of chicken Ig-like receptors (Viertlboeck et al. 2004; Viertlboeck and Göbel 2011). We initially hypothesized that the large number of CHIR could be split in smaller units that functionally represent various mammalian immunoregulatory families. In the meantime, however, we were able to identify various additional immunoreceptor families in the chicken with homology to the mammalian TREM, CD200R, CD300L and Fc receptor families (Viertlboeck et al. 2008; Viertlboeck et al. 2006; Viertlboeck et al. 2009). Here we have focused on the identification of the chicken SLAM family of receptors and characterized five members of the family, as well as the SAP adaptors.

Methods

Database searches

The identification of SLAM family members in chickens was accomplished by either keyword search using the gene database (http://www.ncbi.nlm.nih.gov) and the genome assemblies of chicken (http://www.ensembl.org/Gallus_gallus /index; 2.1 May 2006 release, and PreEnsembl chicken Galgal4, Nov 2011), turkey (Turkey_2.01, Sep 2010), zebrafinch (taeGut3.2.4, Aug 2008), and duck (PreEnsembl, version 1.0) or by BLAST searches (Altschul et al. 1997) with human nucleotide sequences of SLAM family receptors in the above databases or EST databases. The sequences found in the ENSEMBL database or NCBI database were successively subjected to homology search using the BLAST program limited to the "gallus gallus" EST database. Resulting ESTs were assembled into contigs and manually refined using the Lasergene software package (DNASTAR, Madison, USA). The longest open reading frame (ORF) was used for primer design. All oligonucleotides were custom synthesized by Eurofins, Ebersberg, Germany.

Next, bioinformatic approaches were conducted to further characterize putative protein sequences. Structural elements such as signal peptides, immunoglobulin domains and transmembrane regions were identified using SMART (http://smart.embl-heidelberg.de/) and SignalP4.0 (http://www.cbs.dtu.dk/ services/SignalP/) (Bendtsen et al. 2004; Letunic et al. 2012; Schultz et al. 1998). Protein alignments were conducted with CLUSTALW using the DNASTAR Lasergene software package (Madison, USA) and phylogenetic analyses were performed using the MEGA4 software (Tamura et al. 2007).

Genomic sequences for chicken SLAMF1, SLAMF2, SLAMF3like and SLAMF5like were obtained from the gene database while the genomic structure of SLAMF4 was amplified from DNA using specific primers (see **Table 1**).

Animals and cell preparation

Fertilized eggs of the chicken line M11 (MHC Haplotype B2, a kind gift from S. Weigend, Mariensee, Germany) were hatched at the Institute of Animal Physiology, Munich, Germany and used for experiments at the age of 6 to 10 weeks. For RNA preparation, 100 mg of bursa, thymus, spleen and caecal tonsils were taken, frozen in liquid nitrogen and stored at -80°C. Peripheral blood leukocytes (PBL) from heparinized blood were prepared by slow-speed centrifugation as described before (Göbel et al. 1996b). Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using density gradient centrifugation on Ficoll-

Hypaque (Biochrom, Berlin, Germany) (Viertlboeck and Göbel 2007).

Table 1 Oligonucleotides used for cloning.

Number	Sequence	O[a]	Specifity
17	TACCACAATGTACCCTGGC	S	Actin
18	CTCGTCTTGTTTTATGCGC	AS	Actin
1698	AGTGCACTGTTCTGGCTGTTCATCT	S	SLAMF1
1683	GCTGATGGGCAGCATCACGC	AS	SLAMF1
1239	GCTGAGAGGGGTTGTTTGAG	S	SLAMF2
1240	GGAATGTGACAGTGGTTGGA	AS	SLAMF2
1694	ACTCCACTATGCTGTGGGGTCG	S	SLAMF3like
1695	GTGGGGTGGGGGAGCACATC	AS	SLAMF3like
1241	GTCTCCCAGCTCTGTCTCCA	S	SLAMF4
1242	CCAAGTCCGGATGTTCTCAT	AS	SLAMF4
1692	TGGGGATGGAGAGATGGGGCAAC	S	SLAMF5like
1693	CCGTGCTCCTGTGTGGGGAGG	AS	SLAMF5like
1677	GCTGTGCCTCGTGCTGGGAG	S	genomic SLAMF4
1678	GATGCCGCTGTCTGCCTGGG	AS	genomic SLAMF4
1484	GGCAGACAGCGGCATCTATTAT	S	genomic SLAMF4
1449	AAGTGTCTCCAGGCGTGGGC	AS	genomic SLAMF4
1679	GGGCCACCGCTGTGTCCTAC	S	genomic SLAMF4
1680	GCAGCAACACGGTCACTGCG	AS	genomic SLAMF4
1681	CGCAGTGACCGTGTTGCTGC	S	genomic SLAMF4
1242	CCAAGTCCGGATGTTCTCAT	AS	genomic SLAMF4

[a] O = Orientation indicated as *S* sense and *AS* antisense

Cloning procedures

Total RNA was extracted using Trizol reagent (PEQLAB, Erlangen, Germany). The RNA quality was determined with the 2100 Bioanalyzer (Agilent Technologies, Waldbronn,

Germany). RNA with an integrity number above eight was treated with DNase I (Fermentas, St. Leon-Rot, Germany) and reverse-transcribed into cDNA using the Thermoscript RT-PCR system (Invitrogen, Karlsruhe, Germany). Herculase II Fusion DNA Polymerase (Agilent, Santa Clara, USA) was used for PCR. The cycling conditions were 2 min at 95°C for denaturation, followed by 30 cycles of 95°C for 40 s, an annealing time of 40 s at the primer specific temperature, 2 min at 72°C, and a final extension step of 5 min at 72°C to terminate reaction. Primer sequences used for cloning are summarized in **Table 1**. Each primer pair was tested on the different cDNAs and the amplicon with the best result was used for cloning. To verify the identity of the PCR products, they were cloned into the pCR2.1® Topo vector (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The inserts were sequenced using vector-specific primers (GATC, Konstanz, Germany).

Results and Discussion

Identification of five chicken SLAM family members

In order to identify chicken homologues of the mammalian SLAM family, the genomic and EST databases were searched for annotated SLAM genes. Three chicken SLAM genes (designated as SLAMF1, SLAMF3like, SLAMF5like) were readily identified on chromosome 25 in the chicken genome. Corresponding EST sequences that originated from various tissues such as bursa, gut, activated T cells, and macrophages were found (**Tab. 2**). This indicates the wide expression of these SLAM receptors. Two additional chicken SLAM genes with homology to mammalian SLAMF2 and SLAMF4 sequences could initially only be identified in EST databases derived from intestine and pooled bursa, spleen and peyer's patch cDNA, respectively, by searching with the respective human nucleotide sequence. As a next step we gathered all available sequence information coming from genomic predictions and various EST clones and aligned these sequences in order to obtain full length contigs. These were analyzed for the open reading frames that encoded the respective SLAM receptor and used this sequence to design specific oligonucleotides (**Table 1**). A panel of cDNA derived from total RNA isolated from different chicken tissues, such as bursa, thymus, PBL, PBMC, spleen, and caecal tonsils was then amplified with these SLAM specific primer pairs. In most cases, the oligonucleotides amplified a product of the expected size in all tissues, also indicating a wide expression of SLAM family members (**suppl. Fig. 1**). SLAMF4, however, could not be detected in bursa and SLAMF5 like was only weakly expressed in bursa and PBL and not detectable in PBMC, demonstrating that individual SLAM receptors may display differences

in the expression on leukocyte subsets. The amplicons obtained from the specific PCR were subcloned and sequenced to verify the predicted sequences. The full length sequences were transcribed into proteins which were further characterized and compared to human and mouse sequences.

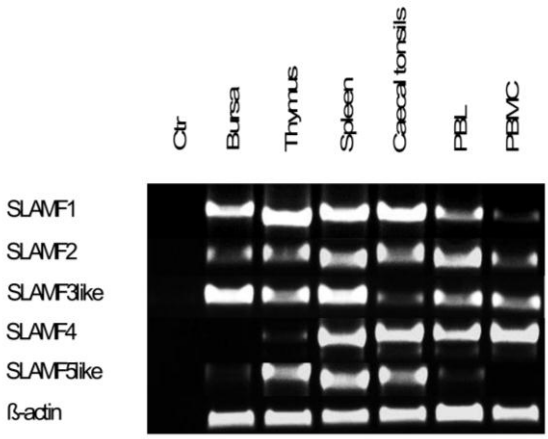
Table 2 Accession numbers and origins of chicken SLAM receptors and SAP adaptors.

	Ensembl	Location	EST	
			Accession	Tissue
SLAMF1	ENSGALG00000024118	Chromosome 25: 1,379,024-1,381,974	AJ443246	Bursa
			CD727802	Gut
			AI981267	ConA activated splenic T cell
			BQ485029	Macrophage
			CD728602	Intestinal lymphocyte
SLAMF2	ENSGALT00000036080_1[a]	Scaffold AADN03026464.1: 11,300-11,692		
SLAMF3like	ENSGALT00000010070	Chromosome 25: 1,373,488-1,374,310	BG710309.1	Liver
			CD736639.1	Intestinal lymphocyte
SLAMF4	ENSGALT00000035707	Un_random: 43,489,589-43,490,956	AM068249.1	Bursa, spleen, Peyer`s patch (pooled)
SLAMF5like	ENSGALT00000040863	Chromosome 25: 790,089-792,997	BU303121	Small intestine
			CD731137	Intestine
			AM066068.1	Bursa, spleen, peyers patch (pooled)

[a] This record was derived from Pre-Ensembl (Gallus Gallus).

Supplementary Figure 1

SLAM genes are expressed in various tissues. The cDNA of the tissues indicated was amplified with chicken SLAM specific primers (**Tab. 1**) and analyzed on agarose gel. The identity of the amplicons was confirmed by sequencing.



Five chicken SLAM receptors share common properties

Common to all of the five chicken SLAM family members was the typical organization of the extracellular domain composed of a two Ig-like structure with an N-terminal V-like domain and a membrane-proximal C2-like domain (**Figs. 1 to 5**). As observed for all SLAM family

members, the V-like domain lacked conserved cysteine residues to form an intradomain disulfide bond, whereas the C2-like domain contained four conserved residues that allow the formation of two intradomain disulfide bonds. These cysteine residues were regularly spaced, the first and second were always separated by five non conserved residues and the third and fourth by 17 (SLAMF1) or 18 residues. Out of the five chicken SLAM genes, three of them could be assigned to potential human counterparts and were therefore named accordingly as SLAMF1, SLAMF2 and SLAMF4 (**Figs. 1 to 3**). Although they only shared around 24 to 28 % identity with the mammalian genes, they were identified by typical sequence features including highly conserved cytoplasmic motifs in the case of SLAMF1 and SLAMF4. With the exception of SLAMF2, the other members displayed a single transmembrane region devoid of basic or acidic residues. SLAMF2, however, lacked a detectable transmembrane region and most likely represents a GPI-anchored protein as has been described for mammalian SLAMF2 (Boles et al. 2001). The two and four ITSM present in SLAMF1 and SLAMF4, respectively, have been entirely conserved and they are all equally spaced (**Fig. 1, 2**). Moreover, an additional tyrosine residue in SLAMF1 located between the two ITSM with the sequence TIYVAA was found to be identical in the chicken. Two additional chicken SLAM receptors (**Figs. 4, 5**) could be isolated and were designated as chicken SLAMF3like and chicken SLAMF5like. This nomenclature was chosen to stress the most likely assignment to mammalian homologues; however, this was not as obvious as for the other three SLAM members. The chicken SLAMF3like gene was also identified on chromosome 25, where it is annotated as SLAMF3. It shares about equal homology to both mammalian SLAMF3 and SLAMF6. In contrast to SLAMF3 that is the only mammalian SLAM with a duplicated V and C domain, the chicken SLAMF3like has only two Ig domains (**Fig. 4**). Chicken SLAMF3like has a single ITSM and a rather short cytoplasmic region, as opposed to two ITSM in mammalian SLAMF3 and SLAMF6. Chicken SLAMF3like is located in the genome between SLAMF1 and the conserved gene VANGL2 (**Fig. 7A**). The corresponding region in the mammalian genome encodes for SLAMF5 and SLAMF6. In summary, chicken SLAMF3like lacks the Ig domain duplication, has a single ITSM and is located in a position of mammalian SLAMF5 and SLAMF6, therefore it could represent mammalian SLAMF3 as it is currently annotated, but also SLAMF6. This will be clarified in the future, once the entire genomic locus has been sequenced. Meanwhile we suggest designating it SLAMF3like. A similar situation is found for the fifth chicken SLAM gene. It has been annotated as SLAMF8 and is found at a similar genomic position as mammalian SLAMF8 (**Fig. 7A**). The detailed sequence analyses have revealed that it harbours a single C-terminal ITSM that has been conserved in mammalian SLAMF5. Moreover, in the location of the membrane proximal ITSM of

mammalian SLAMF5 there is a conserved tyrosine in the chicken, but the ITSM consensus is not completely matched due to a proline at position -2 instead of a threonine (**Fig. 5**). In contrast, mammalian SLAMF8 has a short cytoplasmic region lacking ITSM (**Fig. 5**). Therefore, we suggest naming this chicken SLAM as SLAMF5like. Since the ITSM in four chicken SLAM receptors have been remarkably conserved, we next searched the genome for the presence of the SAP genes. Both, chicken SAP and EAT-2 were readily identified (**Fig. 6**). The alignment with human and mouse homologues revealed that EAT-2 was similar in all three species and chicken EAT-2 shared around 70 % identity with its mammalian counterparts (**Fig.6**). Chicken SAP was most similar to mouse SAP and shared an almost identical length and features defining the SRC homology domain, however, human SAP is much shorter (**Fig. 6**). These results confirm the presence of at least five chicken SLAM receptors and two SAP adaptor proteins and suggest that the chicken SLAM receptors utilize similar signal transduction pathways as described for mammals.

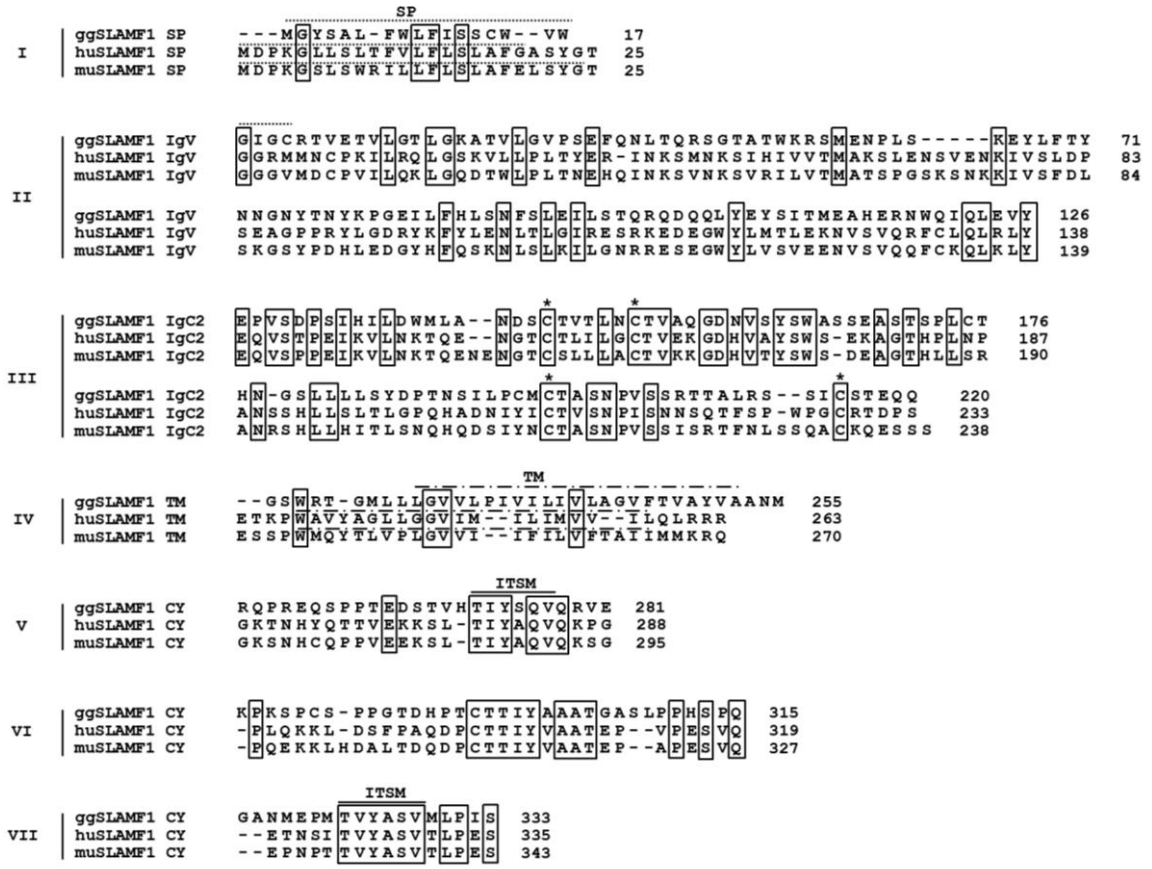


Figure 1 Alignment of SLAMF1 sequences.

The alignment was performed separately for the different domains as identified by exon boundaries as indicated by roman numbering with CLUSTAL W. The Ig domains are depicted in two lines due to space limitations. The signal peptide (SP), transmembrane region (TM), ITSM and cytsteine residues are marked above the sequence. Identical residues are boxed. Accession numbers: human, NP_003028.1; mouse, NP_038758.2; chicken, JX483812.

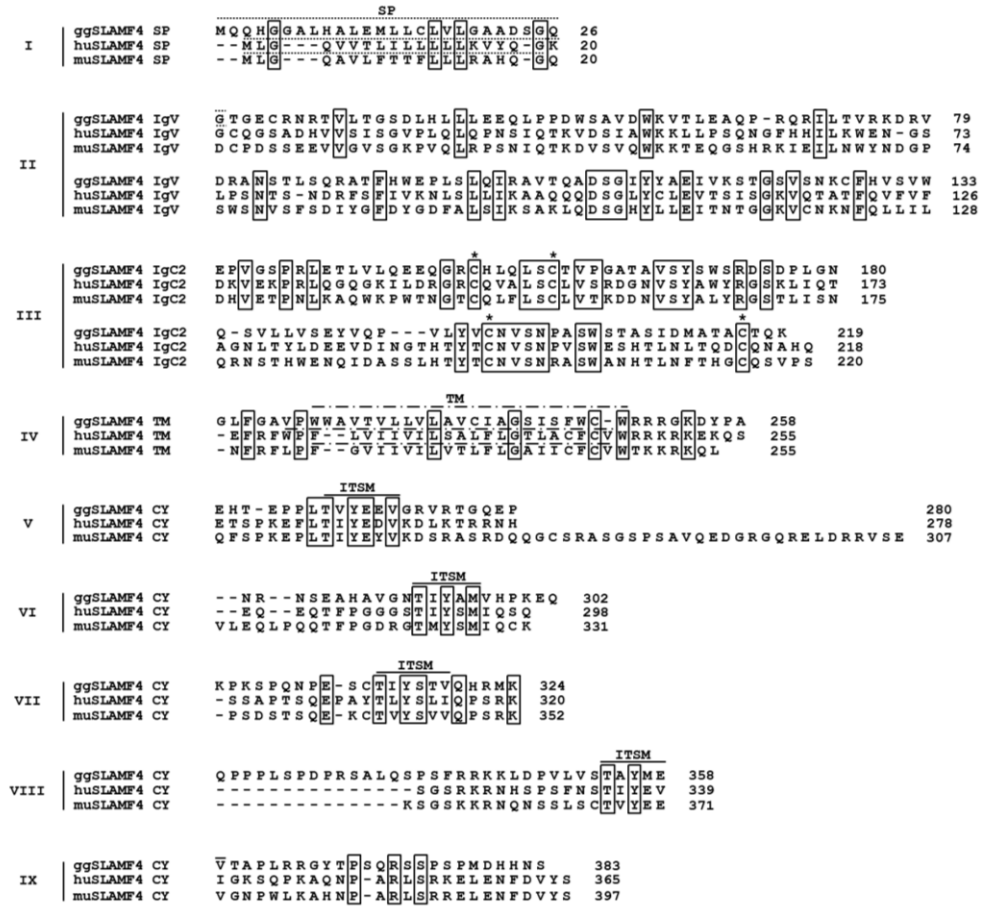


Figure 2 Alignment of SLAMF4 sequences.

Decorations as in Fig. 1. Accession numbers: human, NP_057466.1; mouse, NP_061199.2; chicken, JX483813.

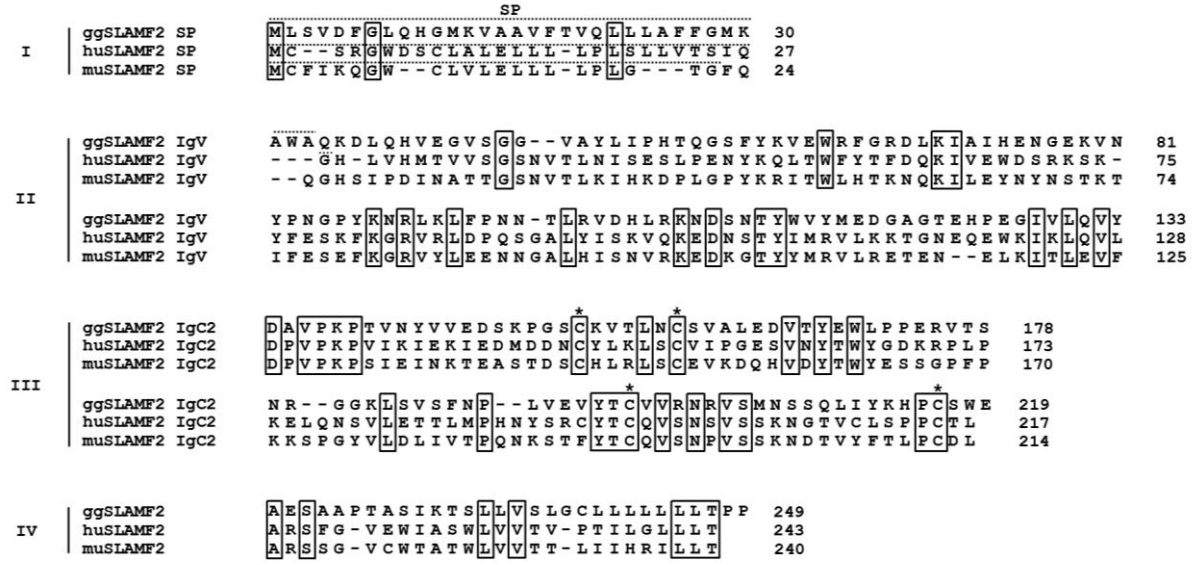


Figure 3 Alignment of SLAMF2 sequences.

Decorations as in Fig. 1. Accession numbers: human, NP_001769.2; mouse, NP_031675.1; chicken, JX483810.

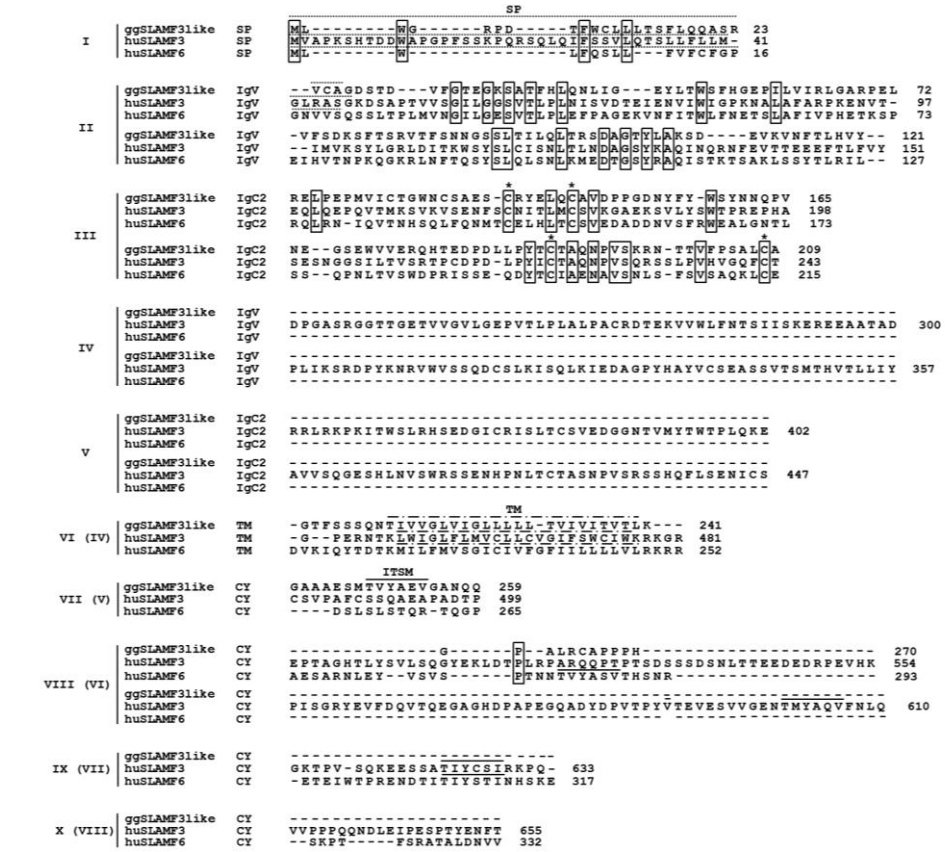


Figure 4 Alignment of chicken SLAMF3like with mammalian SLAMF3 and SLAMF6.

Decorations as in Fig. 1. Numbering of exons according to the human SLAMF3 with corresponding exons for SLAMF3like and SLAMF6 given in brackets. Accession numbers: human SLAMF3, NP_002339.2; chicken SLAMF3like, JX679216; human SLAMF6, NP_001171643.1.

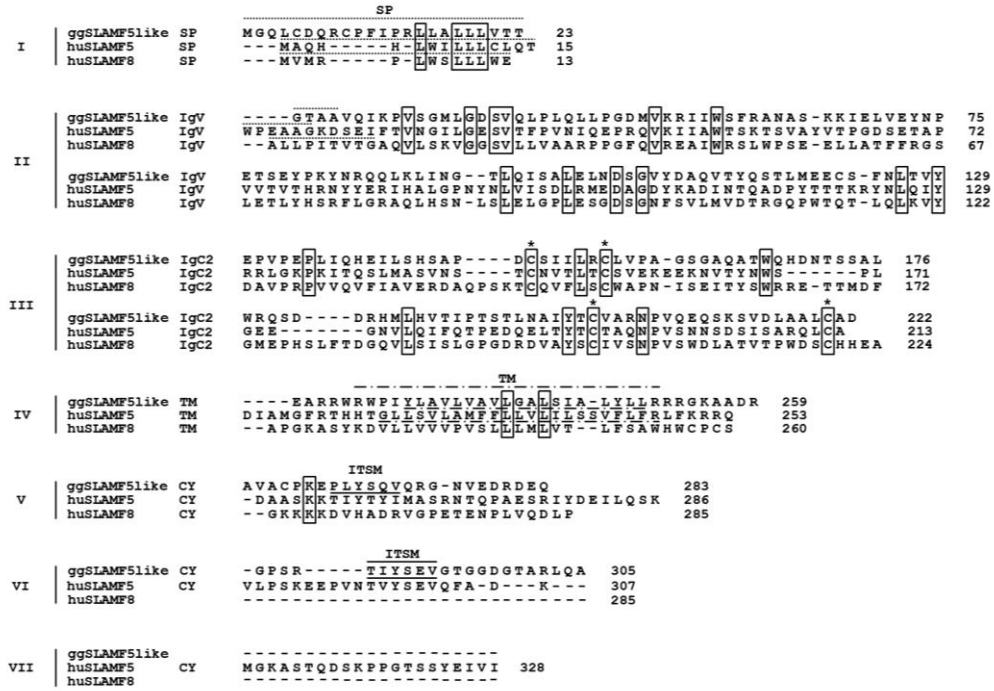


Figure 5 Alignment of chicken SLAMF5like with mammalian SLAMF5 and SLAMF8.

Decorations as in Fig. 1. Accession numbers: human SLAMF5, NP_003865.1; chicken SLAMF5like, JX483811; human SLAMF8, AAI09195.1.

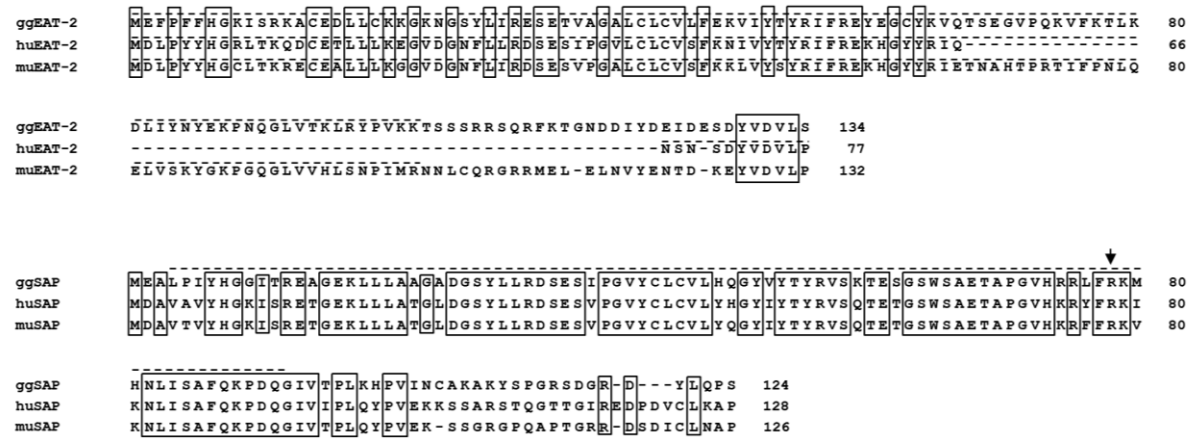


Figure 6 Alignment of EAT-2 and SAP sequences.

The Src homology-2 (SH2) domains are indicated by dotted lines. The recruitment site in SAP for the protein tyrosine kinase Fyn, a motif centered on arginine 78 (R78) is highlighted by an arrow. Accession numbers are: human EAT-2, AAH66595.1; mouse EAT-2, NP_036139.3; chicken EAT-2, ENSMGAT00000016176_1; human SAP, NP_002342.1; mouse SAP, AAI47368.1; chicken SAP, ENSGALT00000013763.

Genomic organization of the chicken SLAM receptor genes

The chicken SLAMF1, SLAMF3like and SLAMF5like genes were identified on chromosome 25 (**Fig. 7A**). Moreover, SLAMF1 homologues could also be identified in the genomes from zebra finch (chromosome 25, ENSTGUT00000004485), turkey (ENSMGAT00000012089) and duck (ENSAPLT00000015980). Likewise, SLAMF5like was also identified on turkey chromosome 16 (ENSMGAT00000019933). For SLAMF2 and SLAMF4, we performed BLAST searches with the confirmed chicken sequences in the genome databases. For SLAMF2 we could find a matching entry in the pre-Ensembl of the chicken which was utilized to obtain the genomic sequence. For SLAMF4, several short matches on an unaligned contig were found. A concise sequence analyses revealed that at least two fragments (encoding the first exon) on the unaligned contig contained identical SLAMF4 sequences. A partial sequence was also found in the turkey genome (ENSMGAT00000020842). In order to obtain a complete genomic sequence, potential exons were identified in the chicken SLAMF4 cDNA sequence according to the information regarding the human sequence. Oligonucleotides located at exon boundaries amplifying each intron were designed (**Tab. 1**) and amplicons cloned and sequenced. In this way, we could finally assemble a genomic sequence of SLAMF4 (**supplementary Tab. 1**). The newly established chicken cDNA sequences for each SLAM receptor were used in order to reanalyze the genomic sequences and to identify the genomic structure. All exon boundaries followed the GT-AG rule. The SLAMF2 gene consisted of four exons, with separate exons encoding the signal peptide, Ig domains and a C-terminus (**Fig.**

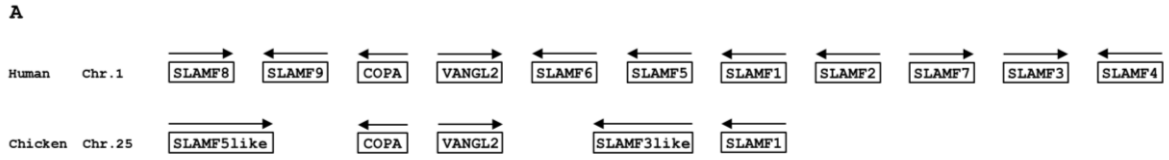
7B). For the other four chicken SLAM receptors, the signal peptide and the two Ig domains were also encoded by three separate exons, followed by one exon for the transmembrane region and a variable number of exons encoding parts of the cytoplasmic region (**Fig. 7B**). Each ITSM or likewise the tyrosine residues in SLAMF5like and SLAMF1 that did not conform to the consensus motif were all encoded by separate exons. In the case of the chicken SLAMF1, SLAMF2, SLAMF4 genes the number and boundaries of the exons is identical to those in man and mouse (**Fig. 1 to 3, 7B**). The chicken SLAMF3like is composed of six exons, whereas human SLAMF3 and SLAMF6 have 10 and 8 exons, respectively (**Fig. 4**). The chicken SLAMF5like is composed of 6 exons, while human SLAMF5 has 7 and human SLAMF8 has 5 exons (**Fig. 5**). In man and mouse, the genes encoding the SLAM receptors are located within a 400 kb cluster on chromosome 1 and it has been argued that the genes arose by successive gene duplication events. The situation in the chicken is not finally resolved, but it is presumed that the location on chromosome 25 where SLAMF1, SLAMF3like and SLAMF5like are located resembles the SLAM gene cluster in the chicken. The sequence identified as SLAMF4 genomic region is on an unassigned chromosome, which needs to be assembled into the SLAM cluster and SLAMF2 is only present in the new, pre-Ensembl version. Finally, the chicken CD2 gene, a distantly related member of the SLAM family is located on chromosome 1 in the chicken (ENSGALG00000015463), thus reflecting a situation similar to the mouse were the SLAM and CD2 genes are present on chromosomes 1 and 3, respectively.

Phylogeny of the SLAM/CD2 family

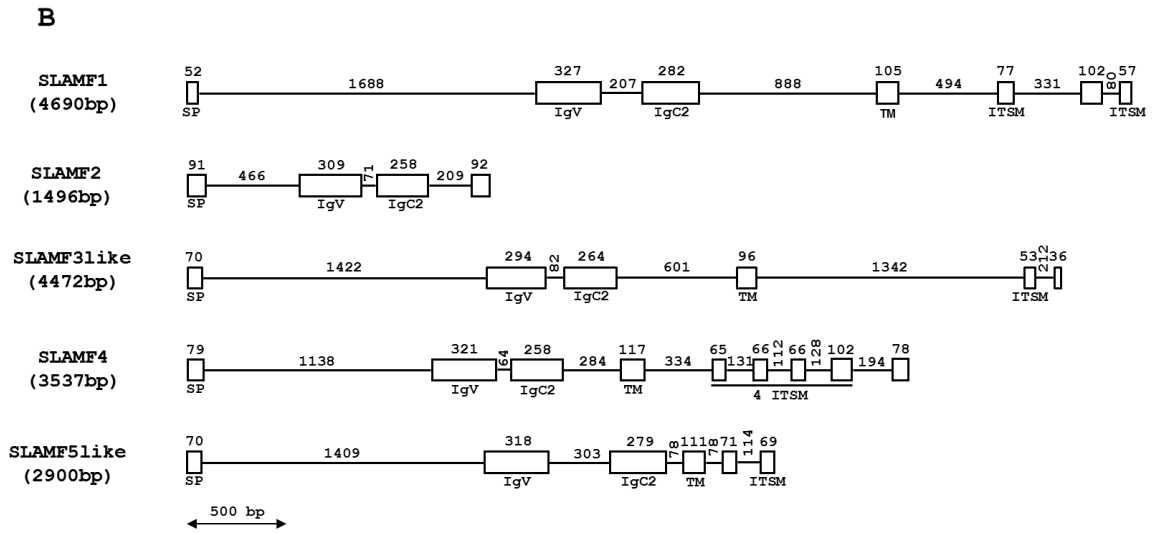
The overall identity between the chicken and mammalian SLAM receptors was rather low. In order to infer the phylogenetic relationships of the chicken and mammalian genes, a number of phylogenetic trees were generated using the MEGA4 software. Since either full length comparisons or separate alignments of the different domains of the proteins produced similar clustering with only slight differences in bootstrapping values, we concentrated on the full length comparison (**Fig. 8**). Besides the human, mouse and chicken SLAM receptors we also included the distantly related chicken CD2 gene identified on chromosome 1 and genes from S. tropicalis, where a large family of SLAM/CD2 like genes has been described, recently (Guselnikov et al. 2011). From those we picked two genes with close homology to CD2 (sCD2-14) and SLAMF1 (sCD2-6), respectively (**Fig. 8**). As expected the CD2 genes formed a separate cluster. The SLAM genes were all related and two major branches of genes could be

distinguished. One branch contained SLAMF1, SLAMF2, SLAMF4 and SLAMF8 genes (**Fig. 8**). SLAMF1 and SLAMF4 genes from all species both formed subgroups, reemphasizing the correct assignment of these chicken SLAMF receptors. Mammalian SLAMF2 and SLAMF8 both formed separate subgroups and chicken SLAMF2 seemed to be closer to the SLAMF8 cluster, however, due to the unique sequence lacking transmembrane and cytoplasmic domains, we are confident that it resembles SLAMF2 rather than SLAMF8. The second branch contained the other SLAM genes, where the mammalian SLAMF3, SLAMF5, SLAMF6 and SLAMF7 appeared to be closely related, while the chicken SLAMF3like and SLAMF5 like genes founded singletons outside this SLAM cluster (**Fig. 8**). In summary, the phylogenetic analysis confirmed the assignment of the various chicken SLAM receptors and emphasized both the close relationship of the SLAM receptors and the unique properties displayed by the individual SLAM groups.

Figure 7 Genomic organization of the SLAM genes.



(A) Schematic representation of the SLAM gene organization in man versus chicken. Note that the scheme is not drawn to scale.



(B) The exon- intron structure of for SLAM genes is illustrated with differentially structured boxes representing exons encoding the signal peptide (SP), Ig-V-like (IgV) and C-2 like (IgC2) domains, transmembrane region (TM) and exons encoding an ITSM. The accession numbers for the genomic sequences are listed in Tab. 2, except for genomic SLAMF4, that was entirely sequenced as described in the text (accession number: JX483814).

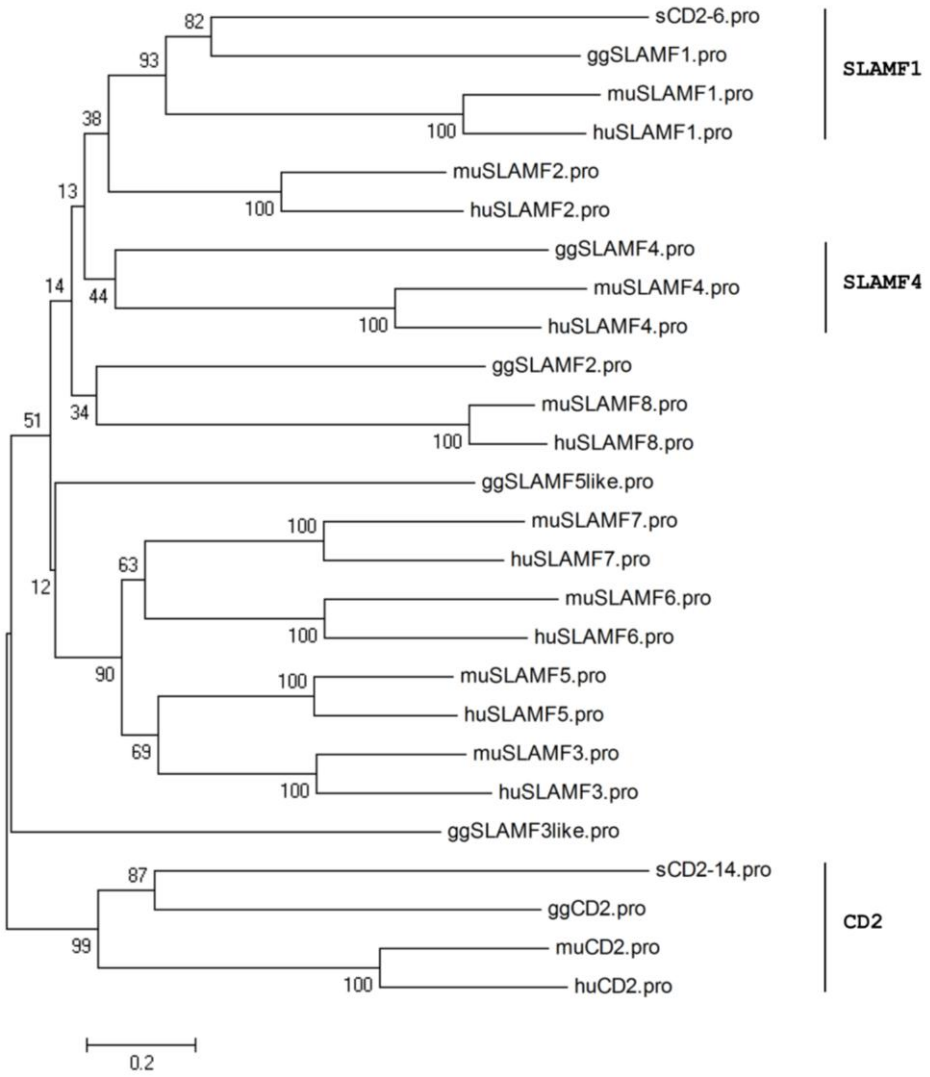


Figure 8 Neighbour-joining phylogenetic tree of the entire proteins of the SLAM/CD2 family in chicken, man, mouse and amphibian (*S.tropicalis*).

Concluding remarks

Here we present the first conclusive characterization of the chicken SLAM family and the SAP adaptors. Without any doubt the identification of homologous genes in non-mammalian vertebrates has become far easier, nevertheless limitations of databases still demand the critical analyses of all sources available. Although the primary amino acid identity was low as expected for a comparison of mammalian and chicken sequences, the SLAM receptors could be identified by their highly conserved properties such as the two Ig domain structure with unique Ig domains and their cytoplasmic regions. It is remarkable how stringent the cytoplasmic ITSM are conserved, but beyond that, even tyrosines and their adjacent residues that do not confirm to the ITSM consensus are also strikingly conserved as in the case of the second tyrosine in SLAMF1 (TIYVAA). In inhibitory or activating signaling molecules it is commonly seen that not only the residues with the consensus motifs, but also the adjacent

amino acid are highly conserved (Göbel and Bolliger 1998; Viertlboeck et al. 2005; Viertlboeck et al. 2007). The presence of both SAP and EAT-2 in the chicken genome also suggests that the SLAM receptors in the chicken have the ability to modulate the immune response in similar fashion as in mammals. It is of interest to characterize immune modulatory receptors on NK cells. So as next step in the analysis of the chicken SLAM family we will produce mab against chicken SLAMF4. Due to the large phylogenetic distance and the resulting low overall identity, this should be a feasible task, and will ultimatively allow studying the expression and function of SLAMF4 on NK cells in more detail.

Supplementary Table 1

Exon – Intron Organization of chicken CD244.

3' Intron	Exon					5' Intron
		ATG	I	CAA	G	GTGAG
		Met	(79)	Gln	G	(1138)
CCCAG	GG	ACT	II	TGG	G	GTGAG
	ly	Thr	(321)	Trp	G	(64)
TGCAG	AG	CCC	III	AAA	G	GTGAT
	lu	Pro	(258)	Lys	G	(284)
CCCAG	GG	GTG	IV	GCA	G	GTTTG
	ly	Leu	(117)	Ala	G	(334)
CTCAG	AA	CAC	V	CCT		GTGAG
	lu	His	(65)	Pro		(131)
TGCAG		AAC	(VI)	CAG		GTGGG
		Asn	(66)	Gln		(112)
GGCAG		AAG	(VII)	AAG		GTGAG
		Lys	(66)	Lys		(128)
GGCAG		CAG	VIII	GAG		GTAAC
		Gln	(102)	Glu		(194)
TGCAG		GTG	IX	TGA		
		Val	(78)	STOP		

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402

Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340:783-795

Boles KS, Stepp SE, Bennett M, Kumar V, Mathew PA (2001) 2B4 (CD244) and CS1: novel members of the CD2 subset of the immunoglobulin superfamily molecules expressed on natural killer cells and other leukocytes. Immunol. Rev. 181:234-249

Cannons JL, Tangye SG, Schwartzberg PL (2011) SLAM family receptors and SAP adaptors in immunity. Annu. Rev. Immunol. 29:665-705

Colonna M (1997) Specificity and function of immunoglobulin superfamily NK cell inhibitory and stimulatory receptors. Immunol. Rev. 155:127-33

Daeron M, Jaeger S, Du Pasquier L, Vivier E (2008) Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol. Rev. 224:11-43

Dong Z, Davidson D, Perez-Quintero LA, Kurosaki T, Swat W, Veillette A (2012) The Adaptor SAP Controls NK Cell Activation by Regulating the Enzymes Vav-1 and SHIP-1 and by Enhancing Conjugates with Target Cells. Immunity 36:974-985

Göbel TW, Bolliger L (1998) The chicken TCR z-chain restores the function of a mouse T cell hybridoma. J. Immunol. 160:1552-4

Göbel TW, Chen CH, Cooper MD (1996a) Avian natural killer cells. Curr. Top. Microbiol. Immunol. 212:107-17

Göbel TW, Chen CH, Cooper MD (1996b) Expression of an avian CD6 candidate is restricted to ab T cells, splenic CD8+ gd T cells and embryonic natural killer cells. Eur. J. Immunol. 26:1743-7

Guselnikov SV, Laktionov PP, Najakshin AM, Baranov KO, Taranin AV (2011) Expansion and diversification of the signaling capabilities of the CD2/SLAM family in Xenopodinae amphibians. Immunogenet. 63:679-689

Kageyama R, Cannons JL, Zhao F, Yusuf I, Lao C, Locci M, Schwartzberg PL, Crotty S (2012) The Receptor Ly108 Functions as a SAP Adaptor-Dependent On-Off Switch for T Cell Help to B Cells and NKT Cell Development. Immunity 36:986-1002

Letunic I, Doerks T, Bork P (2012) SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res. 40:D302-305

Long EO (1999) Regulation of immune responses through inhibitory receptors. Annu. Rev. Immunol. 17:875-904

Neulen ML, Göbel TW (2012) Identification of a chicken CLEC-2 homologue, an activating C-type lectin expressed by thrombocytes. Immunogenet. 64:389-397

Nimmerjahn F, Ravetch JV (2008) Fcgamma receptors as regulators of immune responses. Nat. Rev. Immunol. 8:34-47

Odorizzi PM, Wherry EJ (2012) Inhibitory receptors on lymphocytes: insights from infections. J. Immunol. 188:2957-2965

Ravetch JV, Lanier LL (2000) Immune inhibitory receptors. Science 290:84-89

Reth M (1989) Antigen receptor tail clue. Nature 338:383-384

Rhee I, Veillette A (2012) Protein tyrosine phosphatases in lymphocyte activation and autoimmunity. Nat. Immunol. 13:439-447

Rogers SL, Viertlboeck BC, Göbel TW, Kaufman J (2008) Avian NK activities, cells and receptors. Semin.

Immunol. 20:353-360

Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. U S A 95:5857-5864

Shlapatska LM, Mikhalap SV, Berdova AG, Zelensky OM, Yun TJ, Nichols KE, Clark EA, Sidorenko SP (2001) CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J. Immunol. 166:5480-5487

Sidorenko SP, Clark EA (2003) The dual-function CD150 receptor subfamily: the viral attraction. Nat. Immunol. 4:19-24

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599

Veillette A (2010) SLAM-family receptors: immune regulators with or without SAP-family adaptors. Cold Spring Harb. Perspect. Biol. 2:1-15

Viertlboeck BC, Crooijmans RP, Groenen MA, Göbel TW (2004) Chicken Ig-Like Receptor B2, a Member of a Multigene Family, Is Mainly Expressed on B Lymphocytes, Recruits Both Src Homology 2 Domain Containing Protein Tyrosine Phosphatase (SHP)-1 and SHP-2, and Inhibits Proliferation. J. Immunol. 173:7385-7393

Viertlboeck BC, Göbel TW (2007) Chicken thrombocytes express the CD51/CD61 integrin. Vet. Immunol. Immunopathol. 119:137-141

Viertlboeck BC, Göbel TW (2011) The chicken leukocyte receptor cluster. Vet Immunol Immunopathol 144:1-10

Viertlboeck BC, Habermann FA, Schmitt R, Groenen MA, Du Pasquier L, Göbel TW (2005) The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types. J. Immunol. 175:385-393

Viertlboeck BC, Hanczaruk MA, Schmitt FC, Schmitt R, Göbel TW (2008) Characterization of the chicken CD200 receptor family. Mol. Immunol. 45:2097-2105

Viertlboeck BC, Schmitt R, Göbel TW (2006) The chicken immunoregulatory receptor families SIRP, TREM, and CMRF35/CD300L. Immunogenet. 58:180-190

Viertlboeck BC, Schmitt R, Hanczaruk MA, Crooijmans RP, Groenen MA, Göbel TW (2009) A novel activating chicken IgY FcR is related to leukocyte receptor complex (LRC) genes but is located on a chromosomal region distinct from the LRC and FcR gene clusters. J. Immunol. 182:1533-1540

Viertlboeck BC, Schweinsberg S, Hanczaruk MA, Schmitt R, Du Pasquier L, Herberg FW, Göbel TW (2007) The chicken leukocyte receptor complex encodes a primordial, activating, high-affinity IgY Fc receptor. Proc. Natl. Acad. Sci. U S A 104:11718-11723

Zhao F, Cannons JL, Dutta M, Griffiths GM, Schwartzberg PL (2012) Positive and Negative Signaling through SLAM Receptors Regulate Synapse Organization and Thresholds of Cytolysis. Immunity 36:1003-1016

Zhu Y, Yao S, Chen L (2011) Cell surface signaling molecules in the control of immune responses: a tide model. Immunity 34:466-478

Chicken SLAMF4 (CD244, 2B4), a receptor expressed on thrombocytes, monocytes, NK cells, and subsets of αβ-, γδ-T cells and B cells binds SLAMF2

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Abstract

The SLAM family of membrane receptors is involved in the regulation of immune responses by controlling cytokine production, cytotoxicity as well as cell development, differentiation and proliferation, but has only been described in chickens, recently. The aim of this study was to characterize the avian homologue to mammalian SLAMF4 (CD244, 2B4), a cell surface molecule which belongs to the SLAM family of membrane receptors. We generated a SLAMF4 specific monoclonal antibody (mab) designated 8C7 and analyzed the SLAMF4 expression on cells isolated from various lymphoid organs. Subsets of αβ and γδ T cells found in peripheral blood lymphocytes (PBL) and spleen coexpressed SLAMF4. The expression was restricted to $CD8\alpha^+$ T cells, whereas $CD4^+$ T cells and all thymocytes showed little or no reactivity upon staining with the 8C7 mab. Blood and splenic γδ T cells could be further differentiated according to their expression levels of SLAMF4 into two and three subsets, respectively. SLAMF4 was absent from bursal and splenic B cells, however, it was expressed by a distinct fraction of circulating B cells that were characterized by high level expression of Bu1, Ig, and CD40. SLAMF4 was also present on NK cells isolated from intestine of adult chickens or embryonic splenocytes identified by their coexpression of the 28-4 NK cell marker. Moreover, SLAMF4 expression was found on thrombocytes and monocytes. The interaction of SLAMF4 with SLAMF2 was proven by a reporter assay and could be blocked with the 8C7 mab. In conclusion, the avian SLAMF4 expression markedly differs from mammals; it binds to SLAMF2 and will be an important tool to discriminate several γδ T cell subsets.

Keywords

SLAM, CD244, 2B4, CD48, γδ T cell, Chicken

Abbreviations

SLAM, signaling lymphocytic activation molecule

SAP, SLAM associated protein-family of adaptors

ITSM, immunoreceptor tyrosine-based switch motif

Introduction

SLAMF4 (CD244, 2B4) is a member of the signaling lymphocyte activation molecule (SLAM) family of membrane receptors that belongs to the CD2 subset of the immunoglobulin (Ig) superfamily (Cannons et al., 2011). Receptors related to this family also include SLAMF1 (CD150, SLAM), SLAMF2 (CD48), SLAMF3 (Ly9, CD229), SLAMF5 (CD84), SLAMF6 (NTB-A, CD352), SLAMF7 (CRACC, CD319) and SLAMF8 (BLAME, CD353) and family affiliation is primarily based on homologies of the extracellular domain, each consisting of an amino-terminal IgV-like domain and a membrane-proximal IgC2-like domain (Cannons et al., 2011; Davis and van der Merwe, 1996; Schwartzberg et al., 2009). In addition, SLAMF1, SLAMF3, SLAMF4, SLAMF5, SLAMF6 and SLAMF7 share homologies within their cytoplasmic domains, in particular repeats of immunoreceptor-tyrosine based switch motifs (ITSM) (Shlapatska et al., 2001; Sidorenko and Clark, 2003). Like most other SLAM family members, SLAMF4 interacts through its cytoplasmic motifs with SLAM-associated protein (SAP)-related molecules (Sayos et al., 1998). SAP-related adaptors include SAP, EAT-2 and ERT, the latter being a pseudogene in humans. They are composed of a single Src homology 2 (SH2) domain followed by a short C-terminal tail. Unlike most SLAM family members which form homophilic interactions, SLAMF4 interacts with SLAMF2, a glycosyl-phosphatidyl-inositol (GPI)-anchored protein expressed on virtually all hematopoietic cells. The role of SLAMF4 has been best characterized in natural killer (NK) cells, where it is recognized to act both as activating and inhibitory receptor upon engagement by SLAMF2, although the nature of this bi-functional character is not completely solved. Initial studies showed that ligation of SLAMF4 with anti-SLAMF4 antibodies or its ligand SLAMF2 increased cytotoxicity and IFN-γ secretion exerted by NK cells (Tangye et al., 2000). New reports indicate that the level of surface SLAMF4 expression and the amount of intracellular SAP are the critical parameters involved in either activatory or inhibitory responses by NK cells (Zhao et al., 2012). Expression of SLAMF4 is not restricted to NK cells, but extends to a subset of activated $CD8^+$ αβ T cells, γδ T cells, eosinophils, basophils and monocytes (Munitz et al., 2005; Nakajima et al., 1999; Schlaphoff et al., 2011; Schuhmachers et al., 1995). The dual function of SLAMF4 also applies to $CD8^+$ T cells where cross-linking of SLAMF4 mediates either inhibition or activation, again depending on the levels of expression of SLAMF4 and SAP, respectively (Schlaphoff et al., 2011). In contrast, SLAMF4 present on human eosinophils seems to have a predominantly activating character. Engagement of SLAMF4 resulted in cytotoxic effects toward the $SLAMF2^+$ 721.221 B cell lymphoma and the release of eosinophil peroxidase, a recognized marker of eosinophil activation (Munitz et al., 2005). The expression of SLAMF4

on γδ T cells has been mainly studied on murine epidermal γδ T cells. Here, ligation with soluble anti-SLAMF4 mab caused rapid secretion of IFN-γ and enhanced the capacity of these cells to lyse YAC-1 target cells (Schuhmachers et al., 1995). These findings only refer to the current situation in mammals. The characterization of this receptor in non-mammalian vertebrates may provide novel aspects regarding expression and function. Previously, we have identified several members of the chicken SLAM family including SLAMF4 and SLAMF2, as well as the intracellular adaptor molecules SAP and EAT-2 (Straub et al., 2013). In the present study, we delineate the expression pattern of chicken SLAMF4 on leukocytes with a specific mab and confirm the conservation of the SLAMF4-SLAMF2 interaction in non-mammalian vertebrates. Our studies reveal that SLAMF4 levels discriminate distinct γδ T cell subsets.

Materials and methods

Animals

Fertilized eggs of the chicken line M11 were kindly provided by S. Weigend (Federal Research Institute for Animal Health, Mariensee, Germany). Eggs were incubated and hatched at the Institute for Animal Physiology, University of Munich and chicks were used for experiments at the age of 3 to 10 weeks. Embryonic day 14 (ED14) was estimated by the duration of incubation. Balb/c mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany) and maintained at the institute facilities. All of the experimental procedures were in accordance with institutional, state and federal guidelines on animal welfare.

Cell preparation

Leukocytes were isolated from heparinized whole blood samples by two distinct methods. Peripheral blood lymphocytes (PBL) were prepared by slow-speed centrifugation whereas peripheral blood mononuclear cells (PBMC, thrombocytes, lymphocytes and monocytes) were segregated by density gradient centrifugation on Biocoll-Hypaque (Biochrom, Berlin, Germany) (Viertlboeck and Göbel, 2007). Single cell suspensions of thymus, bursa and spleen were obtained by passage through a stainless steel mesh and mononuclear cells (lymphocytes and macrophages) were isolated by density centrifugation over Biocoll-Hypaque (density 1,077 g/ml; Biochrom, Berlin, Germany). Intestinal intraepithelial lymphocytes were isolated

from the duodenal loop according to a standard DTT/EDTA based protocol as described previously (Göbel, 2000).

Cloning, Cell lines and Transfections

Expression constructs containing the extracellular region of SLAMF1, SLAMF2, SLAMF3like, SLAMF4 and SLAMF5like were generated using the genes described in a previous study (Straub et al., 2013). The plasmids for SLAMF1, SLAMF3like and SLAMF5like were amplified by PCR with primers designed to employ the Gibson Assembly ™ Master Mix (New England BioLabs Inc., Massachusetts, USA). Briefly, primers were devised to amplify residues encoding the respective extracellular domain flanked by sequences recognizing residues of the cloning vector at the 5`UTR and the 3`UTR, respectively and thus creating overlapping ends. Resulting DNA fragments were gel extracted and linked to a modified pcDNA3.1/V5-His expression vector (Invitrogen, Karlsruhe, Germany) (Viertlboeck et al., 2004) by the Gibson Assembly ™ Master Mix (New England BioLabs Inc., Massachusetts, USA), resulting in an N-terminally FLAG-tagged extracellular region of the respective SLAM member fused to the transmembrane region of chicken CD8α and the cytoplasmic domain of murine CD3ζ. In contrast, expression constructs encoding the extracellular region for SLAMF2 and SLAMF4 were PCR-amplified using primers with EcoRI sites (SLAMF2: 1249-1250; SLAMF4: 1251-1252), EcoRI digested and ligated in the modified pcDNA3.1/V5-His Topo Vector. Primer sequences used for cloning are summarized in **Table 1**. All inserts were sequenced using vector-specific primers (GATC, Konstanz, Germany). For immunization and binding studies, a stable cell line was generated transfecting the mouse BWZ.36 reporter cell line by electroporation (Iizuka et al., 2003) with the SLAMF4-FLAG expression construct. After 24 h, cells were plated at 3×10^6 cells/well in a 96-well flat-bottom plate and cultured with RPMI 1640 medium supplemented with 10% low IgG FCS and 1% penicillin/streptomycin under standard growth conditions for a duration of 10 days. G418 (Biochrom AG, Berlin, Germany) in a concentration of 0.8 mg/ml was added as selective antibiotic. Additional cell lines were established to perform molecular binding assays and to exclude mab cross-reactivity with other SLAM family members. For this purpose, HEK-293 cells were stably transduced with all five expression constructs using the Metafectene liposomal transfection reagent according to the manufacturer`s protocol (Biontex, Planegg, Germany). Transfected cells were incubated for 24 h (37°C, 5% CO_2), subsequently seeded in a 96-well flat bottom plate and cultured with medium containing 0.8 mg/ml G418

(Biochrom AG, Berlin, Germany) for 2 weeks. Single colonies were screened by flow cytometry (FACS Canto II, Beckton Dickinson, USA) for expression of the FLAG epitope.

Mab production

The anti-chicken SLAMF4 mab was generated as described previously (Viertlboeck et al., 2004) by repeatedly immunization of a Balb/c mouse with the SLAMF4-FLAG BWZ.36 cell line. Hybridoma supernatants were successively screened on SLAMF4-FLAG transfected HEK-293 cells. Our further studies were conducted with mab 8C7 (mouse IgG1). The mab was affinity purified on protein G coupled agarose from concentrated supernatants using standard procedures (Milipore, MA, USA) and subsequent flow cytometric analysis were performed with the unconjugated mab 8C7.

Mab

Mab used for staining were specific for γδ T cells [TCR1 (Sowder et al., 1988), SBA, Birmingham, USA)], different subsets of αβ T cells [TCR2; TCR3 (Chen et al., 1989; Cihak et al., 1988), SBA, Birmingham, USA], chicken homologues to CD8α, CD4 and CD3 [CT8; CT4; CT3 (Chan et al., 1988; Chen et al., 1986), SBA, Birmingham, USA], monocytes and macrophages [KUL01 (Mast et al., 1998), SBA, Birmingham, USA], B cell marker Bu1 [AV20 (Rothwell et al., 1996; Tregaskes et al., 1996), SBA, Birmingham, USA], intestinal NK cells [28-4, mouse IgG3; (Göbel et al., 2001)], thrombocytes [8G8; mouse IgG2a (Neulen and Göbel, 2012)], CD40 [AV79; mouse IgG2a (Kothlow et al., 2008)], L chain [11C6; mouse IgG3 (Ratcliffe, 1989)], MHCII [2G11 (Guillemot et al., 1986), SBA, Birmingham, USA], and IgM [M2; mouse IgG2a (Erhard et al., 1992)]. All commercially available mab were used as fluorochrome conjugates and IgG1 isotypes except where indicated.

Staining procedures immunofluorescence analysis

For single-cell staining, cells were incubated with the unconjugated anti-SLAMF4 mab, followed by an allophycocyanin-conjugated goat-anti-mouse IgG1 antibody (SBA, Birmingham, USA).

For multi-color immunofluorescence analysis using mab of the IgG1 isotype, the cells were stained with the anti-SLAMF4 mab followed by incubation of an anti-mouse IgG1-APC

conjugate (SBA, Birmingham, USA). Free binding sites of the APC-conjugated second step were blocked by incubation with normal mouse serum (JacksonImmuno Research, West Grove, USA) before addition of the corresponding phycoerythrin-conjugated mab and/or fluorescein conjugated mab.

When appropriate, cells were first treated with a mixture of primary mab, followed by incubation of a mixture of anti-mouse IgG1-APC and, depending on the isotype of the second mab either phycoerythrin anti-mouse IgG2a or IgG3 (all SBA, Birmingham, USA). The fluorochrome combinations as well as the staining procedure are summarized in **Table 2**. For each staining, appropriate isotype-matched controls were used. Additional controls were performed to exclude the possibility of crossreactivity of the anti-mouse IgG2a and IgG3 conjugates with the IgG1 mab. Dead cells were stained with 7-amino-actinomycin D (7-AAD, Sigma-Aldrich, Germany) at 25 µg/ml and the living cell population was analyzed by flow cytometry (FACS Canto II, Becton Dickinson, Heidelberg, Germany) applying the analysis software BD FACS DIVA 6.1.3 and FlowJo (Tree Star Inc., Oregon, USA). All figures are shown biexponentially.

Table 1 Oligonucleotides used for cloning.

Number	Sequence	O[a]	Specifity
1742	GGACGATGACGATAAGTGTAGGACAGTGGAGACAG	S	SLAMF1
1743	TGGATATCTGCAGAATTCCAGCAGCATCCCTGTCC	AS	SLAMF1
1249	ATGAATTCCAGAAGGACCTGCAA	S	SLAMF2
1250	ATGAATTCGATGGAGGCAGTGGG	AS	SLAMF2
1246	GGACGATGACGATAAGGGTGATAGCACGGATGTAT	S	SLAMF3like
1247	TGGATATCTGCAGAATTCATTTTGGCTGCTGGAGA	AS	SLAMF3like
1251	ATGAATTCGGGACTGGAGAGTGC	S	SLAMF4
1252	ATGAATTCCCATGGGACGGCACC	AS	SLAMF4
1744	GGACGATGACGATAAGGTTCAGATCAAGCCAGTAA	S	SLAMF5like
1745	TGGATATCTGCAGAATTCCCACCTGCGTGCCTCGT	AS	SLAMF5like

[a] Primers were designed to be specific for the various SLAM genes indicated (sequence underlined) and also containing overlapping regions to the cloning vector according to the manufacturers protocol (Gibson Assembly™ Master Mix, New England Biolabs Inc., USA); in case of SLAMF2 and SLAMF4, subcloning with restriction endonucleases was used (restriction sites are underlined).

Table 2 Staining procedures.

Primary mouse mab	Secondary anti-mouse conjugates	Blocking step	Direct conjugates
8C7 (IgG1)	IgG1-APC	NMS	TCR1-PE
8C7 (IgG1)	IgG1-APC	NMS	TCR2-PE
8C7 (IgG1)	IgG1-APC	NMS	TCR3-PE
8C7 (IgG1)	IgG1-APC	NMS	CT3-PE
8C7 (IgG1)	IgG1-APC	NMS	CT4-PE
8C7 (IgG1)	IgG1-APC	NMS	CT8-PE
8C7 (IgG1)	IgG1-APC	NMS	AV20-PE
8C7 (IgG1)	IgG1-APC	NMS	TCR1-PE + CT8-FITC
8C7 (IgG1) + AV79 (IgG2a)	IgG1-APC + IgG2a-FITC	NMS	AV20-PE
8C7 (IgG1) + M2 (IgG2a)	IgG1-APC + IgG2a-FITC	NMS	AV20-PE
8C7 (IgG1) + 11C6 (IgG3)	IgG1-APC + IgG3-FITC	NMS	AV20-PE
8C7 (IgG1)	IgG1-APC	NMS	AV20-PE + 2G11-FITC
8C7 (IgG1) + 8G8 (IgG2a)	IgG1-APC + IgG2a-PE		
8C7 (IgG1)	IgG1-APC	NMS	KUL01-PE
8C7 (IgG1) + 28-4 (IgG3)	IgG1-APC + IgG3-PE		
8C7 (IgG1) + 28-4 (IgG3)	IgG1-APC + IgG3-FITC	NMS	CT3-PE

Abbreviations: NMS, Normal Mouse Serum; mab, monoclonal antibody.

BWZ.36 reporter assay

The experimental setup was as follows: a total of 3 x 10^5 cells were seeded in 24 well cell culture plates. The SLAMF4-FLAG BWZ.36 reporter cells were coincubated with stably expressing SLAMF2 Flag–HEK-293 cells at a concentration of 1.5 x 10^5 cells each. As controls, we used both a mock-transfected HEK-293 cell line to exclude unspecific binding reactivity and addition of anti-SLAMF4 mab to block specific binding sites. Additionally, the reporter cell line was incubated in plates coated with the anti-FLAG mab (Sigma–Aldrich, Munich, Germany). After 24 hours, cells were lysed on ice and the β-galactosidase activity was measured using as substrate 130 µl/well of chlorophenolred-ß-D-galactopyranosid (Roche, Mannheim, Germany) and quantified by optical density reading at 575 nm 18 hours after incubation. All experiments were performed in triplicates.

Results

Generation of a mab specific for chicken SLAMF4

For immunization and screening, two stable cell lines (HEK-293 cells and BWZ.36 cells) were generated by transfection of the extracellular SLAM4 domains linked to unrelated transmembrane and cytoplasmic domains. The N-terminal FLAG epitope allowed selection of positive clones by cytometric staining. Following immunization with SLAMF4-FLAG BWZ.36 cells and fusion, the hybridoma supernatants were differentially screened on HEK 293-SLAMF4 cells and untransfected cells to identify SLAMF4 specific clones. The hybridoma producing the 8C7 mab (mouse IgG1) was identified by this strategy (**Fig. 1**). Since the different SLAM receptors as members of a superfamily share structural features, we next tested the ability of mab 8C7 to cross-react with other chicken SLAM family members. For this purpose, chicken SLAMF1, SLAMF2, SLAMF3like and SLAMF5like were stably transfected into HEK-293 cells as FLAG-tagged versions. Whereas anti-FLAG staining revealed surface expression of all receptors, none of these cell lines reacted with the 8C7 mab thus demonstrating that 8C7 is a SLAMF4 specific mab (**Fig. 1**).

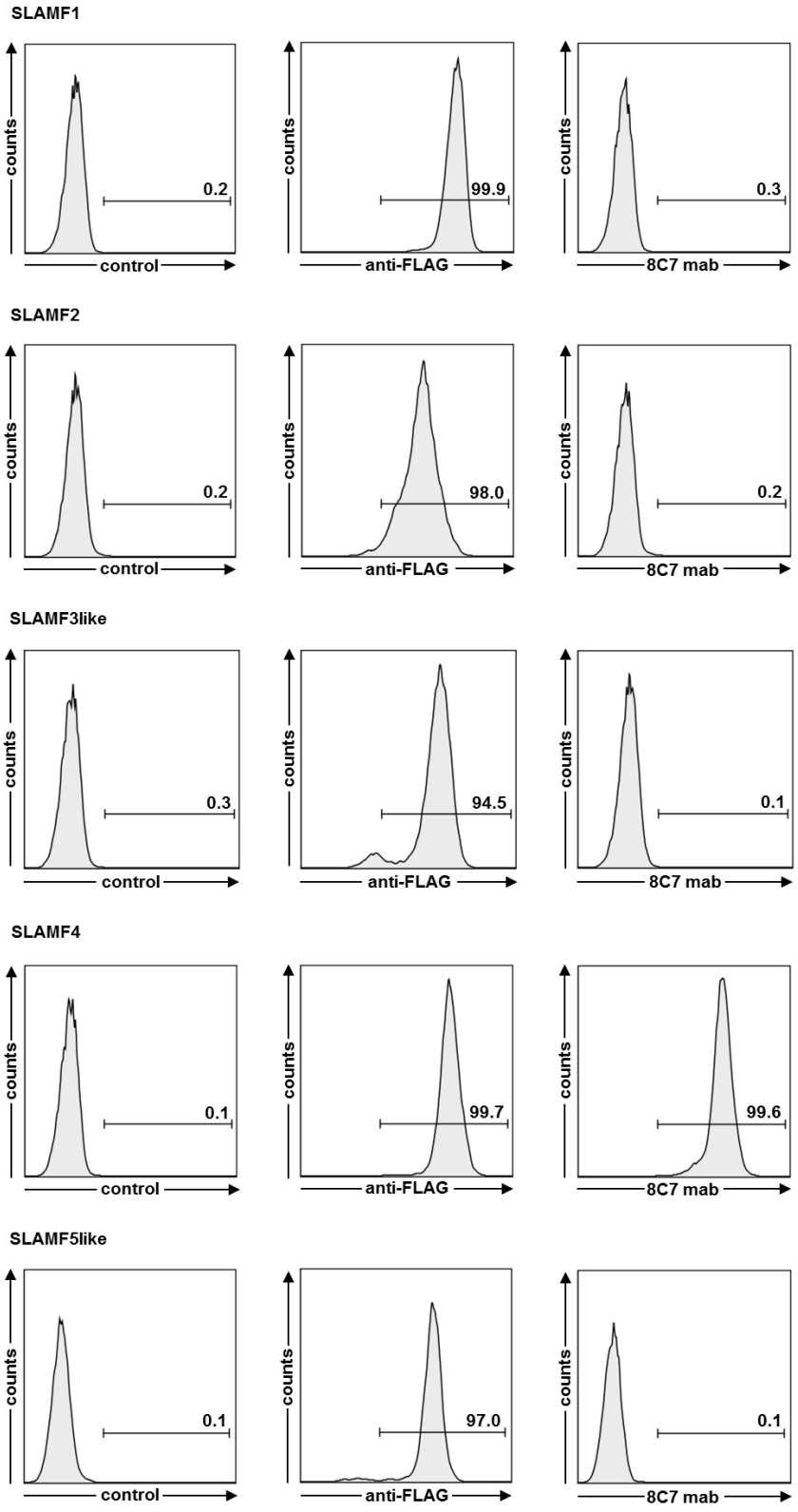


Figure 1 8C7 specifically binds SLAMF4.

HEK-293 cells stably expressing the FLAG epitope tagged extracellular regions of SLAMF1, SLAMF2, SLAMF3like, SLAMF4 and SLAMF5like, respectively, were incubated with an isotype matched control (left panels), an anti-FLAG mab as expression control (middle panels) and the 8C7 mab (right panels). One representative experiment of five is shown.

Chicken SLAMF4 is expressed on subsets of T and B cells

In order to examine the tissue distribution of SLAMF4 in the chicken, we first analyzed lymphocyte preparations obtained from bursa, thymus, blood, and spleen. Thymocytes and bursal cells showed no reactivity upon staining with the 8C7 mab (data not shown). In PBL, SLAMF4 expression divided the $CD3^+$ cells into three distinct subsets, namely $CD3^+$ $SLAMF4^-$ cells, $CD3^+$ $SLAMF4^{dim}$ cells and $CD3^+$ $SLAMF4^{bright}$ cells (**Fig. 2A**). The analysis with TCR specific mab demonstrated that absent, dim or bright SLAMF4 expression was found in both αβ- and γδ T cell subsets; however, most αβ cells were either $SLAMF4^-$ or $SLAMF4^{bright}$, whereas the γδ T cells were mostly $SLAMF4^{dim}$ or $SLAMF4^{bright}$ (**Fig. 2A**). SLAMF4 was further confined to the $CD8\alpha^+$ lymphocytes, while it was barely detectable on $CD4^+$ cells. Finally a subset of B-lymphocytes coexpressed SLAMF4 and this subset consistently expressed higher levels of the B cell marker Bu1 as compared to the $SLAMF4^-$ B cell fraction (**Fig. 2A**).

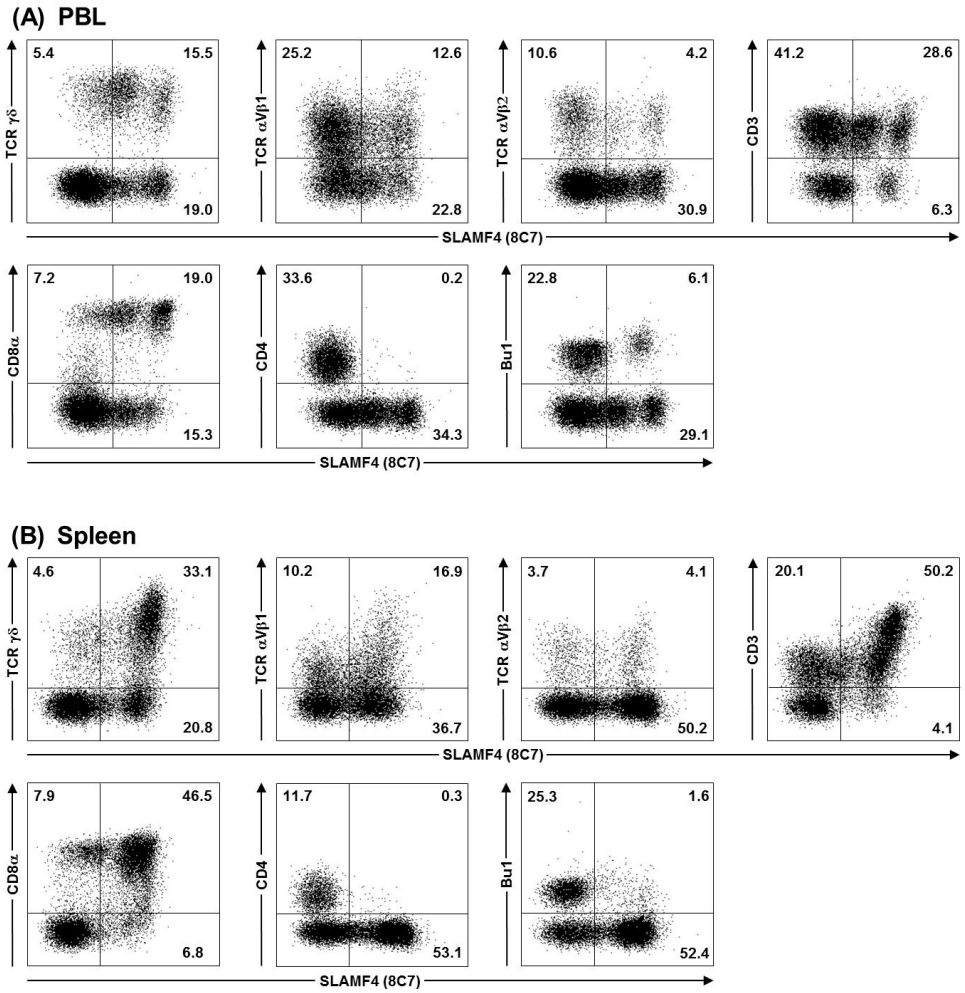


Figure 2 SLAMF4 is expressed on $CD8\alpha^+$ lymphocytes and a blood B cell subset.

Immunofluorescence analyses of the lymphocyte sized cells of (A) PBL and (B) splenocytes using the SLAMF4 specific 8C7 mab in combination with several mab as indicated. Numbers indicate the percentage of cells in the respective quadrants. The markers were set according to isotype-matched negative controls. One representative experiment of five is shown.

The SLAMF4 expression on splenocytes differed in various aspects from that of PBL. SLAMF4 was detectable only on a minute fraction of B cells (**Fig. 2B**). Most of the CD8α$^+$ T cells expressed either dim or bright levels of SLAMF4. The αβ T cells could be divided into two subpopulations of SLAMF4$^+$ and SLAMF4$^-$ cells, whereas most of the γδ T cells expressed SLAMF4 at high levels. As can be seen from the TCR staining and the CD3 staining in **Fig. 2B**, there was a correlation of SLAMF4 expression and TCR expression. In conclusion, SLAMF4 is expressed by CD8 T-lymphocytes at different levels and on a fraction of blood B cells.

Subsets of γδ T cells found in blood and spleen differ in their SLAMF4 expression level

It has been shown that avian γδ T cells can be classified on the basis of the degree of CD8 expression into various subpopulations (Pieper et al., 2011). To elucidate whether SLAMF4 expression on γδ T cells differs between the subpopulations defined by CD8, we performed triple immunofluorescence analyses. In all analyses, we employed the CT8 mab recognizing the CD8α chain, so both CD8αα homodimer and CD8αβ heterodimer expressing cells are stained. In PBL and spleen, γδ T cells were divided into two subpopulations depending on presence or absence of CD8α (**Fig. 3**). In blood, the CD8α$^-$ γδ T cell subset expressed lower SLAMF4 cell surface levels (**Fig. 3**, gate I; MFI 710) as the CD8α$^+$ cells (**Fig. 3**, gate II; MFI 3500). Likewise, the CD8α$^+$ γδ T cells in the spleen expressed high SLAMF4 levels (**Fig. 3**, gate IV; MFI 2066). Within the subset of CD8α$^-$ γδ splenocytes, two subsets based on low and high SLAMF4 expression could be further distinguished (**Fig. 3**, gate III, MFI 363 and MFI 1243, respectively). In conclusion, two and three γδ T cell subpopulations can be defined in blood and spleen, respectively. In blood, these subpopulations represent CD8α$^-$ SLAMF4^{dim} cells and CD8α$^+$ SLAMF4^{bright} cells, whereas in spleen, two subsets of CD8α$^-$ with either dim or intermediate SLAMF4 expression can be distinguished from a third population of cells expressing CD8α and bright SLAMF4 levels.

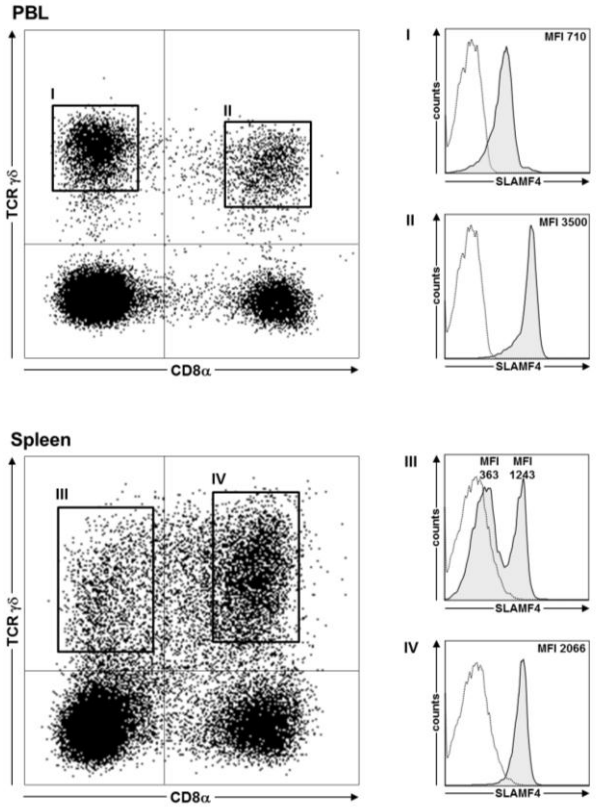


Figure 3 Subsets of γδ T cells differ in their SLAMF4 expression level.

γδ T cells from (A) blood and (B) spleen were triple stained with mab directed against γδ TCR, CD8α and SLAMF4. Gates (I to IV) were set on $CD8\alpha^-$ and $CD8\alpha^+$ γδ T cells and cells within gates were analyzed for their SLAM4 expression (filled histograms) as opposed to a negative control (open histograms). The mean fluorescence intensity (MFI) of SLAMF4 staining is indicated. One representative experiment of five is shown.

The $SLAMF4^+$ B cells display high surface Ig levels

In blood but not in spleen, a fraction of B cells coexpressed SLAMF4. These cells were defined as B cells using the Bu1 marker and the plots revealed that the expression of Bu1 was higher on the $SLAMF4^+$ B cells as compared to the $SLAMF4^-$ fraction. To further characterize this B cell subset, we employed three color immunofluorescence analysis by gating on Bu1 single positive and Bu1/SLAMF4 double positive cell populations, respectively (**Fig. 4**, gates I and II). Both populations expressed MHC class II at similar levels. In contrast, the $SLAMF4^+$ B cells expressed higher levels of IgM, Ig light chain and CD40. These results show that SLAMF4 is expressed on a B cells subset with high CD40 and Ig surface levels.

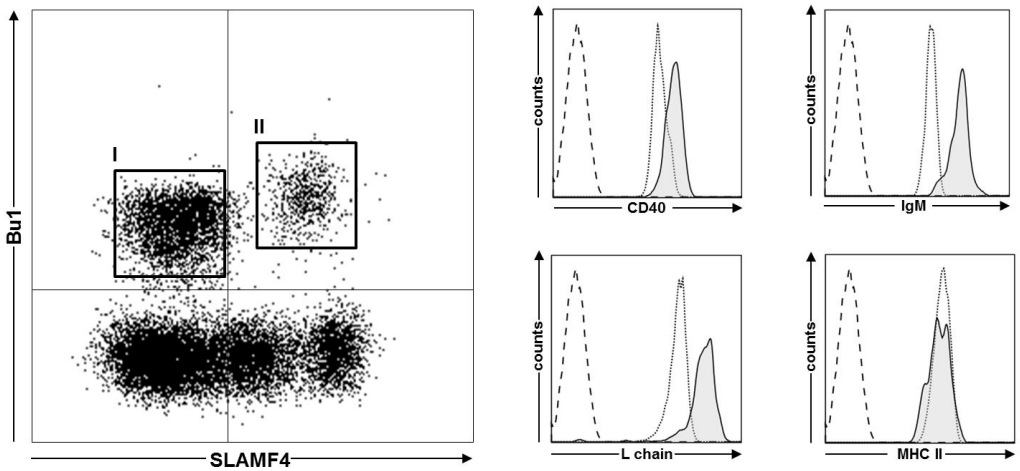


Figure 4 Differences between SLAMF4^{+} and SLAMF4^{-} B cells.

B cells were gated according to their SLAMF4 and Bu1 expression into either Bu1 single positive (gate I) or double positive cells (gate II) and analyzed for various markers as indicated below the histograms. Dashed lines represent isotype matched control staining, dotted lines cells in gate I and shaded histograms double positive B cells in gate II. One representative experiment of five is shown.

Expression of chicken SLAMF4 on thrombocytes and monocytes

We next analyzed the expression profile of SLAMF4 on thrombocytes and monocytes. For this purpose, we prepared blood samples by density gradient centrifugation to obtain a cell suspension enriched with mononuclear cells, in particular nucleated thrombocytes and monocytes. Following gating on thrombocytes by characteristic forward/side scatter properties, SLAMF4 was coexpressed on virtually all cells labeled with the mab against CLEC-2 (**Fig. 5**), a molecule expressed on chicken thrombocytes (Neulen and Göbel, 2012). Likewise, gating on monocytes and successive analysis of the cells for the coexpression of SLAMF4 and KUL01, a known monocyte/macrophage marker revealed that virtually all monocytes expressed SLAMF4 (**Fig. 5**). These results indicate that SLAMF4 is not confined to the lymphocyte lineage, but is also expressed by other leukocytes.

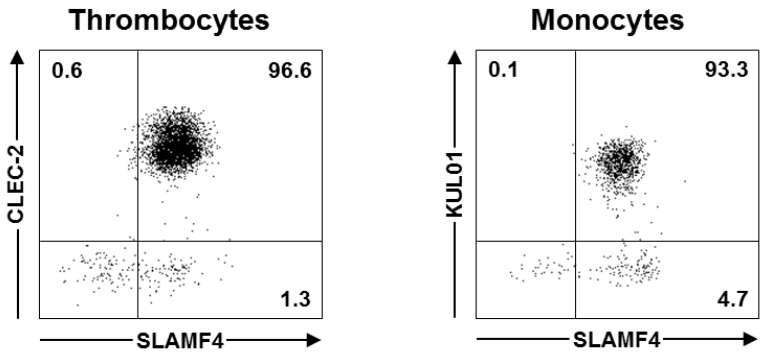


Figure 5 SLAMF4 expression on thrombocytes and monocytes.

Mononuclear blood leukocytes were isolated by density centrifugation and gates were set on thrombocytes and monocytes according to forward/side scatter properties (**suppl. Fig.1**). SLAMF4 staining was combined with either the thrombocyte specific marker CLEC-2 (left panel) or the monocyte/macrophage specific marker KUL01 (right panel). One representative experiment of five is shown.

SLAMF4 expression on NK cells

In previous studies, avian NK cells have been mainly found in two distinct sites, the intestinal epithelium (IEL) and embryonic spleen (Göbel et al., 1994; Göbel et al., 2001). The 28-4 marker specifically recognizes NK cells in these organs (Göbel et al., 2001). In embryonic spleen, most of the 28-4$^+$ cells coexpressed SLAMF4, whereas a minority of cells displayed low or undetectable levels (**Fig.5A**). There was an additional subset of SLAMF4$^+$ 28-4$^-$ cells not positive for T cell or B cell markers. They might represent hematopoietic precursors present in embryonic spleen. In the IEL population, we performed a three color staining to analyze SLAMF4 levels on CD3$^+$ T cells and 28-4$^+$ NK cells (**Fig. 5B**). The 28-4$^+$ NK cells were all found to express SLAMF4 (**Fig. 5B**, gate I), and likewise the CD3$^+$ IEL cells all expressed SLAMF4 (**Fig. 5B**, gate II). These CD3$^+$ IEL cells mainly represent intestinal γδ T cells and with a low percentage of αβ T cells. Likewise, the low number of CD4$^+$ IEL did not express SLAMF4 (data not shown). In conclusion, SLAMF4 is present on all major NK cell populations in the chicken and it is also expressed by intestinal T cells.

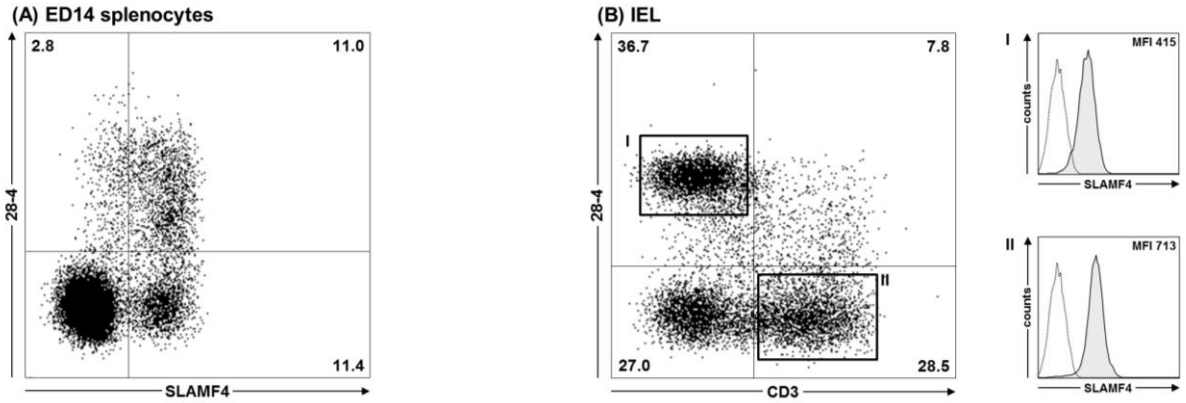


Figure 6 SLAMF4 expression on NK cells and intestinal T cells.

(A) ED14 splenocytes were analyzed for the dual expression of the NK cell marker 28-4 and SLAMF4. (B) Three color-imaging of IEL using mAbs specific for CD3, the 28-4 antigen and SLAMF4. Gates were set according to the 28-4/CD3 staining and analyzed for SLAMF4 expression (filled histograms) as compared to the negative control (open histograms). MFI of SLAMF4 is indicated. One representative experiment of five is shown.

Conservation of SLAMF2 as counter-ligand of SLAMF4

SLAMF2 has been identified as the high-affinity ligand of SLAMF4 in mammalian species (Brown et al., 1998). To test, whether chicken SLAMF4 is able to interact with SLAMF2, we employed a previously established reporter assay using SLAMF4 transfected BWZ.36 cells. As expected, plate bound anti-FLAG mab induced strong β-galactosidase activity as a control of the assay (**Fig. 7**). When SLAMF4-BWZ.36 cells were coincubated with SLAMF2-293

cells, enzyme activity was higher as compared to the incubation of SLAMF4-BWZ.36 cells cocultured with 293 cells transfected with an irrelevant plasmid. Moreover, this interaction could be blocked by the addition of the SLAMF4 specific 8C7 mab (**Fig. 7**). In conclusion, our experiments demonstrate that avian SLAMF4 is the counter-ligand of SLAMF2.

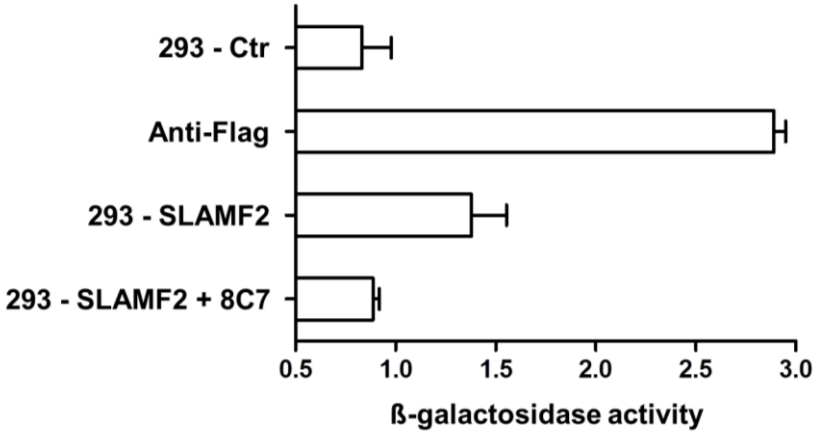


Figure 7 SLAMF4 binds to SLAMF2.

The SLAMF4-FLAG BWZ.36 reporter cell line was incubated with plate bound anti-FLAG mab as a positive control (anti-FLAG), with 293 cells expressing an irrelevant plasmid (293-Ctr) and with 293 cells expressing SLAMF2 (293-SLAMF2), either in the absence or in the presence of SLAMF4 specific mab (293-SLAMF2+8C7). The β-galactosidase activity was measured using as substrate CPRG (Roche, Mannheim, Germany) and quantified by optical density reading at 575 nm. Mean ± SD of five independent assays is shown.

Discussion

In this study, we generated a SLAMF4 specific mab to study its cellular distribution. The mammalian SLAM receptors are found on a wide variety of leukocytes (Cannons et al., 2011). Each receptor has a characteristic expression profile. Some of them like SLAMF2 have a wide expression pattern on hematopoietic cells. SLAMF4 in particular is expressed on NK cells, some γδ T cell subsets, $CD8^+$ αβ T cells, basophils, eosinophils, and monocytes (Nakajima et al., 1999). Using the novel SLAMF4 specific mab, we establish that chicken SLAMF4 has an even wider expression pattern as compared to its mammalian homologue. The similarities of chicken and mammalian SLAMF4 expression include a $CD8\alpha^+$ αβ T cell subset, NK cells and the monocyte/macrophage lineage. An interesting finding is the expression of SLAMF4 on thrombocytes. While several SLAM members have been documented on mammalian platelets such as SLAMF1 (CD150) and SLAMF5 (CD84), this is not the case for SLAMF4. Thrombocytes are the nucleated homologue of mammalian platelets. We and others have recently documented that thrombocytes carry a number of distinct surface receptors involved in immune reactions such as a TREM molecule, CLEC-2, a chicken Fc receptor and several other Ig-like receptors and CD40 ligand (Neulen and Göbel, 2012; Tregaskes et al., 2005;

Viertlboeck et al., 2013; Viertlboeck et al., 2009; Windau et al., 2013). These findings point to a potential involvement of this very abundant cell type during immune responses in the chicken. In addition, we could also show in preliminary experiments an expression of SLAMF4 on a cell population that most likely resembles chicken heterophils. Due to the lack of a specific mab recognizing heterophils, these results have to be confirmed.

In contrast to mammals, we found that a small subset of peripheral B cells expressed SLAMF4. This subset was further characterized as a population expressing high IgM levels and CD40 as compared to the SLAMF4 negative B cell subset. Studies evaluating SLAM expression on mammalian B cells have established that the majority of SLAM can be expressed by several B cell subsets with the exception of SLAMF4 that to our knowledge has not been reported on mammalian B cells. In summary, these expression differences may indicate that individual SLAM receptors in different species may have overlapping and partly redundant functions. It is important to note that in the chicken only five SLAM family members have been identified (Straub et al., 2013). This may either indicate that functions of several mammalian SLAM receptors are combined to one chicken receptor or that there are still more chicken SLAM members to be identified. Since SLAM genes form a tight cluster on chicken chromosome 25, it is very well possible that the higher number of genes found in mammals arose by gene duplications.

A special feature of most SLAM receptors is their homophilic interaction. SLAMF4 is the only known exception interacting with SLAMF2 (CD48) (Brown et al., 1998). Since both receptors are present in the chicken, it was of importance to delineate whether the same interaction is true for the chicken counterparts. Using the BWZ.36 reporter assay we could demonstrate an interaction between chicken SLAMF4 and SLAMF2, which could be blocked by the addition of the 8C7 mab. This illustrates the conservation of a highly conserved receptor ligand pair. Although no chicken SLAMF2 specific mab is available, preliminary PCR data suggests that it is a widely expressed surface antigen (Straub et al., 2013), as is the case in mammals. This implies that the SLAMF4 ligand is expressed by most tissues and as a consequence there is a good chance of SLAMF4 to frequently bind its ligand. While initially SLAMF4 was characterized as an activatory receptor or a costimulatory receptor especially on NK cells important for granule exocytosis and cytokine secretion, recent publications have emphasized a dual SLAMF4 function, dependent on the amount of SLAMF4 surface expression as well as on the availability of the intracellular adaptor molecules SAP and EAT-2 (Waggoner and Kumar, 2012; Zhao et al., 2012).

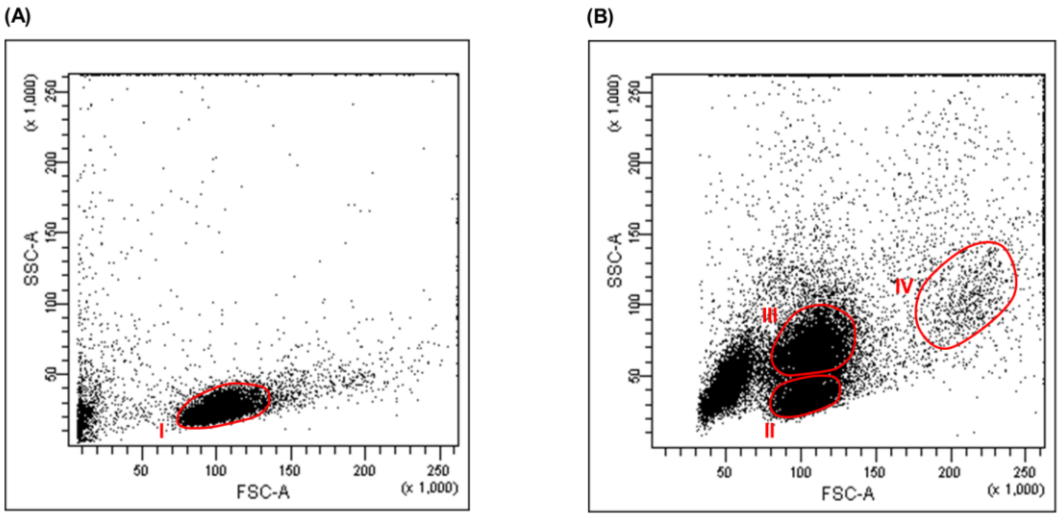
In this respect, the differential SLAMF4 expression on avian γδ T cells is an important finding. In contrast to mice and men, where γδ T cells are a low abundant T cell subset, chickens

display a high γδ T cell frequency in peripheral tissues (Sowder et al., 1988). The function of these cells is largely unknown; however, their high abundance suggests an important role in immune responses. With the help of the novel SLAMF4 mab, we could distinguish two and three γδ T cell subsets in blood and spleen, respectively. Blood γδ T cells were differentiated into $CD8\alpha^-$ $SLAMF4^{dim}$ and $CD8\alpha^+$ $SLAMF4^{bright}$ cells. The initial reports on chicken γδ T cell specific mab TCR1 reported a lack of CD4 and CD8 on blood γδ T cells (Sowder et al., 1988). This is in contrast to our finding of a $CD8\alpha^+$ γδ T cell subset in blood. Other reports also confirmed the finding of two γδ T cell subsets in blood (Pieper et al., 2008). These expression differences of CD8 on blood γδ T cells may be due to different chicken lines used for the experiments, different antigen exposure, or different age. In the spleen, three distinct γδ T cell subsets could be distinguished by various expression levels of CD8 and SLAMF4. Other markers that are differentially regulated on chicken γδ T cells include CD5 with dim expression on blood and bright expression on splenic γδ T cells and CD6 with no expression on blood and high expression on a blood γδ T cell subset (Göbel et al., 1996; Koskinen et al., 1998). Taken together, these phenotypic differences could either identify distinct γδ T cell subsets with different functional properties or different activation states of γδ T cells. We hypothesize that the latter is correct and that the activation of γδ T cells leads to the upregulation of CD8 and SLAMF4. A further support of this hypothesis is the finding that virtually all intestinal T cells most of which represent γδ T cells coexpress CD8 and SLAMF4. Moreover, the CD8 expression on γδ T cells has been used to define cells with high proliferative capacity and high expression levels of FASL, IFN-γ and lymphotactin that are collectively features of activated T cells (Pieper et al., 2008). Similar to mammals, upregulation of SLAMF4 to high density levels on these $CD8\alpha^+$ γδ T cells could be responsible for an inhibitory effect on the cells thus terminating an activation program.

In conclusion, we have characterized the tissue distribution of chicken SLAMF4 using a novel mab. The differential expression of SLAMF4 together with CD8 allows the delineation of several γδ T cell subsets that will be the focus of future studies.

Acknowledgements

We would like to thank Dr. S. Härtle for help and support with flow cytometric analyses and for providing some reagents and B. Sperling, M. Kohn and B. Schaerer for expert technical help.



Supplementary Figure 1

FSC/SSC dot blots of (A) PBL and (B) PBMC. (I) Lymphocyte gate in a PBL preparation. (II) Lymphocyte, (III) thrombocyte, and (IV) monocyte gate in a PBMC preparation.

References

Brown, M.H., Boles, K., van der Merwe, P.A., Kumar, V., Mathew, P.A., Barclay, A.N., 1998. 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. J Exp Med 188, 2083-2090.

Cannons, J.L., Tangye, S.G., Schwartzberg, P.L., 2011. SLAM family receptors and SAP adaptors in immunity. Annu Rev Immunol 29, 665-705.

Chan, M.M., Chen, C.L., Ager, L.L., Cooper, M.D., 1988. Identification of the avian homologues of mammalian CD4 and CD8 antigens. J Immunol 140, 2133-2138.

Chen, C.H., Sowder, J.T., Lahti, J.M., Cihak, J., Losch, U., Cooper, M.D., 1989. TCR3: a third T-cell receptor in the chicken. Proc Natl Acad Sci U S A 86, 2351-2355.

Chen, C.L., Ager, L.L., Gartland, G.L., Cooper, M.D., 1986. Identification of a T3/T cell receptor complex in chickens. J Exp Med 164, 375-380.

Cihak, J., Ziegler-Heitbrock, H.W., Trainer, H., Schranner, I., Merkenschlager, M., Losch, U., 1988. Characterization and functional properties of a novel monoclonal antibody which identifies a T cell receptor in chickens. Eur J Immunol 18, 533-537.

Davis, S.J., van der Merwe, P.A., 1996. The structure and ligand interactions of CD2: implications for T-cell function. Immunol Today 17, 177-187.

Erhard, M.H., Von Quistorp, I., Schranner, I., Jungling, A., Kaspers, B., Schmidt, P., Kuhlmann, R., 1992. Development of specific enzyme-linked immunosorbent antibody assay systems for the detection of chicken immunoglobulins G, M, and A using monoclonal antibodies. Poult Sci 71, 302-310.

Göbel, T.W., 2000. Isolation and analysis of natural killer cells in chickens. Methods Mol Biol 121, 337-345.

Göbel, T.W., Chen, C.H., Cooper, M.D., 1996. Expression of an avian CD6 candidate is restricted to alpha beta T cells, splenic CD8+ gamma delta T cells and embryonic natural killer cells. Eur J Immunol 26, 1743-1747.

Göbel, T.W., Chen, C.L., Shrimpf, J., Grossi, C.E., Bernot, A., Bucy, R.P., Auffray, C., Cooper, M.D., 1994. Characterization of avian natural killer cells and their intracellular CD3 protein complex. Eur J Immunol 24, 1685-1691.

Göbel, T.W., Kaspers, B., Stangassinger, M., 2001. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. Int Immunol 13, 757-762.

Guillemot, F., Turmel, P., Charron, D., Le Douarin, N., Auffray, C., 1986. Structure, biosynthesis, and polymorphism of chicken MHC class II (B-L) antigens and associated molecules. J Immunol 137, 1251-1257.

Iizuka, K., Naidenko, O.V., Plougastel, B.F., Fremont, D.H., Yokoyama, W.M., 2003. Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. Nat Immunol 4, 801-807.

Koskinen, R., Göbel, T.W., Tregaskes, C.A., Young, J.R., Vainio, O., 1998. The structure of avian CD5 implies a conserved function. J Immunol 160, 4943-4950.

Kothlow, S., Morgenroth, I., Tregaskes, C.A., Kaspers, B., Young, J.R., 2008. CD40 ligand supports the long-term maintenance and differentiation of chicken B cells in culture. Dev Comp Immunol 32, 1015-1026.

Mast, J., Goddeeris, B.M., Peeters, K., Vandesande, F., Berghman, L.R., 1998. Characterisation of chicken monocytes, macrophages and interdigitating cells by the monoclonal antibody KUL01. Vet Immunol Immunopathol 61, 343-357.

Munitz, A., Bachelet, I., Fraenkel, S., Katz, G., Mandelboim, O., Simon, H.U., Moretta, L., Colonna, M., Levi-Schaffer, F., 2005. 2B4 (CD244) is expressed and functional on human eosinophils. J Immunol 174, 110-118.

Nakajima, H., Cella, M., Langen, H., Friedlein, A., Colonna, M., 1999. Activating interactions in human NK cell recognition: the role of 2B4-CD48. Eur J Immunol 29, 1676-1683.

Neulen, M.L., Göbel, T.W., 2012. Identification of a chicken CLEC-2 homologue, an activating C-type lectin expressed by thrombocytes. Immunogenetics 64, 389-397.

Pieper, J., Methner, U., Berndt, A., 2008. Heterogeneity of avian gammadelta T cells. Vet Immunol Immunopathol 124, 241-252.

Pieper, J., Methner, U., Berndt, A., 2011. Characterization of avian gammadelta T-cell subsets after Salmonella enterica serovar Typhimurium infection of chicks. Infect Immun 79, 822-829.

Ratcliffe, M.J., 1989. Generation of immunoglobulin heavy chain diversity subsequent to cell surface immunoglobulin expression in the avian bursa of Fabricius. J Exp Med 170, 1165-1173.

Rothwell, C.J., Vervelde, L., Davison, T.F., 1996. Identification of chicken Bu-1 alloantigens using the monoclonal antibody AV20. Vet Immunol Immunopathol 55, 225-234.

Sayos, J., Wu, C., Morra, M., Wang, N., Zhang, X., Allen, D., van Schaik, S., Notarangelo, L., Geha, R., Roncarolo, M.G., Oettgen, H., De Vries, J.E., Aversa, G., Terhorst, C., 1998. The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM. Nature 395, 462-469.

Schlaphoff, V., Lunemann, S., Suneetha, P.V., Jaroszewicz, J., Grabowski, J., Dietz, J., Helfritz, F., Bektas, H., Sarrazin, C., Manns, M.P., Cornberg, M., Wedemeyer, H., 2011. Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells. PLoS Pathog 7, e1002045.

Schuhmachers, G., Ariizumi, K., Mathew, P.A., Bennett, M., Kumar, V., Takashima, A., 1995. Activation of murine epidermal gamma delta T cells through surface 2B4. Eur J Immunol 25, 1117-1120.

Schwartzberg, P.L., Mueller, K.L., Qi, H., Cannons, J.L., 2009. SLAM receptors and SAP influence lymphocyte interactions, development and function. Nat Rev Immunol 9, 39-46.

Shlapatska, L.M., Mikhalap, S.V., Berdova, A.G., Zelensky, O.M., Yun, T.J., Nichols, K.E., Clark, E.A., Sidorenko, S.P., 2001. CD150 association with either the SH2-containing inositol phosphatase or the SH2-containing protein tyrosine phosphatase is regulated by the adaptor protein SH2D1A. J Immunol 166, 5480-5487.

Sidorenko, S.P., Clark, E.A., 2003. The dual-function CD150 receptor subfamily: the viral attraction. Nat Immunol 4, 19-24.

Sowder, J.T., Chen, C.L., Ager, L.L., Chan, M.M., Cooper, M.D., 1988. A large subpopulation of avian T cells express a homologue of the mammalian T gamma/delta receptor. J Exp Med 167, 315-322.

Straub, C., Viertlboeck, B.C., Göbel, T.W., 2013. The chicken SLAM family. Immunogenetics 65, 63-73.

Tangye, S.G., Cherwinski, H., Lanier, L.L., Phillips, J.H., 2000. 2B4-mediated activation of human natural killer cells. Mol Immunol 37, 493-501.

Tregaskes, C.A., Bumstead, N., Davison, T.F., Young, J.R., 1996. Chicken B-cell marker chB6 (Bu-1) is a highly glycosylated protein of unique structure. Immunogenetics 44, 212-217.

Tregaskes, C.A., Glansbeek, H.L., Gill, A.C., Hunt, L.G., Burnside, J., Young, J.R., 2005. Conservation of biological properties of the CD40 ligand, CD154 in a non-mammalian vertebrate. Dev Comp Immunol 29, 361-374.

Viertlboeck, B.C., Crooijmans, R.P., Groenen, M.A., Göbel, T.W., 2004. Chicken Ig-like receptor B2, a member of a multigene family, is mainly expressed on B lymphocytes, recruits both Src homology 2 domain containing protein tyrosine phosphatase (SHP)-1 and SHP-2, and inhibits proliferation. J Immunol 173, 7385-7393.

Viertlboeck, B.C., Göbel, T.W., 2007. Chicken thrombocytes express the CD51/CD61 integrin. Vet Immunol Immunopathol 119, 137-141.

Viertlboeck, B.C., Hanczaruk, M.A., Amann, B., Bader, S.R., Schmitt, R., Sperling, B., Schwarz, S.C., Schmahl, W., Deeg, C.A., Göbel, T.W., 2013. Chicken immunoregulatory Ig-like receptor families: An overview and expression details on ggTREM-A1. Dev Comp Immunol.

Viertlboeck, B.C., Schmitt, R., Hanczaruk, M.A., Crooijmans, R.P., Groenen, M.A., Göbel, T.W., 2009. A novel activating chicken IgY FcR is related to leukocyte receptor complex (LRC) genes but is located on a chromosomal region distinct from the LRC and FcR gene clusters. J Immunol 182, 1533-1540.

Waggoner, S.N., Kumar, V., 2012. Evolving role of 2B4/CD244 in T and NK cell responses during virus infection. Front Immunol 3, 377.

Windau, K., Viertlboeck, B.C., Gobel, T.W., 2013. The Turkey Ig-like receptor family: identification, expression and function. PLoS One 8, e59577.

Zhao, F., Cannons, J.L., Dutta, M., Griffiths, G.M., Schwartzberg, P.L., 2012. Positive and negative signaling through SLAM receptors regulate synapse organization and thresholds of cytolysis. Immunity 36, 1003-1016.

Chicken NK cell receptors

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Abbreviations

CHIR, chicken Ig-like receptor

IEL, intestinal intraepithelial lymphocytes

ITAM, immunoreceptor tyrosine-based activation motif

ITSM, immunoreceptor tyrosine-based switch motif

KIR, killer cell immunoglobulin-like receptors

LRC, leukocyte receptor complex

NKC, natural killer gene complex

NCR, natural cytotoxicity receptors

SLAM, signaling lymphocytic activation molecule

TILR, turkey Ig-like receptor

Abstract

Natural killer cells are innate immune cells that destroy virally infected or transformed cells. They recognize these altered cells by a plethora of diverse receptors and thereby differ from other lymphocytes that use clonally distributed antigen receptors. To date, several receptor families that play a role in either activating or inhibiting NK cells have been identified in mammals. In the chicken, NK cells have been functionally and morphologically defined, however, a conclusive analysis of receptors involved in NK cell mediated functions has not been available. This is partly due to the low frequencies of NK cells in blood or spleen that has hampered their intensive characterization. Here we will review recent progress regarding the diverse NK cell receptor families, with special emphasis on novel families identified in the chicken genome with potential as chicken NK cell receptors.

1. Introduction

Natural killer (NK) cells were first described in 1975 as cytotoxic lymphocytes specifically killing tumor cells without previous sensitization (Kiessling et al., 1975). Since then, they have been categorized as third lymphoid lineage that lack conventional rearranging receptors such as TCR or surface Ig. Instead, they display an arsenal of germline encoded receptors that regulate NK cell function. In general these receptors can be further characterized based on several criteria, such as biochemical nature of the extracellular domain, chromosomal location that defines an affiliation to a receptor family, ligands recognized by the NK cell receptor and their function.

NK cells in man and mice although similar in function use structurally unrelated receptors that evolved independently. The human killer cell immunoglobulin-like receptors (KIR, CD158) are a family of Ig domain containing transmembrane receptors, whereas the Ly49 family in mice resembles type II transmembrane C-type lectins (Natarajan et al., 2002; Yokoyama and Seaman, 1993). The analyses of orthologous genes in mammals showed that either one of the families has been expanded, for instance various primates contain the KIR family, as it is also the case in cattle, while rats and horses have expanded the Ly49 family (Parham, 2005). It has been demonstrated that mice have two KIR genes on the X chromosome that are not expressed in NK cells, whereas the human KIR are located in the leukocyte receptor complex (LRC) on human chromosome 19q13.4 that harbours additional gene families involved in NK cell regulation, for instance leukocyte Ig-like receptors (LILR) (Barrow and Trowsdale, 2008). Vice versa, humans lack a functional Ly49 gene, whereas in mice the corresponding genomic region is heavily expanded. The genomic region containing the Ly49 genes on human chromosome 12p12-13 and mouse chromosome 6 also encodes a number of additional C type lectin NK receptors such as the NKG2D and CD94/NKG2A present in mouse and man and has therefore been designated “natural killer gene complex” (NKC) (Yokoyama and Seaman, 1993). In addition to these two prominent genomic regions encoding many NK cell receptors, additional NK cell receptor genes are found throughout the genome either in the form of small gene families such as the SLAM family of receptors or single receptors as the natural cytotoxicity receptors (NCR). Despite the differences in extracellular domains of KIR and Ly49 receptors, both bind to MHC class I molecules. This is in particular true for the inhibitory receptors that upon MHC binding disarm NK cells and prevent cellular lysis. The corresponding activating receptors bind to diverse set of ligands which in some cases have not yet been molecularly identified. It is generally accepted that the activating receptors of NK cells can bind to three main groups of ligands, namely constitutively expressed molecules,

stress-induced molecules and pathogen derived ligands on target cells (Cheent and Khakoo, 2009; Vivier and Malissen, 2005).

Most of the receptors involved in NK cell function can also be categorized by distinctive features of their transmembrane and intracytoplasmic domains. Activating NK cell receptors generally display a short cytoplasmic tail with no signalling motifs, but instead display a charged transmembrane residue that promotes the association of adaptor molecules such as FcεRIγ, DAP12, or CD3ζ (Campbell and Purdy, 2011). These adaptor proteins mediate signal transduction through a number of immunoreceptor tyrosine-based activation motifs (ITAM) (Reth, 1989). In contrast, inhibitory receptors lack a charged transmembrane residue and instead have a long cytoplasmic tail that contain variable numbers of immunoreceptor tyrosine-based inhibitory motifs (ITIM) or immunoreceptor tyrosine-based switch motifs (ITSM). The downstream signalling cascades of both activating and inhibitory receptors have been the focus of intense research and are summarized elsewhere (Campbell and Colonna, 2001; Daeron et al., 2008; Lanier, 2008; Leibson, 1997).

NK cell recognition of a virally infected cell or an altered self cell, where activating signals dominate over inhibitory signals, leads to a direct cytolytic attack mediated by secretion of cytolytic granules containing perforin and granzymes or by ligation of death domain-containing receptors. NK cells are also potent producers of cytokines; in particular they are a rich source of interferon-γ, tumour necrosis factor-α and granulocyte-macrophage colony-stimulating factor. Thus, NK cell function extends far beyond being a simple killer cell to a magnitude of immunomodulatory activities that influence innate and adaptive immune responses. A concise knowledge of the array of NK cell receptors with antagonistic pathways that are ultimatively integrated is essential to understand the fine tuning of NK cell responses in different settings. Here we will focus on the description of various chicken immunoregulatory receptors with known relevance for NK cells or the potential of being NK cell receptors (**Tab. 1**).

Table 1 Immunomodulatory receptors with proven or probable NK cell expression.

Receptor	Alternative Name	Ligand	Superfamily	Signalling	Chromo-some	mab	Reference
CHIR Family							
CHIR-A2			IgSF	FcεRIγ	31	13E2	(Viertlboeck et al., 2005)
CHIR-B2			IgSF	ITIM	31	3H7	(Viertlboeck et al., 2004)
CHIR-AB1		IgY	IgSF	ITIM	31	8D12	(Viertlboeck et al., 2007)
C type lectins							
B-NK			C-type lectin	ITIM	16	8A11	(Kaufman et al., 1999)
B-Lec			C-type lectin		16		(Kaufman et al., 1999)
CLEC-2	CD94/NKG2		C-type lectin	hemITAM	1	8G8	(Neulen and Göbel, 2012b)
CD69			C-type lectin		1		(Chiang et al., 2007)
SLAM Family							
SLAMF1	CD150		IgSF	ITSM	25		(Straub et al., 2013)
SLAMF2	CD48		IgSF		n.d.		
SLAMF3like			IgSF	ITSM	25		
SLAMF4	CD244, 2B4	CD48	IgSF	ITSM	n.d.	8C7	
SLAMF5like			IgSF	ITSM	25		
Receptors that interact with Nectins							
CRTAM	cTADS	Necl-2			24	8A10	(Ruble and Foster, 2000)
CD96					1		
CD226	DNAM-1				2		
Miscelleanous Receptors							
CD56	NCAM		IgSF		24	4B5	(Neulen and Göbel, 2012a)

2. The chicken leukocyte receptor complex

The chicken LRC has been mapped to microchromosome 31, one of the smallest microchromosome which has not been annotated in the chicken genome project (Hillier et al., 2004; Viertlboeck et al., 2005). So far, only a single multigene receptor family designated chicken Ig-like receptors (CHIR) has been located to this chromosome. The reason for the difficulties in annotation is caused by the highly polygenic nature of the CHIR family, since this chromosomal area is densely covered with many highly homologous CHIR genes and pseudogenes. We and others have not been able to completely assemble the entire CHIR locus and to draw conclusions regarding the number and polymorphism of the chicken LRC (Laun et al., 2006; Lochner et al., 2010). Also, other single genes or gene families corresponding to those represented by the extended LRC including LILR, NKp46, GPVI or DAP12 have not been identified in the chicken, yet. The CHIR family has been the subject of a recent review (Viertlboeck and Göbel, 2011), therefore, we will focus on aspects that identify CHIR as potential NK cell receptors.

2.1 CHIR share features of typical NK cell receptor families

The CHIR gene family is a polygenic and polymorphic gene family. The diversity found in the CHIR complex is still not fully resolved, but seems to be higher compared to KIR and Ly49. The sequence analysis of expressed CHIR in PBMC in two individual animals revealed 70 and 98 different CHIR, respectively. Moreover, when these datasets were compared to each other, there were only few shared CHIR found to be expressed in both individuals (Viertlboeck et al., 2010). These features resemble those of the LRC encoded KIR and the NKC encoded Ly49 genes. As members of the LRC, CHIR are typical type I transmembrane receptors of the Ig superfamily that have one or two C2 type Ig domains. Comparisons of CHIR with related receptor types encoded by the mammalian LRC in terms of amino acid identity, position and nature of the basic transmembrane residue, associated adaptor molecule and genomic structure reveal that CHIR combine features of KIR and LILR. CHIR may thus represent functional homologues of the main receptor families encoded by the LRC (Viertlboeck and Göbel, 2011). One of the three NCR genes, NKp46, is located at the vicinity of the KIR cluster and has drawn special attention as a highly conserved NK cell marker in many species (Walzer et al., 2007). The predicted CHIR structure shows close homology of CHIR and NKp46, so one of the CHIR may also represent a functional NKp46 homologue (Arnon et al., 2008).

KIR genes most likely evolved from a single ancestral gene (KIR3D) that subsequently evolved rapidly to form different KIR types caused by selection driven by pathogens and MHC diversity (Parham, 2008). Likewise, CHIR are highly diversified within chickens, with large sets of different CHIR expressed by individual animals and chicken lines. Moreover, we have recently identified CHIR homologues in turkeys, designated turkey Ig-like receptors, where a first analysis indicates that the number of TILR is smaller compared to chickens (Windau et al., 2013). The TILR have been annotated between highly conserved genes on chromosome 3 (**Fig. 1**). The comparison of this region between all available bird genomes indicates either a genomic rearrangement in the turkey genome placing the LRC into this region or more likely an annotation error. To date we were unable to identify CHIR homologues in other bird genomes, such as ducks and zebra finch. This could indicate that the LRC in these birds has not been extensively expanded or has been completely lost. In this context it is important to note that birds evolved over millions of years, enough time to create a situation similar to mammals where the loss of one NK receptor family is compensated by an alternative.

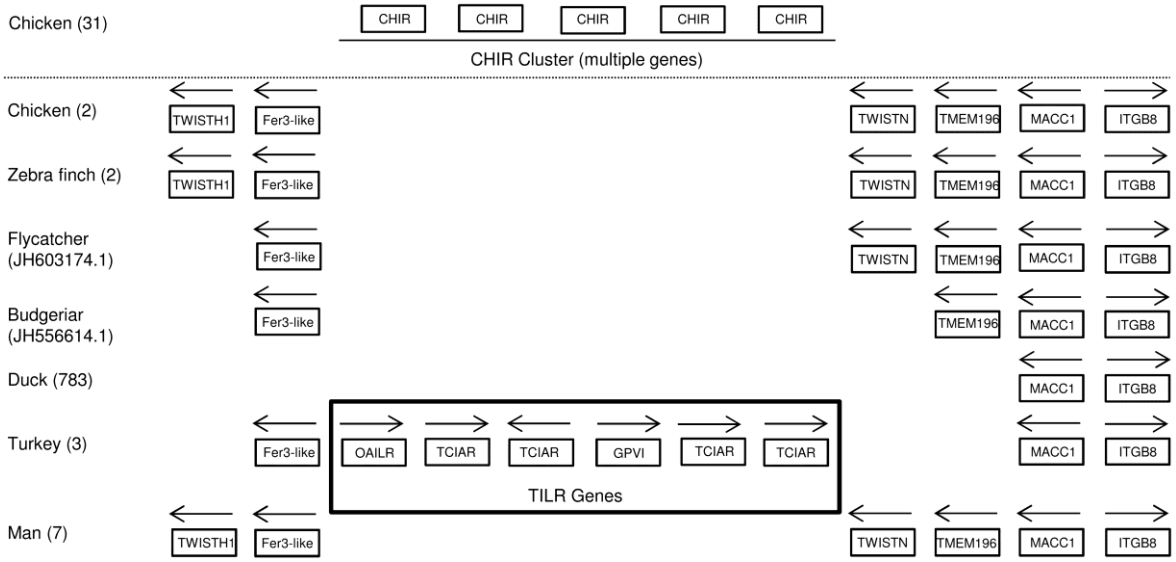

Figure 1 Genomic organization of the CHIR.

The chicken LRC is located on microchromosome 31 (upper part) with a large and variable number of CHIR genes present (for simplicity only five are shown). The turkey genome contains CHIR homologues designated TILR which are currently found on chromosome 3 (boxed region) and annotated with diverse names according to most homologous BLAST hits. As depicted, the syntenic regions in other birds contain several highly conserved genes on both sides of the TILR cluster, but lack any IgSF genes (orientation of genes is indicated by arrows). The number in brackets indicates the identity of the respective chromosome or scaffold. Gene abbreviations are according to the HUGO Gene Nomenclature.

2.2 CHIR types, expression and ligands

CHIR can be classified into inhibitory CHIR-B and activating CHIR-A that display the typical features of corresponding NK cell receptors, namely CHIR-B have cytoplasmic ITIM, whereas CHIR-A have a short cytoplasmic tail and the basic transmembrane residue. A third receptor type, CHIR-AB combines inhibitory and activating features (Viertlboeck and Göbel, 2011; Viertlboeck et al., 2005). The adaptor protein associating with CHIR-A and CHIR-AB has been identified as FcεRIγ chain, but interestingly it is essential for surface expression of CHIR-A2, but not for CHIR-AB1 (Viertlboeck et al., 2007). In contrast to the KIR that are mainly expressed by NK cells, individual CHIR have a much wider expression pattern as judged by specific RT-PCR, a finding closely resembling other LRC encoded families (Viertlboeck et al., 2005). Currently, three mab are available that bind to CHIR, the 8D12 recognizes CHIR-AB1, 3H7 was made against CHIR-B2 and 13E2 was generated against CHIR-A2 expressing cells. Each of the mab has a unique expression pattern that is not limited to NK cells. For instance, the CHIR-B2 reactive 3H7 mab mainly stains B cells (Viertlboeck et al., 2004), whereas the CHIR-AB1 mab 8D12 shows a wider expression pattern, including NK cells (Viertlboeck et al., 2007). It should be noted that some mab against CHIR also crossreact with turkey cells, but interestingly these cells mainly represent thrombocytes (Windau et al., 2013). Given that other LRC members in mammals (GPVI) are platelet receptors (He et al., 2006); this could indicate that some CHIR have evolved as thrombocyte receptors with distinct functions. Alternatively, thrombocytes may not only be involved in hemostasis, but also immune functions as has been reported in terms of TLR expression and cytokine production (Scott and Owens, 2008; St Paul et al., 2012).

The identification of CHIR ligands has been limited to one subset of CHIR, namely the CHIR-AB1, that serves as an Fc receptor for chicken IgY (Viertlboeck et al., 2007; Viertlboeck et al., 2009b). Interestingly, the IgY: CHIR-AB interaction mirrors that of human IgA to FcαRI, a receptor that is also located in the LRC (Pürzel et al., 2009). We are currently investigating the nature of other CHIR ligands with the focus on MHC class I as potential CHIR ligands. In the chicken, the MHC class I molecules can be further separated into BF1 and BF2 molecules. Whereas the BF2 molecules most likely represent the homologues of human HLA-A and HLA-B, the BF1 shares features of HLA-C (O'Neill et al., 2009; Shaw et al., 2007). Since KIR are preferentially binding to HLA-C, BF1 molecules would be the prime candidates as CHIR ligands. In this respect it is interesting to note that the target cell line LSCC-RP9 used widely for functional chicken NK cell assays expresses high levels of MHC class I molecules, an observation that differs from classical mammalian MHC negative NK cell targets and that is in

contrast to the missing self hypothesis (Ljunggren and Karre, 1990). The RP9 cells originated from B^2B^{15} birds, but even MHC matched cells are able to induce cytotoxicity of the RP9 cells (Sharma and Okazaki, 1981). If BF1 molecules are the main restriction elements of NK cells, this puzzling observation can be resolved, because the B^{15} haplotype lack BF1 molecules and the B2 haplotype has an unusual BF1 molecule (Walker et al., 2011). Thus, the RP9 may express BF2 molecules and unusual B^2 derived BF1 molecule, both of which are not the proper restriction elements for inhibitory NK cell receptors. Since NK cell activation depends on the balance between inhibitory and activation signals, alternatively, the RP9 could express high levels of yet undefined ligands for activating NK cell receptors, that induce potent NK cell lysis even in the presence of MHC class I signals.

2.3 CHIR-AB1, an Fc receptor expressed by NK cells

Several features of CHIR-AB1 indicate that it may resemble a prime NK cell receptor candidate. As outlined above it functions as an IgY Fc receptor located within the LRC. Human NK cells also display CD16, a low affinity Fc receptor that is crucial to mediate ADCC responses. The CHIR-AB1 specific 8D12 has been instrumental in the recent characterization of a novel blood NK cell subset. In combination with the 8F2 mab (most likely recognizing a CD11 homologue, see 4.2), a $8D12^{high}$, $8F2^{intermediate}$ peripheral blood subset could be identified that expressed several other NK markers (**Tab. 2**). Moreover, this population exhibited typical NK cell features, such as CD107 expression and IFN-γ secretion (Viertlboeck, et al., submitted for publication). CHIR-AB1 is also expressed on NK cells present in the intestinal epithelium (IEL) and the embryonic spleen (Viertlboeck et al., 2007) (**Tab. 2**). The amino acid alignment of CHIR-AB1 with related human KIR shows some striking similarities in particular to KIR2DL4 (**Fig. 2**).

Table 2 Phenotype of NK cell populations

Antigen	mab	PBMC	IEL	E14
CD8	CT8, 3-298	-	+	+
CD25	AV142	+	+	n.d.
CD56	4B5	-	-	+
CD57	HNK1	+	+	n.d.
CD244	8C7	+	+	+
CHIR-AB1	8D12	+	+	+
n.d	28-4	+	+	+
n.d	20E5	+	+	+
n.d	1G7	+	+	+

Both CHIR-AB1 and KIR2DL4 have a positive transmembrane residue at a similar position and they have a cytoplasmic ITIM, indicative of a putative activating as well as inhibitory function (Viertlboeck and Göbel, 2011). While the activating function has been demonstrated for CHIR-AB1, there is currently no assay available to examine a potential inhibitory capacity. The crystal structure of CHIR-AB1 has reemphasized its particular role as NK cell receptor. The closest structural homologues of CHIR-AB1 have been determined as KIR and NKp46 (Arnon et al., 2008). Another FcR for IgY, ggFcR, is also related to CHIR-AB1. It is located on chromosome 20 and its importance for NK cells has not been examined due to the lack of a specific mab (Viertlboeck et al., 2009a).

In conclusion, various characteristics such as the expression on NK cells, the function as Fc receptor, the chromosomal location within the LRC and the structural homology conclusively identify CHIR-AB1 as an important NK cell receptor. It is likely that other CHIR are also expressed on NK cells.

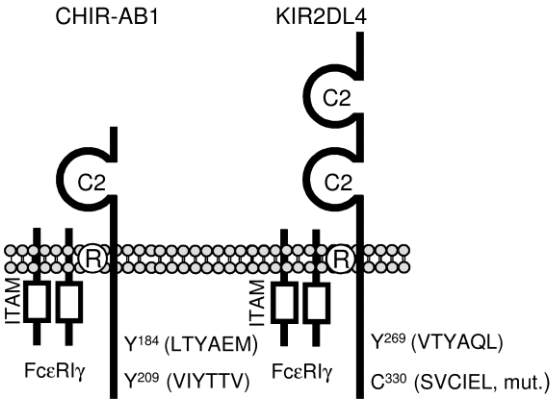

Figure 2 CHIR-AB shares features with KIR2DL4.

The two Ig domains KIR2DL4 which resembles a unique KIR with an arginine instead of a lysine in a different position compared to other KIR and as a consequence binding to the FcεRIγ adaptor instead of DAP12 is compared with the single domain CHIR-AB1. The cytoplasmic motifs found in KIR2DL4 and CHIR are depicted. Note that KIR2DL4 and CHIR-AB1 contain only a single ITIM, while the membrane proximal and –distal ITIM have been mutated in KIR2DL4 and CHIR-AB1, respectively.

3. C-type lectins

The chicken genome contains two distinct chromosomal sites with characterized C-type lectins, namely a syntenic region of the mammalian NKC on chicken chromosome 1 and several C-type lectins that are linked to the chicken MHC on chromosome 16 (Kaufman et al., 1999; Rogers et al., 2008). The large C-type lectin superfamily can be divided into seven groups, where group V represent type II membrane-bound receptors with a CTLD domain that lack critical residues for calcium binding and thus does not function as carbohydrate receptor

(Weis et al., 1998). The C-type lectins present in the two genomic areas all share features of group V C-type lectins. In fact, using the chicken sequences, the group V C-type lectins were further divided into four subgroups (Rogers et al., 2005). Briefly, these include subgroup 1 mainly expressed on NK cells and all with signalling capacity, subgroup 2 representing early lymphocyte activation antigens, subgroup 3 with variable leukocyte expression and subgroup 4 created by multigene families with expression on T- and NK cells and most of them with MHC class I as ligand. Notably, only B-NK has an ITIM and none of the chicken C-type lectins identified so far are reported to have a basic transmembrane residue that would support the association of activating adaptor molecules. A neighbour joining analysis of selected C-type lectins is shown in **Fig. 3**.

Chicken natural killer gene complex

A region on chromosome 1 has been identified as syntenic to the respective NKC on human chromosome 12 and mouse chromosome 6 (Chiang et al., 2007). Two C-type lectins have been characterized on this chromosomal area and according to the latest genome assembly (Dec 2012 version), these two genes are 70 MB apart. Fluorescence in situ hybridization had already predicted a distance of at least 40 MB. Furthermore, when the gene composition in between these two C-type lectins was compared to human and mouse, it became evident that the chicken chromosome has undergone some rearrangements. Therefore, there could be additional C-type lectins that have been missed either within the 70 MB region or outside of this region.

One of the C-type lectins which is actually located right at the beginning of chromosome 1 was identified by means of sequence homology and molecular modelling as homologue to mammalian CD69 (Chiang et al., 2007). This receptor represents one of the early activation antigens. To date, no mab against this molecule has been described.

The second C-type lectin present on chromosome 1 had been originally designated as chicken CD94/NKG2 homologue in order to stress its homology to both mammalian CD94 and NKG2 (Chiang et al., 2007). This observation was based on primary sequence identity of the CTLD domain to human CD94 (62 %) and NKG2A (52 %) as well as phylogenetic trees (**Fig. 3**). In contrast to CD69, which is transcribed in a variety of tissues, the expression of the chicken CD94/NKG2 homologue was more restricted. We have recently generated a novel mab against chicken CD94/NKG2 in order to test its protein expression and its potential role as NK cell receptor. The mab 8G8 specifically recognizes this molecule and reacts almost exclusively with thrombocytes in chickens and turkeys. There may be some residual expression on a small NK cell subset. This expression pattern prompted us to re-evaluate the annotation of chicken

CD94/NKG2 (Neulen and Göbel, 2012b). Interestingly, lectins with similar expression pattern form a unique cluster within the NKC (Dectin cluster) (Bernard et al., 2007; Sattler et al., 2012). The phylogenetic tree in **Fig. 3** indeed shows the position of CD94/NKG2 between the mammalian CD94 and myeloid C-type lectins. The expression on thrombocytes and the presence of a signalling motif in the cytoplasmic domain that was initially classified as ITIM can now be identified as a special so called hemITAM, leading to the activation of thrombocytes upon crosslinking. The 8G8 mab indeed causes thrombocyte activation upon crosslinking. For these reasons, we suggested renaming the CD94/NKG2 as chicken CLEC-2 homologue (Neulen and Göbel, 2012b).

3.1 B-NK and B-Lec

The chicken MHC represents a minimal essential MHC with only 19 genes in a 92 kb region. It was unexpected to find two C-type lectin genes, designated B-NK and B-Lec within this region (Kaufman et al., 1999). They are located next to each other and in opposite transcriptional orientation. Further analysis revealed that B-Lec is similar to mammalian lectin-like transcript 1. It is rapidly upregulated upon cellular activation and like LLTI contains an endocytosis motif (Rogers et al., 2005). Therefore it can be classified as a member of activation induced C-type lectins, such as CD69. Other C-type lectins found in the MHC and RFP-Y region such as chicken 17.5, chYLec-2, Blec1 and Blec3 share significant homology to B-Lec and represent members of this clade, too (**Fig. 3**) (Bernot et al., 1994; Mwangi et al., 2012; Rogers et al., 2003).

In contrast, B-NK is distantly related to the B-Lec group. It is most homologous to human NKRP-1 and it has a functional cytoplasmic ITIM (**Fig. 3**). Initial studies demonstrated mRNA expression an IL-2 expanded NK cells, however, after generating the specific mab 8A11, it was detected on small subsets of T-lymphocytes, on embryonic NK cells as well as on IL-2 in vitro expanded chicken NK cells (Viertlboeck et al., 2008b). So it is still the only chicken C-type lectin with proven expression on chicken NK cells. B-NK and B-Lec thus resemble a conserved pair of C-type lectins such as NKR-P1 and LLT-1 in mammals, respectively. Both pairs are situated side by side in opposite orientation. NKR-P1 is a receptor expressed on NK cells and binds to LLT-1, a ligand found on activated T cells, a situation closely resembling B-NK and B-Lec (Iizuka et al., 2003; Yokoyama and Plougastel, 2003). The direct interaction of B-NK and B-Lec has not been proven so far, but it was demonstrated that B-NK binds to a ligand on activated cells (Viertlboeck et al., 2008b). The inability to directly show the interaction of both molecules may be due to technical reasons, low avidity of binding or the result of the high polymorphic nature of B-NK. In this respect, several studies have revealed

nucleotide exchanges of the B-NK gene, that all represented non-synonymous changes in the coding region leading to amino acid variation with a very high dN/dS ratio, while the adjacent B-Lec gene also displayed a number of nucleotide variations, but most of them were synonymous (Mwangi et al., 2012; Rogers and Kaufman, 2008). Finally, the pair of two lectins is well conserved and also present with some variations in the turkey, pheasant, black grouse and quail MHC (Chaves et al., 2009; Shiina et al., 2004; Wang et al., 2012). The pheasant genome displays two B-NK and one B-Lec homologue (Ye et al., 2012). The quail locus harbours a total of four B-NK homologues (NK1 to NK4) and six B-Lec homologues (Lec1 to Lec6), however, all B-Lec homologues except Lec2 represent pseudogenes. Like for the chicken haplotypes the comparison of the chicken, turkey and quail B-NK homologues reemphasized a very high divergence, a situation also known for mammalian NK cell receptors.

The conservation of the C-type lectin pair adjacent to the MHC has been the reason for the hypothesis that the ancestral MHC has been a major first site containing receptors and ligands involved in innate immune responses, which rapidly evolved under the pressure of pathogen selection. In this respect it is interesting to note that the FPV genome contains several open reading frames that encode C-type lectin sequences (Afonso et al., 2000). Moreover, at least one of them (P14372) has been detected on the surface of infected cells, indicating a potential role as immune evasion ligand that may prevent NK cell lysis (Wilcock et al., 1999). The phylogenetic tree (**Fig. 3**) documents that some of the fowl pox derived C-type lectins share homology to Ly-49. This could indicate that some birds possess homologous genes which were hijacked by the virus. It is not clear at this point, how the C-type lectins function on the surface of infected cells.

In conclusion, most of the diverse C-type lectin groups are represented in the chicken. Homologues of Ly49 are currently only limited to viral ORFs. It could be argued that these receptors are functionally replaced by extended CHIR in the chicken. B-NK in particular seems to be a prime candidate as an important NK cell restriction element and needs further characterization.

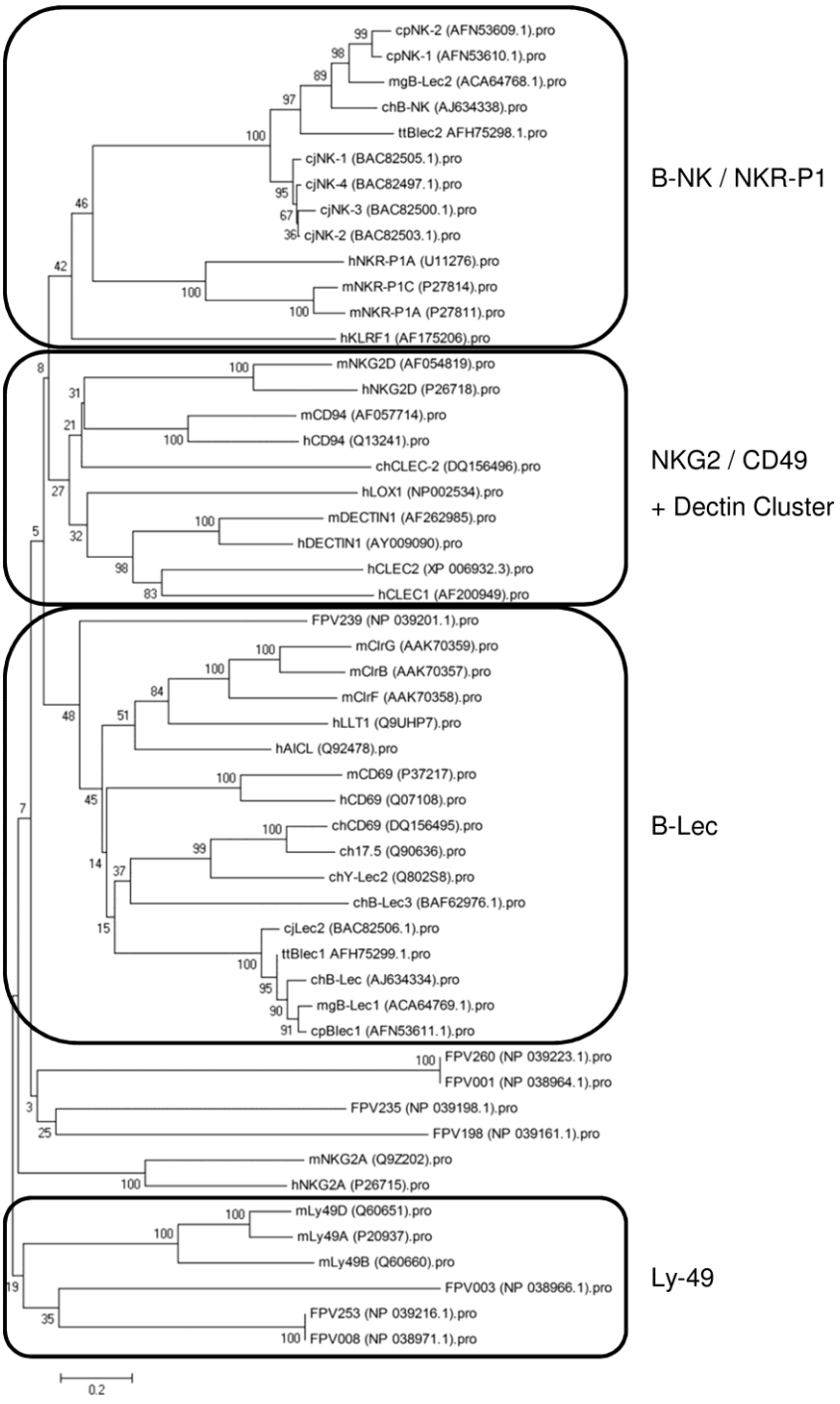

Figure 3 Phylogenetic tree of selected C-type lectins.

Neighbour-joining tree analysis of selected avian and mammalian C-type lectins as indicated. The entire proteins (accession numbers indicated) have been analysed. The four major groups are boxed. Species were denoted as c, chicken; cj, quail; cp, golden pheasant; h, human; m, mouse; mg, turkey; tt, black grouse; FPV, fowl pox virus ORF. Note that Blec2 is commonly used as alternative B-NK designation.

4. Other potential NK cell receptors

4.1 SLAM family

The signaling lymphocytic activation molecule (SLAM) family constitutes a subgroup of the CD2 receptor family and is differentially expressed on leukocytes. Members include SLAMF1 (CD150, SLAMF), SLAMF2 (CD48), SLAMF3 (Ly9, CD229), SLAMF4 (2B4, CD244), SLAMF5 (CD84), SLAMF6 (NTB-A, CD352), SLAMF7 (CRACC, CD319) and SLAMF8 (BLAME, CD353) (Cannons et al., 2011; MacDonald et al., 2008; Veillette et al., 2009). Five SLAM genes have been identified in chickens and three of them were named SLAMF1, SLAMF2 and SLAMF4 due to homologies with their corresponding human counterparts (**Fig. 4**). In contrast, an unambiguous assignment was not possible for two additional chicken SLAM receptors – SLAMF3like and SALMF5like - and therefore, the current nomenclature was chosen to emphasize the most likely mammalian counterparts, SLAMF3 and SLAMF5, respectively (Straub et al., 2013). In man, the SLAM genes are located on the long arm of chromosome 1 whereas the related genes CD2 and CD58 are located on the short arm of chromosome 1. The genomic region encoding the chicken SLAM genes is not totally resolved since SLAMF2 and SLAMF4 are currently located on an unassigned chromosome. However, it may be presumed that the chicken SLAM gene cluster is located on chromosome 25 where genes encoding SLAMF1, SLAMF3like and SLAMF5like could be completely assigned to. The chicken CD2 gene is located on chromosome 1, a situation similar to that of mice where the SLAM and CD2 genes are present on chromosomes 1 and 3, respectively. Avian and mammalian SLAM-associated receptors share several components. First, they are composed of an extracellular region containing an amino-terminal IgV-like domain and a membrane-proximal IgC2-like domain containing two conserved disulfide bonds. Second, all receptors compromise a single transmembrane region, except SLAMF2 which represents a GPI-anchored protein. Third, the cytoplasmic tail contains at least one ITSM. In man, this motif interacts with activating and inhibitory SLAM-associated protein (SAP)-related molecules which in return orchestrate distinct cellular responses. SAP-related adaptors include SAP and EAT-2 which are composed of a single Src homology 2 (SH2) domain followed by short C-terminal tail. Both SLAM-associated adaptor proteins were readily identified in chickens. Chicken SAP shares about 70% identity with mammalian SAP whereas chicken EAT-2 is much longer but still shares 52% identity with its mammalian counterpart (Straub et al., 2013). The identification of both receptors and adaptor molecules indicates a similar role of the SLAM-SAP interaction in non-mammalian vertebrates. SLAM family members generally form homophilic interactions, except SLAMF4 which binds SLAMF2 (Keestra et al., 2008).

SLAMF4 is of particular interest for NK cells. It is expressed on NK cells and it can either mediate activation or inhibition depending on the presence or absence of the adaptor molecules (Munitz et al., 2005; Sivori et al., 2000). We have recently generated a mab against SLAMF4, which stains NK cells in various organs (**Tab. 2**) (Straub, C. et al. in press). The function of SLAMF4 on chicken NK cells needs to be resolved in future studies.

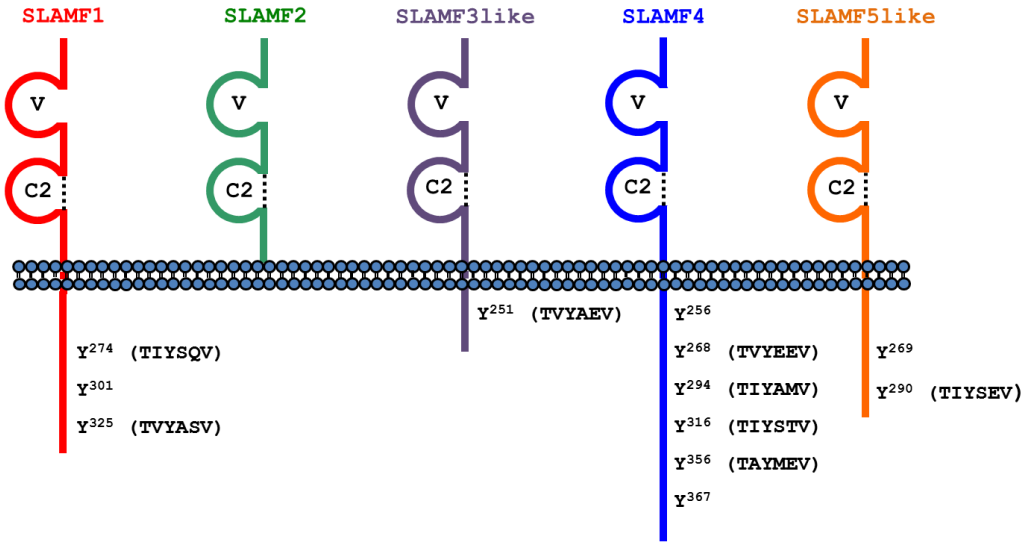

Figure 4 Chicken SLAM family.

Schematic representation of the chicken SLAM family with ITSM sequences indicated.

4.2 Adhesion molecules

Target recognition by NK cells is not only confined to the interaction of activating or inhibitory receptors with their respective ligands, but it is strengthened by a number of adhesion molecules. In the chicken, several of these adhesion molecules have also been documented; however, their contribution to NK cell lysis has not been resolved.

A group of several mab has been identified that seem to recognize an identical molecule expressed by a variety of lymphoid and myeloid cells. The mab were generated in different fusions using macrophages, IEL or in vitro expanded NK cells as immunogens. The group includes mab such as 8F2, 3-6, 6B5 among others. They react with various leukocyte subpopulations including lymphocyte subsets, dendritic cells, monocytes, IEL and also with NK cells (de Geus et al., 2012; Wu et al., 2010). Based on these and biochemical criteria, the antigen recognized by these mab was tentatively assigned as a CD11/CD18 leukocyte integrin (Göbel and Kaspers, unpublished). The final test whether this assignment is correct has been hampered by the lack of a full length annotation of the respective leukocyte integrins in the genome. In fact, we have so far not been able to clone a full length CD11 gene using the

partial information available in several databases. Moreover, it is not clear if the chicken CD11 cluster also contains four different genes as in mammals or if duplications leading to this expansion occurred after the lineage split of chickens and mammals.

Another group of receptors also expressed by NK cells is characterized by its binding to nectin and nectin-like proteins. A total of four receptors have been described to be mainly expressed on $CD8^+$ T lymphocytes and NK cells, including CD96, CD226, TIGIT and CRTAM (Boles et al., 2005; Chan et al., 2012). We have identified homologous of all receptors except TIGIT in various avian genomes. The chicken CRTAM was actually described previously as chicken thymic activation and developmental sequence (cTADS) (Ruble and Foster, 2000). A novel mab generated against chicken CRTAM is currently being characterized. Initial experiments have revealed a staining pattern similar to its mammalian homologue which resembles an activation marker of $CD8^+$ T lymphocytes.

The neural adhesion molecule NCAM-1 or CD56 has been of particular use to characterize various subsets of human NK cells, which show distinct functional characteristics with $CD56^{dim}$ NK cells representing 90 % of the circulating NK cells and $CD56^{bright}$ NK cells. Since the chicken NCAM gene was identified a long time ago (Murray et al., 1984), we have generated a chicken CD56 specific mab and tested its expression on various chicken tissues. In contrast to previously published results that were generated using a polyclonal anti-human CD56 antiserum (Göbel et al., 1996a), CD56 is expressed on some cells in the embryonic spleen and also on lung cells, that may resemble NK cells, however, it is not a universal NK cell marker and its expression on NK cells is highly variable (Neulen and Göbel, 2012a).

The receptors CD5 and CD6 have also been documented to be expressed on chicken NK cell subsets, however, their function is currently unknown (Göbel et al., 1996b; Katevuo et al., 1999; Koskinen et al., 1998).

Finally CD57 also known as HNK1 resembles a terminally sulphated glycan carbohydrate, which is expressed on chronically stimulated T lymphocytes (Brownlie et al., 2009). Recent analyses have revealed expression on a human NK cell subset, representing a mature, terminally differentiated subset that has limited proliferative and cytolytic capacity (Lopez-Verges et al., 2010). The mab also crossreacts with chicken cells and recognizes the blood NK cell population defined by its expression of various NK cell markers (**Tab. 2**). This may indicate that the chicken blood mainly contains mature, terminally differentiated NK cells. In line with this assumption is the capacity of these cells to produce IFN-γ upon stimulation.

4.3 ggTREM-A1

A cluster of IgSF members has been identified on chicken chromosome 26 (Viertlboeck et al., 2006). The genes were identified as homologues to the mammalian TREM genes. A total of three chicken TREM homologues were identified, with two of them representing inhibitory and one an activating receptor. Our recent analysis with the help of a specific mab indicated that the activating ggTREM-A1 is indeed expressed on a subpopulation of blood NK cells (Viertlboeck et al. submitted for publication). A re-evaluation of its sequence properties together with the novel data concerning expression lead us to propose that it might resemble a molecule with features of both TREM and NKp44. NKp44 as one of three NCR receptors is located adjacent to the mammalian TREM cluster (Allcock et al., 2003). The most intriguing similarity between mammalian NKp44 and the ggTREM-A1 is the position of the basic transmembrane residue, which is located at a central position which differs from the position found in CHIR that is located close to the extracellular domain. This implies that an adaptor molecule different to the FcεRIγ, that assembles to CHIR, is responsible for signal transduction. This most likely could be either the CD3ζ chain that has been identified in the chicken (Göbel and Bolliger, 1998) or the DAP12 adaptor, that is located in the mammalian extended LRC, but has so far not been described in the chicken. Future studies will resolve if the chicken ggTREM-A1 also represents a functional NKp44 homologue. There are several other IgSF families in the chicken genome, such as CD200R, SIRP and CD300L however; so far there is no evidence of expression on NK cells (Viertlboeck et al., 2008a; Viertlboeck et al., 2006).

4.4 Mab against molecularly undefined antigens

We have previously generated different panels of mab that were raised against either IEL or in vitro expanded sorted NK cells (Göbel et al., 2001; Jansen et al., 2010). Among these, three seem to be of particular use, although they are all not uniquely expressed by NK cells. The 28-4 mab has originally been raised against IEL and it specifically reacts with a molecule expressed by $CD8^+$ $CD3^-$ that mediate spontaneous cytotoxicity (Göbel et al., 2001). The antigen recognized by 28-4 has not been molecularly identified and its expression is not limited to IEL, but it is also expressed by blood and embryonic NK cells. It is also upregulated upon activation on B- and T-cells (Göbel, unpublished).

20E5 and 1G7 both were generated in a fusion against in vitro expanded sorted $CD8^+$ $CD3^-$ splenocytes (Jansen et al., 2010). Again the antigens recognized have not been molecularly identified and they are not exclusively expressed by NK cells. The 20E5 mab is present on NK

cell populations in embryonic spleen, IEL and blood and also reacts with macrophages and the chicken cell line HD11, whereas 1G7 antigen expression is not found on IEL. The future identification of the genes encoding the 28-4, 20E5 and 1G7 antigens will be helpful to further discriminate several NK cell subpopulations in the chicken.

5. Future directions

The recent developments in genomic databases have had an enormous impact in the identification of receptor families and single receptors that resemble homologues to mammalian NK cell receptors and therefore may be important chicken NK cell receptors as well (**Tab. 1**). We have just begun to exploit the potential of these receptors for the NK cell characterization, mainly by generating mab to some of these receptors. Nevertheless, even this limited number of mab available, now allows the phenotypic characterization of at least three distinct NK cell subsets (**Tab. 2**). The NK cells in the embryonic spleen were the first to be described (Bucy et al., 1989; Göbel et al., 1994). They are detectable early in the embryo and thus may represent immature cells, but once isolated and cultured, they are cytolytic. Their cytokine production has not been examined so far. The intestinal NK cell population represents the most frequent source of NK cells (Göbel et al., 2001). These cells predominantly found in the IEL population represent most likely a unique population with special functions adapted to the intestinal milieu. Finally the small PBMC NK cell population may turn out as terminally differentiated form as indicated by the CD57 expression (**Tab. 2**). Alternatively, it could represent a special subset since it is the only NK cell population that carries CD4. There are definitively more NK cell subsets to be identified, for example, we have found a small population in lung and liver preparations which are currently under investigation.

As a next important step in the characterization of NK cells, there is a need to address potential cytokines which are either secreted by NK cells or that influence their proliferation, maturation and function. To this end, a multitude of cytokines has been cloned and expressed, recently, so it is only a matter of time to use these cytokines as tools for the NK cell characterization (Staeheli et al., 2001). In particular, this is true for type I and type II interferons, IL-2, IL-12, IL-15, IL-18 and IL-21. Single cytokines or combinations may be helpful to establish a robust in vitro culture system that would allow other types of NK cell assays and to test NK cell activity against various pathogens.

It would also be very helpful to establish more assays to functionally characterize NK cells. Recently, a CD107 degranulation assay could be established that is easier to handle as the

classical chromium release assay (Jansen et al., 2010), however, CD107 surface expression is a relatively nonspecific phenomenon that can be seen in many different cell types. In terms of target cells, the LSCC-RP9 is momentarily the only available robust target cell. Finally, besides Elispot assay to detect IFN-γ, it would be of great importance to further establish other Elispot assays and methods for the intracellular detection of cytokines. All these tools together with a steadily growing number of surface markers ultimately enable the functional NK cell characterization in physiological conditions as well as during infections.

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References

Afonso, C.L., Tulman, E.R., Lu, Z., Zsak, L., Kutish, G.F., Rock, D.L., 2000. The genome of fowlpox virus. J. Virol. 74, 3815-3831.

Allcock, R.J., Barrow, A.D., Forbes, S., Beck, S., Trowsdale, J., 2003. The human TREM gene cluster at 6p21.1 encodes both activating and inhibitory single IgV domain receptors and includes NKp44. Eur. J. Immunol. 33, 567-577.

Arnon, T.I., Kaiser, J.T., West, A.P., Jr., Olson, R., Diskin, R., Viertlboeck, B.C., Göbel, T.W., Bjorkman, P.J., 2008. The crystal structure of CHIR-AB1: a primordial avian classical Fc receptor. J. Mol. Biol. 381, 1012-1024.

Barrow, A.D., Trowsdale, J., 2008. The extended human leukocyte receptor complex: diverse ways of modulating immune responses. Immunol. Rev. 224, 98-123.

Bernard, D., Hansen, J.D., Du Pasquier, L., Lefranc, M.P., Benmansour, A., Boudinot, P., 2007. Costimulatory receptors in jawed vertebrates: conserved CD28, odd CTLA4 and multiple BTLAs. Dev Comp Immunol 31, 255-271.

Bernot, A., Zoorob, R., Auffray, C., 1994. Linkage of a new member of the lectin supergene family to chicken Mhc genes. Immunogenet. 39, 221-229.

Boles, K.S., Barchet, W., Diacovo, T., Cella, M., Colonna, M., 2005. The tumor suppressor TSLC1/NECL-2 triggers NK-cell and CD8+ T-cell responses through the cell-surface receptor CRTAM. Blood 106, 779-786.

Brownlie, R., Zhu, J., Allan, B., Mutwiri, G.K., Babiuk, L.A., Potter, A., Griebel, P., 2009. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol 46, 3163-3170.

Bucy, R.P., Coltey, M., Chen, C.I., Char, D., Le Douarin, N.M., Cooper, M.D., 1989. Cytoplasmic CD3+ surface CD8+ lymphocytes develop as a thymus-independent lineage in chick-quail chimeras. Eur. J. Immunol. 19, 1449-1455.

Campbell, K.S., Colonna, M., 2001. Human natural killer cell receptors and signal transduction. Int. Rev. Immunol. 20, 333-370.

Campbell, K.S., Purdy, A.K., 2011. Structure/function of human killer cell immunoglobulin-like receptors: lessons from polymorphisms, evolution, crystal structures and mutations. Immunology 132, 315-325.

Cannons, J.L., Tangye, S.G., Schwartzberg, P.L., 2011. SLAM family receptors and SAP adaptors in immunity. Annu. Rev. Immunol. 29, 665-705.

Chan, C.J., Andrews, D.M., Smyth, M.J., 2012. Receptors that interact with nectin and nectin-like proteins in the immunosurveillance and immunotherapy of cancer. Curr. Opin. Immunol. 24, 246-251.

Chaves, L.D., Krueth, S.B., Reed, K.M., 2009. Defining the turkey MHC: sequence and genes of the B locus. J. Immunol. 183, 6530-6537.

Cheent, K., Khakoo, S.I., 2009. Natural killer cells: integrating diversity with function. Immunology 126, 449-457.

Chiang, H.I., Zhou, H., Raudsepp, T., Jesudhasan, P.R., Zhu, J.J., 2007. Chicken CD69 and CD94/NKG2-like genes in a chromosomal region syntenic to mammalian natural killer gene complex. Immunogenet. 59, 603-611.

Daeron, M., Jaeger, S., Du Pasquier, L., Vivier, E., 2008. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. Immunol. Rev. 224, 11-43.

de Geus, E.D., Jansen, C.A., Vervelde, L., 2012. Uptake of particulate antigens in a nonmammalian lung: phenotypic and functional characterization of avian respiratory phagocytes using bacterial or viral antigens. J. Immunol. 188, 4516-4526.

Göbel, T.W., Bolliger, L., 1998. The chicken TCR z-chain restores the function of a mouse T cell hybridoma. J. Immunol. 160, 1552-1554.

Göbel, T.W., Chen, C.H., Cooper, M.D., 1996a. Avian natural killer cells. Curr. Top. Microbiol. Immunol. 212, 107-117.

Göbel, T.W., Chen, C.H., Cooper, M.D., 1996b. Expression of an avian CD6 candidate is restricted to ab T cells, splenic CD8+ gd T cells and embryonic natural killer cells. Eur. J. Immunol. 26, 1743-1747.

Göbel, T.W., Chen, C.L., Shrimpf, J., Grossi, C.E., Bernot, A., Bucy, R.P., Auffray, C., Cooper, M.D., 1994. Characterization of avian natural killer cells and their intracellular CD3 protein complex. Eur. J. Immunol. 24, 1685-1691.

Göbel, T.W., Kaspers, B., Stangassinger, M., 2001. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. Int. Immunol. 13, 757-762.

He, H., Genovese, K.J., Nisbet, D.J., Kogut, M.H., 2006. Profile of Toll-like receptor expressions and induction of nitric oxide synthesis by Toll-like receptor agonists in chicken monocytes. Mol. Immunol. 43, 783-789.

Hillier, L.W., Miller, W., Birney, E., Warren, W., Hardison, R.C., Ponting, C.P., Bork, P., Burt, D.W., Groenen, M.A., Delany, M.E., Dodgson, J.B., Fingerprint Map, S., Assembly, G., Chinwalla, A.T., Cliften, P.F., Clifton, S.W., Delehaunty, K.D., Fronick, C., Fulton, R.S., Graves, T.A., Kremitzki, C., Layman, D., Magrini, V., McPherson, J.D., Miner, T.L., Minx, P., Nash, W.E., Nhan, M.N., Nelson, J.O., Oddy, L.G., Pohl, C.S., Randall-Maher, J., Smith, S.M., Wallis, J.W., Yang, S.P., Romanov, M.N., Rondelli, C.M., Paton, B., Smith, J., Morrice, D., Daniels, L., Tempest, H.G., Robertson, L., Masabanda, J.S., Griffin, D.K., Vignal, A., Fillon, V., Jacobbson, L., Kerje, S., Andersson, L., Crooijmans, R.P., Aerts, J., van der Poel, J.J., Ellegren, H., Sequencing, C., Caldwell, R.B., Hubbard, S.J., Grafham, D.V., Kierzek, A.M., McLaren, S.R., Overton, I.M., Arakawa, H., Beattie, K.J., Bezzubov, Y., Boardman, P.E., Bonfield, J.K., Croning, M.D., Davies, R.M., Francis, M.D., Humphray, S.J., Scott, C.E., Taylor, R.G., Tickle, C., Brown, W.R., Rogers, J., Buerstedde, J.M., Wilson, S.A., Sequencing, Libraries, O., Stubbs, L., Ovcharenko, I., Gordon, L., Lucas, S., Miller,

M.M., Inoko, H., Shiina, T., Kaufman, J., Salomonsen, J., Skjoedt, K., Wong, G.K., Wang, J., Liu, B., Yu, J., Yang, H., Nefedov, M., Koriabine, M., Dejong, P.J., And Annotation, A., Goodstadt, L., Webber, C., Dickens, N.J., Letunic, I., Suyama, M., Torrents, D., von Mering, C., Zdobnov, E.M., Makova, K., Nekrutenko, A., Elnitski, L., Eswara, P., King, D.C., Yang, S., Tyekucheva, S., Radakrishnan, A., Harris, R.S., Chiaromonte, F., Taylor, J., He, J., Rijnkels, M., Griffiths-Jones, S., Ureta-Vidal, A., Hoffman, M.M., Severin, J., Searle, S.M., Law, A.S., Speed, D., Waddington, D., Cheng, Z., Tuzun, E., Eichler, E., Bao, Z., Flicek, P., Shteynberg, D.D., Brent, M.R., Bye, J.M., Huckle, E.J., Chatterji, S., Dewey, C., Pachter, L., Kouranov, A., Mourelatos, Z., Hatzigeorgiou, A.G., Paterson, A.H., Ivarie, R., Brandstrom, M., Axelsson, E., Backstrom, N., Berlin, S., Webster, M.T., Pourquie, O., Reymond, A., Ucla, C., Antonarakis, S.E., Long, M., Emerson, J.J., Betran, E., Dupanloup, I., Kaessmann, H., Hinrichs, A.S., Bejerano, G., Furey, T.S., Harte, R.A., Raney, B., Siepel, A., Kent, W.J., Haussler, D., Eyras, E., Castelo, R., Abril, J.F., Castellano, S., Camara, F., Parra, G., Guigo, R., Bourque, G., Tesler, G., Pevzner, P.A., Smit, A., Management, P., Fulton, L.A., Mardis, E.R., Wilson, R.K., 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 432, 695-716.

Iizuka, K., Naidenko, O.V., Plougastel, B.F., Fremont, D.H., Yokoyama, W.M., 2003. Genetically linked C-type lectin-related ligands for the NKRP1 family of natural killer cell receptors. Nat. Immunol. 4, 801-807.

Jansen, C.A., van de Haar, P.M., van Haarlem, D., van Kooten, P., de Wit, S., van Eden, W., Viertlbock, B.C., Göbel, T.W., Vervelde, L., 2010. Identification of new populations of chicken natural killer (NK) cells. Dev. Comp. Immunol. 34, 759-767.

Katevuo, K., Imhof, B.A., Boyd, R., Chidgey, A., Bean, A., Dunon, D., Göbel, T.W., Vainio, O., 1999. ChT1, an Ig superfamily molecule required for T cell differentiation. J Immunol 162, 5685-5694.

Kaufman, J., Milne, S., Göbel, T.W., Walker, B.A., Jacob, J.P., Auffray, C., Zoorob, R., Beck, S., 1999. The chicken B locus is a minimal essential major histocompatibility complex. Nature 401, 923-925.

Keestra, A.M., de Zoete, M.R., van Aubel, R.A., van Putten, J.P., 2008. Functional characterization of chicken TLR5 reveals species-specific recognition of flagellin. Mol Immunol 45, 1298-1307.

Kiessling, R., Klein, E., Wigzell, H., 1975. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. Eur. J. Immunol. 5, 112-117.

Koskinen, R., Göbel, T.W., Tregaskes, C.A., Young, J.R., Vainio, O., 1998. The structure of avian CD5 implies a conserved function. J. Immunol. 160, 4943-4950.

Lanier, L.L., 2008. Up on the tightrope: natural killer cell activation and inhibition. Nat. Immunol. 9, 495-502.

Laun, K., Coggill, P., Palmer, S., Sims, S., Ning, Z., Ragoussis, J., Volpi, E., Wilson, N., Beck, S., Ziegler, A., Volz, A., 2006. The leukocyte receptor complex in chicken is characterized by massive expansion and diversification of immunoglobulin-like Loci. PLoS Genet. 2, e73.

Leibson, P.J., 1997. Signal transduction during natural killer cell activation: inside the mind of a killer. Immunity 6, 655-661.

Ljunggren, H.G., Karre, K., 1990. In search of the 'missing self': MHC molecules and NK cell recognition. Immunol. Today 11, 237-244.

Lochner, K.M., Viertlboeck, B.C., Göbel, T.W., 2010. The red jungle fowl leukocyte receptor complex contains a large, highly diverse number of chicken immunoglobulin-like receptor (CHIR) genes. Mol. Immunol. 47, 1956-1962.

Lopez-Verges, S., Milush, J.M., Pandey, S., York, V.A., Arakawa-Hoyt, J., Pircher, H., Norris, P.J., Nixon, D.F., Lanier, L.L., 2010. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. Blood 116, 3865-3874.

MacDonald, M.R., Xia, J., Smith, A.L., Magor, K.E., 2008. The duck toll like receptor 7: genomic organization, expression and function. Mol Immunol 45, 2055-2061.

Munitz, A., Bachelet, I., Fraenkel, S., Katz, G., Mandelboim, O., Simon, H.U., Moretta, L., Colonna, M., Levi-Schaffer, F., 2005. 2B4 (CD244) is expressed and functional on human eosinophils. J. Immunol. 174, 110-118.

Murray, B.A., Hemperly, J.J., Gallin, W.J., MacGregor, J.S., Edelman, G.M., Cunningham, B.A., 1984. Isolation of cDNA clones for the chicken neural cell adhesion molecule (N-CAM). Proc. Natl. Acad. Sci. U S A 81, 5584-5588.

Mwangi, W.N., Smith, L.P., Baigent, S.J., Smith, A.L., Nair, V., 2012. Induction of lymphomas by inoculation of Marek's disease virus-derived lymphoblastoid cell lines: prevention by CVI988 vaccination. Avian Pathol 41, 589-598.

Natarajan, K., Dimasi, N., Wang, J., Mariuzza, R.A., Margulies, D.H., 2002. Structure and function of natural killer cell receptors: multiple molecular solutions to self, nonself discrimination. Annual Reviews of Immunology 20, 853-885.

Neulen, M.L., Göbel, T.W., 2012a. Chicken CD56 defines NK cell subsets in embryonic spleen and lung. Dev. Comp. Immunol. 38, 410-415.

Neulen, M.L., Göbel, T.W., 2012b. Identification of a chicken CLEC-2 homologue, an activating C-type lectin expressed by thrombocytes. Immunogenet. 64, 389-397.

O'Neill, A.M., Livant, E.J., Ewald, S.J., 2009. The chicken BF1 (classical MHC class I) gene shows evidence of selection for diversity in expression and in promoter and signal peptide regions. Immunogenet. 61, 289-302.

Parham, P., 2005. MHC class I molecules and KIRs in human history, health and survival. Nat. Rev. Immunol. 5, 201-214.

Parham, P., 2008. The genetic and evolutionary balances in human NK cell receptor diversity. Semin. Immunol. 20, 311-316.

Pürzel, J., Schmitt, R., Viertlboeck, B.C., Göbel, T.W., 2009. Chicken IgY binds its receptor at the CH3/CH4 interface similarly as the human IgA: Fc alpha RI interaction. J. Immunol. 183, 4554-4559.

Reth, M., 1989. Antigen receptor tail clue. Nature 338, 383-384.

Rogers, S., Göbel, T.W., Viertlböck, B.C., Milne, S., Beck, S., Kaufman, J., 2005. Characterisation of the C-type lectin-like receptors B-NK and B-lec suggests that the NK complex and the MHC share a common ancestral region. J. Immunol. 174.

Rogers, S., Shaw, I., Ross, N., Nair, V., Rothwell, L., Kaufman, J., Kaiser, P., 2003. Analysis of part of the chicken Rfp-Y region reveals two novel lectin genes, the first complete genomic sequence of a class I alpha-chain gene, a truncated class II beta-chain gene, and a large CR1 repeat. Immunogenet. 55, 100-108.

Rogers, S.L., Kaufman, J., 2008. High allelic polymorphism, moderate sequence diversity and diversifying selection for B-NK but not B-lec, the pair of lectin-like receptor genes in the chicken MHC. Immunogenet. 60, 461-475.

Rogers, S.L., Viertlboeck, B.C., Göbel, T.W., Kaufman, J., 2008. Avian NK activities, cells and receptors. Semin. Immunol. 20, 353-360.

Ruble, D.M., Foster, D.N., 2000. Molecular characterization of a new member of the immunoglobulin superfamily that potentially functions in T-cell activation and development. Immunogenet. 51, 347-357.

Sattler, S., Ghadially, H., Hofer, E., 2012. Evolution of the C-type lectin-like receptor genes of the DECTIN-1 cluster in the NK gene complex. TheScientificWorldJournal 2012, 931386.

Scott, T., Owens, M.D., 2008. Thrombocytes respond to lipopolysaccharide through Toll-like receptor-4, and MAP kinase and NF-kappaB pathways leading to expression of interleukin-6 and cyclooxygenase-2 with production of prostaglandin E2. Mol. Immunol. 45, 1001-1008.

Sharma, J.M., Okazaki, W., 1981. Natural killer cell activity in chickens: target cell analysis and effect of antithymocyte serum on effector cells. Infect. Immun. 31, 1078-1085.

Shaw, I., Powell, T.J., Marston, D.A., Baker, K., van Hateren, A., Riegert, P., Wiles, M.V., Milne, S., Beck, S., Kaufman, J., 2007. Different evolutionary histories of the two classical class I genes BF1 and BF2 illustrate drift and selection within the stable MHC haplotypes of chickens. J. Immunol. 178, 5744-5752.

Shiina, T., Shimizu, S., Hosomichi, K., Kohara, S., Watanabe, S., Hanzawa, K., Beck, S., Kulski, J.K., Inoko, H., 2004. Comparative genomic analysis of two avian (quail and chicken) MHC regions. J. Immunol. 172, 6751-6763.

Sivori, S., Parolini, S., Falco, M., Marcenaro, E., Biassoni, R., Bottino, C., Moretta, L., Moretta, A., 2000. 2B4 functions as a co-receptor in human NK cell activation. Eur. J. Immunol. 30, 787-793.

St Paul, M., Paolucci, S., Barjesteh, N., Wood, R.D., Schat, K.A., Sharif, S., 2012. Characterization of chicken thrombocyte responses to Toll-like receptor ligands. PLoS One 7, e43381.

Staeheli, P., Puehler, F., Schneider, K., Gobel, T.W., Kaspers, B., 2001. Cytokines of birds: conserved functions-a largely different look. J. Interf. Cytok. Res. 21, 993-1010.

Straub, C., Viertlbock, B.C., Göbel, T.W., 2013. The chicken SLAM familiy. Immunogenet. 65, 63-73.

Veillette, A., Dong, Z., Perez-Quintero, L.A., Zhong, M.C., Cruz-Munoz, M.E., 2009. Importance and mechanism of 'switch' function of SAP family adapters. Immunol. Rev. 232, 229-239.

Viertlboeck, B.C., Crooijmans, R.P., Groenen, M.A., Göbel, T.W., 2004. Chicken Ig-Like Receptor B2, a Member of a Multigene Family, Is Mainly Expressed on B Lymphocytes, Recruits Both Src Homology 2 Domain Containing Protein Tyrosine Phosphatase (SHP)-1 and SHP-2, and Inhibits Proliferation. J. Immunol. 173, 7385-7393.

Viertlboeck, B.C., Gick, C.M., Schmitt, R., Du Pasquier, L., Göbel, T.W., 2010. Complexity of expressed CHIR genes. Dev. Comp. Immunol. 34, 866-873.

Viertlboeck, B.C., Göbel, T.W., 2011. The chicken leukocyte receptor cluster. Vet Immunol Immunopathol 144, 1-10.

Viertlboeck, B.C., Habermann, F.A., Schmitt, R., Groenen, M.A., Du Pasquier, L., Göbel, T.W., 2005. The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types. J. Immunol. 175, 385-393.

Viertlboeck, B.C., Hanczaruk, M.A., Schmitt, F.C., Schmitt, R., Göbel, T.W., 2008a. Characterization of the chicken CD200 receptor family. Mol. Immunol. 45, 2097-2105.

Viertlboeck, B.C., Schmitt, R., Göbel, T.W., 2006. The chicken immunoregulatory receptor families SIRP, TREM, and CMRF35/CD300L. Immunogenet. 58, 180-190.

Viertlboeck, B.C., Schmitt, R., Hanczaruk, M.A., Crooijmans, R.P., Groenen, M.A., Göbel, T.W., 2009a. A novel activating chicken IgY FcR is related to leukocyte receptor complex (LRC) genes but is located on a chromosomal region distinct from the LRC and FcR gene clusters. J. Immunol. 182, 1533-1540.

Viertlboeck, B.C., Schweinsberg, S., Hanczaruk, M.A., Schmitt, R., Du Pasquier, L., Herberg, F.W., Göbel, T.W., 2007. The chicken leukocyte receptor complex encodes a primordial, activating, high-affinity IgY Fc receptor. Proc. Natl. Acad. Sci. U S A 104, 11718-11723.

Viertlboeck, B.C., Schweinsberg, S., Schmitt, R., Herberg, F.W., Göbel, T.W., 2009b. The chicken leukocyte receptor complex encodes a family of different affinity FcY receptors. J. Immunol. 182, 6985-6992.

Viertlboeck, B.C., Wortmann, A., Schmitt, R., Plachy, J., Göbel, T.W., 2008b. Chicken C-type lectin-like receptor B-NK, expressed on NK and T cell subsets, binds to a ligand on activated splenocytes. Mol. Immunol. 45, 1398-1404.

Vivier, E., Malissen, B., 2005. Innate and adaptive immunity: specificities and signaling hierarchies revisited. Nat. Immunol. 6, 17-21.

Walker, B.A., Hunt, L.G., Sowa, A.K., Skjodt, K., Göbel, T.W., Lehner, P.J., Kaufman, J., 2011. The dominantly expressed class I molecule of the chicken MHC is explained by coevolution with the polymorphic peptide transporter (TAP) genes. Proc. Natl. Acad. Sci. U S A 108, 8396-8401.

Walzer, T., Jaeger, S., Chaix, J., Vivier, E., 2007. Natural killer cells: from CD3 (-)NKp46(+) to post-genomics meta-analyses. Curr. Opin. Immunol. 19, 365-372.

Wang, B., Ekblom, R., Strand, T.M., Portela-Bens, S., Hoglund, J., 2012. Sequencing of the core MHC region of black grouse (Tetrao tetrix) and comparative genomics of the galliform MHC. BMC genomics 13, 553.

Weis, W.I., Taylor, M.E., Drickamer, K., 1998. The C-type lectin superfamily in the immune system. Immunol. Rev. 163, 19-34.

Wilcock, D., Duncan, S.A., Traktman, P., Zhang, W.H., Smith, G.L., 1999. The vaccinia virus A4OR gene product is a nonstructural, type II membrane glycoprotein that is expressed at the cell surface. J. Gen. Virol. 80 (Pt 8), 2137-2148.

Windau, K., Viertlbock, B.C., Göbel, T.W., 2013. The turkey Ig-like receptor family: identification, expression and function. PLoS One 8, e59577.

Wu, Z., Rothwell, L., Young, J.R., Kaufman, J., Butter, C., Kaiser, P., 2010. Generation and characterization of chicken bone marrow-derived dendritic cells. Immunology 129, 133-145.

Ye, Q., He, K., Wu, S.Y., Wan, Q.H., 2012. Isolation of a 97-kb minimal essential MHC B locus from a new reverse-4D BAC library of the golden pheasant. PLoS One 7, e32154.

Yokoyama, W.M., Plougastel, B.F., 2003. Immune functions encoded by the natural killer gene complex. Nat. Rev. Immunol. 3, 304-316.

Yokoyama, W.M., Seaman, W.E., 1993. The Ly-49 and NKR-P1 gene families encoding lectin-like receptors on natural killer cells: the NK gene complex. Annu. Rev. Immunol. 11, 613-635.

5. DISCUSSION

The chicken genome was analyzed for annotated SLAM genes and five putative SLAM genes could be identified by this strategy. Three of them (named SLAMF1, SLAMF3like, and SLAMF5like) were mapped to chromosome 25, whereas genes encoding SLAMF2 and SLAMF4 were only identified in Expressed Sequence Tag (EST) databases and thus allocated to unassigned chromosomes.

Given that the chicken genome harbors three SLAM genes on chromosome 25, it is reasonable to presume the entire SLAM locus on chromosome 25. This assumption is supported by findings made in the zebra finch genome, where SLAMF1 has also been allocated to chromosome 25, albeit this locus lacks other SLAM genes.

In man and mice, all SLAM-encoding genes are tightly clustered within a 400 kb range on chromosome 1 (**Fig. 2**) and it has been argued that these genes arose by successive gene duplications. The existing chicken SLAM genes may thus combine functional properties which must have been in place prior to the divergence of both species 300 million years ago. During evolution, selection-pressure may have caused gene duplication events in mammals in order to battle a novel quality and quantity of pathogens. On the other hand, it cannot be ruled out that other genes may exist on yet unassigned chromosomes.

Although overall identity of the chicken SLAM genes with their mammalian counterparts was rather low, ranging from 24 to 28%, an unambiguous assignment to the SLAM family was possible due to several structural analogies. Firstly, all chicken SLAM receptors are composed of an extracellular domain displaying the typical two-Ig-like structure, that is, an N-terminal Ig variable (V)-like domain lacking disulfide bonds and a membrane-proximal Ig constant 2 (C2)-like domain with an additional pair of disulfide bonds. Secondly, all chicken receptors comprise a single transmembrane region and at least one ITSM motif in their cytoplasmic tail, except SLAMF2, which lacks a transmembrane domain and instead represents a GPI-anchored protein (**Fig. 3**).

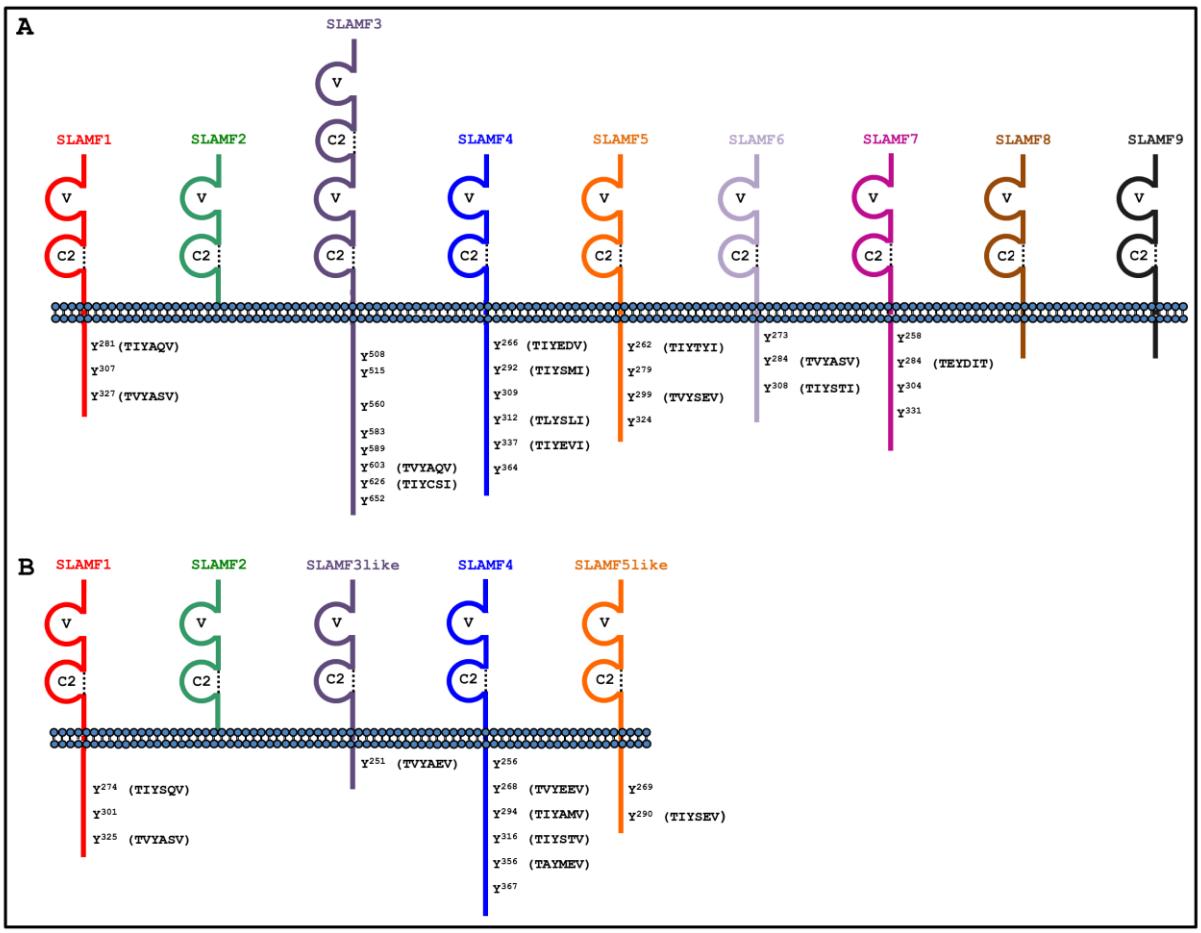

Figure 3 Comparison of SLAM family receptors in (A) man and (B) chicken.

Human and chicken SLAM receptors share several structural analogies, in particular an extracellular two Ig-like structure, a single transmembrane region and cytoplasmic ITSM motifs. Modified according to [18].

In this respect, three genes (SLAMF1, SLAMF2 and SLAMF4) could be univocally assigned to their mammalian orthologues which was not feasible for two additional SLAM receptors. Sequence comparisons revealed an almost equal homology of chicken SLAMF3like to both mammalian SLAMF3 and SLAMF6. SLAMF3like was initially annotated as SLAMF3 on chromosome 25. The syntenic region in the mammalian genome however encodes SLAMF6 and SLAMF5, respectively. Furthermore, mammalian SLAMF3 is unique by displaying a duplication of the extracellular V-C2-like domain whereas chicken SLAMF3like only encompasses two Ig-like domains. In addition, chicken SLAMF3like comprises a single cytoplasmic ITSM motif, as opposed to two ITSMs in mammalian SLAMF3 and SLAMF6, respectively. Therefore, SLAMF3like could resemble both mammalian SLAMF3 and SLAMF5 and we chose the current nomenclature to emphasize its most likely counterpart, mammalian SLAMF3.

A similar situation applied for SLAMF5like, originally annotated as SLAMF8 and mapped to the syntenic locus of mammalian SLAMF8. Chicken SLAMF5like comprises a single ITSM

in its C-terminal tail that has been conserved in mammalian SLAMF5. Moreover, at the position of the membrane-proximal ITSM of mammalian SLAMF5, chicken SLAMF5like harbors a conserved tyrosine residue, albeit the ITSM consensus is not completely matched due to a proline at position 1 instead of a threonine. Since mammalian SLAMF8 displays a rather short cytoplasmic tail devoid of typical ITSM motifs, we suggested naming it SLAMF5like.

Unresolved questions concerning both the number and correct annotation of chicken SLAM genes may be addressed once the entire genomic region has been sequenced.

The existence of several SLAM genes in the chicken genome consequently led to the identification of chicken orthologues to mammalian SAP and EAT-2, respectively. Sequence analyses revealed that chicken EAT-2 is most homologous to murine EAT-2 whereas human EAT-2 is much shorter. Mammalian EAT-2 seems to transduce intracellular signals through two tyrosine residues located at the C-terminus [111] and markedly, both tyrosine residues are conserved in chickens. Chicken SAP shares an almost identical length as well as structural features to human and murine SAP, in particular a SH2 domain and a short C-terminal tail. In addition, the recruitment site in SAP for the protein tyrosine kinase Fyn, a motif centered on arginine 78 (R78), has been highly conserved in chickens.

In conclusion, both SAP and EAT-2 are preserved in the chicken genome, results that imply similar signal transduction pathways as in mammals, the latter supported by the conservation of key amino acids involved in SLAM receptor signaling.

Another feature of mammalian SLAM family members is their expression on a variety of immune cells. Mammalian SLAMF4, for instance, is expressed on NK cells, some γδ T cell subsets, $CD8^+$ αβ T cells, basophils, eosinophils, and monocytes [88]. In this respect, preliminary PCR analyses of various lymphoid tissues also indicate a wide expression of chicken SLAM receptors. SLAMF4 in particular could not be detected in bursal cDNA and was weakly expressed in thymic cDNA. Using a novel SLAMF4 specific mab, flow cytometric analyses established an even wider tissue distribution of chicken SLAMF4 as its mammalian homologue. Similarities of both chicken and mammalian SLAMF4 expression include a $CD8\alpha^+$ αβ T cell subset, γδ T cells, NK cells, and monocytes.

Furthermore, we found chicken SLAMF4 to be expressed on thrombocytes, the nucleated homologue to mammalian platelets. In mammals, only SLAMF1 and SLAMF5 are expressed on platelets, while SLAMF4 expression has not been documented [52]. Given that chicken thrombocytes carry a plethora of immunoregulatory receptors, including a TREM molecule, CLEC2, an Fc receptor, CD40 ligand and several other Ig-like receptors [22, 122-124], these findings indicate an involvement of chicken thrombocytes in immune regulation.

Another difference of chicken SLAMF4 was its detection on a small subset of peripheral B cells since mammalian SLAMF4 expression has not been reported on B cells. In addition, this B cell subpopulation was also marked by high levels of surface IgM and CD40, respectively.

The expression differences of SLAMF4 in chickens and mammals may therefore indicate that chicken SLAMF4 combines functions exerted by several mammalian SLAM receptors. As discussed earlier, these findings support the notion that the higher number of mammalian SLAM genes arose by gene duplication events. Most mammalian SLAM receptors are self-ligands whereas SLAMF4 binds with SLAMF2 [37], an interaction which could also be established in chickens. Although no SLAMF2 specific mab exists, preliminary PCR analyses indicate a wide tissue distribution. Since both chicken SLAMF4 and SLAMF2 seem to be expressed on a variety of leukocytes, it is conceivable that chicken SLAMF4 frequently encounters its ligand SLAMF2.

Mammalian SLAMF4 was originally considered an activatory receptor on NK cells due to its capacity to enhance IFN-γ secretion and cytotoxicity [82-84]. In contrast, recent studies indicate that the level of surface SLAMF4 expression and the availability of intracellular SAP molecules are the critical parameters involved in either activatory or inhibitory cellular responses [39, 85]. Experiments with human $CD8^+$ T cells demonstrated that a $SLAMF4^{high}$ SAPlow phenotype is predominantly inhibitory, whereas $SLAMF4^{low}$ SAP^{high} expression levels markedly promote cytotoxicity and cytokine production [86].

In this context, the expression of chicken SLAMF4 on different γδ T cell subsets is an important finding. In contrast to mice and men, where γδ T cells only constitute 1-10% of total T cells, chickens display a high γδ T cell frequency in peripheral tissues [125].

Albeit little is known about γδ T cell function in chickens, the high abundance itself indicates an important role in immune regulation. The novel SLAMF4 specific mab allowed the differentiation of several γδ T cell subsets in blood and spleen, respectively, which also varied in the expression of CD8α. Accordingly, blood γδ T cells were differentiated into $CD8\alpha^-$ $SLAMF4^{dim}$ and $CD8\alpha^+$ SLAMF4bright cells, whereas splenic γδ T cells were defined into three subsets, namely $CD8\alpha^+$ SLAMF4bright, $CD8\alpha^-$ $SLAMF4^{dim}$ and $CD8\alpha^-$ $SLAMF4^-$ γδ T cells.

The CD8 expression has been used to define γδ T cells in terms of proliferation capacity and gene expression. Both splenic and blood $CD8\alpha^{+high}$ γδ T cells have been considered as activated due to their high expression levels of FasL, IFN-γ and lymphotactin, respectively [126]. Our results may thus hallmark a similar situation to mammals where high SLAMF4 surface levels in conjunction with low SAP content result in cellular inhibition [86].

6. SUMMARY

The chicken signaling lymphocytic activation molecule (SLAM) family

The signaling lymphocyte activation molecule (SLAM) family falls within the CD2 subset of the greater immunoglobulin superfamily and plays a crucial role in the immune regulation of leukocytes. Receptors related to this family have been best characterised in mouse and man. The first part of our study was aimed to search the chicken genome for the presence of SLAM-related genes and SLAM-associated adaptor proteins, respectively. We found three family members homologous to mammalian SLAMF1 (SLAM, CD150, IPO-3), SLAMF2 (CD48) and SLAMF4 (2B4, CD244). Two additional receptors lacked an unambiguous assignment and were therefore named SLAMF3like (Ly-9, CD229) and SLAMF5like (CD84) to stress their most likely mammalian counterparts. Several structural features are conserved across species, in particular an extracellular domain comprising an amino-terminal IgV-like domain and a membrane proximal IgC2-like domain, a single transmembrane region and a cytoplasmic domain with several tyrosine-based switch motifs. Moreover, chicken homologues to the intracellular adaptor molecules SLAM-associated adaptor protein and Ewing`s sarcoma-associated transcript-2, respectively, were found to be highly conserved in the chicken genome. The second part was aimed to study the role of the cell surface molecule SLAMF4 (CD244, 2B4) in chicken immunity. We generated a novel SLAMF4 specific monoclonal antibody (named 8C7) and analyzed its expression on distinct immune cells. The expression was restricted to both $CD8^+$ αβ and γδ T cells, whereas $CD4^+$ T helper cells and all thymocytes showed little or no reactivity when stained with the 8C7 monoclonal antibody. Blood and splenic γδ T cells could be further discriminated into several subsets according to their SLAMF4 and CD8 expression. SLAMF4 was absent on B cells isolated from bursa, spleen and caecal tonsils, whereas a small fraction of peripheral B cells coexpressed SLAMF4 which also displayed high expression levels of Bu1, Ig, and CD40, respectively. SLAMF4 was also present on NK cells isolated from intestine of adult chickens or embryonic splenocytes as marked by coexpression of the 28-4 NK cell marker. Moreover, SLAMF4 was also detected on myeloid cells such as thrombocytes and monocytes. In addition, the heterotypic interaction of SLAMF4 and SLAMF2 was proven by a reporter assay.

The novel SLAMF4 specific monoclonal antibody will be important in future experiments to distinguish several γδ T cell subsets as well as to gain new insights into chicken γδ T cell function.

7. ZUSAMMENFASSUNG

Die signalgebende Lymphozyten-aktivierende Molekülfamilie (SLAM) des Haushuhns

Die signalgebende Lymphozyten-aktivierende Molekülfamilie (SLAM) gehört als Teil der größeren Immunglobulin-Superfamilie der Unterfamilie der CD2-ähnlichen Rezeptoren an und leistet entscheidende Beiträge zur Immunregulation von Leukozyten. Die ihr zugeordneten Rezeptoren wurden am besten bei Menschen und Mäusen untersucht. Der erste Teil unserer Studie zielte darauf ab, das Hühnergenom auf Gene zu untersuchen, die der signalgebenden Lymphozyten-aktivierenden Molekülfamilie beziehungsweise den SLAM-assoziierten Adapterproteinen angehören. Drei Familienmitglieder konnten auf diese Weise gefunden werden, die große Ähnlichkeiten zu den Rezeptoren SLAMF1 (SLAM, CD150, IPO-3), SLAMF2 (CD48) und SLAMF4 (CD244, 2B4) bei Säugetieren aufwiesen. Bei zwei weiteren Rezeptoren konnte keine eindeutige Verwandtschaftsbeziehung hergestellt werden, so dass sie als SLAMF3-ähnlich (Ly-9, CD229) beziehungsweise SLAMF5-ähnlich (CD84) bezeichnet wurden, um mögliche Verwandtschaften hervorzuheben. Es konnte gezeigt werden, dass verschiedene Strukturmerkmale artübergreifend konserviert sind, insbesondere die extrazelluläre Domäne mit einer N-terminalen IgV-ähnlichen Kette und einer membran-proximalen IgC2-ähnlichen Kette, außerdem eine einzelne Transmembranregion sowie ein zytoplasmatischen Teil, der mehrere Immunrezeptor Tyrosin-basierende Wechselmotive (ITSM) enthält. Desweiteren gelang es uns, die beiden für die intrazelluläre Signalweiterleitung verantwortlichen Moleküle SLAM-assoziiertes Adapterprotein (SAP) beziehungsweise Ewing`s Sarkoma-assoziiertes Transkript 2 (EAT-2) zu identifizieren, die hoch konserviert im Hühnergenom vorliegen. Der zweite Teil verfolgte das Ziel, die Rolle, die das Zelloberflächenmolekül SLAMF4 (CD244, 2B4) im Immunsystem des Huhnes spielt, näher zu untersuchen. Hierfür wurde der spezifische monoklonale Antikörper 8C7 erzeugt und die Oberflächenexpression von SLAMF4 auf verschiedenen Immunzellen untersucht. Die Expression war auf $CD8^+$ αβ und γδ T-Zellen beschränkt, während $CD4^+$ T-Helferzellen sowie Thymozyten kaum oder nur in sehr geringem Maße von dem monoklonalen Antikörper 8C7 angefärbt wurden. Zusätzlich konnten γδ T-Zellen im Blut und der Milz anhand der Oberflächenmarker SLAMF4 und CD8 in mehrere Subpopulationen unterteilt werden. Auf B-Zellen, die aus der Bursa, der Milz sowie den Caecaltonsillen gewonnen wurden, konnte der Rezeptor nicht nachgewiesen werden. Im Gegensatz dazu exprimierte nur ein geringer Teil der zirkulierenden B-Zellen SLAMF4, wobei diese Subpopulation zusätzlich durch eine hohe

Expression der Oberflächenmoleküle Bu1, Ig sowie CD40 gekennzeichnet war. Mithilfe des Antikörpers 28-4 gelang es uns auch, SLAMF4 auf Natürlichen Killerzellen nachzuweisen, die insbesondere im Darm erwachsener Hühner sowie in Embryonalmilzen zahlreich vorkommen. Ebenso konnte SLAMF4 auf der Zelloberfläche myeloider Zellen wie etwa Thrombozyten und Monozyten nachgewiesen werden und SLAMF2 als Ligand bestätigt werden. Der neue SLAMF4 spezifische monoklonale Antikörper wird es zukünftigen Arbeiten ermöglichen, verschiedene Populationen von γδ T-Zellen zu unterscheiden und so neue Einblicke in die Funktion der γδ T-Zellen des Haushuhns zu gewinnen.

8. BIBLIOGRAPHY

1. Suto, Y., et al., *The human natural killer gene complex (NKC) is located on chromosome 12p13.1-p13.2*. Immunogenetics, 1997. **46**(2): p. 159-62.
2. Yokoyama, W.M., et al., *cDNA cloning of mouse NKR-P1 and genetic linkage with LY-49. Identification of a natural killer cell gene complex on mouse chromosome 6*. J Immunol, 1991. **147**(9): p. 3229-36.
3. Yokoyama, W.M. and B.F. Plougastel, *Immune functions encoded by the natural killer gene complex*. Nat Rev Immunol, 2003. **3**(4): p. 304-16.
4. Barrow, A.D. and J. Trowsdale, *The extended human leukocyte receptor complex: diverse ways of modulating immune responses*. Immunol Rev, 2008. **224**: p. 98-123.
5. Martin, A.M., et al., *Leukocyte Ig-like receptor complex (LRC) in mice and men.* Trends Immunol, 2002. **23**(2): p. 81-8.
6. Reth, M., *Antigen receptor tail clue*. Nature, 1989. **338**(6214): p. 383-4.
7. Wu, J., et al., *An activating immunoreceptor complex formed by NKG2D and DAP10*. Science, 1999. **285**(5428): p. 730-2.
8. Tomasello, E., et al., *Gene structure, expression pattern, and biological activity of mouse killer cell activating receptor-associated protein (KARAP)/DAP-12*. J Biol Chem, 1998. **273**(51): p. 34115-9.
9. Lanier, L.L., et al., *Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells*. Nature, 1998. **391**(6668): p. 703-7.
10. Smith, K.M., et al., *Ly-49D and Ly-49H associate with mouse DAP12 and form activating receptors*. J Immunol, 1998. **161**(1): p. 7-10.
11. Arase, N., et al., *Association with FcRgamma is essential for activation signal through NKR-P1 (CD161) in natural killer (NK) cells and NK1.1+ T cells*. J Exp Med, 1997. **186**(12): p. 1957-63.
12. Muta, T., et al., *A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling*. Nature, 1994. **368**(6466): p. 70-3.
13. Burshtyn, D.N., et al., *Recruitment of tyrosine phosphatase HCP by the killer cell inhibitor receptor.* Immunity, 1996. **4**(1): p. 77-85.

14. Ono, M., et al., *Deletion of SHIP or SHP-1 reveals two distinct pathways for inhibitory signaling*. Cell, 1997. **90**(2): p. 293-301.

15. Ravetch, J.V. and L.L. Lanier, *Immune inhibitory receptors*. Science, 2000. **290**(5489): p. 84-9.

16. Koch, J., et al., *Activating natural cytotoxicity receptors of natural killer cells in cancer and infection*. Trends Immunol, 2013. **34**(4): p. 182-91.

17. Eckert, C., et al., *Mapping of the human P84 gene to the subtelomeric region of chromosome 20p*. Somat Cell Mol Genet, 1997. **23**(4): p. 297-301.

18. Cannons, J.L., S.G. Tangye, and P.L. Schwartzberg, *SLAM family receptors and SAP adaptors in immunity*. Annu Rev Immunol, 2011. **29**: p. 665-705.

19. Colonna, M., *TREMs in the immune system and beyond*. Nat Rev Immunol, 2003. **3**(6): p. 445-53.

20. Borrego, F., *The CD300 molecules: an emerging family of regulators of the immune system*. Blood, 2013. **121**(11): p. 1951-60.

21. Chiang, H.I., et al., *Chicken CD69 and CD94/NKG2-like genes in a chromosomal region syntenic to mammalian natural killer gene complex*. Immunogenetics, 2007. **59**(7): p. 603-11.

22. Neulen, M.L. and T.W. Göbel, *Identification of a chicken CLEC-2 homologue, an activating C-type lectin expressed by thrombocytes*. Immunogenetics, 2012. **64**(5): p. 389-97.

23. Kaufman, J., et al., *The chicken B locus is a minimal essential major histocompatibility complex*. Nature, 1999. **401**(6756): p. 923-5.

24. Rogers, S.L., et al., *Characterization of the chicken C-type lectin-like receptors B-NK and B-lec suggests that the NK complex and the MHC share a common ancestral region*. J Immunol, 2005. **174**(6): p. 3475-83.

25. International Chicken Genome Sequencing, C., *Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution*. Nature, 2004. **432**(7018): p. 695-716.

26. Viertlböck, B.C., et al., *The chicken leukocyte receptor complex encodes a primordial, activating, high-affinity IgY Fc receptor*. Proc Natl Acad Sci U S A, 2007. **104**(28): p.

11718-23.

27. Laun, K., et al., *The leukocyte receptor complex in chicken is characterized by massive expansion and diversification of immunoglobulin-like Loci*. PLoS Genet, 2006. **2**(5): p. e73.

28. Viertlböck, B.C., et al., *The chicken leukocyte receptor complex: a highly diverse multigene family encoding at least six structurally distinct receptor types*. J Immunol, 2005. **175**(1): p. 385-93.

29. Dennis, G., Jr., H. Kubagawa, and M.D. Cooper, *Paired Ig-like receptor homologs in birds and mammals share a common ancestor with mammalian Fc receptors*. Proc Natl Acad Sci U S A, 2000. **97**(24): p. 13245-50.

30. Viertlböck, B.C. and T.W. Göbel, *The chicken leukocyte receptor cluster*. Vet Immunol Immunopathol, 2011. **144**(1-2): p. 1-10.

31. Viertlböck, B.C., et al., *Chicken immunoregulatory Ig-like receptor families: An overview and expression details on ggTREM-A1*. Dev Comp Immunol, 2013.

32. Viertlböck, B.C., et al., *Characterization of the chicken CD200 receptor family*. Mol Immunol, 2008. **45**(7): p. 2097-105.

33. Viertlböck, B.C., R. Schmitt, and T.W. Göbel, *The chicken immunoregulatory receptor families SIRP, TREM, and CMRF35/CD300L*. Immunogenetics, 2006. **58**(2-3): p. 180-90.

34. Schwartzberg, P.L., et al., *SLAM receptors and SAP influence lymphocyte interactions, development and function*. Nat Rev Immunol, 2009. **9**(1): p. 39-46.

35. Guselnikov, S.V., et al., *Expansion and diversification of the signaling capabilities of the CD2/SLAM family in Xenopodinae amphibians*. Immunogenetics, 2011. **63**(10): p. 679-89.

36. Veillette, A., *SLAM-family receptors: immune regulators with or without SAP-family adaptors*. Cold Spring Harb Perspect Biol, 2010. **2**(3): p. a002469.

37. Brown, M.H., et al., *2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48*. J Exp Med, 1998. **188**(11): p. 2083-90.

38. Dong, Z., et al., *The adaptor SAP controls NK cell activation by regulating the enzymes Vav-1 and SHIP-1 and by enhancing conjugates with target cells*. Immunity, 2012.

36(6): p. 974-85.

39. Zhao, F., et al., *Positive and negative signaling through SLAM receptors regulate synapse organization and thresholds of cytolysis*. Immunity, 2012. **36**(6): p. 1003-16.

40. Kageyama, R., et al., *The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T cell help to B cells and NKT cell development*. Immunity, 2012. **36**(6): p. 986-1002.

41. Veillette, A., et al., *Importance and mechanism of 'switch' function of SAP family adapters*. Immunol Rev, 2009. **232**(1): p. 229-39.

42. Wu, C., et al., *Genomic organization and characterization of mouse SAP, the gene that is altered in X-linked lymphoproliferative disease*. Immunogenetics, 2000. **51**(10): p. 805-15.

43. Purtilo, D.T., et al., *X-linked recessive progressive combined variable immunodeficiency (Duncan's disease)*. Lancet, 1975. **1**(7913): p. 935-40.

44. Sayos, J., et al., *The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM*. Nature, 1998. **395**(6701): p. 462-9.

45. Engel, P., M.J. Eck, and C. Terhorst, *The SAP and SLAM families in immune responses and X-linked lymphoproliferative disease.* Nat Rev Immunol, 2003. **3**(10): p. 813-21.

46. Isomaki, P., et al., *Increased expression of signaling lymphocytic activation molecule in patients with rheumatoid arthritis and its role in the regulation of cytokine production in rheumatoid synovium*. J Immunol, 1997. **159**(6): p. 2986-93.

47. Ferrante, P., et al., *Cytokine production and surface marker expression in acute and stable multiple sclerosis: altered IL-12 production and augmented signaling lymphocytic activation molecule (SLAM)-expressing lymphocytes in acute multiple sclerosis*. J Immunol, 1998. **160**(3): p. 1514-21.

48. Valdez, P.A., et al., *NTB-A, a new activating receptor in T cells that regulates autoimmune disease*. J Biol Chem, 2004. **279**(18): p. 18662-9.

49. Sidorenko, S.P. and E.A. Clark, *Characterization of a cell surface glycoprotein IPO-3, expressed on activated human B and T lymphocytes*. J Immunol, 1993. **151**(9): p. 4614-24.

50. Cocks, B.G., et al., *A novel receptor involved in T-cell activation*. Nature, 1995.

376(6537): p. 260-3.

51. Wang, N., et al., *The cell surface receptor SLAM controls T cell and macrophage functions*. J Exp Med, 2004. **199**(9): p. 1255-64.

52. Nanda, N., et al., *Platelet aggregation induces platelet aggregate stability via SLAM family receptor signaling*. Blood, 2005. **106**(9): p. 3028-34.

53. Bleharski, J.R., et al., *Signaling lymphocytic activation molecule is expressed on CD40 ligand-activated dendritic cells and directly augments production of inflammatory cytokines*. J Immunol, 2001. **167**(6): p. 3174-81.

54. Hamalainen, H., S. Meissner, and R. Lahesmaa, *Signaling lymphocytic activation molecule (SLAM) is differentially expressed in human Th1 and Th2 cells*. J Immunol Methods, 2000. **242**(1-2): p. 9-19.

55. Mavaddat, N., et al., *Signaling lymphocytic activation molecule (CDw150) is homophilic but self-associates with very low affinity*. J Biol Chem, 2000. **275**(36): p. 28100-9.

56. Tatsuo, H., et al., *SLAM (CDw150) is a cellular receptor for measles virus*. Nature, 2000. **406**(6798): p. 893-7.

57. Berger, S.B., et al., *SLAM is a microbial sensor that regulates bacterial phagosome functions in macrophages*. Nat Immunol, 2010. **11**(10): p. 920-7.

58. Bhat, R., et al., *Fine-tuning of immune responses by SLAM-related receptors*. J Leukoc Biol, 2006. **79**(3): p. 417-24.

59. Kiel, M.J., et al., *SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells*. Cell, 2005. **121**(7): p. 1109-21.

60. Oguro, H., L. Ding, and S.J. Morrison, *SLAM Family Markers Resolve Functionally Distinct Subpopulations of Hematopoietic Stem Cells and Multipotent Progenitors*. Cell Stem Cell, 2013. **13**(1): p. 102-16.

61. Yilmaz, O.H., M.J. Kiel, and S.J. Morrison, *SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity*. Blood, 2006. **107**(3): p. 924-30.

62. Spangrude, G.J., S. Heimfeld, and I.L. Weissman, *Purification and characterization of mouse hematopoietic stem cells*. Science, 1988. **241**(4861): p. 58-62.

63. Ikuta, K. and I.L. Weissman, *Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation*. Proc Natl Acad Sci U S A, 1992. **89**(4): p. 1502-6.

64. Forsberg, E.C., et al., *Differential expression of novel potential regulators in hematopoietic stem cells*. PLoS Genet, 2005. **1**(3): p. e28.

65. Sandrin, M.S., et al., *CD48 is a low affinity ligand for human CD2*. J Immunol, 1993. **151**(9): p. 4606-13.

66. Kaplan, A.J., et al., *Production and characterization of soluble and transmembrane murine CD2. Demonstration that CD48 is a ligand for CD2 and that CD48 adhesion is regulated by CD2*. J Immunol, 1993. **151**(8): p. 4022-32.

67. Kato, K., et al., *Identification of the T cell surface signal-transducing glycoprotein sgp-60 as CD48, a counter-receptor for mouse CD2*. Eur J Immunol, 1993. **23**(6): p. 1412-5.

68. Henniker, A.J., et al., *A novel non-lineage antigen on human leucocytes: characterization with two CD-48 monoclonal antibodies*. Dis Markers, 1990. **8**(4): p. 179-90.

69. Gonzalez-Cabrero, J., et al., *CD48-deficient mice have a pronounced defect in CD4(+) T cell activation*. Proc Natl Acad Sci U S A, 1999. **96**(3): p. 1019-23.

70. Fukushima, K., Y. Ikehara, and K. Yamashita, *Functional role played by the glycosylphosphatidylinositol anchor glycan of CD48 in interleukin-18-induced interferon-gamma production*. J Biol Chem, 2005. **280**(18): p. 18056-62.

71. Messmer, B., et al., *CD48 stimulation by 2B4 (CD244)-expressing targets activates human NK cells*. J Immunol, 2006. ***176***(8): p. 4646-50.

72. Dalton, D.K., et al., *Multiple defects of immune cell function in mice with disrupted interferon-gamma gene*s. Science, 1993. **259**(5102): p. 1739-42.

73. Isaacs, A. and J. Lindenmann, *Virus interference. I. The interferon*. Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 258-67.

74. Zoumbos, N.C., et al., *Interferon is a mediator of hematopoietic suppression in aplastic anemia in vitro and possibly in vivo*. Proc Natl Acad Sci U S A, 1985. **82**(1): p. 188-92.

75. Kawano, Y., et al., *Synergistic effect of recombinant interferon-gamma and interleukin-*

3 on the growth of immature human hematopoietic progenitors. Blood, 1991. **77**(10): p. 2118-21.

76. Boles, N.C., et al., *CD48 on hematopoietic progenitors regulates stem cells and suppresses tumor formation*. Blood, 2011. **118**(1): p. 80-7.

77. Wang, R.A., et al., *PAK1 hyperactivation is sufficient for mammary gland tumor formation*. Oncogene, 2006. **25**(20): p. 2931-6.

78. Dummler, B., et al., *Pak protein kinases and their role in cancer*. Cancer Metastasis Rev, 2009. **28**(1-2): p. 51-63.

79. Sintes, J., et al., *Characterization of mouse CD229 (Ly9), a leukocyte cell surface molecule of the CD150 (SLAM) family*. Tissue Antigens, 2007. **70**(5): p. 355-62.

80. Romero, X., et al., *CD229 (Ly9) lymphocyte cell surface receptor interacts homophilically through its N-terminal domain and relocalizes to the immunological synapse*. J Immunol, 2005. **174**(11): p. 7033-42.

81. Sintes, J., et al., *Cutting edge: Ly9 (CD229), a SLAM family receptor, negatively regulates the development of thymic innate memory-like CD8+ T and invariant NKT cells*. J Immunol, 2013. **190**(1): p. 21-6.

82. Valiante, N.M. and G. Trinchieri, *Identification of a novel signal transduction surface molecule on human cytotoxic lymphocytes*. J Exp Med, 1993. **178**(4): p. 1397-406.

83. Garni-Wagner, B.A., et al., *A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells*. J Immunol, 1993. **151(**1): p. 60-70.

84. Tangye, S.G., et al., *2B4-mediated activation of human natural killer cells*. Mol Immunol, 2000. **37**(9): p. 493-501.

85. Bloch-Queyrat, C., et al., *Regulation of natural cytotoxicity by the adaptor SAP and the Src-related kinase Fyn*. J Exp Med, 2005. **202**(1): p. 181-92.

86. Schlaphoff, V., et al., *Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells*. PLoS Pathog, 2011. **7**(5): p. e1002045.

87. Schuhmachers, G., et al., *2B4, a new member of the immunoglobulin gene superfamily, is expressed on murine dendritic epidermal T cells and plays a functional role in their killing of skin tumors*. J Invest Dermatol, 1995. **105**(4): p. 592-6.

88. Nakajima, H., et al., *Activating interactions in human NK cell recognition: the role of 2B4-CD48*. Eur J Immunol, 1999. **29**(5): p. 1676-83.

89. Munitz, A., et al., *2B4 (CD244) is expressed and functional on human eosinophils*. J Immunol, 2005. **174**(1): p. 110-8.

90. Nagy, N., et al., *SH2D1A and SLAM protein expression in human lymphocytes and derived cell lines*. Int J Cancer, 2000. **88**(3): p. 439-47.

91. Schuhmachers, G., et al., *Activation of murine epidermal gamma delta T cells through surface 2B4*. Eur J Immunol, 1995. **25**(4): p. 1117-20.

92. Martin, M., et al., *CD84 functions as a homophilic adhesion molecule and enhances IFN-gamma secretion: adhesion is mediated by Ig-like domain 1*. J Immunol, 2001. **167**(7): p. 3668-76.

93. Krause, S.W., et al., *Characterization of MAX.3 antigen, a glycoprotein expressed on mature macrophages, dendritic cells and blood platelets: identity with CD84*. Biochem J, 2000. **346 Pt 3**: p. 729-36.

94. Sayos, J., et al., *Cell surface receptors Ly-9 and CD84 recruit the X-linked lymphoproliferative disease gene product SAP*. Blood, 2001. **97**(12): p. 3867-74.

95. Tangye, S.G., et al., *CD84 is up-regulated on a major population of human memory B cells and recruits the SH2 domain containing proteins SAP and EAT-2*. Eur J Immunol, 2002. **32**(6): p. 1640-9.

96. Tangye, S.G., et al., *Functional requirements for interactions between CD84 and Src homology 2 domain-containing proteins and their contribution to human T cell activation*. J Immunol, 2003. **171**(5): p. 2485-95.

97. Bottino, C., et al., *NTB-A [correction of GNTB-A], a novel SH2D1A-associated surface molecule contributing to the inability of natural killer cells to kill Epstein-Barr virus-infected B cells in X-linked lymphoproliferative disease*. J Exp Med, 2001. **194**(3): p. 235-46.

98. Fraser, C.C., et al., *Identification and characterization of SF2000 and SF2001, two new members of the immune receptor SLAM/CD2 family*. Immunogenetics, 2002. **53**(10-11): p. 843-50.

99. Falco, M., et al., *Homophilic interaction of NTBA, a member of the CD2 molecular*

family: induction of cytotoxicity and cytokine release in human NK cells. Eur J Immunol, 2004. **34**(6): p. 1663-72.

100. Flaig, R.M., S. Stark, and C. Watzl, *Cutting edge: NTB-A activates NK cells via homophilic interaction*. J Immunol, 2004. **172**(11): p. 6524-7.

101. Bouchon, A., et al., *Activation of NK cell-mediated cytotoxicity by a SAP-independent receptor of the CD2 family*. J Immunol, 2001. **167**(10): p. 5517-21.

102. Cruz-Munoz, M.E., et al., *Influence of CRACC, a SLAM family receptor coupled to the adaptor EAT-2, on natural killer cell function*. Nat Immunol, 2009. **10**(3): p. 297-305.

103. Hsi, E.D., et al., *CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma*. Clin Cancer Res, 2008. **14**(9): p. 2775-84.

104. Kumaresan, P.R., et al., *CS1, a novel member of the CD2 family, is homophilic and regulates NK cell function*. Mol Immunol, 2002. **39**(1-2): p. 1-8.

105. Tassi, I. and M. Colonna, *The cytotoxicity receptor CRACC (CS-1) recruits EAT-2 and activates the PI3K and phospholipase Cgamma signaling pathways in human NK cells*. J Immunol, 2005. **175**(12): p. 7996-8002.

106. Lee, J.K., et al., *CS1 (CRACC, CD319) induces proliferation and autocrine cytokine expression on human B lymphocytes*. J Immunol, 2007. **179**(7): p. 4672-8.

107. Kingsbury, G.A., et al., *Cloning, expression, and function of BLAME, a novel member of the CD2 family*. J Immunol, 2001. **166**(9): p. 5675-80.

108. Wang, G., et al., *Cutting edge: Slamf8 is a negative regulator of Nox2 activity in macrophages*. J Immunol, 2012. **188**(12): p. 5829-32.

109. Zhang, W., et al., *Genetic approach to insight into the immunobiology of human dendritic cells and identification of CD84-H1, a novel CD84 homologue*. Clin Cancer Res, 2001. **7**(3 Suppl): p. 822s-829s.

110. Veillette, A., *Immune regulation by SLAM family receptors and SAP-related adaptors*. Nat Rev Immunol, 2006. **6**(1): p. 56-66.

111. Roncagalli, R., et al., *Negative regulation of natural killer cell function by EAT-2, a SAP-related adaptor*. Nat Immunol, 2005. **6**(10): p. 1002-10.

112. Sayos, J., et al., *Potential pathways for regulation of NK and T cell responses: differential X-linked lymphoproliferative syndrome gene product SAP interactions with*

SLAM and 2B4. Int Immunol, 2000. **12**(12): p. 1749-57.

113. Mikhalap, S.V., et al., *CDw150 associates with src-homology 2-containing inositol phosphatase and modulates CD95-mediated apoptosis*. J Immunol, 1999. **162**(10): p. 5719-27.

114. Morra, M., et al., *Defective B cell responses in the absence of SH2D1A*. Proc Natl Acad Sci U S A, 2005. **102**(13): p. 4819-23.

115. Nichols, K.E., et al., *Regulation of NKT cell development by SAP, the protein defective in XLP*. Nat Med, 2005. **11**(3): p. 340-5.

116. Cannons, J.L., et al., *Biochemical and genetic evidence for a SAP-PKC-theta interaction contributing to IL-4 regulation*. J Immunol, 2010. **185**(5): p. 2819-27.

117. Li, C., D. Schibli, and S.S. Li, *The XLP syndrome protein SAP interacts with SH3 proteins to regulate T cell signaling and proliferation*. Cell Signal, 2009. **21**(1): p. 111-9.

118. Gu, C., et al., *The X-linked lymphoproliferative disease gene product SAP associates with PAK-interacting exchange factor and participates in T cell activation*. Proc Natl Acad Sci U S A, 2006. **103**(39): p. 14447-52.

119. Davidson, D., et al., *Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation*. Immunity, 2004. **21**(5): p. 707-17.

120. Morra, M., et al., *Structural basis for the interaction of the free SH2 domain EAT-2 with SLAM receptors in hematopoietic cells*. EMBO J, 2001. **20**(21): p. 5840-52.

121. Tangye, S.G., et al., *Cutting edge: human 2B4, an activating NK cell receptor, recruits the protein tyrosine phosphatase SHP-2 and the adaptor signaling protein SAP*. J Immunol, 1999. **162**(12): p. 6981-5.

122. Tregaskes, C.A., et al., *Conservation of biological properties of the CD40 ligand, CD154 in a non-mammalian vertebrate*. Dev Comp Immunol, 2005. **29**(4): p. 361-74.

123. Viertlböck, B.C., et al., *Chicken immunoregulatory Ig-like receptor families: An overview and expression details on ggTREM-A1*. Dev Comp Immunol, 2013. **41**(3): p. 403-12.

124. Viertlböck, B.C., et al., *A novel activating chicken IgY FcR is related to leukocyte*

receptor complex (LRC) genes but is located on a chromosomal region distinct from the LRC and FcR gene clusters. J Immunol, 2009. **182**(3): p. 1533-40.

125. Sowder, J.T., et al., A large subpopulation of avian T cells express a homologue of the mammalian T gamma/delta receptor. J Exp Med, 1988. 167(2): p. 315-22.

126. Pieper, J., U. Methner, and A. Berndt, *Heterogeneity of avian gammadelta T cells*. Vet Immunol Immunopathol, 2008. **124**(3-4): p. 241-52.

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