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Trojan: a novel avian lymphocyte surface protein, involved in T-cell development

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Table of Contents

List of abbreviations

AP1	activator proteins 1
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma protein 2
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
CD	cluster of diversification
cDNA	complementary DNA
CLP	common lymphoid progenitor
COS	CV-1 (simian) in Origin, carrying the SV40 genetic material
CTP	circulating T cell progenitor
D	diversity region
DAG	diacylglycerol
DC	dendritic cells
DL	delta-like
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DN	double negative
DNA	deoxyribonucleic acid
DOC	sodium deoxycholate
DP	double positive
E day	embryonic day
ECL	enhanced chemiluminescence
Egr-1	early growth response-1
Elk-1	E-26-like protein 1
ELP	early lymphoid progenitors
ERK	extracellular signal-regulated protein kinase
ETP	early T lineage progenitor
Ets	erythroblastosis virus E26 oncogene homolog
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Flt3	fms-like tyrosine kinase 3
Fos	FBJ murine osteosarcoma viral oncogene homolog
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
Gads	Grb2- related adaptor downstream of Shc

GCN5	general control of amino-acid synthesis 5-like
GEF	guanine exchange factor
Grb2	Growth factor receptor-bound protein 2
GTP	guanosine triphosphate
HDAC7	histone deacetylase 7
HEB	HeLa E box-binding
HEMCAM	hemopoietic cell adhesion molecule
HLH	helix-loop-helix
HSC	hematopoietic stem cell
Id-proteins	inhibitors of DNA binding proteins
Ig	immunoglobulin
IKK	IκB kinase
IMDM	Iscove's modified DMEM
IP_3	inositol 1,4,5-trisphosphate
iSP	immature single positive
ITAM	immunoreceptor tyrosine-based activation motifs
IκB	inhibitor of NF-κB
J	joining region
JNK	C-Jun N-terminal kinase
Jun	jun oncogene
kDa	kilodalton
LAT	linker for activation of T cells
Lck	leukocyte-specific protein tyrosine kinase
LDS	lithium dodecyl sulfate
Lin	lineage cocktail containing a mix of mAb against: CD8, TCRβ, TCRγ, NK1.1, CD11b, Gr1, CD19, CD11c and Ter119
LSK	Lin^- Ska^- c-Kit^-
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MDCC-CU	Mareck's disease chicken cell line developed at Cornell University
MDV	Mareck's disease virus
MEF2D	myocyte enhancer factor 2D
MEK	MAPK/ERK kinase
MHC	major histocompatibility complex
MPP	multipotent progenitors
NCS	normal chicken serum
NF-κB	nuclear factor κB

NFAT	nuclear factor of activated T cells
NotchIC	Notch intracellular domain
NP-40	nonidet P40
NTP	nucleotides triphosphate
Nur77	nuclear receptor 77
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCAF	P300/CBP-associated factor
PCR	polymerase chain reaction
PIP_2	phosphatidyl-inositol-4,5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PLC	phoshpolipase C
PMSF	phenylmethansulfonylfluoride
POZ/BTB	Pox virus and zinc finger/bric-a-brac tramtrack broad complex
PTP	ptrotein tyrosine phosphatase
Raf	v-raf-leukemia viral oncogene
Ras	rat sarcoma viral oncogene
RasGRP	Ras guanyl nucleotide- releasing protein
RBP-J	Recombination signal binding protein for immunoglobulin - J region
REL	reticuloendotheliosis viral oncogene homolog
Rho	rho-type guanine exchange factor
RIPA	RadioImmuno Precipitation Assay
RNA	ribonucleic acid
Rsk	ribosomal s6 kinase
RT	reverse transcriptase
RT-PCR	reverse transcription polymerase chain reaction
SA-HRP	streptavidin conjugated to horseradish peroxidase
SAP1	sphingolipid activator protein 1
SAP2	sphingolipid activator protein 2
Sca	stem cell antigen 1
SCZ	sub-capsular zone
SDS	sodium dodecyl sulfate
SH2	Src homology 2
SLP76	SH2-domain containing leukocyte protein of 76 kDa
Sos	son of sevenless

SP	single positive
Src	Rous sarcoma oncogene
TAE	Tris-acetate-EDTA
TBS-T	tris buffered saline containing TWEEN-20
TCF	ternary complex factor
TCR	T cell receptor
TEC	thymic epithelial cells
Th-POK	T helper inducing POK factor
V	variable region
Vav	vav guanine nucleotide exchange factor
YS	yolk sac
ZAP-70	ζ-chain associated protein kinase of 70 kDa

I BACKGROUND

1. Introduction

Immune system is responsive for the protection of an individual from the various pathogens found in the surrounding environment. The different components of the immune system act in a regulated and orchestrated way, interacting with each other and complementing each others functions. Immune system has two major components – the innate immunity, which represents the first barrier against infection and the adaptive immunity which provides more versatile defence machanisms and to provides an increased host protection upon subsequent reinfectation with a same pathogen. T cells are an important component of the adaptive immunity, and together with the other components of the immune system function to protect the host against infection.

Thymus is the place of T cell maturation and represents an essential organ for the development and function of the immune system. Thymic stromal cells provide a highly specific microenvironment, which is of fundamental importance for T cell maturation. During their maturation in thymus, T cells migrate through the different subcompartments of the thymic microenvironment, interacting with the thymic stromal cells. Their migration ensures the proper type and timing of interactions between developing thymocytes and thymic microenvironment. These interactions are the basis for successful T cell receptor (TCR) gene rearrangement and elimination of potential self-reactive thymocytes, the processes known as positive and negative selection. Cell-cell interactions involve different receptors and their ligands, as well as adhesion molecules. Signals received through cell surface receptors are transmitted to cell interior via tightly regulated signalling pathways, which have different effects on cell behaviour and cell fate.

Understanding the mechanisms of T cell development, requires detailed knowledge on various signalling molecules involved and their mutual interactions.

Trojan

In order to characterize molecules that are involved in T cell development, monoclonal antibodies (mAb) have been raised against chicken embryonic day 13 (E13) thymocytes by Dr. Olli Vainio. By flow cytometry analyses, an antibody termed 11-7-3, showed specific staining pattern of embryonic thymocytes. cDNA library from chicken E13 bone marrow was prepared

and cloned into pcDNA3 expression vector by Drs. Olli Vainio and Beat A. Imhof. The library was transfected into COS cells by Jussi Salmi, M.D. and the subsequent cell surface antigen expression was screened with 11-7-3 monoclonal antibody. A positive cell was isolated, its plasmid was extracted and 11-7-3 was confirmed to react with the protein encoded in the isolated cDNA. In further analysis, another three mAb: 11-5, 11-10-4 and 11-35 were found to recognise the same antigen. The cDNA coding for this molecule was sequenced and the novel protein that it encodes was named as Trojan.

2. Literature Review

2.1 T cell development during embryogenesis

T cells are of fundamental significance for immune system, being a vital component of the adaptive immunity. Components of the hematopoietic and lymphoid systems like erythrocytes or T cells, develop from hematopoietic stem cells. However, their development during embryogenesis is more complicated than in adult life and involves the sequential and overlapping shift of several major hematopoietic sites. Embryonic T cell development involves the organised migration of progenitor cells from their sites of origin to the embryonic thymus. The overall development of the hematopoietic system during embryogenesis is very similar between mammals and aves, despite of the absence of fetal liver hematopoiesis in birds (Jaffredo *et al.* 2003). Chicken embryos are often more appropriate to study the early events of hematopoietic development due to their relatively big size and easy access. In chicken, embryonic development last for about 21 days. Primitive hematopoiesis develops outside of the embryo in the yolk sac (YS) of one day old embryos and temporarily gives rise to primitive erythrocytes, megacaryocytes and macrophages. Later, definitive hematiopoiesis begins to develop between embryonic day 3 (E3) and embryonic day 4 (E4) in the aortic region, by hematopoietic stem cells (HSC) protruding into the lumen of the aorta to form intraaortic clusters with hematopoietic characters. Between E6 and E8, hematopoiesis shifts outside of the aorta to the paraaortic foci and at E10, hematopoiesis shifts to the bone marrow. Embryonic thymus is colonised by thymocyte precursors in three sequental waves. Each wave lasts for about two days and the periods between them are restricted for colonisation and characterised with the production of thymocyte progeny. The first wave takes place between E6 and E8 and the cells participating in it originate from the paraaortic foci (Dunon D *et al.* 1998) . The second wave occurs between E12 and E14, while the third wave starts at E18 and prolongs till after hatching. The cells of the second and third waves originate from the bone marrow and colonise embryonic thymus via the blood stream (Dunon D *et al.* 1999). However, not much is known about the internal clock that directs thymus colonisation. Cell surface phenotype of chicken embryonic T cell precursors include the expression of c-Kit, HEMCAM, BEN, alphaIIbeta3 integrin, ChT1, MHC class II, CD44 and thrombomucin. Some of these molecules are involved in adhesion and/or signal transduction (Ody *et al.* 2000).

2.2 Phenotype of the early T cell progenitors

Directing multipotent progenitor cells towards a certain lineage fate, requires the delivery of specific signals by the present microenvironment. Further differentiation may require the micorenvironment of another locale, specialised for the development of that particular cell type (Bhandoola *et al.* 2007). Commitment of bone marrow hematopoietic stem cells (HSC) towards the T cell lineage represents a complex process, characterised by phenotypically distinct developmental stages of different lineage potentials. Progenitor cells destined for the T lineage, migrate to thymus which provides the unique microenvironment for their progressive differentiation, selection and maturation into T cells. The identification and characterisation of the progenitors migrating from bone marrow to thymus has proven to be a difficult task (Shortman & Wu 1996, Bhandoola *et al.* 2003). The expression of various cell surface antigens is used to classify and distinguish between the different developing cell populations in bone marrow, blood and thymus.

In bone marrow, HSC are found within the highly heterogeneous population of LSK (Lin^-, $Sca\text{-}1^+$, Kit^{hi}) cells. The self-renewing and multipotent HSC (Lin^-, $c\text{-}Kit^{hi}$, $Sca\text{-}1^{hi}$, $Flt3^-$) are believed to differentiate into multipotent progenitors (MPP), which represent another subpopulation of LSK, but are unable of self-renewal (Adolfsson *et al.* 2001, Christensen & Weissman 2001). Based on the levels of expression of the cytokine receptor Flt3, MPP (Lin^-, $c\text{-}Kit^{hi}$, $Sca\text{-}1^{hi}$, $Flt3^+$) can be subdivided into MPP-$Flt3^{lo/+}$ cells, that have erythroid potential and MPP-$Flt3^{hi}$ cells having reduced potential for megacaryocytes and erythrocytes. A subset of these MPP-$Flt3^{hi}$ initiates the transcription of the genes encoding Rag (recombination-activating genes) and is referred to as early lymphoid progenitors (ELP) (Igarashi *et al.* 2002). These ELP (Lin^-, $c\text{-}Kit^{hi}$, $Sca\text{-}1^{hi}$, $Rag1^+$), are believed to further give rise to lymphoid-restricted, non-self renewing common lymphoid progenitor (CLP) and CLP-2, capable to give rise to B and T cells. Progenitors of T lineage potential that are known to circulate in bloodstream are the bone marrow derived MPPs and ELP (Schwarz & Bhandoola 2004; Perry *et al.* 2006), and the recently identified circulating T cell progenitor (CTP) (Carlyle & Zuniga-Pflucker 1998, Krueger & von Boehmer 2007), which origin is unclear. T cell development in thymus requires the periodic or continuous input of progenitor cells. A tiny, intrathymic cell population termed “early T-lineage progenitor” (ETP) has been identified, comprising ~0.01% of all thymocytes. ETP (Lin^-, $c\text{-}Kit^{hi}$, $Sca\text{-}1^{hi}$, $CD25^-$, $CD44^{hi}$) have the potential to become T cells, less potential to become B cells, compared to CLP (Wang *et al.* 1996) and are believed to represent the earliest T cell progenitors inside the thymus (Allman *et al.* 2003).

2.3 Phenotypes of maturing thymocytes and their migration through the thymus

Thymic microenvironment is built up by dendritic cells (DC) and thymic epithelial cells (TEC), which are the major components of thymic stroma. The stroma itself can be subdivided into outer part (cortex) and inner part (medulla), composed of cortical thymic epithelial cells (cTEC) and medullary thymic epithelial cells (mTEC), respectively. Maturation of thymocytes involves their polarised migration through the thymic stroma, mediated by chemokines and adhesion molecules. During their maturation in adult murine thymus, thymocytes go through a series of stages, each phenotypically characterised by a specific expression pattern of cell surface antigens (Figure 1). Based on the expression of the CD4 and CD8 co-receptors, maturing thymocytes can be divided into four major sub-populations (Ceredig *et al.* 1983): ~5% express neither CD4 nor CD8 (termed double negative, or DN cells), ~80% express both CD4 and CD8 (termed double positive, or DP cells), ~10% express only CD4 (termed CD4 single-positive, or CD4 SP cells) and ~5% express only CD8 (termed CD8 single-positive, or CD8 SP cells). Based on the expression of CD44 and CD25, DN cells can be further divided into four, consecutive, sub-populations (Pear *et al.* 2004): DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^+$), DN3 ($CD44^-$ $CD25^+$) and DN4 ($CD44^-$ $CD25^-$).

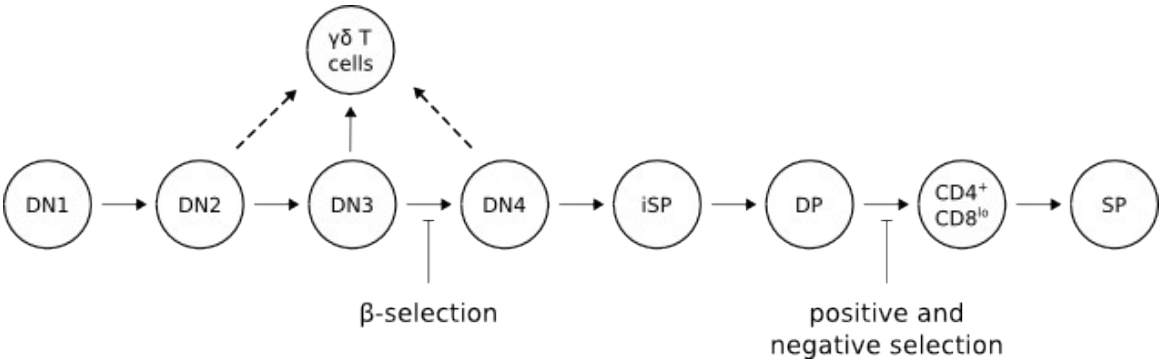


Figure 1. Stages of T cell maturation.

T cell precursors enter the thymus at the corticomedullary junction from the blood vessels and differentiation to the DN1 stage occurs close to the site of entrance. DN1 cells represent a highly heterogeneous population, comprising the ETP and CD4lo cells, where the latter passively acquire a transient surface expression of CD4 (Wu et al. 1991). DN1 cells start their migration through thymic stroma and become DN2 cells till the time they reach the mid-

cortex. There, DN2 cells begin to rearrange the V(D)J gene fragments of their TCR γ, δ and β loci. The rearrangement, together with the addition or removal of nucleotides at the V(D)J joining sites is in the basis of αβ and γδ T cell repertoire generation. Only one out of three attempts in the rearrangement process results in a functional product. It is presumed that between DN2 and DN4 stages γδ-lineage T cells diverge from the αβ-lineage T cells (Petrie et al. 1992) and in the case of γδ TCR, these "failed" thymocytes are still able to shift towards the αβ T cell lineage (Dudley et al. 1995). DN3 cells accumulate at the subcapsular zone (SCZ) and those destined for the αβ T cell lineage undergo a check point termed β-selection, for the successful rearrangement of their β-chain genes. Only those DN3 cells that express a productively rearranged β-chain paired with the invariable pre-Tα chain to form a pre-TCR (Capone et al. 1998), are allowed to pass the check point, otherwise they die by apoptosis. Proper signalling through the pre-TCR inhibits further TCR-β rearrangements, initiates the rearrangement and expression of TCR-α chain and allows cells to survive, proliferate and differentiate towards the next DN4 step. Still at the SCZ, DN4 cells acquire small amounts of CD8 and become immature single positive cells (iSP) whose importance is unclear. Rapidly after the iSP stage, cells upregulate the expression of CD4 to become DP cells (Hayday & Pennington 2007). DP cells complete the rearrangement of TCR-α and express the αβTCR on their surface. They reverse the migration polarity and head towards the medulla, undergoing positive and negative selections as they follow their way. Positive selection tests the ability of TCR to recognise self MHC molecules and takes place in the cortex, driven by the cTECs (Bevan 1997). Cells that have generated TCR incapable to recognise the self MHC molecules, fail to undergo positive selection and are deprived of survival signals leading to their death by neglect. Cells that are positively selected reach the corticomedullary junction entering the medulla, where they are subjected to negative selection, mediated by mTEC and DC. Cells that recognise self MHC molecules with too high affinity are considered potentially autoreactive. Majority of DP cells are negatively selected and receive a signal to undergo apoptosis. Hence, only the small fraction of cells that recognise self MHC molecules with moderate affinity pass both positive and negative selection and are allowed to continue their development (Bommhardt et al. 2004). These thymocytes that survive, go through the intermediate CD4+CD8lo stage and eventually downregulate either CD4 or CD8 to become SP cells which are later exported from thymus to the periphery.

2.4 Coupling TCR signals to transcription factors in the nucleus

The receptor that plays a major role in thymocyte maturation is the TCR together with the associated CD3 complex and the coreceptors CD4 and CD8. The strength of the signal transmitted from the TCR is presumed to direct thymocytes towards the proper lineage choice and to lead them through the required checkpoints. TCR is a rare example of a single receptor, capable to transmit differentially altered signals, which direct the cell towards different and often opposing fates. The intracellular signalling pathways, that underlay TCR (Figure 2), represent a complex and not completely understood network of interacting kinases, phosphatases, adaptor molecules and other components (Mustelin & Tasken 2003). Their function is regulated by different level of phosphorylation of adaptor molecules, divergent temporal execution of the required signalling cascades and varied subcellular localisation of key interacting molecules. As a result, signals originating from the TCR are taken up and transmitted by this signalling network in a tightly regulated fashion, eventually arriving in the nucleus where they mediate gene transcription.

The early, membrane-proximal events of TCR signal transmission, involve the activation of the Src family tyrosine kinase Lck, which induces the phosphorylation of the CD3 complex on its ITAMs (immunoreceptor tyrosine-based activation motifs). Phosphorylated ITAMs serve as docking sites for the tandem SH2 (Src homology 2) domains of the Syc family tyrosine kinase ZAP-70 (ζ-chain associated protein kinase of 70 kDa) recruiting it in close proximity to the TCR and the activated Lck. Lck then phosphorylates and activates the recruited ZAP-70 (Chan *et al.* 1995, Wange et al 1995), which in turn phosphorylates the adaptor protein LAT (linker for activation of T cells) on its numerous ITAMs (Zhang *et al.* 1998, Zhang *et al.* 1999). The strength of the signal originating from the TCR is believed to induce correspondingly different kinetics of the downstream signalling cascades reflecting in the levels of LAT ITAM phosphorylation. The varying degrees of LAT phosphorylation represent an important junction point for the further signalling cascades. LAT phosphorylated ITAMs induce the specific recruitment of additional kinases and adaptor proteins, which result in the branching of the downstream signals.

An important downstream cascade that is believed to play a critical role in thymocyte development as well as in activation of mature T cells, is the ERK (extracellular signal-regulated protein kinase) activation pathway. The recruitment of the adaptor protein Grb2 (Asada *et al.* 1999) to LAT leads to the activation of Sos (Son of sevenless), a guanine nucleotide exchange factor (GEF). Sos activates the small GTP-binding protein Ras (rat

sarcoma viral oncogene) which results in the subsequent recruitment and activation of Raf, a serine/threonine kinase. Once active, Raf phospho-activates the dual specificity tyrosine/threonine kinase MEK (MAPK/ERK kinase), that in turn phosphorylates and activates ERK. ERK proteins (ERK-1 and ERK-2) are serine/threonine kinases having cytosolic and nuclear substrates. Phosphorylated ERK can physically translocate to the nucleus, where it induces the activation of various transcription factors.

The recruitment of another adaptor protein Gads to LAT is followed by the recruitment of the adaptor protein SLP76 (SH2-domain containing leukocyte protein of 76 kDa) to Gads (Liu *et al.* 1999) and the subsequent phosphorylation of SLP76 by ZAP-70 (Wardenburg *et al.* 1996). Phosphorylated SLP76 induces cytoskeletal rearrangements through the activation of VaV, a guanine nucleotide exchange factor for the Rho family of small G-proteins. Another phosphorylation target of ZAP-70 is PLCγ (phospholipase Cγ), which associates to LAT through its SH2 domain. Activated PLCγ induces the primary, transient release of Ca^{++} from intracellular stores like endoplasmic reticulum (ER) through the PIP_2 - DAG - IP_3 pathway. PLCγ hydrolyses PIP_2 (phosphatidyl-inositol-4,5 bisphosphate), generating IP_3 (inositol 1,4,5-trisphosphate) and DAG (diacylglycerol). IP_3 is responsible for the opening of Ca^{++} channels in the ER leading to increased Ca^{++} concentration in the cytosol (Jordan *et al.* 2993). The increased cytoplasmic Ca^{++} concentration leads to calmodulin activation and its binding to calcineurin. Activated calcineurin docks to the transcription factor NFAT (nuclear factor of activated T cells) and activates it by dephosphorylation, which exposes its nuclear localisation signal. NFAT subsequently translocates to the nucleus, where it interacts with AP1 (activator proteins 1) and other transcriptional partners to promote gene transcription (Macian 2005). Ca^{++} concentration also induce the relocalisation of the Ras guanine exchange factor, RasGRP to intracellular membranes, e.g, Golgi, which in turn activates the Ras signalling cascade in a Ca^{++} mediated manner (Mor A & Philips 2006). The increased Ca^{++} concentration also triggers a poorly characterised mechanism that leads to the additional opening of plasma membrane Ca^{++} channels. This leads to a secondary, sustained elevation of intracellular Ca^{++} concentration. During the secondary increase of Ca^{++} concentration, Ca^{++} and DAG induce the anchoring of PKC (protein kinase C) to the plasma membrane and its activation. Activated PKC activates IKK (IκB kinase), which in turn triggers the degradation of IκB (inhibitor of NF-κB) from the NF-κB:IκB complex. The degradation of IκB frees the transcription factor NF-κB (nuclear factor κB) to enter the nucleus and initiate gene transcription (Siebenlist *et al.* 2005).

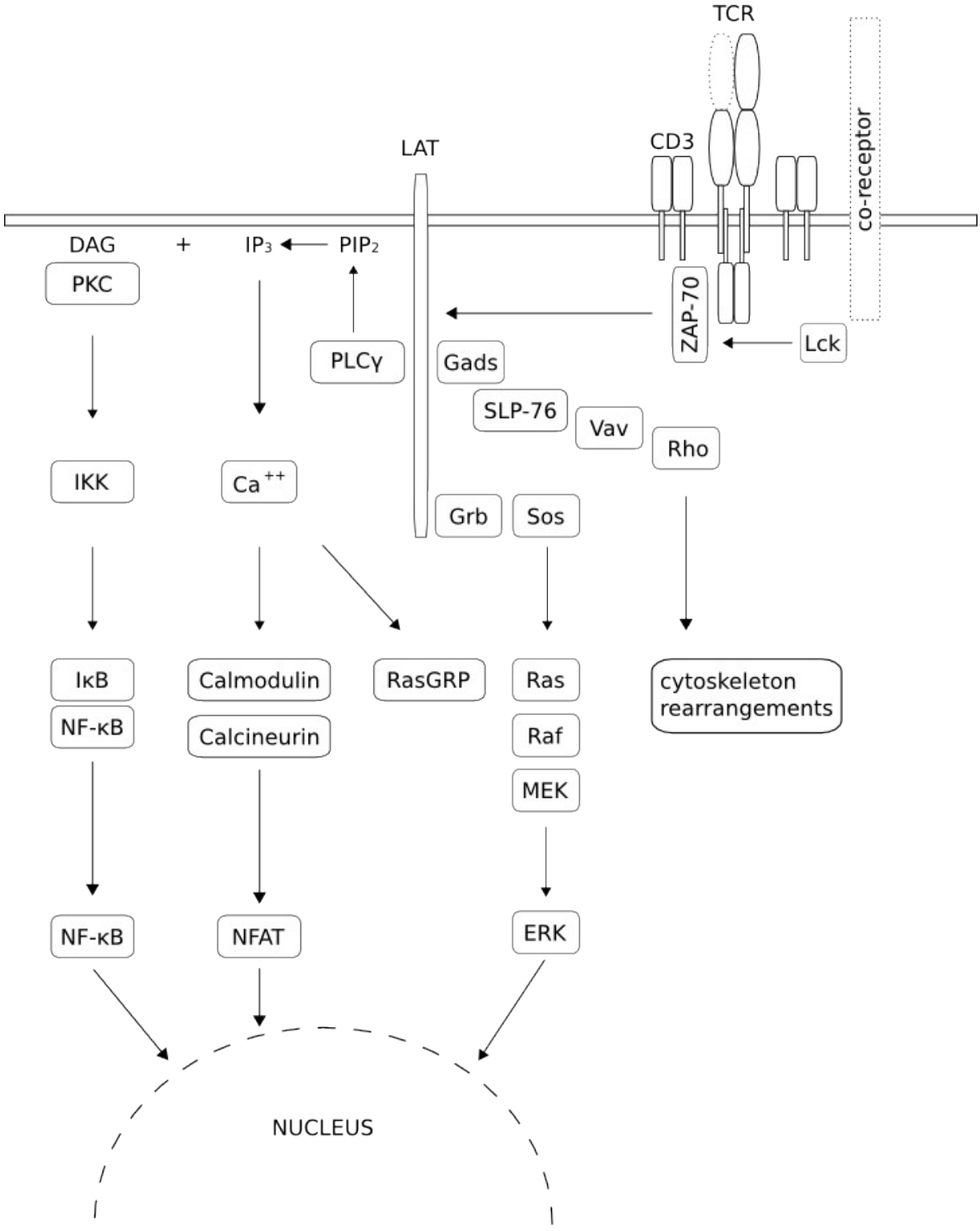


Figure 2. Intracellular pathways coupling TCR (pre-TCR, γδ-TCR or αβ-TCR) to the nucleus. *In the case of pre-TCR, pT-α chain lacks the domain shown in a dashed line. CD4 or CD8 are collectively represented as "co-receptor" and since their expression is not present at all developmental steps, they are denoted by a dashed line.*

The signals transmitted to the nucleus by the intracellular signalling network ultimately induce gene transcription (Figure 3). Transcription initiates the upregulation of required genes, some of them coding for additional transcription factors and/or transcription factor regulators. The outcome of these events eventually directs the cell towards the appropriate fate during maturation. E-proteins are transcription factors, that play a crucial role in T cell development. They mediate the transcription of genes with E-box regulatory sequences. E-box sequences are found in the enhancer elements of the TCR-β, pre-Tα, TCR-α, CD4 and CD8 genes. Activity of E-proteins, in turn, is mediated by Ets, Egr and Id proteins in response to the appropriate cytoplasmic signals arriving in the nucleus.

The Ets proteins represent a large family of transcription factors that share a common helix-loop-helix DNA-binding domain and regulate gene expression and cellular differentiation in a large number of species (Sharrocks 2001). Elk-1, SAP1 and SAP2 belong to the TCF subfamily of Ets transcription factors and represent direct phosphorylation targets of ERK. Upon ERK-mediated activation, Elk-1 increases its activity (Gille *et al.* 1995) and contributes to the transcriptional regulation of Egr-1 transcription factor.

The Egr proteins comprise a family of transcription factors expressed in diverse cell types, regulating different genetic programs of cell growth and proliferation. The family has four members: Egr1, Egr2, Egr3 and Egr4, from which only Egr4 is not expressed in T -lineage cells. Egr1 is activated by ERK in an Elk-1 dependent manner, while Egr2 and Egr3 are activated by NFAT (Rengarajan *et al.* 2000, Basson *et al.* 2000, Shao *et al.* 1999). Egr1 has been shown to activate the transcription of Id3 helix-loop-helix proteins (Bain *et al.* 2001).

The Id proteins represent a family of four transcription factor-regulators: Id1, Id2, Id3 and Id4 (Massari & Murre 2000), that possess HLH but lack DNA-binding basic domain. Hence, they are able to form heterodimers with HLH transcription factors and inactivate their function. Id proteins have been shown to interact and block the activity of E-proteins (Norton *et al.* 1998).

The E-proteins represent a yet another family of transcription factors. They are widely expressed, basic helix-loop-helix (bHLP) proteins that bind specifically to DNA E-box consensus sequences (Lazorchak *et al.* 2005; Sun 2004), as mentioned earlier. The family consists of four members, encoded in three genes: HEB, E2-2, E12 and E47, where the latter two are encoded in the same gene (E2A) and are produced by differential splicing. All four members of the E-protein family are expressed in developing thymocytes, where E47 and HEB form heterodimers (Murre 2005).

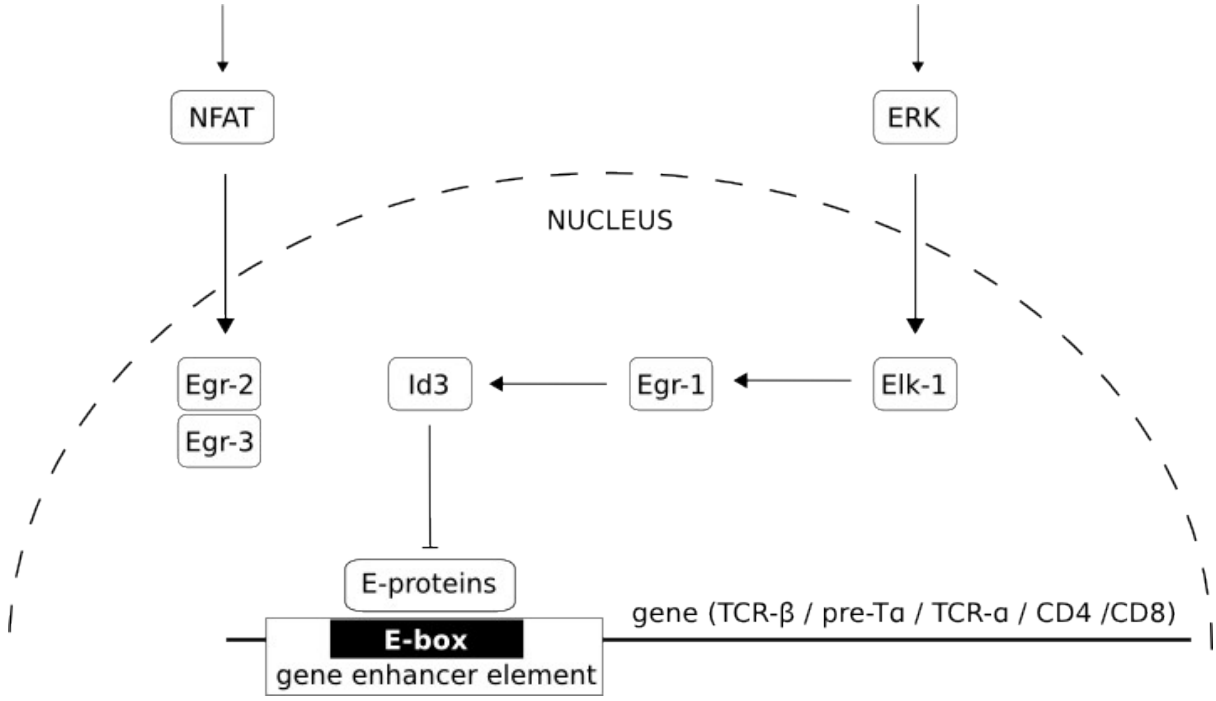


Figure 3. Regulation of E-proteins activity by cytoplasmic signalling.

Among the transcription factors of importance for thymocyte development are the structurally and evolutionary related NFAT and NF-κB. NFAT proteins represent a family of transcription factors expressed in various tissues (Crabtree & Olson 2002) playing an important role in immune system development. NFAT proteins are involved in the control of thymocyte development, differentiation, establishment of self tolerance and T cell activation (Macian 2005). The family consists of five members: NFAT1, NFAT2, NFAT3, NFAT4 and NFAT5, from which only NFAT3 is not expressed in the immune system and NFAT5 is not regulated by calcium signalling. The DNA-binding domain of NFAT proteins is structurally related to that of NF-κB proteins (also known as REL-family transcription factors) implying a similar DNA-binding specificity. NFAT proteins interact with different transcription-factor partners in the nucleus resulting in the integration of calcium signalling with other signalling patways. The main transcriptional partners of NFAT proteins are AP1, representing dimers of Fos and Jun. AP1 expression is induced by the RAS-MAPK pathway. Calcium-calcineurin signalling is involved in the regulation of thymocyte proliferation and the development of immature DN thymocytes into mature SP cells. In addition signalling through the pre-TCR leads to an increase of intracellular calcium concentration resulting in the activation of both NFAT and NF-κB (Aifantis *et al.* 2001). However, the exact gene targets of NFAT and NF-κB in immature thymocytes have not been identified.

NF-κB proteins comprise a family of transcription factors playing essential roles in both innate and adaptive immunity. The family consists of five members: NF-κB1, NF-κB2, REL, REL-A and REL-B. These proteins form homo- and heterodimers to generate functional NF-κB complex. In the absence of signalling stimulus, most of the NF-κB dimers are kept inactive by association with one of the following inhibitors: IκBα, IκBβ or IκBε. Upon activation signal, the inhibotors are targeted for degradation by IKK and the released NF-κB is allowed to enter nucleus and exert its function (Siebenlist *et al.* 2005). NF-κB it is believed to play a role in thymocyte development by promoting mainly, but not exclusively, cell survival. NF-κB has particularly high expression at late DN3 and DN4, suggesting an involvement in β-selection transition.

2.5 Role of Notch in T cell lineage commitment

Thymus provides the highly specialised microenvironment for the commitment of early thymocyte progenitors into the T cell lineage and development of other lineages practically does not stand a chance. Thymic stromal cells ensure the various cytokines, adhesion molecules and receptor ligands required for thymocyte development. Among the different receptor ligands, stromal cells express the ligands for the Notch-family of receptors, expressed on developing thymocytes. Notch signalling is believed to play an important role in the direction of thymocyte progenitors towards the T cell lineage by being able to directly determine cell fate or make cells competent to respond to other factors.

Notch proteins represent a highly conserved family of transmembrane receptors, which participate in the regulation of various processes including decisions of cell fate, survival and proliferation (Kimble & Simpson 1997). The mammalian Notch family has four members – Notch1, Notch2, Notch3 and Notch4 which are highly expressed in different tissues, including hematopoietic (Kojika & Griffin 2001). Notch receptors can interact with at least five ligands – two Serrate-like (Jagged1, Jagged2), and three Delta-like (DL1, DL3, DL4). Upon ligand-binding, Notch receptors undergo a proteolytic cleavage by γ-secretase, releasing the Notch intracellular domain (NotchIC) to the cytoplasm (Schroeter *et al.* 1998, Struhl & Adachi 1998). NotchIC translocates to the nucleus, where it forms a heterodimer with the DNA-binding protein RBP-J (Kato *et al.* 1996) and the coactivators PCAF and GCN5 (Kurooka & Honjo 2000). This triggers the transcription of Notch target genes of the HES family of basic helix-loop-helix proteins (Jarriault *et al.* 1995). Notch proteins have also been proposed to interact with NF-κB as nuclear binding partners or competing for the same DNA-binding sites,

thus regulating the expression of gene targets. Additionally it has been shown that Notch1 can transactivate the gene encoding for NF-κB2 (Oswald *et al.* 1998) and to regulate the basal levels of IκB (Oakley *et al.* 2003). In developing thymocytes, Notch cooperates with E2A to express other target genes. It is suggested that Notch signalling is involved in the expression of the pT-α chain and in the rearrangement of the TCR-γ, TCR-δ and TCR-β genes at the DN2-DN3 transition.

2.6 γδ vs αβ TCR lineage choice and β-selection

Thymocytes at the DN3 stage that have rearranged their TCR genes express either γδ TCR or pre-TCR and can differentiate into γδ or αβ T cell lineages. The mechanism of lineage decision is presumably based on TCR signal strength and irrespective of the receptor from which the signalling originates (Hayes *et al.* 2003). In the case of productive rearrangement of the TCR loci, γδ-TCRs would transmit stronger cytoplasmic signals than pre-TCRs. The strength difference is due to the lower surface expression levels of pre-TCR and its ligand-independent signalling versus the higher levels of γδ-TCR expression and ligand-induced signalling (Lauritsen *et al.* 2006). Thymocytes that non-productively rearranged their γδ-TCR genes, still have the chance to shift towards the αβ-lineage. Hence, signalling from a pre-TCR is always associated with αβ lineage choice, while γδ-TCR signalling is compatible with either γδ or αβ T cell lineage choice, depending on the signal intensity (Garbe & von Boehmer 2007). Strong signals from a γδ-TCR would induce proportionally strong activation of the ERK - Ets – Egr – Id3 pathway resulting in stronger suppression of E-proteins (Bain *et al.* 2001). Weak signals from a pre-TCR or from a unsuccessfully rearranged γδ TCR would result in a weaker activation of ERK – Ets – Egr – Id pathway and weaker suppression of E-proteins. Since E-proteins are required for the β-chain gene rearrangements and pre-T-α expression, it is presumed that the magnitude of E-protein suppression directs the cell towards γδ or αβ T cell lineage. Strong signals would nearly extinguish E-protein activity, halt the β-chain and pre-T-α expression, and direct the cell towards the γδ T cell lineage. Weaker signals would result in more modest reduction of E-protein activity, which is permissive for αβ lineage development (Engel & Murre 2001). Curiously, the products of the E2A gene have opposing effects during early thymocyte development. At the DN2-DN3 transition, or the stage of bifurcation of γδ from αβ T cell lineages, E2A is required for the promotion of TCR-β gene rearrangement. On the other hand, at late DN3 when β selection takes place, E2A mediates the inhibition of further thymocyte differentiation in the absence of pre-TCR signalling (Michie & Zuniga-

Pflucker 2002).

In addition to the TCR "strength model", a role of Notch in γδ vs αβ T cell lineage choice has been suggested. According to this "TCR-Notch synergy model", signalling from a pre-TCR in synergy with a moderate Notch signal leads to αβ lineage choice. Strong signalling through the γδ-TCR results in γδ T cell lineage choice and is Notch independent. Weak signalling through the γδ TCR, but coupled to stronger Notch signalling directs the cell towards the αβ T lineage (Garbe & von Boehmer 2007).

Thymocytes that have been directed towards the αβ T cell lineage, undergo a checkpoint between stages DN3-DN4 for the productive rearrangement of their β-chain, expressed as pre-TCR. If its rearrangement was productive, the expressed pre-TCR is capable of transmitting signals and the downstream cascades activate the ERK – Ets – Egr – Id3 pathway resulting in the suppression of E-proteins, including products of the E2A. At β-selection the absence of pre-TCR signalling does not lead to upregulation of Id proteins and in the absence of inhition, E2A gene products would arrest further thymocyte differentiation (Michie & Zuniga-Pflucker 2002). Hence, cells that have not expressed a productively rearranged pre-TCR do not pass the β-selection checkpoint and die by apoptosis. The transcription factors NFAT and NF-κB also have a role in β-selection, providing additional survival signal for thymocytes that pass successfully the check point (Aifantis *et al.* 2001, Voll *et al.* 2000).

2.7 Positive and negative selections

The second important checkpoint in thymocyte development occurs at the DP stage, when thymocytes have already expressed an αβ TCR. Positive and negative selections are mediated by thymic stromal cells, expressing MHC molecules with self peptides. The affinity of MHC/peptide binding to the TCR expressed by DP thymocytes is proposed to result in proportionally different kinetics of the downstream signalling cascades. The signal kinetics are believed to induce positive or negative selection through the correspondingly different activation levels of key signalling molecules, including ERK and downstream transcription factors. Thymocytes expressing TCRs, unable to recognise self MHC/ligand molecules would not transmit signals to the cytoplasm, resulting in their death by neglect, because no survival signals would be passed to the cells. TCRs that bind MHC/ligands with moderate affinity would induce slow and sustained activation of ERK and lead to positive selection of the cell. Thymocytes, expressing TCRs that bind MHC/ligands with very high affinity, would receive strong and transient activation of ERK resulting in their negative selection by apoptosis

(Werlen *et al.* 2000, Mariathasan *et al.* 2001). It is believed that the slow and sustained activation of ERK leading to positive selection is induced through the PLCγ – IP_3 – Ca^{++} -- RasGRP – Ras pathway. In the case of a high affinity negatively selecting signal, ERK activation is additionally augmented through the Grb2 – Sos – Ras pathway (Werlen *et al.* 2003). The transient activity of ERK in response to a high affinity signal is possibly achieved by ERK induced phosphorylation of a negatively regulatory site of Lck. The timing of ERK activation in respect with the activation of other signalling molecules of the MAPK family like JNK (Jun terminal kinase) is also thought to be involved. Moderate signalling through TCR leads to ERK activation after the activation of JNK, while strong signalling activates ERK before JNK. Additionally, the subcellular localization of Ras and ERK as a result of different lipid modifications is believed to play a role in the processes of positive and negative selection (Mor & Philips 2006). The genetic mechanisms that mediate positive and negative selection are poorly understood. The negative regulator of E-protein activity, Id3 is important for positive selection, since its inactivation leads to a block of positive selection. However, the targets of the E proteins during positive selection are unknown (Alberola-Ila & Hernandez-Hoyos 2003). It is believed that sustained activation of ERK would lead to activation of Rsk, an ERK effector kinase. Rsk would phosphorylate the pro-apoptotic protein BAD and diminish its activity, hence favouring thymocyte survival (Bonni *et al.* 1999; Mok *et al.* 1999). On the other hand the strong, but transient activation of ERK induced by a negative selecting signal might not allow a sufficiently long phosphorylation of BAD to promote cell survival. The precise mechanism leading to apoptosis during negative selection is unclear, but is believed to involve activation of protein kinase D (PKD) probably by the strongly activated ERK. Active PKD induces the phosphorylation followed by nuclear exclusion of the histone deacetylase 7 (HDAC7). If not phosphorylated by PKD, HDAC7 has a suppressive role on the transcription factor myocyte enhancer factor 2D (MEF2D). Nuclear exclusion of HDAC7 activates MEF2D to initiate transcription of a nuclear receptor – Nur77. Nur77 is believed to have pro-apoptotic function by transcriptional modifications of other genes and by interacting with Bcl-2. Binding of Nur77 to Bcl-2 is believed to suppress its anti-apoptotic functions. Similar process of HDAC7 exclusion may also activate the transcription of Bim through derepression of the E2F1 transcription factor (Siggs *et al.* 2006). Bim, together with Bax and Bak act to disrupt mitochnodrial stability and induce apoptosis. Additionally, NF-κB is suggested to be involved in the transition between DP and SP stages, having anti-apoptotic role in positive selection (Mora A *et al.* 2001) and pro-apoptotic role in negative selection (Kim *et al.* 1999, Kim *et al.* 2002).

2.8 CD4 vs CD8 TCR lineage choice

After successfully passing through positive and negative selection, developing thymocytes have to take another decision – the CD4 versus CD8 T cell lineage choice. It is proposed that, again, quantitative differences of the signals transmitted through the TCR are responsible for the lineage decision. Two models exist – the classical signal strength and the kinetic signalling model, which are not mutually exclusive and in fact could complement each other (He & Kappes 2006). In both models stronger signalling from the TCR induces CD4 lineage choice while the weaker induces CD8 lineage choice. The models differ in the molecular basis for the stronger signalling from MHC class II-restricted TCR and the developmental stage at which the lineage choice occurs. The classical model implies that there are inherently higher amounts of Lck associated with CD4 than CD8, leading to stronger signals transmitted from MHC class II-restricted TCR (Matechak *et al.* 1996). In this model, lineage choice could occur as early as the DP stage. The kinetic model suggests that a developmentally programmed downmodulation of CD8 expression at the $CD4^{+}CD8^{lo}$ stage results in impaired signalling from MHC class I-restricted TCR (Brugnera *et al.* 2000). Hence, in this model lineage choice occurs not before the $CD4^{+}CD8^{lo}$ stage.

The intracellular signalling events that mediate CD4 vs CD8 lineage choice are believed to involve the activation of the Ras – Mek – ERK signalling patway. However, the detailed signalling mechanism and its coupling to the required transcription factors remain unclear. A transcriptional regulator, believed to play an important role for lineage commitment is Th-POK (T helper inducing POK factor) (He & Kappes 2006). Th-POK belongs to a family of POK transcription factors, characterised by a regulatory POZ/BTB domain that mediates interactions with other transcription factors and a Zn-finger DNA-binding domain. Th-POK is believed to induce transcriptional repression by recruitment of histone deacetylase and corepressors (Melnick *et al.* 2002). A candidate for Th-POK mediated repression is the transcription factor Runx3. Runx3 itself is believed to negatively regulate CD4 expression and positively regulate CD8 expression for example by upregulating CD8 gene during the CD4+CD8lo to SP CD8 transition (Taniuchi *et al.* 2002, Woolf *et al.* 2003, Sato *et al.* 2005). The transcription factor Gata-3 has also been demonstrated to promote CD4 development indirectly (Hernandez-Hoyos *et al.* 2003), by blocking the development of MHC class I-restricted thymocytes. After taking the lineage choice, SP cells proliferate briefly in thymus, after which they emigrate to the periphery as mature, but naïve αβ T cells.

II EXPERIMENTAL PART

3. Objectives of the study

Despite the considerable amount of study, there still remain the processes on thymus development, including lineage choice, selection and apoptosis survival. Understanding the mechanisms of T cell development, requires detailed knowledge on complexed signalling molecules and their mutual interactions. Characterisation of novel molecules and their involvement in the signalling events is an important step to widen the knowledge of one of the key processes in the immune system development – generating functional T cells.

Aim of this study is to biochemically characterise a novel, lymphocyte surface molecule termed “Trojan” and to investigate its function and possible involvement in T cell development during embryogenesis and adult life. Chicken was used as an animal model through the entire project, because of the advantages on the study of immune cell development – chicken immune system develops and functions in a way very similar to mammals. Development of T and B cells takes place in distinct organs – the thymus and the bursa of Fabricious, respectively. Embryonic manipulation is facilitated by the easy access to the embryo, its relatively big size and the ease to obtain large numbers of precisely staged embryos.

A key aspect for functional analysis is the search for Trojan-interacting molecules and their subsequent identification. This, together with the search for possible tissue-specific isoforms would suggest a place of Trojan in the complicated signalling mechanisms of developing T cells.

4. Materials and Methods

4.1 Animals

White Leghorn chickens of lines H.B.2 and H.B.15 from the colonies of the Department of Medical Microbiology, Oulu University were used as a source of eggs and primary cells. Post hatched chickens of days 16 or 18 were sacrificed for tissue isolation. Eggs were incubated at 38^{o} C for 13 days with gentle swaying, after which embryos were extracted and used for tissue isolation.

4.2 Cell manipulation

4.2.1 Primary cells isolation

Tissues isolated from chicken embryos of day 13 were briefly rinsed with ice cold PBS and kept in PBS solution on ice, while tissues isolated from a post hatched chicken of days 16 or 18 were kept on ice without rinsing. Tissues were placed on a metal grid covered by a 100 μm nylon filter (Millipore) and gently smashed with a syringe pestle. Cell suspensions were prepared by washing the smashed tissue with ice-cold PBS and filtered through a 100 μm nylon filter. Cells were then counted using Neubauer hemocytometer and analysed by flow cytometry or processed for surface biotinylation.

4.2.2 Cell lines

All cell lines were obtained from frozen stocks stored at the Department of Medical Microbiology at -80^{o} C. 11-7-3 is a mouse hybridoma cell line, secreting mAb 11-7-3 specific for Trojan antigen. MDCC-CU42 is a MDV (Mareck's disease virus) transformed chicken $CD4^{+}$ T cell line.

4.2.3 Reagents for cell culture

Liquid and powdered Dulbecco's Modified Eagle's Medium (DMEM), 1M Hepes and 7.5% $NaHCO_3$ were from Gibco Life Technologies. Fetal calf serum (FCS) was from Cambrex

and was heat-inactivated at 56° C for 1 h. Normal chicken serum (NCS) was from Jackson Immuno Research Laboratories and was also inactivated at 56° C for 1 h. Gentamycin (2 mg/mL) and the following reagents were from Sigma: L-alanine, L-asparagine, L-asparatic acid, L-glutamic acid, L-proline, Na-pyruvate, d-Biotin, Vitamin B12, HCl, L-cysteine, β-mercaptoethanol, Bovine Serum Albumin (BSA).

Chicken Iscove's Modified Dulbecco's Medium (IMDM) was prepared from powdered DMEM (Lassila *et al.* 1988), supplemented with: 0.25 % $NaHCO_3$, 10 mM Hepes, 224 µM L-alanine, 150 µM L-asparagine, 180 µM L-asparatic acid, 354 µM L-glutamic acid, 278 µM L-proline, 800 µM Na-pyruvate, 0.3 µM d-Biotin, 0.06 µM Vitamin B12, 280 µM HCl, 39.8 µM L-cysteine, 0.00035 % β-mercaptoethanol and 0.05 % BSA.

4.2.4 Mammalian cell culture

Mouse hybridoma cell line 11-7-3 was grown as suspension in DMEM, supplemented with 10% FCS and 50 µg/mL gentamycin, at 37° C in a 5% CO_2 humidifying incubator. Culture was maintained by ~2 passages per week, diluting ~10 times with fresh medium. For collection of supernatant, 11-7-3 cell line was cultured till maximum confluency was reached, after which medium was collected and centrifuged at 1500 g, for 10 min, at 4° C. Supernatant was transferred to a fresh tube, NaN_3 was added to a final concentration of 0.02% and supernatant was stored at 4° C.

4.2.5 Avian cell culture

Chicken cell line MDCC-CU42 was grown as suspension in IMDM supplemented with 5% FCS, 1% NCS and 50 µg/mL gentamycin, at 40° C in a 5% CO_2 humidifying incubator. Culture was maintained by 2 passages per week, diluting ~5 times with fresh culture medium. For flow cytometry analyses or surface biotinylation, cells were grown at a confluency of ~2 x 10^6/mL, counted by Neubauer hemocytometer and collected by centrifugation at ~500 g, for 10 min at 4° C.

4.2.6 Cell thawing and freezing

Cryotubes with frozen cells were thawed at 37° C in a water bath. Cells of each tube were mixed with 5 mL in culture medium and centrifuged at 500 g, for 5 min, at room temperature. After centrifugation, supernatant was discarded, and cell pellet was resuspended in 5 mL fresh medium. The cell suspension was transferred to a culture flask and placed in an incubator for up to 1 week without passaging until cells recover from the freezing storage.

For freezing, cells were suspended in FCS supplemented with 10% dimethyl-sulfoxide at a cell density of ~5x10^6/mL and divided in 1 mL cryotube aliquotes. The cryotubes were placed in a Cryo -1 Container (NALGENE) at –80° C over night to achieve gradual decrease of temperature.

4.3 Antibodies

Mouse-anti-chicken mAb were in the form of hybridoma culture supernatant: F21-21 (IgG1) against MHC class I α-chain, 11G2 (IgG1) against Bu1b, 3-298 (IgG2b) against CD8α and 11-7-3 (IgG2a), 11-5-3 (IgG2b), 11-35 (IgG2a), 11-10-4 (IgM) against Trojan. Purified mAb 11-7-3 was used only for immunoprecipitation and had initial concentration of 2 μg/μL. Antibodies are summarised in Table 1.

Antibody	Isotype	Specificity	Used for	Stored as
F21-21	IgG1	MHC I α	flow cytometry, immunoprecipitation	hybridoma supernatant
11G2	IgG1	Bu1b	flow cytometry, immunoprecipitation	hybridoma supernatant
3-298	IgG2b	CD8α	flow cytometry, immunoprecipitation	hybridoma supernatant
11-7-3	IgG2a	Trojan	flow cytometry, immunoprecipitation	hybridoma supernatant
11-7-3	IgG2a	Trojan	immunoprecipitation	purified
11-5-3	IgG2b	Trojan	flow cytometry	hybridoma supernatant
11-35	IgG2a	Trojan	flow cytometry	hybridoma supernatant
11-10-4	IgM	Trojan	flow cytometry	hybridoma supernatant

Table 1. List of monoclonal antibodies (mAb) used.

4.4 Cell staining

4.4.1 Reagents for flow cytometry

Flow cytometry buffer	PBS, 2% FCS
Primary mAb	hybridoma culture supernatants
Secondary mAb solution	PBS, 2% FCS, 2.5 µg/mL goat-anti-mouse IgG+IgA+IgM (H+L) FITC conjugate (Southern Biotech)

4.4.2 Flow cytometry

MDCC-CU42 cells were stained with the following mAb: 11-7-3, 11-5-3, 11-35, 11-10-4, F21-21 and 3-298. Cells from thymus, spleen, bursa and bone marrow of chicken embryos at day 13 were stained with 11-7-3. Cells from thymus of a day 18 post-hatched chicken were analysed with 11-7-3, F21-21 and 11G2. As negative controls, cells from each type were resuspended in flow cytometry buffer in stead of primary antibody.

The following procedures were performed in polypropylene U-bottomed 96 well plates kept on ice. All washings were done in ice-cold flow cytometry buffer with centrifugations at 1000 g, for 1 min, at 4^{o} C. Cells were dispensed at a density of 0.5 x 10^{6} cells per well and sedimented by centrifugation. Supernatant was discarded, cell pellets were resuspended in primary antibody at a volume of 100 µL per well and cells were then incubated for 30 min on ice. After incubation, cells were washed 3 times and resuspended in the secondary mAb solution at a volume of 100 µL/well. Cells were incubated on ice for 30 min, followed by 3 washings and resuspended in flow cytometry buffer at a volume of 500 µL per well. Cells were filtered through 100 µm nylon filter, transferred to flow cytometry tubes (Falcon) and analysed by FACScan (Becton Dickinson) using Cell Quest 5 program. The acquired data was later additionally analysed by Weasel 2.4 software, which was used to export the graphics presented in the results section.

4.5 Protein analysis Techniques

4.5.1 Reagents for protein analysis

Biotinylation solution	PBS pH 8.0, ~1.0 mg/mL (w/v) EZ-Link Sulfo-NHS-LC-Biotin (Pierce)
Biotin washing solution	PBS pH 8.0, 100 mM glycin
RIPA	50 mM Tris-Cl pH7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 1mM PMSF
Sepharose beads	Protein-A sepharose CL-4B beads (Amersham Biosciences)
Beads blocking solution	PBS, 0.1% (w/v) BSA and 0.01% (w/v) NaN_3
(4x) NuPAGE LDS Sample Buffer, pH 8.5	106 mM Tris HCl, 141 mM Tris base, 2% LDS, 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM Phenol Red
(20x) MOPS SDS running buffer, pH 7.7	50 mM MOPS, 50 mM Tris base, 0.1% SDS, 1 mM EDTA
(20x) Transfer buffer, pH 7.2	25 mM Bicine, 25 mM Bis-Tris, 1 mM EDTA
membrane blocking solution	100 mM Tris-Cl, 150 mM NaCl, 0.1% TWEEN-20, 0.2% casein
membrane staining solution	3 µg/mL SA-HRP (Pierce), 100 mM Tris-Cl, 150 mM NaCl, 0.1% TWEEN-20, 0.2% casein
TBS-T	20 mM Tris-Cl, 150 mM NaCl, 0.1% TWEEN-20

4.5.2 Cell surface biotinylation

Surface biotinylated cells were: MDCC-CU42 cells, primary cells from thymus, spleen and bone marrow of chicken embryo at day 13 and cells from thymus of a day 18 post-hatched chicken. Cells were washed 3 times in ice-cold PBS, pH 8.0, centrifuging between the washings at 500 g, for 10 min, at 4^{o} C. Cell pellet was suspended at a concentration of ~25 x 10^6 cells/mL in biotinylation solution. The suspension was then incubated for 30 min at 4^{o} C with gentle swirling of the tube. After biotinylation, per 1 mL of reaction volume 2 mL of biotin washing solution were added, mixed and incubated for additional 5 min at 4^{o} C with gentle rolling. Cells were then washed 3 times with ice cold biotin washing solution and once

with PBS, centrifuging between washings at 1000 g, for 10 min, at 4° C. The supernatant was removed and cell pellets were lysed immediately or stored at -80° C for later lysis.

4.5.3 Cell lysis

Total cell lysates were made prior to immunoprecipitation from biotinylated cells. Biotinylated cells were suspended in fresh RIPA at a concentration of 10^7 cells/mL and incubated for 30 min at 4° C with gentle shaking. Following incubation, the cell lysate was additionally disrupted by ~30-90 passages through a 21 gauge needle, several times until no protein aggregates were visible. Lysate was then centrifuged at 12 000 g, for 10 min, at 4° C to pellet cell debris and the supernatant was transferred to a fresh microcentrifuge tube.

4.5.4 Immunoprecipitation

All steps were carried out at 4° C in a cold room and all washings were with centrifugations at 12 000 g for 30 sec. Sepharose beads were kept at 4° C as a 1:1 (v/v) slurry in beads blocking solution. Prior to immunoprecipitation, beads were washed three times with fresh RIPA and resuspended in RIPA to make a 1:1 (v/v) slurry.

Lysate was precleared three times with beads slurry, to reduce the amount of proteins that would unspecifically bind to sepharose. For the first preclearing step, for 1 mL of lysate 100 µL beads were added, mixed and incubated for 1 h on a shaker. After incubation, lysate was centrifuged and supernatant was transferred to a fresh tube. For the second preclearing step, for 1 mL of lysate 100 µL beads were added, mixed and incubated for 1.5 h on a shaker. After incubation, lysate was again centrifuged and transferred to a fresh tube. For the third preclearing step, for 1 mL of lysate 50 µL beads were added, mixed and incubated for 30 min on a shaker. After incubation, the precleared lysate was centrifuged and transferred to a fresh tube, ready for immunoprecipitation.

During the incubation time of the second preclearing step, mAb were coupled to sepharose beads as follows: 100 µL mAb supernatant was mixed with 50 µL beads slurry in 500 µL RIPA or in the case when purified mAb was used, 4 µg mAb were mixed with 50 µL beads slurry in 600 µL RIPA. The reaction was incubated for 1 h on a shaker, after which the mAb-beads complexes were washed 3 times in RIPA.

The following mAb were used for immunoprecipitation from MDCC-CU42 cell line:

purified 11-7-3, F21-21 and 3-298. For immunoprecipitation from cells of thymus, spleen and bone marrow of chicken embryos at day 13, purified 11-7-3 was used. For immunoprecipitation from thymocytes of a day 18 chicken purified 11-7-3, F21-21 and 11G2 were used.

Antigen was precipitated by adding 500 μL of protein lysates per tube of washed mAb-beads complexes, followed by incubation for 1 h on a shaker. After incubation, immunoprecipitates were washed 4 times with RIPA and twice with 50 mM Tris-Cl, pH 7.0. Supernatant was removed and the pellet, containing the immunoprecipitated protein was stored at -20^{o} C.

4.5.5 Sodimdodecylsulfate polyacrylamide gel electrophoresis

Each immunoprecipitate was suspended in 15 μL (1x) sample buffer, prepared from (4x) NuPAGE LDS Sample Buffer. Immunoprecipitates to be run under reducing conditions were suspended in the same sample buffer supplemented with 10% β-mercaptoethanol (β-ME). Samples were heated at 70^{o} C for 10 min and centrifuged at 12 000 g, for 30 sec at 4^{o} C to pellet the beads. Following centrifugation, supernatant was carefully taken and loaded on a 10% or 4-12% polyacrylamide gel (NuPAGE Bis-Tris 10 well, Invitrogen).

Immunoprecipitates from MDCC-CU42 cell line were run under reducing conditions and their migration was compared to ECL DualValue Western Blotting Markers (Amersham Biosciences). The migration of the other immunoprecipitates was compared to Prestained Protein Molecular Weight Marker (Fermentas). Immunoprecipitates from thymus cells of a day 16 post-hatched chicken were run under non-reducing condintions and in a later experiment were run under reducing and non-reducing conditions on the same gel. Immunoprecipitates from thymus cells of chicken embryos at day 13 were run under reducing conditions.

Gels were run for 60-70 min, 200V, 125 mA in (1x) running buffer, prepared fresh by diluting (x20) MOPS SDS Running Buffer.

4.5.6 Western Blotting

Proteins separated on a polyacrylamide gel were electrophoretically blotted on a nitrocellulose membrane (Hybond ECL, Amersham Biosciences) in an XcellSureLock apparatus (Invitrogen), according to manufacturer's instructions. Blotting was performed for 1

h, at 30V, 175 mA in 600 mL fresh (1x) transfer buffer diluted from (20x) Transfer Buffer, and supplemented with 10% methanol and 120 µL β-ME.

4.5.7 Membrane blocking, staining and visualisation

After protein transfer, membrane was placed in fresh membrane blocking solution for 1 h at room temperature with gentle agitation. Membrane was then rinsed twice with TBS-T and incubated for 45 min at room temperature in 30 mL membrane staining solution with gentle agitation. After staining, the membrane was rinsed twice with TBS-T, followed by one washing in TBS-T for 15 min and three more washings of 5 min each. Result was visualised using ECL Plus (Amersham Biosciences), as follows: 50 µL of solution A were mixed with 2 mL of solution B and were poured on a piece of parafilm. The nitrocellulose membrane was placed on the liquid with proteins side down and incubated for 5 min. Following incubation, the membrane was sealed in a plastic transparent foil and placed in a photo-developing cassette together with a photo film (Hyperfilm, Amersham Biosciences). Exposure time depended on the strength of signal, so multiple exposures were performed with increasing amount of time in the range of 10 sec to 10 min.

4.6 Computational analyses

Open reading frame (ORF) of the cDNA sequence of Trojan was determined by the PLOTORF program, part of EMBOSS suite installed on a local computer. The cDNA sequence was then translated into amino acid sequence using the SIXPACK program, also part of EMBOSS suite. The molecular weight of the amino acid chain was predicted by Compute pI/Mw tool (http://expasy.org/tools/pi_tool.html).

Topology organization of Trojan protein was predicted from its amino acid sequences, using the SMART (Simple Modular Architecture Research Tool) web site in "normal mode" (http://smart.embl-heidelberg.de/) and the tools at PROSITE database web site, (http://www.expasy.ch/prosite/).

Search for putative N-glycosylation sites was performed by the tools available at PROSITE and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc) using default settings. Existence of possible phosphorylation sites was first investigated by the tools at PROSITE, where the option "exclude patterns with a high probability of occurrence" was

unchecked to detect all possibilities. The same prediction result was acquired by the tool available at NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) using default parameters. The kinase specificity for the putative phosphorylation sites was additionally investigated by NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/).

A model of the putative three dimensional structure of the FNIII domains of Trojan was generated by the tool at CPHmodels 2.0 (http://www.cbs.dtu.dk/services/CPHmodels/) web site. A second model of the three dimensional structure of the transmembrane region of Trojan and its cytoplasmic tail was generated using the tools at the Protein Structure Prediction Meta Server (http://pcons.net/) web site. The acquired 3D structure models were in .pdb format and were visualised by UCSF Chimera software.

Homology search for the amino acid sequence of Trojan was performed by protein BLAST (Basic Local Alignment Tool) tool at the NCBI (National Center for Biotechnology Information) web site, using the default parameters (http://www.ncbi.nlm.nih.gov/). Detailed analysis for the homology of Trojan to three example molecules was performed by SIM program (http://expasy.org/tools/sim-prot.html) using gap open penalty of 11 and gap extension penalty of 4, same as the protein BLAST defaults. The alignment graphics were generated by Lalnview software, installed on a local machine.

The place of Trojan gene in the chicken genome database was investigated by the mega BLAST tool at the NCBI web site. Search was performed with the cDNA sequence of Trojan, using the default parameters. The topology organization of the hypothetical protein coded in LOC427414 of the chicken genome database was predicted using the same tools, as for Trojan.

4.7 RNA and DNA manipulation techniques

4.7.1 Reagents for RNA and DNA manipulation

(10x) StrataScript buffer	0.5M Tris-HCl pH 8.3, 0.75M KCl, 0.03M $MgCl_2$
(10x) DyNAzyme II Reaction buffer	10mM Tris-HCl pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-10
(1x) TAE buffer pH 8.0	40 mM Tris base, 20 mM CH_3COOH, 1 mM EDTA

4.7.2 Total RNA isolation

Total RNA was isolated from thymus, bursa, spleen, bone marrow, liver and brain of chicken embryos at day 13. From each tissue, 50-100 µg were placed in a microcentrifuge tube and homogenized in 1 mL TRIzol (Invitrogen) by passage through a 21 gauge needle. Dissolved tissues were incubated for 5 min at room temperature and 200 µL chloroform was added per tube, followed by vigorous shaking and vortexing. Tubes were left at room temperature for 5 min and centrifuged at 12 000 g, for 15 min, at 4^{o} C. The solution separates into upper aqueous phase, containing mainly RNA, middle phase, containing proteins and lower phenol-chloroform phase containing DNA. Approximately 500 µL of the RNA-containing aqueous phase were transferred to a fresh microcentrifuge tube and mixed with 500 µL isopropanol. Tubes were vortexed briefly, left at room temperature for 10 min and centrifugated at 12 000 g, for 10 min, at 4^{o} C. RNA forms a gel-like pellet on the bottom of the tube, which was washed twice in 1 mL ethanol (75%), centrifuging between washings at 7600 g, for 5 min, at 4^{o} C. Washed pellet was air-dried and resuspended in 20-40 µL RNase-free deionised water, depending on the pellet size.

4.7.3 Treatment of RNA with DNase

RNA isolated from liver and brain was additionally treated with DNase as follows: 64 µg of RNA were mixed with 10 µL RQ1 DNase (Promega), 10 µL (10x) RQ1 DNase buffer (Promega) and RNase-free deionised water to a final volume of 100 µL. Reaction was incubated at 37^{o} C for 5 min. The DNase-treated RNA was purified by TRIzol isolation as described earlier.

4.7.4 cDNA synthesis

First strand cDNA was synthesized from isolated total RNA using StrataScript kit (Stratagene), as follows: 10 µg of total RNA were mixed with 3 µL of (100 ng/µL) Oligo(dT) primer and RNase-free deionised water to a final volume of 40 µL. Suspension was heated at 65o C for 5 min followed by slow cooling at room temperature for 10 min. The following components were added to the mixture: 5 µL of (10x) StrataScript buffer, 1 µL of (40 U/µL) RNase Block Ribonuclease Inhibitor (Rnasin, Promega), 2 µL of (100 mM) dNTP mix

(Amersham Biosciences) and 2 µL of (50 U/µL) StrataScript Reverse Transcriptase. The reagents were mixed gently and incubated at 42o C for 1 h, followed by heating at 90o C for 5 min to inactivate the reaction. The product cDNA was placed on ice, ready to be used as PCR template.

4.7.5 Primers

Primers described (Table 2) were purchased from Oligomer. Primers *fn3_fwd* and *fn3_rvs* were designed from the cDNA sequence of Trojan and were used to amplify a 576-bp region that comprises the two FNIII domains. Primers *trjn_fwd* and *trjn_rvs* were also designed from the cDNA sequence of Trojan and were used to amplify a 467-bp region between the second FNIII domain and the transmembrane part. The primer *fn3_fwd* was additionally combined with *trjn_rvs* in a third combination of primers. Primer *ptp_rvs* was designed from the putative mRNA (XM_424987.2) sequence of LOC427414 from a region coding for a suggested PTP domain. A combination of the Trojan-specidic primer *trjn_fwd* and *ptp_rvs,* was used to search for a possible isoform of Trojan, containing the PTP domain from LOC427414. Primers *cd45_fwd* and *cd45*_rvs were designed from the cDNA sequence of chicken CD45 and were used as control, amlifying a 457-bp region. Primers *g3pdh_fwd* and *g3pdh_rvs* amplifying a 497-bp region of G3PDH (Glyceraldehyde 3-Phosphate Dehydrogenase) were used as and additional control.

Name	**Sequence**	**Position**	**Direction**	**Designed from**
fn3_fwd	5'-GGAGTGTGTGGAGGTGCTACAAG-3	345-367	forward	Trojan cDNA
fn3_rvs	5'-GTCTCTGAGCCGTTGGTGTGG-3	941-921	reverse	Trojan cDNA
trjn_fwd	5'-CACACCAACGGCTCAGAGACC-3'	922-942	forward	Trojan cDNA
trjn_rvs	5'-ACAACCACCCCAATCACAATCC-3'	1388-1367	reverse	Trojan cDNA
ptp_rvs	5'-CACTCTTGTTCACCATTTCCACC-3'	1708-1730	reverse	XM_424987.2
cd45_fwd	5'-AGGAAGGAAACAGGAACAAATG-3'	2153-2174	forward	chicken CD45 cDNA
cd45_rvs	5'-GATGGATAAGGATGTACTGTGAC-3'	2608-2586	reverse	chicken CD45 cDNA
g3pdh_fwd	5'- ATCAATGGGCACGCCATCACTA-3'	228-249	forward	chicken G3PDH cDNA
g3pdh_rvs	5'- CACACGGAAAGCCATTCCAGTA -3'	728-705	reverse	chicken G3PDH cDNA

Table 2. List of primers used in PCR

4.7.6 Polymerase chain reaction

cDNA synthesised from total RNA of thymus, bursa, spleen and bone marrow was analysed by PCR, using the following three primer pairs: 1) *trjn_fwd* and *trjn_rvs*; 2) *trjn_fwd*

and *ptp_rvs*; 3) *cd45_fwd* and *cd45_rvs*. As a RT- control for possible genomic DNA contamination of the RNA used for cDNA synthesis, the primer pair *cd45_fwd* and *cd45_rvs was* used in a PCR having RNA as template.

Per reaction, the following PCR reagents were mixed in deionised water to a final volume of 50 µL: 5 µL (10x) DyNAzyme II Reaction buffer, 1 µL (10 mM) dNTPs, 1 µL (10 µM) forward primer, 1 µL (10 µM) reverse primer and 1 µL (2U/µL) DyNAzyme II DNA Polymerase. Templates were added last as follows: 1 µL cDNA synthesised from thymus and spleen, 2 µL cDNA synthesised from bursa and bone marrow and 2 µL of RNA isolated from thymus, spleen, bursa and bone marrow. PCR was performed using the following program: 94^{o} C for 1 min, then 35 cycles of 94^{o} for 30 sec, 52^{o} C for 15 sec and 72^{o} C for 2 min.

cDNA synthesised from total RNA of liver and brain was analysed by PCR, using the following five primer pairs: 1) *fn3_fwd* and *fn3_rvs*; 2) *trjn_fwd* and *trjn_rvs*; 3) *fn3_fwd* and *trjn_rvs*; 4) *cd45_fwd* and *cd45_rvs*; 5) *g3pdh_fwd* and *g3pdh_rvs*. The primer combination of *fn3_fwd* and *fn3_rvs* was additionally used in a PCR reaction having as template Trojan plasmid cDNA (insert in pcDNA3 vector). As a control for possible genomic DNA contamination of the RNA used for cDNA synthesis, two primer combinations were used in PCR having RNA as template: 1) *cd45_fwd* and *cd45_rvs* ; 2) *g3pdh_fwd* and *g3pdh_rvs*.

Per reaction, the following PCR reagents were mixed in deionised water to a final volume of 50 µL: 5 µL (10x) Optimized DyNAzyme II Reaction buffer, 1 µL (10 mM) dNTPs, 1 µL (10 µM) forward primer, 1 µL (10 µM) reverse primer and 1 µL (2U/µL) DyNAzyme II DNA Polymerase. Template was added last as follows: 1 µL cDNA synthesised from liver and brain, 1 µL (10 ng/µL) pcDNA3 plasmid and 2 µL RNA isolated from liver and brain. PCR was performed under the following conditions: 94^{o} C for 30 sec, then 35 cycles of 94^{o} for 30 sec, 52^{o} C for 20 sec and 72^{o} C for 1 min.

4.7.7 Agarose gel electrophoresis

Agarose 1% (w/v) was dissolved by boiling in (1x) TAE buffer pH 8.0 and 5 ng/mL $EthBr_2$ was added. PCR product was kept at 4^{o} C and 5 µL of each sample was mixed with 1 µL (6x) Loading dye (Fermentas). Gels were run at 100 V for 10 min in (1x) TAE. The size of DNA fragments was measured by reference to 1 kbp DNA ladder (Fermentas). The results were visualised by trans-illumination of the gel with UV light and photographed.

5. Results

5.1 Trojan expression during chicken embryogenesis.

To characterise the expression pattern of Trojan throughout chicken embryonic development, flow cytometry analyses of thymus and bone marrow cells were performed by Mrs. Taina Kirjonen. Results showed that, percentage of Trojan positive cells peaked at embryonic days 13 in thymus and 12 in bone marrow, coinciding with the period of second wave of thymic colonisation by precursors from bone marrow (Figure 4). After the peak, expression of Trojan on embryonic thymocytes gradually decreased till E18 and stayed relatively constant till the day of hatching. This expression pattern suggested involvement in the process of T cell development and this possibility was further investigated.

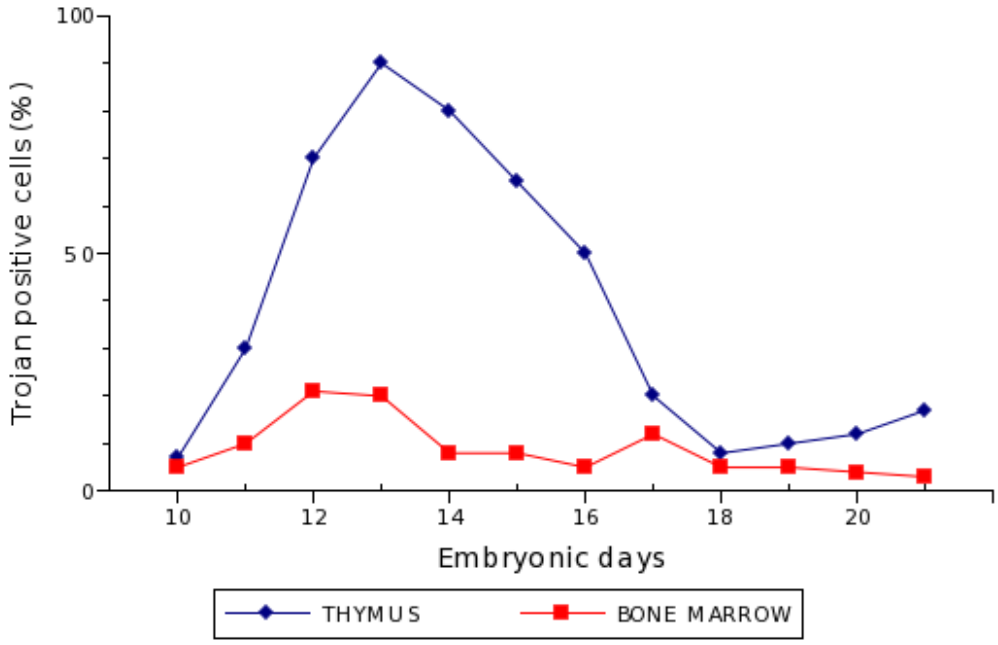


Figure 4. Expression of Trojan during chicken embryogenesis. *Charts show the variation in expression on cells isolated from embryonic thymus (blue line) and embryonic bone marrow (red line). X-axis represents embryonic days, starting from embryonic day 10 and continuing till day 21 – the day of hatching. Y-axis represents percentage of cells expressing Trojan. The peak of Trojan expression is between E12-E14 in thymus and E11-E13 on bone marrow cells.*

5.2 Selection of anti-Trojan and control antibodies

To select and optimal mAb for later experiments, MDCC-CU42 cell line was stained by flow cytometry, using anti-Trojan mAbs 11-7-3, 11-5-3, 11-35 and 11-10-4. Staining with mAb 11-7-3 showed the highest fluorescence intensity (Figure 5) and 11-7-3 was selected for further experiments.

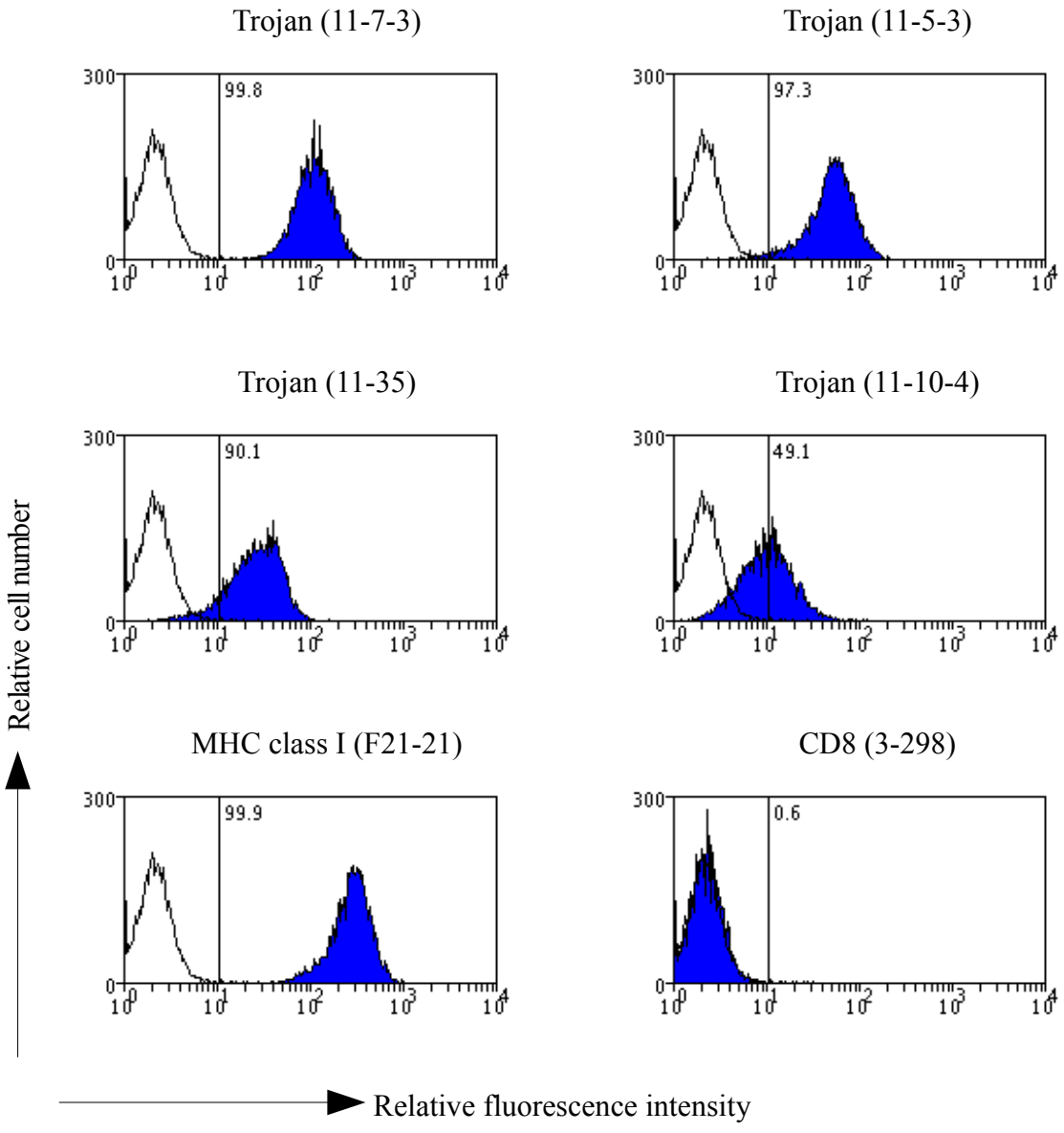


Figure 5. Staining of MDCC-CU42 cell line with mAb against Trojan, MHC class I and CD8. *Filled histograms represent mAb stainings, while blank histograms represent non-stained cells, used as negative control. Percentage of cells expressing each analysed antigen is shown to the right of the histogram marker.*

The same cell line was also analysed with mAb F21-21 (against MHC class I) and 3-298 (against CD8) to test if they are suitable controls for later experiments. Results showed that all cells express MHC class I with high intensity, while there was no expression of CD8 (Figure 5). In further biochemical analyses using MDCC-CU42, F21-21 was used as a positive control and 3-298 was used as a negative control.

5.3 Trojan expression on embryonic tissues

To compare the expression levels of Trojan on thymocytes, bursal cells and progenitor cells from bone marrow, cells were isolated from thymus, bursa, bone marrow and spleen of chicken embryos of day 13 and analysed by flow cytometry (Figure 6). Results showed that in thymus around 70% of the cells expressed Trojan, in spleen – around 30%, while in bursa and bone marrow – 19% and 15%, respectively.

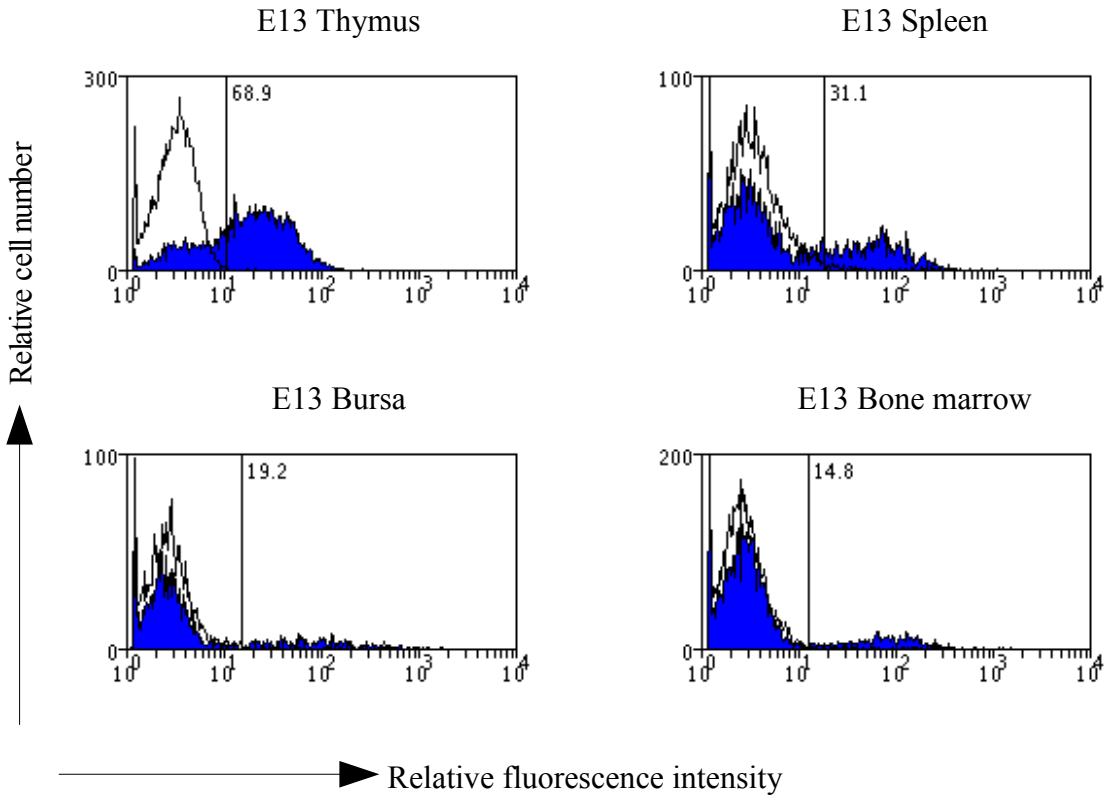


Figure 6. Expression of Trojan on primary cells isolated from thymus, spleen, bursa and bone marrow of chicken embryos at day 13. *Filled histograms show Trojan staining, while blank histogram show non-stained cells, used as negative control. Percentage of cells expressing Trojan is shown right to the histogram marker.*

The highest percentage of positive cells in thymus and the low in bursa suggested that, at this stage of embryonic development, Trojan was expressed mostly on T cells and to a lesser extent on B cells. Compared to thymus and bursa, spleen had a somewhat mean value, possibly due to the presence of both T and B cells within. Trojan expression on early progenitor cells was low, as bone marrow data suggested, but possibly increased as cells became committed towards the T lineage.

5.4 Trojan expression on adult thymocytes

To investigate the expression level of Trojan on thymocytes from a young adult chicken, cells were isolated from the thymus of a day 18 post-hatch chicken and analysed by flow cytometry.

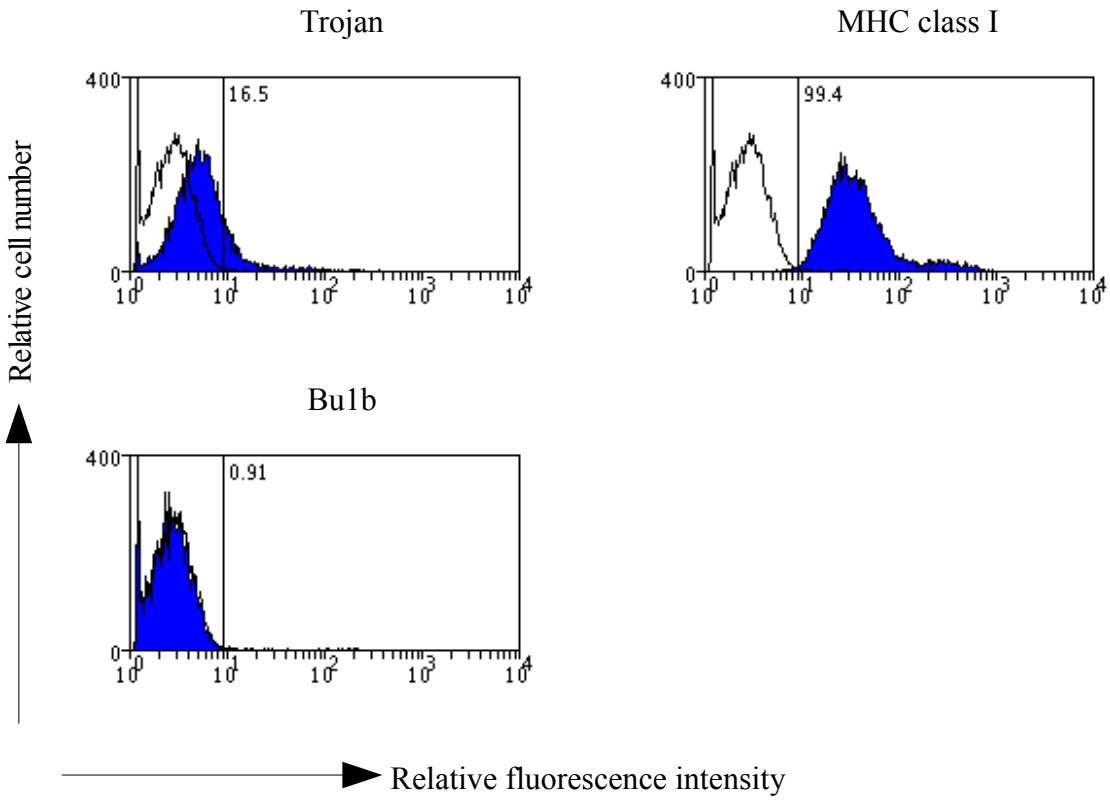


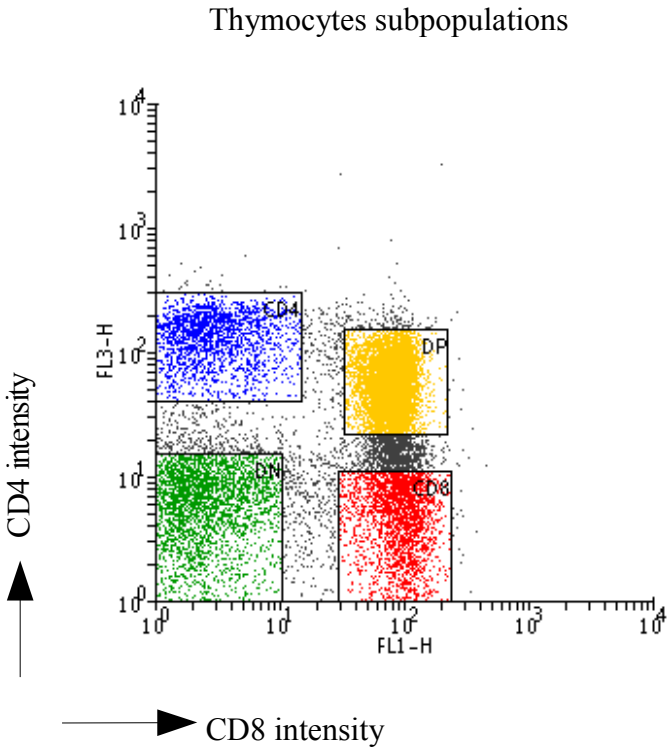
Figure 7. Expression of Trojan, MHC class I and Bu1b on thymocytes isolated from chicken at day 18, post hatch. *Filled histograms show mAb stainings, while blank histograms represent non-stained cells, used as negative control. Percentage of positive cells is shown right to the histogram marker.*

Result showed (Figure 7) that around 15% of adult thymocytes were Trojan positive, which is a much lower value, compared to thymocytes of embryonic day 13. This difference, implied that Trojan might have a more specialised function during embryogenesis.

Thymocytes of day 18 were also analysed with mAb F21-21 and 11G2 (against the B cell antigen Bu1b). Results showed (Figure 7) that all thymocytes expressed MHC class I while expression of the B cell-specific marker, Bu1b, was not detected. In further experiments using thymocytes, F21-21 and 11G2 were as positive and negative control, respectively.

5.5 Expression of Trojan on different subpopulations of adult thymocytes

To characterise Trojan expression on thymocytes at separate developmental stages, three colour flow cytometry analysis for Trojan, CD4 and CD8 was performed on cells from thymus of a day 16 post hatch chicken. Expression of Trojan was investigated on double negative ($CD4^-CD8^-$), double positive ($CD4^+CD8^+$) and single positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) subpopulations by Dr. Maki Motobu. The result showed that Trojan was expressed on double negative and single positive cells, but had almost no expression on double positive cells (Figure 8), suggesting involvement in thymocyte development.



Population	Percentage from all thymocytes
DN	15.5 %
CD4 SP	9 %
CD8 SP	22 %
DP	40 %

Table 3. Percentage of each population from the total amount of thymocytes.

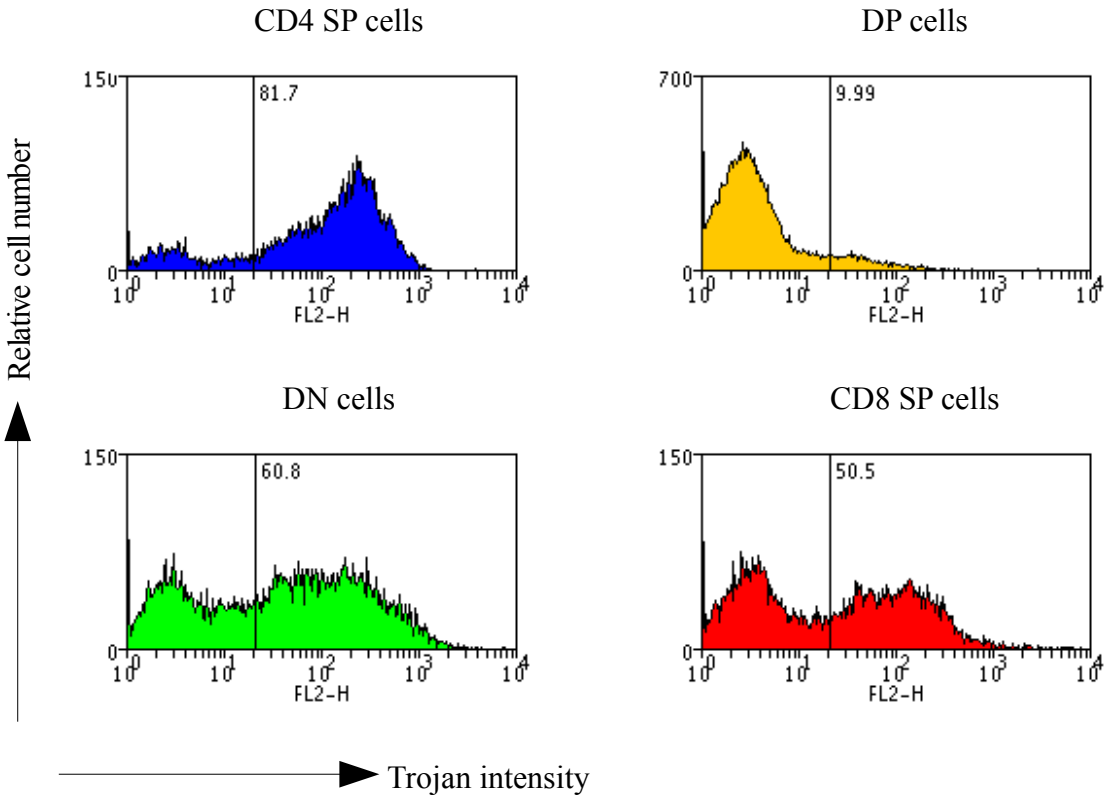


Figure 8. Expression of Trojan on different thymocytes subpopulations from a chicken at day 16, post-hatch. *A) Analysis of thymocytes for the expression of CD4 and CD8, using isotype-specific secondary mAb conjugated to PerCP and FITC, respectively. The dot-plot shows four distinct populations – DN: double negative cells (green), DP: double positive cells (orange), CD4 SP: CD4 single positive cells (blue) and CD8 SP: CD8 single positive cells (red). The relative percentage of each analysed population from the total amount of thymocytes is shown in Table 3, right to the dot-plot. Each subpopulation was gated and analysed for the expression of Trojan detected by an isotype-specific secondary mAb conjugated to PE. B) Analysis of each population of thymocytes showed that Trojan was expressed on DN (green histogram), CD4 SP (blue histogram) and CD8 SP (red histogram) thymocytes, but had much lower expression on DP cells. The percentage of cells expressing Trojan is shown to the right of the histogram marker.*

5.6 Immunoprecipitation

To perform basic biochemical characterisation of Trojan protein, several immunoprecipitations were carried out. Trojan was immunoprecipitated from MDCC-CU42 cell line using mAb 11-7-3 and as positive and negative controls mAb F21-21 (against MHC class I) and 3-298 (against CD8) were used, respectively. Immunoprecipitates were run on SDS polyacrylamide gel under reducing conditions and the result showed a single, clear band of Trojan at around ~75 kDa (Figure 9 A), as compared to ECL DualValue Western Blotting Markers (Amersham Biosciences).

To search for possible interacting molecules, Trojan was immunoprecipited from a natural source – primary cells isolated from thymus of a day 16 post-hatched chicken. Immunoprecipitates were run on a non-reducing SDS polyacrylamide gel, to search for Trojan homodimers or interacting molecules of a similar to Trojan size, which would otherwise migrate at the same rate as Trojan molecule. Antibodies F21-21 and 11G2 (against Bu1b) were used as positive and negative controls, respectively. Result showed a single band of Trojan at around 85-90 kDa (Figure 9 B), as compared to Prestained Protein Molecular Weight Marker (Fermentas). The relative molecular weight (MW) was about 10-15 kDa higher than the MW of the band immunoprecipitated previously from MDCC-CU42 cell line. However, the difference was too small to imply an existence of interacting molecule(s), and was rather due to the different MW standards used in the two experiments.

To compare the relative MW of Trojan expressed in different lymphoid organs, Trojan was immunoprecipitated from primary cells isolated from spleen, bone marrow and thymus of chicken embryos of day 13. Samples were run on SDS polyacrylamide gel under reducing conditions and the result showed single bands of apparently equal size (Figure 9 D). Relative molecular weight was estimated to be around ~90-95 kDa, as compared to Prestained Protein Molecular Weight Marker (Fermentas).

In the performed experiments, the migration of Trojan molecule showed slight difference when run under reducing and non reducing conditions. For a more precise comparison, Trojan was again immunoprecipitated from cells isolated from thymus of day 16 post hatched chicken. Immunoprecipitates were run under reducing and non-reducing conditions on the same SDS polyacrylamide gel, using Prestained Protein Molecular Weight Marker (Fermentas). The obtained result showed a small shift in migration of the two bands. Trojan protein had run a bit slower under reducing conditions (Figure 9 C), suggesting the existence of disulfide bond(s).

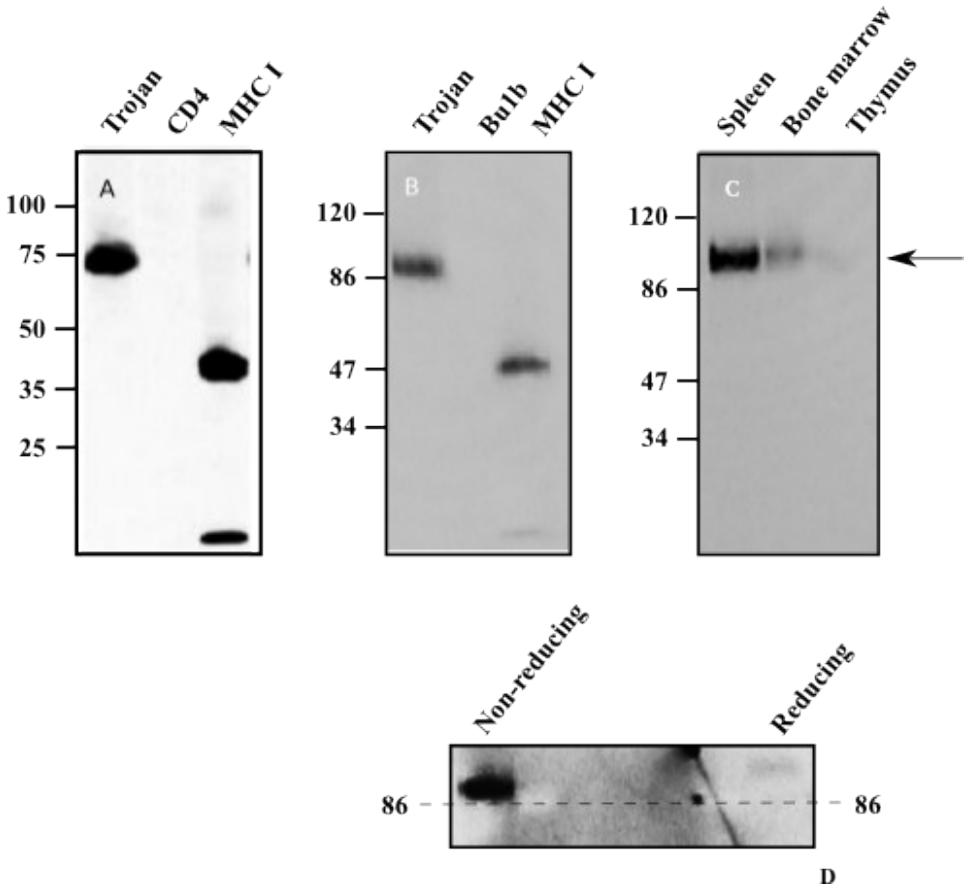


Figure 9. Immunoprecipitations of Trojan and controls followed by SDS-PAGE, showing the relative MW in kDa. *A) Immunoprecipitates from MDCC-CU42 cells line, run under reducing conditions. The relative MW of Trojan-band was estimated to be around 75 kDa; B) Immunoprecipitates from day 16 post-hatch thymus, run under non-reducing conditions. The relative MW of Trojan-band was estimated to be around 85-90 kDa. C) Immunoprecipitates from spleen, bone marrow and thymus of E13 embryos, run under reducing conditions. The bands had apparently the same MW of around 90-95 kDa. Position of Trojan band in thymus is pointed with an arrow. D) Immunoprecipitates from day 16 post-hatch thymus, run under reducing and non-reducing conditions on the same gell. A small shift in migration was observed, as compared to MW markers (dashed line).*

5.7 Sequence analyses

The cloned cDNA sequence of Trojan (Figure 11) was about 2.1 kbp long and coded for a polypeptide chain of 494 amino acids, with a predicted molecular weight of ~54 kDa. Computational analysis of Trojan amino acid sequence (Figure 10) revealed that Trojan was a

transmembrane protein, having a leading peptide (amino acids 1 to 22), two fibronectin type three (FNIII) domains (between amino acids 122-207 and between amino acids 217-300), a transmembrane part (amino acids 457-479) and an intracellular tail of 15 amino acids (480-494). Searches for putative protein glycosylation, predicted four most probable N-glycosylation sites: at amino acids 138, 250, 310 and 361. The cytoplasmic tail had a predicted positive charge, especially at amino acids 481-484: Arg – Arg – Arg – Lys (RRRK). Bioinformatics analyses for possible phosphorylation, pointed out two potential phosphorylation sites within the intracellular tail, on serines 485 and 487. The first (Ser 485) was a putative PKC phosphorylation target, while the second (Ser 487) was a putative cAMP- or cGMP-dependent protein kinase (PKA) phosphorylation site.

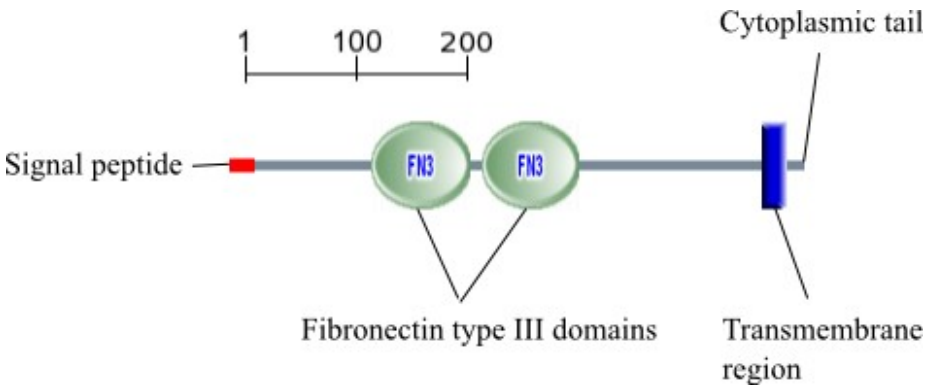


Figure 10. Predicted topology organisation of Trojan molecule. *The ruler indicates amino acids number.*

5.8 Protein structure prediction

A putative model of the three dimensional structure of Trojan FNIII domains was generated (Figure 12). It suggested the first FNIII domain to be composed of a pair of three anti-parallel β-sheet sets, linked by a disulfide bridge between cysteines 154 and 184. This putative bond is in agreement with the immunoprecipitation results, which also suggested the existence of a disulfide bridge. The second FNIII domain had a putative structure that resembles a sandwich of three anti-parallel β-sheets situated against two anti-parallel β-sheets. A model of the three dimensional structure of Trojan transmembrane part and cytoplasmic tail was also generated (Figure 13). It represented the transmembrane part as an α-helix, followed by the cytoplasmic tail, kinked in a Z-like shape to form two loops.

```
   1 ATGGCCCTGCAGTTCCTGGCTCTGACCTTTCTCCTGATCCTTTTTCCTCTGCTGGTAGCACAAGGCCAGGAAGAACCCTA  80
   1 M  A  L  Q  F  L  A  L  T  F  L  L  I  L  F  P  L  L  V  A  Q  G  Q  E  E  P  Y  27
  81 TTCCGTGGTCCAAGGAGCACCCCAAGAAATAGAAACGTGCCAAAGGCCCCAGTGGGACCCAGGACTCCGCCTGGCACCAG 160
  28  S  V  V  Q  G  A  P  Q  E  I  E  T  C  Q  R  P  Q  W  D  P  G  L  R  L  A  P  D  54
 161 ATCTGCAGGAGTATAAGAAAAATGAAGAAGTGACTCTGAGCTGCCCGGAGGGTTTACAGCCATCCTACACCCACATCAGA 240
  55   L  Q  E  Y  K  K  N  E  E  V  T  L  S  C  P  E  G  L  Q  P  S  Y  T  H  I  R   80
 241 TGTTCAAGAGAAGTTCAGACTATCGTTCATGGGAAACCTGTGTACAGGGAAGTTTGGCTGGGAAGAGCCAGCAGCGGTTC 320
  81 C  S  R  E  V  Q  T  I  V  H  G  K  P  V  Y  R  E  V  W  L  G  R  A  S  S  G  S  107
 321 CTGGATCCGCATTCGGTCCAGAGTGGAGTGTGTGGAGGTGCTACAAGTTGTCCCTGGGACCCTGGAGGTTTCCAGCACCA 400
 108  W  I  R  I  R  S  R  V  E  C  V  E  V  L  Q  V  V  P  G  T  L  E  V  S  S  T  S 134
 401 GCATCAAACTGAACTGGACCTGCAGCTTCCCTGATGCCTGCCAGCACACGGAGGCCACGTGCCGGCTGGCAGGGCCTTCC 480
 135   I  K  L  N  W  T  C  S  F  P  D  A  C  Q  H  T  E  A  T  C  R  L  A  G  P  S  160
 481 TCACCTGCCTGTGAGGCTGAGGAGGTCTCTGGAGTGGAGCTCCTGCACGGCCAGAGGGGAACATTCACCTGCACTTCCCT 560
 161 S  P  A  C  E  A  E  E  V  S  G  V  E  L  L  H  G  Q  R  G  T  F  T  C  T  S  L  187
 561 GCAGCCCTTCACTGACTACACCGTCACCATCTCAGTGCCCCCCAGCACAGTGCTGTTTTCATGGGTGATCAGGACAAAGG 640
 188  Q  P  F  T  D  Y  T  V  T  I  S  V  P  P  S  T  V  L  F  S  W  V  I  R  T  K  E 214
 641 AAGCAGTGCCGCAGAAGCCGGAGCAGCTGCAGCTGGATGCCCGCACGGGGACTCTCAGGTGGAAGGCGCTGCCCTCCTGC 720
 215   A  V  P  Q  K  P  E  Q  L  Q  L  D  A  R  T  G  T  L  R  W  K  A  L  P  S  C  240
 721 CGAGGGGAGATCGTTGGGTACCAGCTGAACATCACGGCCTGGGCCTCGCAGGACGGCGGCTTCCTGCACATGGAGCGGCT 800
 241 R  G  E  I  V  G  Y  Q  L  N  I  T  A  W  A  S  Q  D  G  G  F  L  H  M  E  R  L  267
 801 GAGGCTGAGCGGGCAGAGCACGGAGCACCGGCTGCCCACATACGGCCCCGGCACCAGATATGTGGTGGCCGTGCGGGGCC 880
 268  R  L  S  G  Q  S  T  E  H  R  L  P  T  Y  G  P  G  T  R  Y  V  V  A  V  R  G  L 294
 881 TCACGGCTGCGGGTCCTGGAGCTGCGTCGCTGTGGGAGTCCCACACCAACGGCTCAGAGACCCCAGGGCCGCCCCATGGC 960
 295   T  A  A  G  P  G  A  A  S  L  W  E  S  H  T  N  G  S  E  T  P  G  P  P  H  G  320
 961 TGCTCCCGCCTCGTGCTCGATACCTCTCCATCTCAGGGGACGGCCGTGCTTCCCCTGCACCCCATCTCCCAGCCCCCCGA 1040
 321 C  S  R  L  V  L  D  T  S  P  S  Q  G  T  A  V  L  P  L  H  P  I  S  Q  P  P  E  347
1041 GGCAGTGAGTGAGCACCAGCTGCTCGTGGCCGTGACGCACAACAGCACGGTGCTGGAAGACGCCTGCTCGGGGGAGCTGC 1120
 348  A  V  S  E  H  Q  L  L  V  A  V  T  H  N  S  T  V  L  E  D  A  C  S  G  E  L  Q 374
1121 AGCCCTCCAACCACAGCCACCCGCCTGACCCCTACGTGGCCGCCGTGCTCAACCTCAGCGCACCCACGGACTTCGTGCTG 1200
 375   P  S  N  H  S  H  P  P  D  P  Y  V  A  A  V  L  N  L  S  A  P  T  D  F  V  L  400
1201 GGTGACGGGACCCGTGGGCACGGCTTCCACAACGCTCCCCTGCACCCGGGATGGGACTACAGCGCCCTTCTGCGCCTCGC 1280
 401 G  D  G  T  R  G  H  G  F  H  N  A  P  L  H  P  G  W  D  Y  S  A  L  L  R  L  A  427
1281 CCGGCGCTCACCGCAGGCAGAGACGTTCACCTGCGTGTGCTACAGCTTTTCCATGGTTGCAGGGCAGTCATCATACCCAT 1360
 428  R  R  S  P  Q  A  E  T  F  T  C  V  C  Y  S  F  S  M  V  A  G  Q  S  S  Y  P  W 454
1361 GGCCAGGGATTGTGATTGGGGTGGTTGTGCTGTTGGTGCTGGTCCTCGTGTTTGCAGGCATCGTGTGGTTTTGTGCTGTCC 1440
 455   P  G  I  V  I  G  V  V  V  L  L  V  L  V  L  V  F  A  G  I  V  W  F  V  L  S   480
1441 AGGAGAAGGAAGTCTGTGTCTGTCAAAGCTAAGGAGAATAATTAAAAAGATAGAGAAAAGATGGAGCCAGGAGATTCTGC 1520
 481  R  R  R  K  S  V  S  V  K  A  K  E  N  N  *
1521 CCCTCGGGAGAATCATGTGCTCATCCTCTTTCGGACATGTCCCCCTGTGCCCCAGCCAGGCTGGAAAGCTGCTCTCTCAG 1600
1601 CTCCTGCCTGTCCTGGAGTTCTTGGAACAGTAACAGCAGTGATGCAGAGAAGATGGTGTGGTTGTACCTGGATCAACACT 1680
1681 GGCTCTCCTGTGACCATCACCATGCTAAATGTCCCATCCATTTCAGCAGAAAGTGGGAAATCAAGGAGGGTCATAAACGT 1760
1761 TTTCTTCCCAGCTTTTATTTACCCTTGTTGTAAGTTCTCGAACTTTTCAATGGGATTTTCTGCAGTTCCTTTTTTTAGAC 1840
1841 TTTGATTTTGCTTTCTTGAACGTGAGAATTGTACACAGGAATTCCCATTCTTTGCCTCAGGGTGGCTCAGGCTGAAGGGC 1920
1921 AGTCTGAGCTCCTGCCATGTCCTGGGTTCCTCACTCCATTTCCCCCCCTGTGCTGAAATCCATTCAAACCCAGTGCTCTG 2000
2001 CTTGAGCTTGATGTGACTGCATTGCCCTTGCCAACCTGTCTGCCCTGTCCTGAAAACCAATAAATGCAGTTTGGAGGTGG 2080
2081 CTGCCTGCCATGCTAAAAAAAAAAAAAAAAAA 2112
```

Figure 11. Trojan cDNA with the putative translated protein sequence. *Leader peptide sequence is shown in red, the sequence of the FNIII domains in green, transmembrane region in blue, the basic regions is coloured in yellow, the possible phosphorylation sites in purple (serine 485) and orange (serine 487), the possible N-glycosylation sites are underlined, and the stop codon is shown in red.*

A B

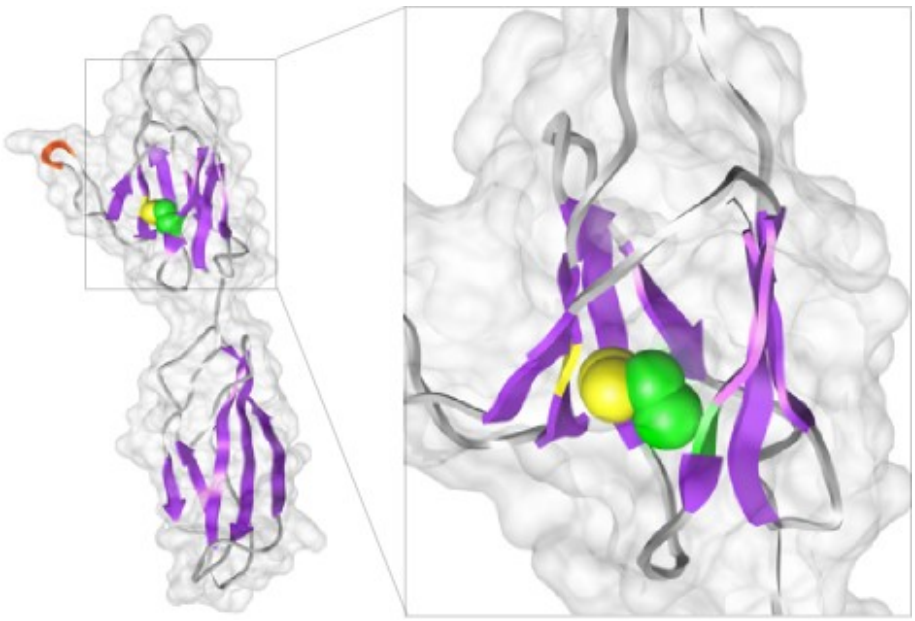


Figure 12. The predicted three dimensional protein structure of Trojan FNIII domains. *A) Both FNIII domains; B) A closer look of the first FNIII domain, where a possible disulfide bond was suggested.*

A B

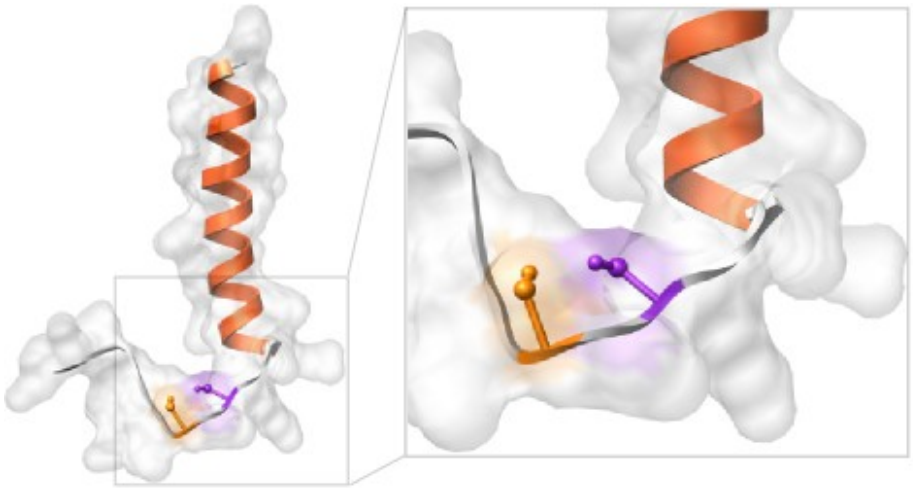
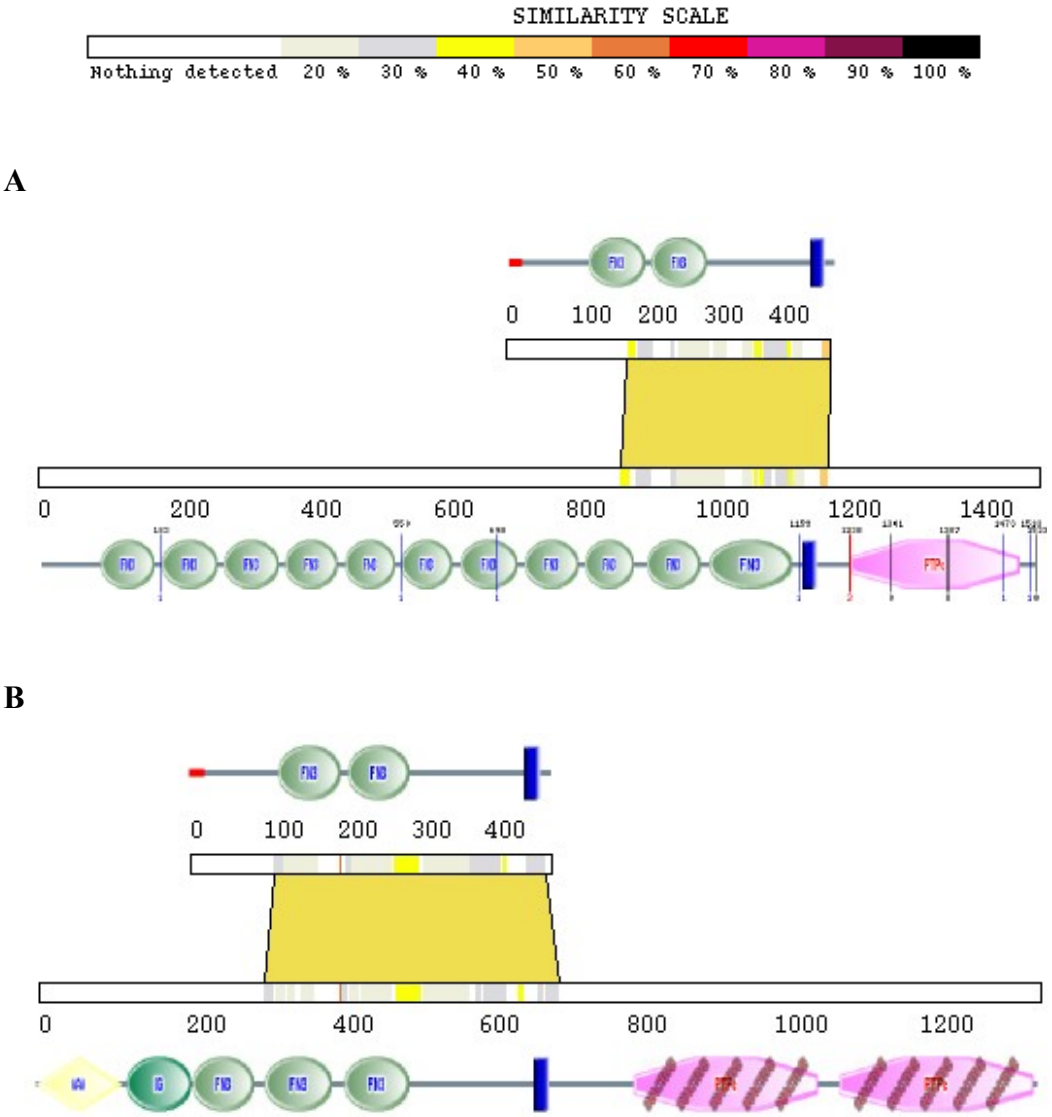


Figure 13. The predicted three dimensional structure of Trojan transmembrane and intracellular parts. *A) The two possible phosphorylation sites are shown within the intracellular part: serine 485 (purple) and serine 487 (orange); B) A closer look at the two serines and part of the transmembrane α-helix.*

5.9 Homology analysis

To identify possible homologues of Trojan in other species, protein BLAST search was performed for Trojan amino acid sequence. The results showed partial homology of Trojan to receptor-type protein tyrosine phosphatases (rPTPs) from different species. Similarities were at the region of the FNIII domains and/or between the second FNIII domain till the transmembrane part, yet no full-length homologue of Trojan was found. In Figure 14, three molecules that gave partial homology to Trojan are shown as an example: – rPTP 10d from *Aedes aegypti* (yellow fever mosquito) with 24% sequence identity, rPTP U from *Danio rerio* (zebra fish) with 23% sequence identity and rPTP M from *Homo sapiens* (human) with 25% sequence identity. Although our cDNA clone does not code for a PTP domain, the similarity to rPTPs was intriguing.



C

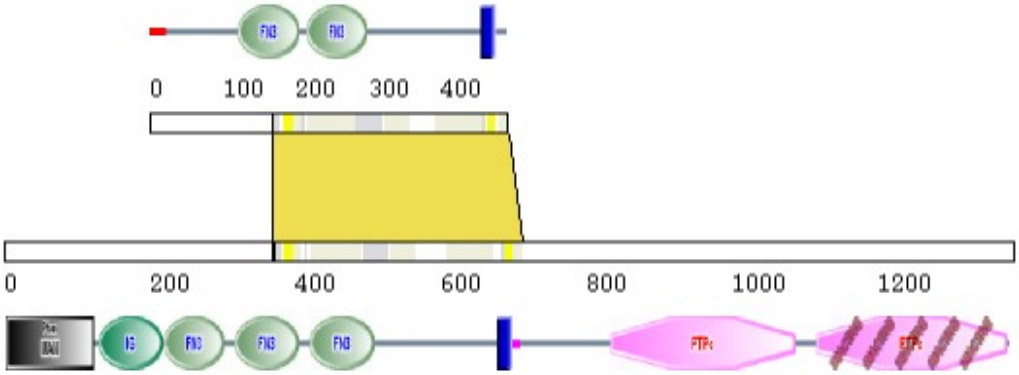


Figure 14. Example of the partial homology of Trojan amino acid sequence to molecules from different species. *The amino acid length of each molecule is indicated as a numbered bar, next to the scheme of its predicted domain organisation. The similarity of the alignment is indicated with the colour code shown in the similarity scale. A) Alignemnt of Trojan (top) to rPTP 10d from Aedes aegypti (yellow fever mosquito); B) Alignment of Trojan (top) to rPTP U from Danio rerio (zebra fish); C) Alignment of Trojan (top) to rPTP M from Homo sapiens (human).*

5.10 Genomic database analysis

Search of the chicken genome database with Trojan cDNA sequence, localised Trojan within a putative locus on Z chromosome, termed LOC427414. This locus encoded an uncharacterised protein tyrosine phosphatase (PTP) gene, to which Trojan cDNA matched only partially. The 582 nucleotides region coding for the two putative FNIII domains of Trojan had ~99% identity to three sequential exons of LOC427414 (Figure 15 – match 1). Immediately downstream the second FNIII domain of Trojan, a 125- nucleotides region (Figure 15 – match 2) had 97% identity to a sequence, represented as an intron in the database. Close to the 3' end of Trojan cDNA, a 183- nucleotides long region (Figure 15 – match 3), which resides after the stop codon, was 95% homologous to a part of the genomic minus strand, outside and after LOC427414. The other regions of Trojan cDNA had no match to the database sequence. However, the sequence of LOC427414 was incomplete and had several regions of unidentified nucleotides (Figure 15 – gaps) and may be misorientation. This might explain the only partial match of Trojan cDNA to the chicken genome database sequence.

The translated hypothetical PTP encoded in LOC427414, had a predicted topology organisation including a region of internal repeat (RPT), a complement control protein (CCP) domain, two FNIII domains, a PTP domain and a motif resembling an incomplete PTP domain (Figure 15). Although the homology of Trojan sequence to the database sequence is yet incomplete, Trojan would probably be mapped in LOC427414. The PTP domain coded in this putative locus raised the possibility that a PTP-domain containing isoform of Trojan exists.

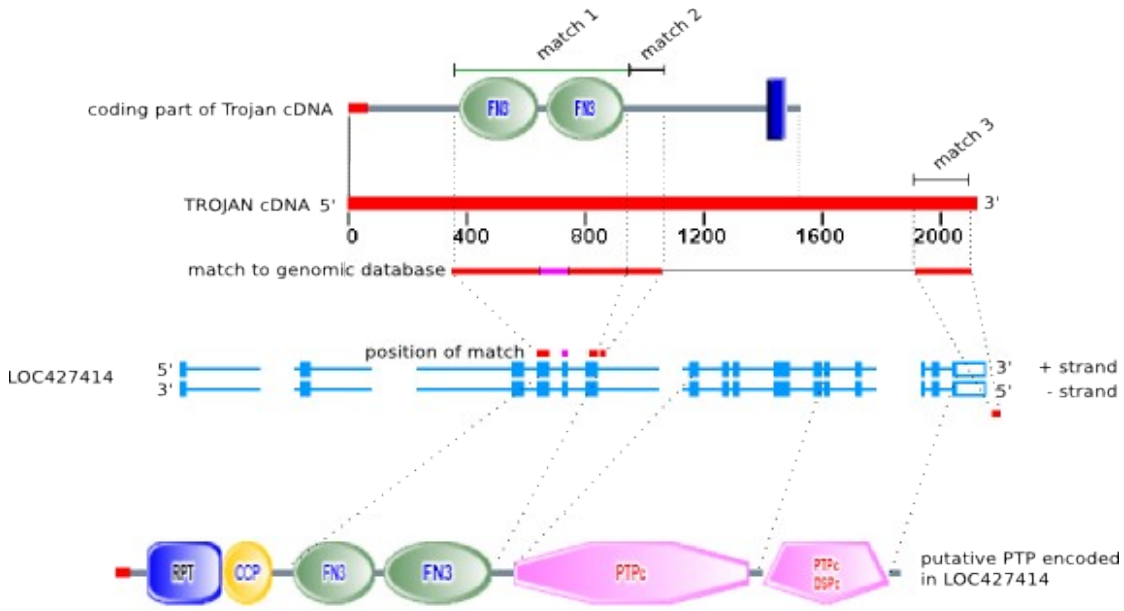


Figure 15. Localisation of the cDNA of Trojan to the chicken genome database locus LOC427414*. The coding part of Trojan cDNA is represented as a scheme of Trojan protein and is aligned to the full length Trojan cDNA. The exons of LOC427414 are represented as blue squares and the regions of unidentified nucleotides are shown as gaps. The predicted domain organisation of the putative PTP encoded in LOC427414 is shown at the bottom of the scheme.*

5.11 Trojan isoform search in lymphoid tissues

Since Trojan has homology to rPTPs and localises in a putative PTP locus, the existence of an isoform, possessing the PTP domain from LOC427414 was investigated. Search was performed by RT-PCR analysis of spleen, thymus, bone marrow and bursa of chicken embryos at day 13. Trojan expression was detected by a Trojan-specific primer set, amplifying the

region between the second FNIII domain and the trans-membrane part (Figure 16 A).

The forward primer was designed to bind a sequence downstream the second FNIII domain, in order to reduce the chance of possible misannealing to a FNIII sequence of other molecules. The reverse primer was designed to recognise part of the transmembrane sequence. The same forward primer was also combined with a reverse primer designed from the PTP domain sequence of LOC427414 and the pair was used to search for a Trojan PTP isoform in the tissues analysed (Figure 16 A).

Our PCR results showed amplification of Trojan in all tissues, but no amplification was detected of the supposed PTP isoform (Figure 16 B), suggesting that a Trojan isoform containing that particular PTP domain does not exist in the analysed tissues.

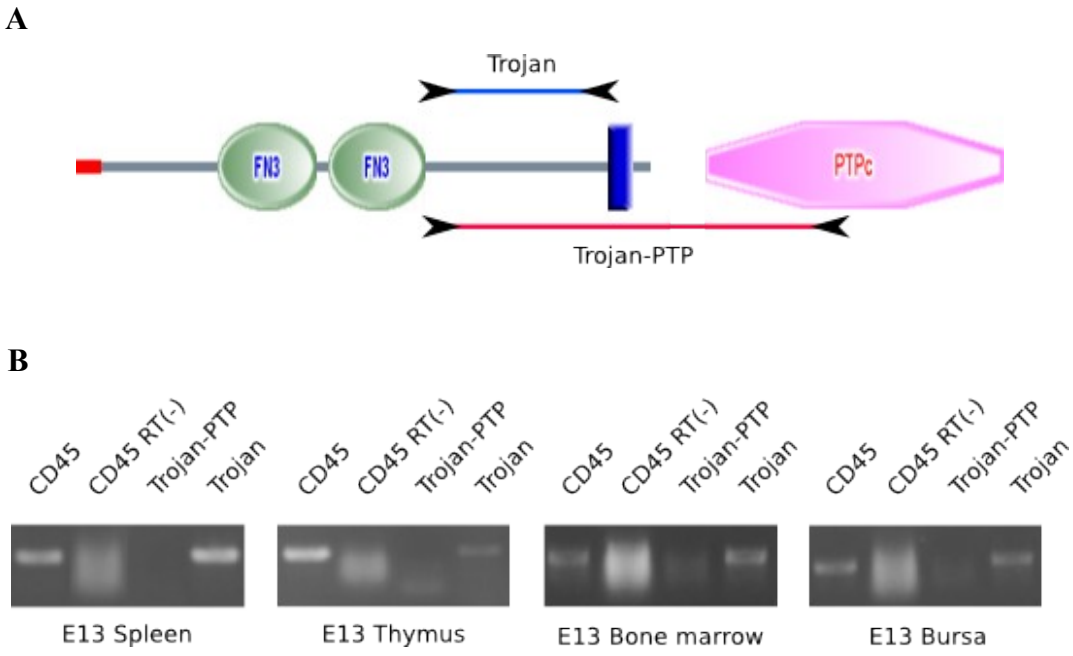


Figure 16. Trojan RT-PCR from embryonic lymphoid tissues. *A) Primer pairs used are represented as black arrows and the expected amplification products, are shown as colour lines between the primers. Trojan specific primers that amplify the region from the second FNIII domain till the transmembrane part are show connected with a blue line, named "Trojan". The primers used to search for a putative PTP isoform are shown connected by a red line, named "Trojan-PTP". B) Trojan expression was detected in all tissues (Trojan), while no band was observed of the suggested isoform (Trojan-PTP). Positive control CD45 product was amplified in all tissues and no genomic DNA contamination was detected in the negative control (CD45 RT-).*

5.12 Expression analysis of Trojan in non-lymphoid tissues

Trojan expression in non-lymphoid tissues was investigated by RT-PCR analysis of liver and brain of chicken embryos at day 13. Two primer sets were used to amplify two contiguous parts of Trojan sequence.

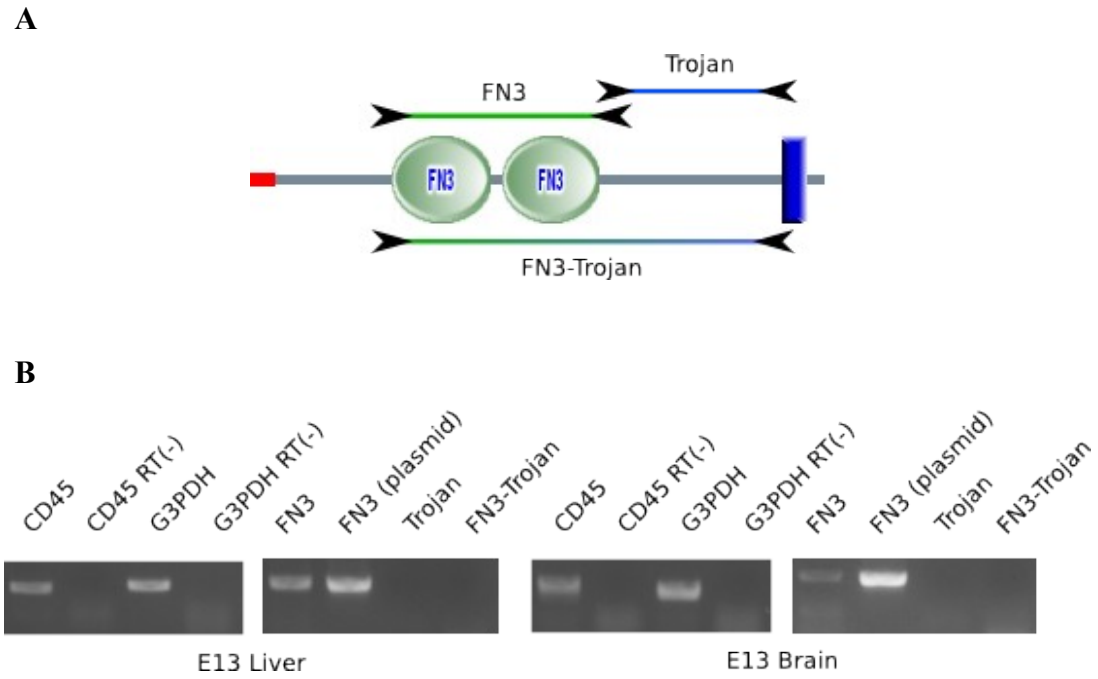


Figure 17. Trojan RT-PCR analysis of embryonic non-lymphoid tissues. *A) Primer pairs are represented as black arrows and the expected amplification products, are shown as colour lines between the primers. Primers that amplify the region of the FNIII domains are shown connected with a green line, named "FN3". Primers that would amplify the region from the second FNIII domain till the transmembrane part are show connected with a blue line, named "Trojan". In a third combination, the left primer upstream the first FNIII domain is connected with the right primer from the transmembrane part by a blue-green line, named "FN3-Trojan".B) Trojan RT-PCR products of the FNIII domains (FN3) were amplified in both tissues, while no amplification was detected of the expected products from the second FNIII domain till the transmembrane part (Trojan) and from the first FNIII domain till the transmembrane part (FN3-Trojan). The positive controls CD45 and G3PDH PCR products were amplified in both tissues and no genomic DNA contamination was detected in the negative controls (CD45 RT- and G3PDH RT-).*

The primers of the first set were designed to amplify the region comprising the FNIII domains of Trojan. The primers were designed to bind upstream and downstream the FNIII domains, to reduce the chance of possible misannealing to FNIII sequence of other molecules (Figure 17 A). The second set of primers was the one described in the previous section, amplifying the region between the second FNIII domain and the trans-membrane part.

Our PCR results (Figure 17 B) showed amplification of Trojan FNIII domains sequence from both liver and brain, while there was no amplification of the region between the second FNIII and the transmembrane part. The right primer from the transmembrane part was additionally combined with the left primer upstream the FNIII domains, but no amplification was detected either.

The lymphoid-specific CD45 was amplified in liver and brain (Figure 17 B), suggesting that leucocytes are present in these tissues. It is likely that the Trojan FNIII sequence was amplified from them, rather then hepatocytes or neurones. The lack of amplification by primers specific for the transmembrane sequence opened the question if a transcript lacking that particular part exists.

6. Discussion

In this study, we analysed a novel, avian lymphocyte cell surface molecule, termed "Trojan", aiming to examine its possible role in T cell development. We investigated Trojan expression and basic biochemical characteristics in lymphoid tissues of embryos or post hatch chickens. Sequence analyses were performed, followed by a search for possible isoforms in lymphoid and non lymphoid embryonic tissues.

During chicken embryogenesis, expression level of Trojan in thymus and bone marrow is not constant but instead varies with time. In these organs, the highest expression occurs at the period of second wave of thymic colonisation. The wave consists of T cell precursors that originate in the bone marrow to seed the thymus. During this embryonic period, Trojan has highest expression on thymocytes, compared to B cells and bone marrow progenitor cells. These expression patterns suggested a more T cell-specific function of Trojan at this developmental stage and a possible involvement in thymocyte maturation during embryogenesis.

During chicken adult life, Trojan shows variant levels of expression on thymocyte subpopulations at different maturation stages. The molecule has high surface expression on double negative (DN) and single positive (SP) cells, but very low or almost none on double positive (DP) cells. Thymocytes at the DP stage are subject of positive and negative selections and the majority of them die by apoptosis. The selection is mediated by thymic microenvironment via interactions between stromal cells and developing thymocytes. The consequent cell fate is implemented by complex intracellular signalling, which outcome is cell survival or cell death. The specific pattern of Trojan expression, could be used to draw a parallel to a similarly expressed molecule, with already identified function. A hint about a possible function of Trojan can be obtained from the role of such molecule in the processes of thymocytes development. An example of a transmembrane protein having similar expression pattern to Trojan is the interleukin 7 receptor (IL-7R), which is expressed on DN and SP cells but not on DP cells. During thymocyte maturation, IL-7R regulates thymocyte proliferation, differentiation and survival (Porter & Malek, 2000). The receptor exerts a pro-survival effect by inducing the expression of the anti-apoptotic protein Bcl-2 and downregulating the pro-apoptotic protein Bax (Kim *et al.* 1998). The expression pattern of Bcl-2 during thymocyte maturation closely resembles that of IL-7R (Veis *et al.* 1993), and therefore that of Trojan. This similarity in the expression of Trojan and proteins that play pro-survival roles may

suggest that Trojan has a similar function. However, a putative regulation of apoptosis in a Trojan-dependent manner would probably require signalling from plasma membrane to cell interior. The mechanism of such process would depend on the type of signalling that Trojan might have — a field that remains to be investigated.

Trojan has two predicted extracellular fibronectin type 3 domains (FNIII), but such domains are present in a variety of different proteins, making it difficult to suggest a function based on them. Similarity searches by computational analysis of the putative translated sequence of Trojan, failed to identify a full-length homologue in other organisms. However, partial homology to receptor type protein tyrosine phosphatases (rPTPs) from various species was observed. Analyses of the chicken genome database, localised Trojan cDNA to a putative locus, coding for a uncharacterised PTP molecule. Since our cDNA does not code for a PTP domain, we searched by PCR analysis of embryonic lymphoid tissues for an isoform of Trojan, containing the PTP domain from that genomic locus. Such an isoform might participate in the phospho-signalling events underlying thymocyte development, possibly having a pro-survival role, as suggested above. However, our PCR results from thymus, bone marrow bursa or spleen, failed to detect an isoform of Trojan containing that particular PTP domain. To investigate if Trojan has an isoform possesing another PTP domain, northern blot, with Trojan-specific probes could be used in further studies. Our immunoprecipitations identified single Trojan bands of apparently same size, from all tissues analysed. The molecular weight was around 30 kDa larger than the predicted MW of the amino acid sequence. Curiously, the PTP domain from chicken genome database has a predicted MW of approximately 30 kDa. However this difference is probably due to glycosylation of Trojan molecule, as Trojan has four putative N-glycosylation sites. In further studies, a cell line that does not express Trojan by default could be transfected with Trojan cDNA cloned into an expression vector. Hence, by immunoprecipitation from the transfected cells, the MW of Trojan protein coded in our cDNA clone would be obtained. This MW would provide a reliable standard to demonstrate whether 30 kDa size difference comes from a PTP domain or glycosylation.

The question remains open whether Trojan has no isoforms but rather exerts its signalling effects through its intracellular tail, in a cooperation with cytoplasmic molecules. Therefore, a hint about the molecules, that might interact with Trojan cytoplasmic part can be obtained from the amino acid sequence of the tail. The predicted, overall charge of the tail is positive, especially at the first four amino acids, which reside immediately under the inner layer of the plasma membrane. These residues comprise three consequent arginines followed by a lysine to form a basic region — RRRK. Intracellular signalling molecules, like MAP

kinases, are known to have highly acidic patch within their substrate-docking sites (Liu *et al.* 2006). The docking site helps to associate the kinase to its targets via electrostatic interactions. Hence, we performed computational analysis the tail amino acid sequence for possible sites of kinase phosphorylation. Despite its relatively short size, the cytoplasmic tail of Trojan has two predicted phosphorylation sites on serine residues. The first serine is adjacent to the lysine residue of the RRRK region, and is separated from the second serine by a single, hydrophobic, valine. The first and second serine residues are predicted as potential phosphorylation targets of cAMP protein kinase (PKA) and protein kinase C (PKC), respectively. These kinases are known to participate in the intracellular signalling of T cells, where they play important roles in TCR signal transduction and regulation (Mustelin & Tasken 2003). Thus, Trojan might serve as an integrating protein for signals mediated by cAMP via PKA and by Ca^{++} via PKC. However, the possibility of Trojan serine phosphorylation will be investigated by immunoprecipitation, followed western blotting with anti-phospho-serine antibodies.

Trojan may also associate with another transmembrane molecule and transmit signals to cell interior in a mutually regulated manner. Such process might be similar to the signalling principle of IL-7R, which has same expression pattern as Trojan, as described earlier. The receptor is a complex of two transmembrane chains: α, which is specific for IL-7R and γ, which is also found in other interleukin receptors. Both α and γ chains have a FN3 domain in their extracellular regions and possess cytoplasmic tails of about 200 and 100 amino acids, respectively. Upon ligand binding, the two chains form a dimer and bring together the cytoplasmic molecules associated with their intracellular tails (Porter & Malek 2000). The tail of the α chain possesses a serine-rich region which serves to associate with JAK1 (Janus kinase1) (Foxwell et al 1995), while the tail of the γ chain associates with JAK3 (Janus Kinase 3). It is possible that a cytoplasmic molecule associates with the serine residues of Trojan, in a way similar to IL-7R α chain. The interactions between IL-7R tail-associated molecules, initiates intracellular signal transduction cascade. Similarly, Trojan signalling may require ligand binding followed by receptor dimerisation to induce downstream signal transduction. In our co-immunoprecipitation experiment we failed to detect dimers of Trojan with another protein. If a ligand binding is required for dimerisation to occur, this would explain why we detected only Trojan from non-stimulated cells, isolated from adult thymus. To verify this possibility, further immunoprecipitation from stimulated cells will be performed. Mimicking Trojan ligand binding, Trojan will be crosslinked with the monoclonal antibody prior to immunoprecipitation.

Our PCR analyses of embryonic non-lymphoid tissues, showed amplification of Trojan

FNIII region in both liver and brain. However, there was no amplification when primer sets, specific for the transmembrane sequence were used. It is possible that a transcript of Trojan lacking this particular part exists and might represent an isoform, without a transmembrane region. Such an isoform may stand for a secreted protein, possibly acting as a regulator of Trojan functioning. Some cytokine receptors, like IL-1R (Jensen & Whitehead 2003), have secreted isoforms, which negatively regulate their function by competitive inhibition. Hence, Trojan function might also be regulated in such a manner, by an isoform that serves to quench its putative ligand. However, the detection of the secreted isoform only in the non lymphoid tissues of brain and liver, rises the question whether such putative isoform has a specific tissue distribution. It is likely that lymphocytes, residing in these organs express this secreted Trojan isoform, rather than neurones or hepatocytes. However this possibility and its meaning should be further investigated by more detailed PCR analysis.

In the project described, we investigated Trojan — a novel lymphocyte cell surface protein. Based on our results, several, not mutually exclusive assumptions can be made about its putative role. Trojan might have an isoform possessing a PTP domain, mediating cellular signalling or may transmit a signal by its cytoplasmic tail with or without the help of another transmembrane protein. A possible way to regulate Trojan function might be by a secreted isoform, as suggested. At a later stage of research, the possibilities discussed by now will be used as starting points for deeper investigations.

7. Summary

T cell development in thymus is triggered and regulated by complex interactions of surface molecules expressed on T cell precursors and thymic microenvironment. Signals received through cell surface receptors are transmitted to cell interior via tightly regulated intracellular signaling pathways, which have different effects on behavior and fate of T cell precursors.

"Trojan" is a novel avian cell surface protein cloned from embryonic thymocytes on the purpose to identify molecules that are involved in T-cell differentiation. Flow cytometry analysis of adult thymocytes shows expression of Trojan on single positive ($CD4^+$ or $CD8^+$) and double negative ($CD4^-CD8^-$) T cells, while there is almost no expression on double positive ($CD4^+CD8^+$) T cells. Molecules with similar expression pattern, like IL-7R and Bcl-2, are known to play pro-survival functions in developing thymocytes, implying that Trojan might have a similar role. By immunoprecipitations followed by SDS-PAGE, the relative molecular weight of Trojan was determined to be ~85-90 kDa, which is about 30 kDa larger than the molecular weight predicted from the translated amino acid sequence. This difference is probably due to glycosylation, since Trojan has four putative N-glycosylation sites. Computational sequence analyses reveal that Trojan is a transmembrane protein, having two Fibronectin type III domains and a short cytoplasmic tail. Homology searches of the putative translated sequence of Trojan show partial similarities with protein tyrosine phosphatases (PTPs) from several species. Furthermore analysis of the chicken genomic database localizes Trojan in a locus on Z chromosome where an uncharacterized receptor-type protein tyrosine phosphatase (rPTP) is encoded. The existence of a Trojan isoform containing a PTP domain was investigated by the designed RT-PCR, but no evidence was obtained. The cytoplasmic amino acid sequence of Trojan has a positively charged region (RRRK) and two putative serine phosphorylation sites. The positively charged region might mediate the interaction of cytoplasmic molecules with the two serines, possibly leading to intracellular signalling. Finally, our RT-PCR analyses of non-lymphoid tissues suggested a potential secreted isoform of Trojan. The isoform might serve to regulate the function of Trojan in a manner similar to other receptors like IL-1R, which is regulated by competitive inhibition via a secreted isoform.

In the presented study, it was suggested that a novel surface molecule, Trojan, plays a role in T cell development in thymus possibly as a signal mediator. However, further studies of its signalling potential are required to provide a clear insight of Trojan functions in T cell development.

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