Estudi de la regulació transcripcional del gen de CD69

Berta Vàzquez Prat

Facultat de Biologia

Departament de Fisiologia

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Tesi Doctoral

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Memòria presentada per **Berta Vàzquez Prat**,

llicenciada en Biologia, per optar al grau de

Doctora per la Universitat de Barcelona.

Tesi realitzada sota la direcció de la doctora Pilar Lauzurica Gómez,

professora titular a la Universitat de Barcelona

i investigadora titutar a l'Instituto de Salud Carlos III,

i la tutoria del doctor Luis Palacios Raufats, catedràtic de Fisiologia

a la Universitat de Barcelona.

Tesi adscrita al Departament de Fisiologia, Facultat de Biologia,

Universitat de Barcelona

Programa de doctorat de Biomedicina, bienni 2002-2004

Director	Tutor	Autor
Pilar Lauzurica Gómez	Luis Palacios Raufast	Berta Vàzquez Prat

AL ROGER,

PER TOT

AGRAÏMENTS

En primer lugar quiero agradecer a la Dra.Pilar Lauzurica por haberme aceptado en su laboratorio y haberme apoyado durante todos estos años. Querría agradecerle el enseñarme la importancia de la colaboración y la discusión científica y el hecho de que todo es posible, si uno se lo propone.

I would like to thank Dr. Mike Krangel for believing in this project and always welcome me in his lab. Thanks for the support and guidance during the past years.

A tots els meus companys de laboratori a Barcelona: al Xavi, l'Eli, el Jordi i a l'Enric, pel munt d'hores que hem passat dins el laboratori, la nostra segona casa, i per totes les vivències viscudes fora. To my lab mates at Duke, Chrys, Iratxe, Juan, Ryan, Han-yu, Zanchun and Jose. Thanks for making my stay at Duke so comfortable and easy! To Chrys and Iratxe, for their friendship. A Juan, por enseñarme tantos experimentos y trucos de laboratio. Thanks Zanchun and Chrys for helping me with the mice colony. A todas las chicas de Madrid: Tere, Sheila, Almudena, Isa y Cris, por todos los cotilleos, risas y momentos divertidos. Gracias Sheila por mantener y genotipar la colonia de ratones.

A tots els meus companys del Departament de Fisiologia: a l'Elisa i la Núria per ser tan bones amigues en tot moment i escoltar-me sempre que ho he necessitat; a la Bàrbara, el Joanet, la Nahir, l'Àngela, el Maxi, el David, la Marta, el Pablito, la Núria, el Jordi… Al laboratori de la Dra. María Macías, per acollir-me en els meus intents de ficar-me en el món de les proteïnes. Gràcies

Lídia, Bego, Roman i Ximena per tractar-me com una més quan vaig estar al vostre lab i per demostrar-me que és possible fer clonatges en dos dies!

A la meva mare, que sempre m'ha estimat, escoltat i recolzat en les meves decisions. A mi padre, que, aunque no hayamos podido estar cerca, sé que me quiere y está orgulloso de mí. A mis hermanos y a Ana. Y a mi abuela, que aunque ya no estés, muy a menudo estás en mis recuerdos y sé que también estarías orgullosa de mí. A tota la família Noguerol, pel seu recolzament i estima en tot moment.

A tots, dir-vos que moltes gràcies!!

INDEX

ABBREVIATIONS

AA: Aminoacid

BM: Bone marrow

CII: Collagen type II

CAT: Chloramphenicol acetyl transferase

CAIA: CII-antibody induced arthitis

CIA: Collagen induced-arthritis

Chip: Chromatin immunoprecipitation assay

CHX: Cycloheximide

CNS: Conserved non coding sequence

CTLD: C-type lectin domain

CsA: Cyclosporine A

DN: Double negative

DP: Double positive

HAT: Histone aceltytransferase

HDAC: Histone deacetylase

Io: Ionomycin

HS: Hypersensitive site

LN: Lymph node

LCR: Locus control region

LPS: Lipopolysaccharide

pDC: Plasmacytoid dendritic cells

PolI: Polymerase II

PKC: Protein kinase C

PTM: Post-translational modification

mAb: Monoclonal antibody

MBP: Mannose binding protein

MCP-1: Monocyte chemoattractant protein 1

NF-AT: Nuclear factor of activated T cells

NK: Natural killer

PMA: 12-myristate 13-acetate

PRG: Primary response gene

TBP: TATA binding protein

TCR: T cell receptor

Tg: Transgenic

TGFβ: Transforming growth factor β

TLR: Toll like receptor

RA: Rheumatoid arthritis

Rag: Recombination activating genes

S1P: Sphingosine-1-phosphate

SLE: Systemic lupus erythematosus

SP: Single positive

UTR: Untranslated region

I. INTRODUCTION

1.- CD69: A MOLECULE OF THE IMMUNE SYSTEM

1.1.- CD69 molecular structure

CD69 is a transmembrane C-type lectin originally identified as a molecule reactive with specific monoclonal antibodies (mAb) raised against antigens expressed in activated peripheral blood cells (Cebrian et al., 1988; Cosulich et al., 1987; Hara et al., 1986; Lanier et al., 1988). CD69 was previously known as AIM (activation inducer molecule), EA-1 (early activation antigen), MLR-3 or Leu-23. Immunoprecipitation assays revealed that CD69 molecule is a 60 kDa disulfide-linked homodimer. Both subunits are constitutively phosphorylated and present variable glycosylation. The molecular cloning of CD69 cDNA showed that the protein contained 199 amino acids (AA) and presented a type-II orientation. The 40 initial AA corresponded to the intracellular region, the next 21 AA to the transmembrane region and the last 138 to the extracellular domain.

CD69 extracellular domain was expressed and purified from *E.coli* cultures and the three dimensional structure was determined by crystallization (Llera et al., 2001; Natarajan et al., 2000). CD69 extracellular folding was similar to other C-type lectins such as mannose binding protein (MBP) or Ly49. The monomer is composed of two α helices and two β sheets (Fig. 1A). One β sheet contains β0, β1 and β5 chains and another β2, β2', β3 and β4 chains. β0 is implicated in the non-covalent dimerization of CD69. There are three intrachain disulfide bonds in the C-type lectin domain (CTLD) corresponding to Cys_{85}-Cys_{96},

Cys_{113}-Cys_{194} and Cys_{173}-Cys_{186}. Cys_{113}-Cys_{194} and Cys_{173}-Cys_{186} are conserved among C-type lectins. Cys_{68}, located at the stalk region, is important for the dimerization of CD69 monomers through disulfide bond formation.

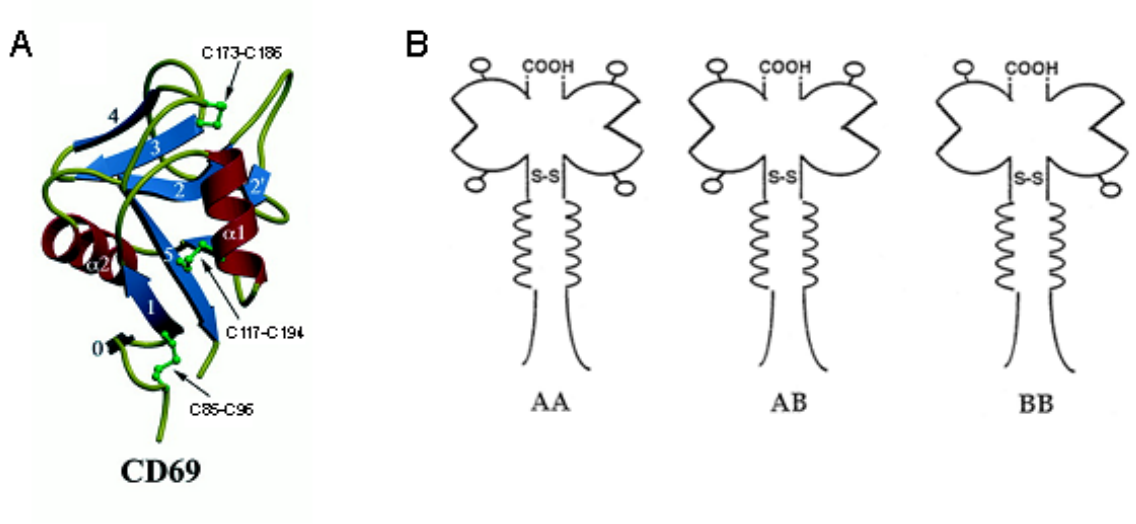


Figure 1: **CD69 protein structure**. (A) Human CD69 monomer showing the fold of the C-type lectin domain. The α helices are shown in red and β strands are shown in blue (Llera et al., 2001). (B) Multiple dimeric forms of human CD69 molecules result from variable modification of typical and atypical glycosylation motifs (Vance et al., 1997).

CD69 protein presents differential glycosylation of the 24kDa core monomer (Bjorndahl et al., 1988). Human CD69 has one N-glycan addition sequence in the extracellular domain while mouse CD69 presents three. Human CD69 monomers, however, exist as two glycoforms of 28 and 32 kDa. Site directed mutagenesis showed that this heterogeneity is achieved by variable N-glycans addition to the consensus glycosylation motif Asn_{166}-Val_{167}-Thr_{168} and the atypical glycosilation motif Asn_{111}-Ala_{112}-Cys_{113} (Vance et al., 1997). Analysis of CD69 mutant molcules lacking typical and atypical N-glycan attachment

motifs indicated that N-glycosylation is not necessary for CD69 dimerization nor intracellular transport to the plasma membrane (Vance et al., 1999)

1.2.- CD69 protein expression

CD69 is broadly expressed in bone marrow-derived cells. Platelets, monocytes, epidermal Langerhans cells, plasmacytoid dendritic cells (pDC) and a minor fraction of unstimulated lymphocytes constitutively express CD69 (Bieber et al., 1992; De Maria et al., 1994; Gao et al., 2009; Testi et al., 1990). Importantly, CD69 expression is inducible in many immune cells. In the thymus, CD69 is expressed in the surface of approximately 10-15% of immature $CD4^+CD8^+$ double positive (DP) thymocytes that are undergoing positive selection and in transitional $CD4^+CD8^-$ single positive (SP) and $CD4^-CD8^+$SP thymocytes (Swat et al., 1993; Yamashita et al., 1993) and it is the consequence of specific MHC-TCR interactions (Lucas and Germain, 1996). Major lymphoid population express CD69 in virus and bacterial infection and antitumoral responses. *In vitro*, stimulation of lymphocytes with anti-CD3/CD28, anti-CD2/CD28, anti-IgM and anti-CD16 mAb (Muse et al., 2007; Testi et al., 1989), IL-2, TNFα and IFNα/β cytokines (Shiow et al., 2006), viral and bacterial superantigens and tumour cells (Le Bon et al., 1996; Lina et al., 1998; North et al., 2007), a heat shock treatment (Muse et al., 2007) or phorphol esters also results in rapid induction of CD69. In bone marrow derived murine macrophages, lypopolissacharide (LPS) is also a potent inducer of CD69 (Marzio et al., 1997) and human peripheral eosinophils acquire CD69 expression after GM-CSF or IL-13 stimulation (Hartnell et al., 1993; Luttmann et al., 1996).

Notably, altered levels of CD69 are observed in infiltrating lymphocytes at inflammation sites. The expression is high in NK cells and T lymphocytes in tumour infiltrates (Van den Hove et al., 1997), bronchoalveolar eosinophils from patients with asthma and eosinophilic pneumonia (Hartnell et al., 1993; Nishikawa et al., 1992) and synovial T cells and neutrophils in rheumatoid arthritis (RA) (Atzeni et al., 2004; Iannone et al., 1996). Anti-CD69 autoantibodies have been detected in sera from patients with RA, systemic lupus erythematosus (SLE) and chronic hepatitis and have been associated with disease severity (Atzeni et al., 2004; Yui et al., 2001). Expression of CD69 has also been observed in a large percentage of patients with B-cell lymphocytic leukemia and correlates with poor disease prognosis (D'Arena et al., 2001) and recently it has been reported an increased T cell regulatory population with diminished CD69 expression and suppressive function in patients with systemic sclerosis (Radstake et al., 2009).

1.3.- Regulation of CD69 expression

a) Signaling pathways

The best-characterized signaling pathways leading to CD69 expression are the ones initiated by 12-myristate 13-acetate (PMA) and ionomycin (Io) treatment and anti-CD3 antibodies.

Protein kinase C (PKC) activation was shown to be the primary signaling pathway involved in CD69 expression (Cebrian et al., 1989; Villalba et al., 2000a). Transient transfection assays in Jurkat cells indicated that Ras, Raf and Vav also played a key role in the induccion of CD69 (D'Ambrosio et al., 1994;

Taylor-Fishwick and Siegel, 1995; Villalba et al., 2000a; Villalba et al., 2000b). A constitutively active mutant of any of the former molecules was sufficient to induce CD69 expression while a negative mutant abolished it. Vav-1 deficiency results in the absence of CD69 induction in TCR-stimulated T cells (Fischer et al., 1995). Induction in B lymphocytes, however, is normal and suggests the existence of different signaling pathways governig CD69 expression in lymphocyte subtypes.

Calcium-dependent signaling pathways stimulate CD69 expression. Moreover, they also potentiate the PKC-induced CD69 expression (Bjorndahl et al., 1988). Based on the fact that calcium-dependent CD69 up-regulation is inhibited with Cyclosporine A (CsA), some reports discuss the possible participation of the Nuclear factor of activated T cells (NF-AT) transcription factor members.

Finally, the signaling pathways leading to CD69 expression in NK cells involves a PKC-independent activation of protein tyrosine kinases (Borrego et al., 1993)

b) Gene structure and regulation

The molecular cloning of the CD69 cDNA was achieved by several groups (Hamann et al., 1993; Lopez-Cabrera et al., 1993; Ziegler et al., 1993). Chromosomal mapping showed that CD69 was a single copy gene located in the NK complex in chromosome 6 and 12 of mouse and human respectively (Lopez-Cabrera et al., 1993; Schnittger et al., 1993; Ziegler et al., 1994).

CD69 gene structure is highly conserved between human and mouse. Mouse CD69 has approximately 7.5Kb and contains 5 exons. Exon I and II, with 64bp and 123bp respectively, codify for the intracellular and transmembrane regions. Exon III, IV and V, with 200bp, 104bp and 106bp respectively, codify for the extracellular domain.

CD69 expression is strongly controlled at the transcriptional level (Cebrian et al., 1989; Sutcliffe et al., 2009; Testi et al., 1989; Ziegler et al., 1994). Treatment of cells with the protein synthesis inhibitor Cycloheximide (CHX) or the RNA synthesis inhibitor Actinomycin D results in the abolishment of CD69 expression. Stimulation of cells with PMA or anti-CD3 antibodies results in a rapid increase of transcripts. They are detected in 30-60 minutes, being maximum at 4-6 hours and after this period they start to decrease. Protein expression at the cell surface presents a slower kinetics. It is detected 2-3 hours after stimulation and is maximally expressed 12-24 hours after.

The 3' untranslated region (UTR) is highly conserved between human and mouse and presents AU rich sequences involved in the post-transcriptional degradation of the CD69 mRNA (Santis et al., 1995).

Human and mouse CD69 promoters have been cloned. They both contain a consensus TATA box upstream of the transcription start site (TSS) and are able to direct transcription of a reporter in resting and stimulated cells in transient transfection assays (Lopez-Cabrera et al., 1993; Ziegler et al., 1993). C*is*-elements contributing to this inducibility were mapped to the proximal promoter

region and these elements were shown to interact with transcription factors Erg-1, Erg-3, ATF-3/CREB and AP-1 upon stimulation (Castellanos et al., 1997; Castellanos Mdel et al., 2002). Interestingly, Erg-3 binding was inhibited by CsA. Basal CD69 transcription was also detected by transient transfection of mouse and human promoter constructs and was attributed to the -78 to +16 region of the human CD69 gene. Interestingly, the transcription factor Sp1 was shown to constitutively bind to this region at position -56. In another study, an NFκB motif at position -223 of the human CD69 promoter was shown to be required for transcriptional induction of CD69 in response to TNFα (Lopez-Cabrera et al., 1995).

CD69 gene activation is associated with changes in the chromatin structure (Sutcliffe et al., 2009). Chromatin immunoprecipitation (Chip) indicated that RNA polymerase II (Pol II) is preloaded at the CD69 promoter in human $CD4^+$T and Jurkat T cells before transcription (Schones et al., 2008; Sutcliffe et al., 2009). H3.3 and H2A.Z are histone variants that are exchange upon gene induction. In the resting state, CD69 TSS is flanked by H2A.Z and H3 histones and transcription activation results in their depletion and a concomitant deposition of H3.3. A sequential Chip showed an increase in nucleosomes with H3.3 and H2A.Z histones, which are highly unstable and are thought to play an active role in maintaining accessible chromatin (Jin and Felsenfeld, 2007).

1.4.- CD69 function

a) *In vitro* CD69 targeting

Due to the fact that no extrinsic ligand has been identified for CD69, many of its immunological functions have been analysed by its crosslinking with monoclonal antibodies.

In lymphocytes, CD69 targeting with mAb in PMA-preactivated cells induces a proliferative response (Cebrian et al., 1988) and production of cytokines such as TNFα and INFβ (Santis et al., 1992). Despite no recognizable sequences for connection to intracellular effectors are present in the CD69 intracellular domain, CD69-mediated signal transduction involves a pertussis toxin-sensitive G protein pathway (Sancho et al., 2000). CD69 crosslinking has been reported to induce intracellular ERK activation in $CD69^+$ transfectants and primary $CD69^+CD4^+CD25^-$T regulatory cells (Han et al., 2009; Zingoni et al., 2000). Importantly, sustained ERK activation in $CD69^+CD4^+CD25^-$T regulatory cells was required to maintain high levels of membrane-bound TGFβ1 (Han et al., 2009).

In human IL-2 preactivated NK cells, the lytic activity positively correlated with CD69 expression levels (Lanier et al., 1988). Moreover, anti-CD69 mAb treatment enhanced the proliferative response of PMA, IL-12 or IL-2 preactivated NK cells and induced a strong release of TNFα (Borrego et al., 1999) . Interestingly, CD69-mediated citotoxicity was blocked by anti-CD94 mAb (Borrego et al., 1999). CD69-induced cytoxicity in NK cells is dependent

on the selective activation of Lck and Syk kinases and subsequent phosphorylation and activation of PLCγ2 and Vav1 (Pisegna et al., 2002).

Crosslinking of CD69 on human monocytes results in intracellular Ca^{2+} influx, PLA_2 activation and NO-dependent cytotoxic activity (De Maria et al., 1994). Similarly, in platelets it generates Ca^{2+} influx, PLA_2 activation, release of PGE_2 and TXB_2, aggregation and degranulation (Testi et al., 1990; Testi et al., 1992). CD69 targeting in human eosinophils and LPS-preactivated human monocytes results in apoptosis induction (Ramirez-Carrozzi et al., 2009; Walsh et al., 1996). CD69-induced monocyte apoptosis in this system involves at least three independent signalling pathways: PLA_2/lipoxenase pathways, NO synthesis and G_i-protein-dependent signal transduction (Ramirez-Carrozzi et al., 2009).

b) *In vivo* CD69 targeting

Mouse anti-mouse CD69 mAb were generated by immunizing $CD69^{-/-}$ mice with $CD69^{+}$ cells (Esplugues et al., 2005). Characterization of the specific anti-murine CD69 mAb 2.2 indicated that neither it bound the complement system nor Fc receptors and its *in vivo* administration resulted in CD69 internalization with no alterations of immune populations. The therapeutic potential of mAb 2.2 was evaluated in mice bearing MHC-I low tumors. Anti-CD69 treated mice presented an enhanced survival that correlated with augmented and diminished IFNγ and TGFβ1 mRNA levels respectively and with an increased cytotoxic activity of NK cells.

The most commonly used animal model for rheumatoid arthritis is the collagen-induced arthritis (CIA). Targeting CD69 in CIA with different anti-CD69 mAb resulted in variation in the disease progression (Sancho et al., 2006). 2.2 anti-mouse CD69 mAb exacerbated CIA while 2.3 mAb inhibited its development. The mechanism through which 2.3 mAb ameliorated the course of the disease was a partial depletion of $CD69^+$ cells, diminished proliferation of type-II collagen-specific lymphocytes and reduced IFNβ production.

In a murine model of asthma, CD69 targeting resulted in the inhibition of ovoalbumin (OVA) -induced airway inflammation associated with reduced accumulation of OVA-specific T cells at the inflammation site (Miki-Hosokawa et al., 2009).

c) CD69 and lymphocyte egress

Analysis of transgenic and different knockout mice suggested that CD69 might participate in the control of lymphocyte egress from lymphoid organs.

Two research groups overexpressed CD69 in T cells by means of transgenesis and found that constitutive expression of CD69 was associated with an accumulation of mature SP thymocytes and a concomitant reduction of peripheral T cells due to a failure in thymocyte egress (Feng et al., 2002; Shinkai et al., 1993). Moreover, generation of mature thymocytes was also inhibited by *in vivo* administration of anti-CD69 mAb (Shinkai et al., 1993).

CD69 upregulation was later shown to inhibit lymphocyte egress from lymphoid organs through its interaction in cis and down modulation of sphingosine-1-phosphate-1 ($S1P_1$) receptor (Shiow et al., 2006). Analysis of fetal liver chimeras, generated by transplanting irradiated wild-type mice with fetal liver cells from $S1P_1$ knockout donors, indicated that $S1P_1$ is required within maturing thymocytes to emigrate from the thymys and within mature T and B cells for their egress from peripheral lymphoids organs (Matloubian et al., 2004). Importantly, CD69 expression was elevated in $S1P_1$ deficient T and B cells and $S1P_1$ was identified in a screening for negative regulators of CD69 in activated Jurkat T cells (Chu et al., 2003; Matloubian et al., 2004). Similarly to $S1P_1$ deficiency in hematopoieitic cells, Kruppel-like factor 2 (KLF2) null mice present defective thymocyte emigration and absence of lymphocytes in peripheral organs (Carlson et al., 2006). KLF2 is a zinc finger transcription factor that binds and activates $S1P_1$ promoter and, importantly, CD69 expression is high in $KLF2^{-/-}$ cells.

In another study a potential link between CD69 and $S1P_4$ receptor was suggested (Gao et al., 2009). $S1P_4$ is the predominant receptor expressed on pDC and it is required for their egress from lymph nodes (LN) into the lymph. CD69 is expressed on the surface of pDC and it is upregulated upon viral infection. Whether these molecules physically interact and regulate pDC egress in a similar manner as $S1P_1$ and CD69 in T and B lymphocytes remains to be established. A recent study has provided evidence that $S1P_5$ is required for NK

cell egress from LN and BM (Jenne et al., 2009). Immunoprecipitation and transwell migration assays, however, have highlighted that CD69 does not interact with $S1P_5$ neither inhibits $S1P_5$ function suggesting that this receptor may promote migration of NK cells in a $CD69^+$ effector state.

d) Immune response in CD69 deficient mice

Mouse CD69 gene disruption was accomplished by replacement of the genomic region containing exons II, III, IV and V with a neomycin resistance gene (Lauzurica et al., 2000). CD69 deficient mice showed normal T cell development and only a minor increase in the pre-B cell and immature B cell compartment was observed. T cell function was also normal and only a slight increase in the humoral immune response was reported. Finally, spleen and peritoneal cellularity presented a moderate increase in CD69 null mice that correlated with diminished spontaneous apoptosis (Esplugues et al., 2003).

CD69 deficient mice displayed an enhanced resistance to MHC class-I^- tumour growth associated to an increased recruitment of NK and T cells at the site of tumour challenge and altered cytokine production. At the site of tumour inoculation, there was an increase in the mRNA levels of MCP-1, IL-12, IL-1α and IL-1β and a reduced expression of the anti-inflammatory cytokine TGFβ1.

CD69 deficient mice developed an exacerbated form of CIA (Sancho et al., 2003). At the joints, there was a severe swelling due to enhanced expression of the inflammatory cytokines IL-1β, RANTES, macrophage protein-1α (MIP-1α) and MIP-1β and a reduced expression of TGFβ1 and TGF-β2. In the spleen

there was an increase in the proliferative response of specific lymphocytes and in the levels of Th1-dependent IgG antibodies.

The generation of CD69 null mice was also accomplished by Murata and colleagues by removing the first exon of the CD69 gene that contains the initiation ATG codon (Murata et al., 2003). They evaluated the role of CD69 in a CII-antibody induced arthitis (CAIA) model and found that the severity of the disease was clearly reduced in $CD69^{-/-}$ mice. The number of infiltrated neutrophils at the arthritic joints and levels of proinflammatory cytokines and chemokines IL-1β, IL-6, MIP-1α and MCP-1 and CXCL1 were reduced. Moreover, transfer experiments indicated that CD69 expressing neutrophils were critical in the induction of the disease. In contradiction with this data, another study showed that the arthritis response in $CD69^{-/-}$ mice in the CAIA model was normal. Importantly, neutrophil recruitment in CD69 deficient mice was not altered (Lamana et al., 2006).

A recent report has studied the role of CD69 in the pathogenesis of asthma using a Th2-induced eosinophilic airway inflammation model (Miki-Hosokawa et al., 2009). CD69 deficient mice developed an attenuated form of the disease as a result of decreased number of infiltrating leukocytes and decreased mRNA levels for IL-4, IL-13 and IL-5 in the bronchoalveolar fluids. Lower expression of VCAM-1 protein was also obseved in the asmathic lung. Importantly, *in vitro* assesment of Th2 differentiation revealed and altered IL4/IFNγ profile with reduced and increased IL4 and IFNγ cytokines respectively. This highlights the potential role of CD69 in regulating the balance of Th1/Th2 responses.

e) CD69 and T regulatory cell function

A potential inhibitory role for CD69 in $CD4^+$ regulatory T cells has been suggested (Han et al., 2009; Ishikawa et al., 1998; Radstake et al., 2009; Sobel et al., 1993). Early data described an increased CD69 expression in T lymphocytes that was accompanied with a inhibitory function and an absence of any proliferative response (Ishikawa et al., 1998; Sobel et al., 1993). Immunosupression in chronically infected mice with the Friend retrovirus was also associated with an increase in the $CD4^+CD69^+$ T cell population (Iwashiro et al., 2001). An increased $CD25^+Foxp3^+CD4^+$T regulatory population with diminished CD69 expression has also been described in patients with systemic sclerosis (Radstake et al., 2009). Interestingly, lower CD69 expression correlated with diminished suppressive function and endogenous TGFβ production. Similarly, $CD69^+CD4^+CD25^-$T cells were recently described to be a new subset of T regulatory cells from tumour bearing mice that suppressed $CD4^+$T cell proliferation through membrane bound TGFβ1 (Han et al., 2009).

1.5.- CD69 ligand

Although the extrinsic ligand for CD69 has not been identified, specific cell populations expressing putative ligands have been suggested (McInnes et al., 1997; North et al., 2007). Soluble recombinant human CD69 protein was shown to interact with some tumour cells, but not with normal lymphocytes, resulting in the activation of NK cell cytotoxicity (North et al., 2007).

Protein immunoprecipitation and sequencing identified the N-terminal of calreticulin as a CD69 interacting protein at the surface of lymphocytes (Vance et al., 2005). The implication of CD69:calreticulin interaction, however, has not

been explored. Similarly, and as described above, $S1P_1$ has also been shown to interact in cis with CD69 resulting in the regulation of T and B lymphocyte egress from lymphoid organs (Shiow et al., 2006).

Whether CD69 binds to its putative ligand through a protein or glucidic interaction remains controversial. Despite crystallographic data suggested that CD69 putative binding domain closely resembled a protein-protein interacting domain rather than a carbohydrate domain (Llera et al., 2001), early biochemical studies indicated that N-acetyl-glucosamine and N-acetyl galactosamine were monosaccharide ligands for CD69 (Bezouska et al., 1995). Calcium has been shown to be essential for carbohydrate binding in many C-type lectins and, importantly, CD69 carbohydrate binding was lost in the absence of calcium. Although Asp_{171} is the only amino acid conserved from those that conform the calcium binding site in MBP, CD69 binds calcium under pH neutral conditions through this residue (Pavlicek et al., 2003).

2.- TRANSCRIPTIONAL REGULATION AND CHROMATIN STRUCTURE

Transcription is a fundamental step in gene expression and it can be divided into initiation and elongation. Transcription is initiated at gene promoters but many classes of other transcriptional regulators such as enhancers, silencers, insulators and locus control region (LCR) also participate to achieve a correct spatial and temporal expression. There are many well-documented studies about immune system molecules that are transcriptionally controlled by DNA distal elements. CD4 expression is developmentally controlled by two DNA

elements: a T-cell specific enhancer, located 13kb upstream of the gene (Sawada and Littman, 1991), and a silencer, located at the first intron and responsible for CD4 silencing in double negative (DN) and $CD4^{-}CD8^{+}$ thymocytes (Sawada et al., 1994; Zou et al., 2001). A more complex control involving multiple enhancers appears to regulate the CD8 locus (Hostert et al., 1997). There are examples of bifunctional elements, which function as both enhancers and silencers in a developmental-stage specific way (Bilic et al., 2006; He et al., 2008). Similarly, recombination activating gene (Rag)-1 and Rag-2 gene expression in DP thymocytes is achieved by the action of a distal antisilencer element that counteracts the action of silencer (Yannoutsos et al., 2004).

Transcription in eukaryotes occurs through chromatinized DNA. Chromatin is a highly ordered nucleoprotein assembly and its fundamental unit is the nucleosome. The nucleosome is composed of an octamer of the four core histones (H3, H4, H2A and H2B organized in two dimers H3-H4 and two dimers H2A-H2B) and 146bp of DNA wrapped around (Luger et al., 1997) (fig.2).

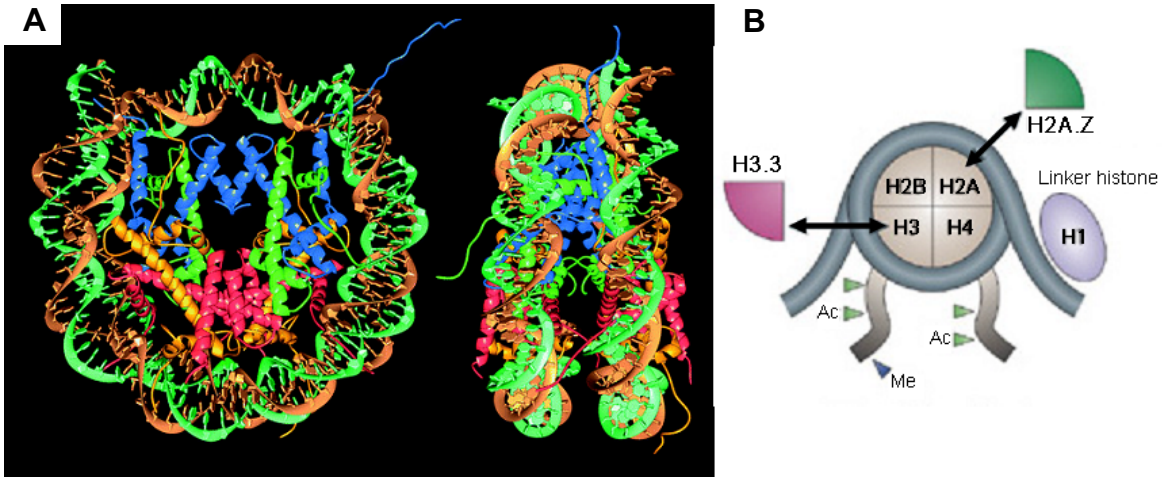


Figure 3: **Nucleosome structure**. (A) Crystal structure of the nucleosome core particle showing 146bp DNA and eight histone proteins. DNA phosphodiester backbones are shown in brown and green, histone H3 in blue, H4 in green, H2A in yellow and H2B in red (Luger et al., 1997). (B) Schematic representation of a nucleosome. Examples of histone tail modifications and histone variants H2A.Z and H3.3 are shown. Ac: acetylation, Me: methylation (Jiang and Pugh, 2009).

Chromatin functions beyond DNA compactation and changes in chromatin structure are required to open or close the DNA fiber and regulate the differential access of transcription factors and chromatin binding proteins with effector functions (Campos and Reinberg, 2009). It is generally accepted that higher order chromatin structures inhibit essential processes such as transcription. Indeed, nucleosomal templates severely inhibit transcription *in vitro* (Izban and Luse, 1991; Orphanides and Reinberg, 2000) and nucleosomal loss has been observed at many promoters upon activation including the mouse mammary tumour virus (Richard-Foy and Hager, 1987) and IL-2 promoters (Chen et al., 2005). Genome wide nucleosome maps have provided information about the organization of nucleosomes around protein-coding genes (Jiang and Pugh, 2009; Ozsolak et al., 2007; Schones et al., 2008). In human $CD4^+$ T cells, nucleosomes near the TSS of expressed or poised genes are well positioned and correlate with polymerase binding, while there is a tendency for random nucleosome positions in unexpressed genes (Ozsolak et al., 2007; Schones et al., 2008). Transcription is accompanied by disruption of histone-DNA contacts as RNA polymerase II moves along followed by reformation of nucleosomes. The mechanisms by which chromatin structure can be modulated to regulate gene expression are discussed below.

2.1.- Covalent modifications of histone tails

The core histones are predominantly globular except for their N-terminal domain, that extends out the central structure and it is often called the N-terminal “tail”. These short tails are subject to multiple post-translational modifications (PTMs) including acetylation and methylation. For the purpose of transcription, they can be divided into those that correlate with activation of gene expression and those that correlate with repression (Kouzarides, 2007).

The strong correlation between gene activation and histone acetylation was noticed long time ago (Allfrey and Mirsky, 1964). Histone acetylation occurs at multiple lysine residues and it is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add or remove acetyl groups to histones respectively. Acetylation is recognized by multiple effector proteins and it also changes the nucleosome charge that results in a more loosen DNA-histone interaction. Analysis of the chromatin structure of 30Mb of the human genome indicated that histone H3 and H4 acetylation is enriched at active promoters and enhancers (Heintzman et al., 2007). Moreover, a recent genome wide mapping have highlighted that not only HATs but also HDACs are enriched at promoters and enhancers of active genes in human $CD4^{+}$T cells and positively correlate with gene expression (Wang et al., 2009).

Methylation occurs at several times (mono-, di- or trymethylation) and the final biological output can be activation or repression of transcription. It is performed by the enzymatic activities of methylases and demethylases. Histone H3 lysine

4 methylation (H3K4me) is associated with activation of gene expression or maintenance of poised states, H3K36me with transcriptional elongation and H3K9me and H3K27me with silent genes (Barski et al., 2007). TAF3, a subnit of the basal transcription factor TFIID, and RAG2, an important mediator of V(D)J recombination, interact with H3K4me3 and are important examples of how histone marks connect to nuclear processes (Matthews et al., 2007; Vermeulen et al., 2007).

In the immune system, the differentiation of $CD4^+$T cells into mature effector cells involves multiple epigenetic changes at remarkable cytokine genes (Akimzhanov et al., 2007; Ansel et al., 2003; Avni et al., 2002; Lee et al., 2006). In Th2 cells, histone acetylation and H3K4 trimethylation are increased at the IL-4 promoter but not at IFNγ and IL-17 promoters. Conversely, in Th1 cells, these marks are enriched at the IFNγ promoter but not at the IL-4 and IL-17 promoters. Finally, under Th17 polarizing conditions, only the IL-17 and IL-17R genes become H3 acetylated and K4 trimethylated.

2.2.- Chromatin remodellers

Chromatin remodelling complexes are ATP-dependent enzymes that alter the position of nucleosomes. In mammals, the most studied chromatin remodellers are SWI/SNF and Mi-2/NurD complexes. SWI/SNF complexes contain as ATPase subunits BRG1 and BRM proteins and Mi-2/NurD complexes contain Mi-2α and Mi-2β subunits (Chi, 2004; de la Serna et al., 2006).

Early studies on inducible transcription in mammalian cells suggested that chromatin remodelling was important for gene activation. It was shown that INFβ gene induction after viral infection required the removing of a positioned nucleosome at the transcriptional initiation site by SWI/SNF complexes (Agalioti et al., 2000). Similarly, the inducible IL-12 promoter contained a positioned nucleosome in the vicinity of the transcription initiation site that was selectively remodelled upon LPS stimulation (Weinmann et al., 1999). Another study, however, reported the existence of early response genes that were independent of chromatin remodelling activities for their activation (Ramirez-Carrozzi et al., 2009). They classified inflammatory induced genes into primary response genes (PRG) such as Cxcl2 (encoding MIP-2), tnf or Ptgs2 (COX-2), that were independent of SWI/SNF activities and presented constitutive accessibility to nucleases, and secondary response genes (SRG), such as Ccl5 (RANTES), Il6, Il12b, Nos2 (iNOS) or IFNb1, that were SWI/SNF dependent, were expressed with delayed kinetics and presented inducible accessibility to nuclease digestion.

2.3.- Histone variants

Incorporation of histone variants is another way of modifying chromatin structure. H3.3 and CENPA are variants for canonical histone H3 while H2A.X, H2A.Z, macroH2A and H2ABBD for canonical H2A (Chang and Aune, 2007). H3.3 and H2A.Z are the best characterized. Recent evidence suggests that H2AZ is enriched at promoters of active or poised genes and H3.3 is associated with active transcription in human cells (Barski et al., 2007; Sutcliffe et al., 2009).

2.4.- DNA methylation

DNA methylation consists in the addition of methyl groups to the cytosine of CpG doublets in DNA. Low amounts of methylation are associated with high levels of gene expression and, conversely, high levels of methylation are associated with low levels of gene expression. Epigenetic regulation by CpG methylation has been shown to be an important regulator of the expression of different cytokines genes such as IL-2 (Bruniquel and Schwartz, 2003) and IL-4 (Lee et al., 2002). Similarly, demethylation of CpG islands at specific sites of the Foxp3 locus is associated with stable expression of FoxP3 in regulatory T cells (Kim and Leonard, 2007; Lal et al., 2009).

2.5.- Conserved non coding sequences

DNA regulatory elements such as enhancers, silencers or insulators can be widely dispersed in the genome and, before whole-genome sequences became available, their identification was accomplished by testing large segment of genomic DNA typically for their ability to drive gene expression in transgenic mice (Hostert et al., 1997). In the past few years, genome sequence comparison between different species has emerged as a useful indicator of dispersed DNA sequences with regulatory activity (Fields et al., 2004; Hatton et al., 2006; Loots et al., 2000; Nardone et al., 2004; Shnyreva et al., 2004). Vista Browser is one of the several comparative genomic programs successfully used to detect conserved non coding sequences (CNSs) (Loots et al., 2000). It is based on whole-genome alignments of many species and it is available at the web page http://www-gsd.lbl.gov/vista. Once a CNS has been identified, its

biological activity may be detected through different approaches (Nardone et al., 2004):

a) DNase I hypersensitivity

Hypersensitive sites (HSs) are genomic regions that present increased sensitivity to cleavage by DNase I as a result of altered nucleosomal structure (Gross and Garrard, 1988). Notably, CNSs often colocalized with constitutive or developmentally regulated HS sites (Fields et al., 2004; Shnyreva et al., 2004).

b) Chromatin immunopreciptation

Chromatin immunoprecipitation (Chip) is a technique in which a DNA binding protein is selectively immunoprecipitated to determine the DNA sequence associated with it. Analysis of histone post-translational modifications or histone variants may be performed on purified nucleosomes preparations (McMurry and Krangel, 2000) or sonicated cross-linked chromatin (Chang and Aune, 2007). Recently, significant effort has been made in characterizing the chromatin structure in a genome wide scale in mammalian cells using Chip assays (Barski et al., 2007; Boyle et al., 2008; Schones et al., 2008; Wang et al., 2009).

c) Reporter assays

To test their function, CNSs are normally linked to a reporter gene and assayed in transient transfection assays. These experiments, however, do not account for potential influences of the chromatin environment. Analysis in a chromosomal context may provide more reliable information about the *in vivo* function and it can be achieved with the generation of transgenic mice

containing CNS elements linked to reporter genes such as lacZ, hCD2 or GFP (Decker et al., 2009; Pennacchio et al., 2006; Sawada et al., 1994).

d) Targeted disruption

Generation of mice containing deletions or mutations of CNSs can be done on the endogenous CNS or in large bacterial or yeast artificial chromosomes. Individual deletions of different CNSs may uncover important developmental- and stage-specific regulatory roles as recently described for the Foxp3 locus (Zheng et al., 2010).

3.- Resum introducció (català)

CD69: UNA MOLÈCULA DEL SISTEMA IMMUNE

Distribució tissular de l'expressió de CD69

CD69 és una proteïna transmembrana identificada a partir de la generació d'anticossos monoclonals específics contra antígens expressats en cèl·lules activades de sang perifèrica (Hara, Jung et al. 1986; Cosulich, Rubartelli et al. 1987; Cebrián, Yague et al. 1988; Lanier, Buck et al. 1988). Assajos d'immunoprecipitació van revelar que CD69 és una proteïna homodimérica unida per ponts disulfur de 60 kDa de pes. A més, les dues subunitats estan constitutivament fosforilades i presenten glicosilació variable.

CD69 s'expressa àmpliament en cèl·lules derivades del moll de l'os. Així, plaquetes, monòcits, cèl·lules de Langerhans, cèl·lules dendrítiques i una una fracció petita de limfòcits no estimulats de sang perifèrica expressen constitutivament CD69 (Testi, Pulcinelli et al. 1990; Bieber, Rieger et al. 1992; De Maria, Cifone et al. 1994; Gao, Majchrzak-Kita et al. 2009). Al tim, CD69 s'expressa en la superfície d'aproximadament el 10-15% de timocits $CD4^+CD8^+$ en processos de selecció positiva i en timocits $CD4^+CD8^-$ i $CD4^-CD8^+$ en processos de maduració (Swat, Dessing et al. 1993; Yamashita, Nagata et al. 1993). In vitro, l'estimulació de cèl·lules T amb anticossos anti-CD3/CD28 (Testi, Phillips et al. 1989), anti-CD2/CD28 (Risso, Smilovich et al. 1991), superantígens virals i bacterians (Lc Bon, Lucas et al. 1996; Lina, Cozon et al. 1998), èsters de forbol i un xoc tèrmic (Risso, Smilovich et al.

1991) també n'indueixen l'expressió. En les cèl·lules NK, CD69 pot ser induït amb IL-2, INFα, anticossos anti-CD16, cèl·lules tumorals (Nord, Bakhsh, et al. 2007) i èsters de forbol. En macròfags derivats de medul·la òssia, amb lipolisacàrid bacterià (Marzio, Jirillo et al. 1997) i en eosinòfils humans amb GM-CSF o IL-13 (Hartnell, Robinson et al. 1993; Luttmann, Knoechel et al. 1996).

L'expressió de CD69 és elevada en les cèl·lules NK i els limfòcits T d'infiltrats tumorals (Van den Hove, Van Gool et al. 1997), en eosinòfils broncoalveolar de pacients amb asma i pneumònia (Nishikawa, Morii et al. 1992; Hartnell, Robinson et al. 1993) i en cèl·lules T i neutròfils d'infiltrats sinovials en artritis reumatoide (Iannone, Corrigal et al. 1996; Atzeni, Del Papa et al. 2004). L'expressió de CD69 també s'ha observat en un gran percentatge de pacients amb leucèmia de cèl·lules B i es correlaciona amb mal pronòstic de la malaltia (D'Arena, Musto et al. 2001). Recentment s'ha descrit que pacients amb esclerosi múltiple presenten un augment de la població de cèl·lules T reguladores amb una expressió de CD69 i capacitat supressora, ambdues disminuïdes (Radstake, Van Bon et al. 2009).

Regulació de l'expressió i estructura del gen de CD69

Diferents estudis han demostrat que les vies de senyalització que condueixen a l'expressió CD69 en limfòcits T inclouen l'activació de les proteïnes cinasa C (PKC), Ras, Vav i Raf i l'activació de vies depenents de calci (Cebrian et al., 1989; Taylor-Fishwick and Siegel, 1995; Villalba et al.,

2000a; Villalba et al., 2000b). D'altra banda, les vies de senyalització en les cèl·lules NK són independents de la PKC i impliquen l'activació de proteïnes tirosina cinases (Borrego, Peña et al. 1993).

El clonatge molecular del cDNA de CD69 va ser assolit per diferent grups (Hamann, Fiebig et al. 1993; López-Cabrera, Santis, et al. 1993; Ziegler, Ramsdell et al. 1993). El seu mapatge cromosòmic va demostrar que CD69 estava codificat en un únic gen i es localitzava en el complex gènic NK en el cromosoma 6 de ratolí i 12 d'humà (López-Cabrera, Santis, et al. 1993; Schnittger, Hamann et al. 1993; Ziegler, Levin et al. 1994). L'estructura del gen CD69 està altament conservada entre humà i ratolí. El gen murí té aproximadament 7.5Kb i conté 5 exons. Els exons I i II, amb 64 i 123 parells de bases respectivament, codifiquen per a les regions intracel·lular i transmembrana. Els exons III, IV i V, amb 200, 104 i 106 parell de bases respectivament, codifiquen pel domini extracel·lular.

L'expressió de CD69 està fortament controlada a nivell de la transcripció (Cebrián, Redondo et al. 1989; Testi, Phillips et al. 1989; Ziegler, Levin et al. 1994; Sutcliffe, Parish et al. 2009). Els transcrits presenten una cinetica ràpida d'expressió. Es detecten 30-60 minuts després de processos d'activació, essent màxims a les 4-6 hores i després d'aquest període comencen a disminuir.

Els promotors d'humà i ratolí han estat clonats, ambdós contenen una

seqüència consens TATA amunt del lloc de l'inici de la transcripció i són capaços de dirigir l'activitat d'un gen reporter en cèl·lules estimulades (López-Cabrera, Santis, et al. 1.993 ; Ziegler, Ramsdell et al. 1993). Els elements en *cis* que contribueixen a aquesta inducció s'assignen a la regió promotora proximal, en la qual interaccionen els factors de transcripció Erg-1, Erg-3, ATF-3/CREB i AP-1 (Castellanos et al., 1997; Castellanos Mdel et al., 2002). Una activitat basal promotora també ha estat descrita per transfecció transitòria i s'atribueix a la regió també proximal amb posicions -78 a +16 del gen humà. Curiosament, el factor de transcripció Sp1 s'uneix en aquesta regió en la posició -56. En un altre estudi, un motiu NFκB en la posició -223 del promotor de CD69 humà es va demostrar que era necessària per a la inducció de la transcripció de CD69 en resposta a TNF (López-Cabrera, Muñoz et al. 1995).

Assaigs d'immunoprecipitació de cromatina han demostrat que la polimerasa II està precarregada al promotor CD69 humà en les cèl·lules $CD4^+$ T I la línea T Jurkat abans de la transcripció (Barski, Cuddapah et al. 2007; Sutcliffe, Parish et al. 2009). L'activació del gen CD69 s'associa amb canvis en l'estructura de la cromatina (Sutcliffe, Parish et al. 2009). En l'estat de repòs, i segurament per mantenir el gen en un estat preparat (Jin i Felsenfeld 2007), el promotor de CD69 està flanquejat per les histones H2AZ i H3. L'activació de la transcripció provoca una depleció de H2AZ i H3 i una incorporació de la histona H3.3, que s'associa amb transcripció activa.

Estudi de la funció *in vivo* de CD69

CD69 i la migració dels limfòcits

Diferents estudis suggereixen que CD69 podria participar en el control de la sortida dels limfòcits dels òrgans limfoides. La sobreexpressió de CD69 en cèl·lules T de ratolins mitjançant transgènesi s'associa amb la retenció timocits madurs i la conseqüent reducció de cèl·lules T en perifèria (Feng, Woodside et al. 2002; Nakayama, Kasprowicz et al. 2002).

D'altra banda, s'ha demostrat que CD69 inhibeix la sortida dels limfòcits dels òrgans limfoides a través de la seva interacció en cis i *down* modulació dels receptors $S1P_1$ a la superfície cel·lular (Shiow, Rosen et al. 2006). És important destacar que l'expressió CD69 és elevada en ratolins deficients per $S1P_1$ i que $S1P_1$ va ser identificat en un *screening* de reguladors negatius de CD69 en cèl·lules Jurkat activades (Chu, Pardo et al. 2003; Matloubian, Lo et al. 2004).

El paper de CD69 durant una resposta immunitària

Tot i que les dades *in vitro* suggerien que CD69 podia tenir un paper proinflamatori, les dades en diferent models animals de malalties humanes en ratolins $CD69^{-/-}$ van revelar un nou paper com a proteïna immunomoduladora.

Els ratolins deficients per CD69 tenen un desenvolupament normal de cèl·lules T i només tenen un petit increment del compartiment pre-B en el

moll de l'òs (Lauzurica, Sancho et al. 2000). Tot i això, tenen una major resistència al creixement de tumors negatius per a la molècula MHC de classe I associada a un major reclutament de cèl·lules NK i limfòcits T i a una producció alterada de les citocines MCP-1, IL-12, IL-1α, IL-1β i TGFβ. En un model d'artritis induïda per col·lagen de tipus II (CIA), els ratolins deficients per CD69 desenvolupaven una forma exacerbada de la malaltia (Sancho, Gómez et al. 2003). A la melsa, hi havia una resposta proliferativa específica de limfòcits T augmentada i uns nivells d'anticossos IgG també augmentats. A les articulacions, els nivells citocines IL-1β, RANTES, proteïna dels macròfags-1α (MIP-1α) i el MIP-1β estaven incrementades i els TGFβ1 i TGF-β2 disminuïts. En un altre model d'artritis reumatoide que consisteix en la injecció d'anticossos anti-col·lagen seguit d'injeccions amb LPS, es va observar que el desenvolupament de la malaltia era menys sever en els ratolins $CD69^{-/-}$ havent-hi un nombre reduïts de neutròfils infiltrants i nivells disminuïts de les citocines proinflamatòries MIP-1α i MCP-1 i CXCL1 a les articulacions artrítiques.

Un recent informe ha estudiat el paper de CD69 en la patogènesi de l'asma utilitzant com a model una inflamació induïda eosinofílica de tipus Th2 (Miki-Hosokawa, Hasegawa et al. 2009). Ratolins deficients en CD69 desenvolupaven una forma atenuada de la malaltia com a conseqüència de la disminució del nombre d'eosinòfils, les citocines IL-4, IL-13 i IL-5 en els líquids broncoalveolar i la proteïna VCAM-1 en el pulmó asmàtic. És important destacar que, en l'avaluació de la diferenciació dels limfòcits cap al llinatge

Th2, es va posar de manifest un perfil IL4/IFNγ alterat amb reducció de IL-4 i augment IFNγ. Aquests resultats posen de relleu el paper potencial de CD69 en la regulació de l'equilibri de les respostes Th1/Th2.

Lligands per CD69

Encara que el lligand extrínsec de CD69 no ha estat identificat, poblacions de cèl·lules que expressen potencials lligands han estat suggerides (McInnes, Leung et al. 1997; Nord, Bakhsh, et al. 2007).

Per immunoprecipitació de proteïnes es va identificar la regió N-terminal de la calreticulina com una proteïna que interaccionava amb CD69 en la superfície dels limfòcits (Vance, Harley et al. 2005). Les implicacions d'aquesta interacció, però, no han estat explorades. Així mateix, $S1P_1$ també interactua amb CD69 a la superfície cel·lular i el resultat d'aquesta interacció és la regulació de la sortida T i limfòcits B dels òrgans limfoides.

Saber del cert si CD69 s'uneix al seu lligand putatiu a través d'una interacció proteica o d'una glucídica segueix essent un tema controvertit. Tot i que les dades cristal·logràfiques suggereixen que el domini d'unió de CD69 al seu lligand s'assembla al domini d'interacció de proteïnes (Llera, Viedma et al. 2001), estudis bioquímics van indicar que l'N-acetil-glucosamina i N-acetil-galactosamina són lligands monosacàrids per CD69 (Bezouska, Nepovim et al. 1995). D'altra banda, la unió a aquests hidrats de carboni es perd en absència de calci. Encara que l'aminoàcid Asp_{171} és l'únic aminoàcid

conservat dels que conformen el lloc d'unió a calci, CD69 s'uneix al calci en condicions de pH neutre a través d'aquest residu (Pavlicek, Sopko et al. 2003).

REGULACIÓ DE LA TRANSCRIPCIÓ I ESTRUCTURA DE LA CROMATINA

La transcripció és un pas fonamental en l'expressió gènica. Malgrat que la transcripció s'inicia al promotors dels gens, la participació d'altres reguladors com *enhancers*, silenciadors o aïllants és clau per aconseguir una correcta expressió espacial i temporal dels gens. Moltes molècules essencials del sistema immune són controlades transcripcionalment per elements distals d'ADN. Un cas molt ben documentat és el del coreceptor CD4, l'expressió del qual està controlat per un *enhancer* específic de cèl·lules T, situat a 13kb sobre el gen (Sawada i Littman 1991), i un silenciador, situat en el primer intró i responsable de silenciar CD4 durant el desenvolupament de les cèl·lules CD8 (Sawada , Scarborough et al. 1994; Zou, Sunshine et al. 2001). Un control més complex que involucra múltiples enhancers sembla regular el locus CD8 (Hostert, Tolaini et al. 1997). Hi ha exemples d'elements bifuncionals, que funcionen com a enhancers i silenciadors de forma específica en el desenvolupament com és el cas del factor de transcripció Th-Pok (Bilic, Koesters et al. 2006; Ell, Park et al. 2008).

La transcripció en eucariotes es produeix a partir de l'ADN organitzat en un complex nucleoproteic anomenat "*cromatina*". La unitat fonamental de la cromatina és el nucleosoma, que es compon d'un octàmer de les quatre

principals histones (H3, H4, H2A i H2B organitzat en dos dímers H3-H4 i dos dímers H2A-H2B) i 146bp d'ADN embolicat (Luger, Mader et al. 1997). Les funcions de la cromatina van més enllà de compactació de l'ADN. De fet, canvis en l'estructura de la cromatina són requerits per obrir o tancar la fibra d'ADN, i regular així l'accés diferencial dels factors de transcripció i altres proteïnes amb funcions efectores (Campos i Reinberg 2009). És generalment acceptat que les estructures d'ordre superior de la cromatina inhibeixen la transcripció. De fet, *arrays* de nucleosomes inhibeixen greument la transcripció *in vitro* (Izban i Luse 1991; Orphanides i Reinberg 2000) i la pèrdua de nucleosomes s'ha observat en molts promotors després de ser activats (Richard-Foy i Hager, 1987) (Chen, Wang et al. 2005). Hi ha una sèrie de mecanismes pels quals l'estructura de la cromatina pot ser modulada i, d'aquesta manera, regular l'accessibilitat d'ADN I l'expressió gènica:

Modificació covalent de les histones

Les histones són predominantment globulars a excepció del seu domini N-terminal, que s'estén cap enfora de l'estructura central i és sovint anomenat "cua" N-terminal. Aquestes cues són objecte de múltiples modificacions post translacionals, incloent-hi l'acetilació i la metilació (Kouzarides 2007).

La correlació entre l'activació gènica i l'acetilació de les histones va ser observada fa molt de temps (Allfrey i Mirsky 1964). L'acetilació de les histones es produeix en múltiples residus de lisina i és controlada per l'acció d'acetiltransferases i deacetilases. L'acetilació canvia la càrrega dels

nucleosomes i resulta en una interacció més laxa entre l'ADN i les histones i en el reconeixement de múltiples proteïnes efectores. La metilació pot produir-se diferents vegades (mono-, bi- o trimetilació) en residus de lisines per l'acció de metilases i demetilases i la conseqüència biològica pot ser d'activació o la repressió de la transcripció. Les metilacions a lee lisines 4 i 36 de la histona H3 (H3K4me i H3K36me) estan associades amb l'activació de l'expressió gènica o el manteniment dels estats a punt d'expressar, mentre que les metilacions H3K9, H3K27 i H4K20 estan associats amb la repressió.

L'anàlisi de l'estructura de la cromatina de 30Mb del genoma humà indica que l'acetilació i la metilació a la lisina 4 de les histones H3 i H4 estan enriquides en promotors actius i *enhancers* (Heintzman, Stuart et al. 2007). A més, un recent mapeig de tot el genoma ha posat en relleu el paper de les deacetilases en el control de gens actius en cèl·lules T $CD4^+$ humanes (Wang, Zang et al . 2009).

Enzims remodeladors de la cromatina:

Es tracta d'enzims que usen l'energia d'hidròlisi de l'ATP per reposicionar els nucleosomes de la cromatina i així fer-la més accessible a les proteïnes d'unió a ADN. En els mamífers, el remodeladors més estudiats són els complexos SWI/SNF i Mi-2/NurD. Estudis sobre la transcripció induïble en cèl·lules de mamífers, van suggerir que la remodelació de la cromatina era important per a l'activació d'aquests gens. Es va demostrar que la inducció

de gen de l'IFNβ després d'una infecció viral requeria l'eliminació d'un nucleosoma posicionat en l'inici de la transcripció per complexos SWI/SNF (Agalioti, Lomvardas et al. 2000). De la mateixa manera, el promotor induïble de la Il-12 conté un nucleosoma ben posicionat en les proximitats de l'inici de la transcripció que és selectivament desplaçat després d'una activació amb LPS. Posteriorment, es va veure que no tots els gens induïbles requereixen una activitat remodeladora en els seus promotors per iniciar la transcripció. La inhibició de l'expressió dels complexes SWI/SNF mitjançant la tècnica del RNA d'interferència, va mostrar que una gran quantitat de gens de resposta primerenca durant una resposta inflamatòria activa, com ho són els genes Cxcl2 (codifica per de MIP-2), Tnf o Ptgs2 (COX-2), són independents de l'activitat SWI/SNF i presenten una accessibilitat constitutiva a les nucleases. Altres gens, com els Ccl5 (RANTES), Il6, Il12b, Nos2 (iNOS) o Ifnb1 depenen d'SWI /SNF, i s'expressen amb una cinètica una mica més lenta i presenten una accessibilitat induïble a la digestió per nucleases (Ramírez-Carrozzi, Nazarian et al. 2006)

Variants d'histones

La majoria dels canvis d'histones es produeixen en les histones H3 i H2A. L'anàlisi del genoma va mostrar que la variant H2A.Z està enriquida en els nucleosomes -2 i +1 de gens actius o a punt d'expressar-se (Barski et al., 2007). Es creu que H2A.Z té un paper en el manteniment dels gens en un estat preparat, mentre que H3.3 s'associa amb la transcripció activa.

Metilació de l'ADN

Aquest procés passa més sovint a la citosina de doblets GC de l'ADN. Baixes quantitats de metilació estan associats a alts nivells d'expressió de gens i, per contra, els alts nivells de metilació estan associats amb baixos nivells d'expressió de gens. La regulació epigenètica per metilació CpG és important en l'expressió de gens de diferents citocines com la IL-2 (Bruniquel i Schwartz 2003) i l'IL-4 (Lee, Agarwal et al. 2002).

Regions conservades no codificants

Els elements d'ADN reguladors de l'expressió gènica, com ara els *enhancers*, silenciadors o aïllants poden ser molt dispersos en el genoma i, abans de disposar de la seqüència del genoma sencer, la seva identificació es realitzava mitjançant el testatge de grans segment d'ADN genòmic, generalment per la seva capacitat per conduir l'expressió gènica en ratolins transgènics. En els darrers anys, la comparació genòmica entre diferents espècies s'ha convertit en un indicador útil d'elements d'ADN amb activitat reguladora, donat que se solen conservar en seqüència (Loots, Locksley et al. 2000; Camps, Lee et al. 2004; Nardone, Lee et al. 2004; Shnyreva, Weaver et al. 2004; Hatton, Harrington et al. 2006). Aquestes regions conservades en seqüència no codificants s'anomenen CNS (*Conserved non coding sequence*) i Vista Browser és un dels diversos programes de genòmica comparativa utilitzat amb èxit per detectar-les (Frazer, Pachtas et al. 2004). Vista Browser es basa fonamentalment en les alineacions del genoma de moltes espècies i està disponible a la pagina web http://www-

gsd.lbl.gov/vista. Una vegada que un CNS ha estat identificat, la seva activitat biològica pot ser detectada a través d'assajos d'hipersensibilitat a la DNasa I, assajos de gens reporters en ratolins transgènics o transfeccions transitòries, delecions especifiques *in vivo* o per immunoprecipitació de la cromatina (Nardone, Lee et al. 2004).

II. OBJECTIVES

The goals of this thesis are:

1. To identify potential DNA elements by cross-species DNA sequence comparison important for the transcriptional regulation of the CD69 gene.

2. To characterize the chromatin structure of the newly identify DNA elements and the CD69 promoter.

3. To analyse the *in vitro* and *in vivo* function of conserved non coding sequences by transient transfection assays and transgenic mice.

III. RESULTS AND DISCUSSION

CHAPTER 1

El gen de CD69 està regulat de manera diferencial en cèl.lules T i B per elements conservats distals al promotor

Resum

CD69 és una lectina tipus C amb orientació tipus II, que participa en la migració de limfòcits i la secreció de citocines. L'expressió de CD69 és un dels indicadors més primerencs disponibles de l'activació dels leucòcits i la seva ràpida inducció es produeix a través de l'activació de la transcripció. En aquest estudi es va examinar el mecanisme molecular subjacent en la regulació transcripcional del gen de CD69 de ratolí *in vivo* en cèl·lules T i B. L'anàlisi de la regió 45kb per sobre del gen CD69 va revelar conservació evolutiva en el promotor i en quatre seqüències no codificants (CNS) que van ser anomenats CNS1, CNS2, CNS3 i CNS4. Es van trobar regions d'hipersensibilitat en experiments d'hipersensibilitat a la digestió per DNasa I i assajos d'immunoprecipitació de cromatina van mostrar modificacions epigenètiques específiques. En assajos de transfecció transitòria en cèl·lules T, CNS2 i CNS4 mostraven una activitat enhancer constitutiva i induïble. Per testar la funció dels diferents CNS es van fer ratolins transgènics i es va trobar que el promotor conferia una expressió regulada durant la selecció positiva dels timocits, però no podia donar suport a una expressió regulada en limfòcits madurs. La inclusió de CNS1 i CNS2 va causar la supressió de l'expressió de CD69 i l'addició de CNS3 i CNS4 va donar lloc a una expressió regulada a les cèl·lules T, però no a les cèl·lules B. Vam concloure que CNS1-4 són elements reguladors importants que interactuen tant positivament com negativament amb el promotor de CD69 i que contribueixen de manera diferent, a l'expressió CD69 a les cèl·lules T i B.

CD69 gene is differentially regulated in T and B cells by evolutionarily conserved promoter-distal elements

Summary

CD69 is a type II C-type lectin involved in lymphocyte migration and cytokine secretion. CD69 expression represents one of the earliest available indicators of leukocyte activation and its rapid induction occurs through transcriptional activation. In this study we examined the molecular mechanism underlying mouse CD69 gene transcription *in vivo* in T and B cells. Analysis of the 45kb region upstream of the CD69 gene revealed evolutionary conservation at the promoter and at four non-coding sequences (CNS) that were called CNS1, CNS2, CNS3 and CNS4. These regions were found to be hypersensitive sites in DNase I digestion experiments and chromatin immunoprecipitation assays showed specific epigenetic modifications. CNS2 and CNS4 displayed constitutive and inducible enhancer activity in transient transfection assays in T cells. Using a transgenic approach to test CNS function, we found that the CD69 promoter conferred developmentally regulated expression during positive selection of thymocytes but could not support regulated expression in mature lymphocytes. Inclusion of CNS1 and CNS2 caused suppression of CD69 expression whereas further addition of CNS3 and CNS4 supported developmental-stage and lineage-specific regulation in T cells but not in B cells. We concluded CNS1-4 are important cis-regulatory elements that interact both positively and negatively with the CD69 promoter and that differentially contribute to CD69 expression in T and B cells.

Introduction

Leukocyte activation involves coordinated changes in the expression of key genes involved in the inflammatory cascade and leukocyte migration to promote effective immune responses against diverse pathogens and malignant cells. Recently, several reports using *in vivo* animal models have highlighted the role of the CD69 membrane molecule in both cytokine gene regulation and cell migration upon leukocyte activation. CD69 has been shown to be involved in the inhibition of lymphocyte egress from lymphoid organs in response to INF$\alpha\beta$ through a mechanism that involves downregulation of $S1P_1$ receptors (Shiow et al., 2006). Overexpression of CD69 in transgenic mice supports a role in thymocyte migration (Feng et al., 2002; Shinkai et al., 1993). CD69 deficient mice display enhanced resistance to MHC class Ĩ tumor growth and increased susceptibility to collagen induced arthritis and *Listeria M*. infection, associated with increased cellular recruitment and altered cytokine production and apoptosis (Esplugues et al., 2003; Sancho et al., 2003). Moreover, *in vivo* blocking of CD69 with monoclonal antibodies resulted in exacerbated autoimmune and antitumor responses (Esplugues et al., 2005). Recently, it has been demonstrated that $CD69^+CD4^+CD25^-$ cells represent a new subset of regulatory T cells involved in tumor-induced immunosuppression (Han et al., 2009). Although CD69 is expressed during lymphocyte development (Yamashita et al., 1993), positive and negative selection of thymocytes is normal in CD69 deficient mice and only minor alterations in the pre-B cell compartment have been detected (Lauzurica et al., 2000).

The CD69 gene is located in the natural killer complex (NKC) on mouse chromosome 6 and human chromosome 12. This complex includes a variety of genes encoding C-type lectins with diverse expression patterns and functions in the immune system (Kelley et al., 2005). CD69 is expressed on the surface of activated leukocytes through a mechanism that involves ras and raf activation and calcium release (D'Ambrosio et al., 1994; Taylor-Fishwick and Siegel, 1995). A variety of agents, including anti-CD3 antibodies, TNFα, INFαβ, polyI:C or phorbol esters, can up-regulate CD69 *in vitro*. Transcripts are detected as early as 30 min after T cell stimulation and cell surface protein is observed 3h later (Ziegler et al., 1994). CD69 transcription, however, is transient and returns to an "off" state at later times (Lopez-Cabrera et al., 1993). Transient transfection experiments showed that both the mouse and human CD69 promoters can direct reporter transcription in cells stimulated with PMA plus ionomycin (Lopez-Cabrera et al., 1993; Ziegler et al., 1993). C*is*-elements contributing to this inducibility were mapped to the proximal promoter region and these elements were shown to interact with transcription factors Erg-1, Erg-3, ATF-3/CREB and AP-1 upon stimulation (Castellanos Mdel et al., 2002). Basal CD69 transcription was also detected by transient transfection of mouse and human promoter constructs and was attributed to the -78 to +16 region of the human CD69 gene. Interestingly, the transcription factor Sp1 was shown to constitutively bind to this region. In another study, an NFκB motif at position -223 of the human CD69 promoter was shown to be required for transcriptional induction of CD69 in response to TNFα (Lopez-Cabrera et al., 1995).

Transient transfection assays do not account for potential influences of the chromatin environment, which may require the action of distal enhancers, silencers and insulators for efficient gene expression, and may require DNA methylation and histone modifications to regulate access to transcription factors. Epigenetic regulation has been shown to be critical for inducible expression of a variety of immune system genes, including IL-4, INFγ, INFβ and IL-12 (Agalioti et al., 2000; Hatton et al., 2006; Lee et al., 2001; Weinmann et al., 1999). Therefore, the goal of this study was to define the epigenetic changes and *cis*-acting elements important for regulated CD69 gene expression in lymphocytes *in vivo*. We searched for distal regulatory elements using cross-species sequence comparison, DNase I hypersensitivity mapping and transcriptional activity analysis in transient transfection assays and we then used a transgenic approach to test the functional significance of candidate *cis*-regulatory elements *in vivo*. Our results indicate that unusual as well as common transcriptional regulatory mechanisms control expression of the CD69 gene in different lymphocyte populations.

Materials and Methods

Comparative genomic analysis

Sequence comparison of mouse, human and dog CD69 was performed using VISTA Browser from the Lawrence Berkeley National Laboratory and available at http://pipeline.lbl.gov/cgi-bin/gateway2.

DNase I Hypersensitivity assay

Rag2$^{-/-}$x *Tcrb* transgenic mice (RXβ) and hemaglutinin (HA)-TCR transgenic mice were described previously (Morgan et al., 1996; Shinkai et al., 1993). Red blood cells were lysed in 0.15M NH_4Cl, 1mM $KHCO_3$, 1mM Na_2-EDTA, pH 7.4 for 3min at 4ºC and subsequently washed in 30ml of cold PBS. Thymocytes (10^7/ml) were permeabilized with 0.067mg/ml lysolecithicin in buffer C (0.15M Sucrose, 80mM KCl, 30mM Hepes pH7.4, 5mM $MgCl_2$, 5m $CaCl_2$) for 5 minutes. DNase I was added at a final concentration of 0, 4, 8, 12, 16, 20, 24, 28, or 32 units/ml for 10min on ice. Reactions were stopped by the addition of EDTA, SDS and Proteinase K to final concentrations of 10mM, 0.4% (w/vol) and 0.4mg/ml, respectively, and were incubated overnight at 37ºC. DNA was purified by phenol, phenol:chloroform and chloroform extractions and ethanol precipitation, taking care not to shear the genomic DNA. Purified DNA (10μg) was incubated overnight at 37ºC with an excess of EcoRI restriction enzyme. Digests were separated by 0.7% (wt/vol) agarose gel electrophoresis and were analysed by Southern blot with ^{32}P-labeled DNA probes (primers to generate probes, Supplementary Table 1).

Reporter constructs and transgenic mice

hCD2 reporter constructs were generated by modification of the mCD8 reporter vector (Bilic et al., 2006). To generate construct 1 (fig. 4B), the promoter of mCD69 (-645/+1) was PCR amplified (Expand High Fidelity PCR System, Roche) and cloned into pstBlue vector (Vector Acceptor Kit, Novagen). Restriction sites introduced during PCR amplification were used to excise the mCD69 promoter from this plasmid with ApaI and ClaI and ligate it to the

ApaI/ClaI digested hCD2 construct. To ensure that we incorporated conserved sequences and DNase I hypersensitive sites, primers for genomic regions encompassing CNS1 (233bp), CNS2 (451bp), CNS3 (439bp) and CNS4 (181bp) were designed (primer sequences, Supplementary table 1). PCR products for CNS1-4 fragments were 807bp, 932bp, 600bp and 707bp in length respectively. To generate construct 2, CNS1 and CNS2 were first combined into pstBlue cloning vector. CNS2/CNS1 fragment was then excised from this construct using ApaI and XbaI and inserted into XbaI/ApaI sites upstream of the mCD69 promoter in construct 1. Similarly, to make construct 3, CNS3 and CNS4 were combined into pstBlue, digested with XbaI and inserted into the XbaI site upstream of CNS2 in construct 2. Importantly, NotI restriction sites were flanking all three hCD2 reporter constructs. Construct 1 had a size of 3.7 kb and was separated from vector backbone using ApaI/NotI digestion. Construct 2 was 5.4 kb and was separated with XbaI/NotI digestion and construct 3, with a size of 6.7 kb, with NotI digestion. Purified DNA was then microinjected into fertilized eggs. Founders were identified by PCR and copy number was determined by quantitative real-time PCR. Input DNA was determined using primers ExonIIIF and ExonIIIR (primers sequences, supplementary table 1). Handling of mice and experimental procedures were in accordance with institutional requirements for animal care and use.

Purification of cell populations and cell culture

To enrich for T cell populations, lymph node cell suspensions were passed through a nylon wool column (Polysciences) following the manufacturer's instructions. T cell purity, determined by flow cytometry, was >85%.

Splenocytes of TCRβ$^{-/-}$TCRδ$^{-/-}$ mice were used to obtain B cells (>95% B220^{+} by flow cytometry).

Prior to chromatin immunoprecipitation analysis of peripheral lymphocyte populations, cells were stimulated *in vitro* with 10ng/ml PMA (Sigma Biochemical) and 0.5μM Calcium Ionophore (Sigma) for 6h at 37ºC. CD69 expression was then analyzed by flow cytometry.

For hCD2 analysis in peripheral lymphocytes, splenocytes of wild-type and transgenic mice were activated by incubation with 10ng/ml PMA and 1μM Io or 5μg/ml plate-bound anti-CD3 (clone 145-2C11, eBiosciences) and anti-CD28 (clone 37.51, eBiosciences) monoclonal antibodies overnight at 37ºC. In experiments using PMA plus ionomycin, data was collected from experiments in which activated lymphocytes were >90% CD69^{+}.

Poly (I:C) treatment

Mice were injected intraperitoneally (i.p.) with 500μg of Poly (I:C) (Sigma). After 18h of treatment, spleens were obtained from animals and lymphocyte suspensions were prepared for FACS analysis.

Chromatin immunoprecipitation

For analysis of thymocytes, immunoprecipitations were performed on purified mononucleosomes as described previously (McMurry and Krangel, 2000). Briefly, thymocytes were lysed and nuclei were treated with microccocal nuclease to produce a partial chromatin digest. After removal of linker histone H1, chromatin was fractionated on a sucrose gradient. For analysis of

peripheral lymphocytes, immunoprecipitations were performed on paraformaldehyde-crosslinked chromatin prepared as described previously (Carabana et al., 2005). Sonication was used to obtain DNA fragments ranging from 300 to 500 bp. With either approach, anti-diacetylated H3, anti-dimethylated H3 K4 and control rabbit-IgG antibodies (Upstate Biotechnology) were used for immunoprecipitation and bound and input fractions were quantified using SYBR green real-time PCR (Roche). Analysis of the constitutively active carbamoyl transferase dihydrorotase (CAD) gene was used to normalize values of different samples. Primer sequences are provided in supplementary Table 1.

Flow cytometry

Cell suspensions from spleen, thymus and bone marrow were stained with FITC-CD69 (BD Bioscience), PE-hCD2 (Caltag), PerCP-CD4 (BD Bioscience), APC-CD8 (BD Bioscience), FITC-CD24 (HSA, eBiosciences), PE-Cy5 IgM (eBioscience) or APC-B220 (eBioscience) antibodies. Data was collected on a FACSCalibur or FACSCanto (BD Bioscience) and was analyzed using Flow Jo software.

Luciferase reporter constructs and cell transfections

For the firefly luciferase vector we used the pXPG vector (Bert et al., 2000). Primers used to amplify fragments were the same as for the hCD2 reporter constructs with modifications in the restriction sites (PromF contained a XhoI restriction site and other primers a BamHI restriction site). The CD69 promoter was digested with XhoI and BamHI and ligated to XhoI/BamHI digested pXPG-Luc vector. CNS1-4 fragments were digested with BamHI and ligated to BamHI

digested CD69 prom-pXPG-Luc vector. Construct sequences were confirmed by restriction enzyme digestion and sequence analysis.

For transient transfection assays, a total of $5x10^5$ Jurkat cells were plated into a 24-well plate and transfected with 1μg of specific firefly luciferase test plasmid, 20ng of pRL-TK renilla luciferase control plasmid and 4μl of Superfect (Qiagen) according to manufacturer's instructions. Twenty-four hours after transfection, cells were stimulated with 10ng/ml PMA and 1μM Io or 5μg/ml plate bound anti-CD3 (clone OKT3, eBiosciences) and anti-CD28 (clone CD28.2, eBiosciences) monoclonal antibodies or were mock incubated. A luciferase assay was performed twenty-four hours later using the Dual-Luciferase Reporter Assay System (Promega). Transfections were performed in duplicate and values were normalized to Renilla luciferase activity.

Results

Conservation of the CD69 locus

CD69 is a type II transmembrane C-type lectin encoded in the NK complex on mouse chr6 and human chr12 (Fig. 1A). The gene spans approximately 7.5 kb and contains 5 exons. The first two exons encode the cytoplasmic and transmembrane domains and exons III, IV and V encode the extracellular portions of the molecule. The defined murine CD69 promoter (-656 to +1 relative to the transcription start site) is the only *cis*-acting element known to regulate CD69 expression (Ziegler et al., 1994). We wanted to identify other potential *cis*-acting sequences involved in CD69 gene regulation. As cross-

species genome analysis has been useful for this purpose (Loots et al., 2000), we compared mouse, human and dog genomic sequences by the means of VISTA Browser (Loots et al., 2000). Using the default parameters for defining a conserved non-coding sequence (CNS) element (70% identity over 100bp length), four elements upstream of the CD69 gene were identified (Fig. 1B). CNS1was upstream of and contiguous to the promoter and CNS2, CNS3 and CNS4 were 9 kb, 28 kb and 40 kb away from the main site of transcription initiation, respectively. Marked conservation was also observed at the promoter. As there are substantial differences in genomic organization of the human and mouse NK complexes upstream of CD69 (Fig. 1A), we hypothesized that the four CNSs may regulate CD69 rather than neighbouring gene expression.

Accessibility of the CD69 locus

Chromatin regulatory regions are typically characterized by DNase I hypersensitive sites (HSs), that reflect nuclear factor binding and disrupted nucleosome organization. We conducted DNase I HS assays and southern blots to determine whether CNS1-4 represented DNase HSs. CD69 is upregulated by thymocyte positive selection and different TCR transgenic mouse strains express characteristic quantities of CD69 based on both the selecting background and the strength of TCR-MHC:peptide interactions (Puls et al., 2002). We used thymocytes from B10.D2 H-2K^{d} mice expressing a transgenic TCR specific for a hemagglutinin peptide presented by H-2K^{d} (HA) to isolate thymocytes with high CD69 expression and thymocytes from Rag2$^{-/-}$mice that express a *Tcrb* transgene (Rxβ) to isolate thymocytes with low CD69 expression (Fig. 2A). Total thymocytes from these strains were treated with

varying concentrations of DNase I and genomic DNA was extracted, digested with EcoRI, and analyzed by southern blot (Fig. 2B). A site within the inactive trypsinogen gene was used as an internal control to compare chromatin digestion in different samples. Probes 1 and 2, which hybridized to different ends of the same genomic EcoRI fragment (Fig. 2B), detected five distinct HSs (Fig. 2C, upper panels). HS I and II corresponded to the CD69 promoter and CNS1, respectively, whereas HSIV and HSV mapped to CNS2. HSIII was evident as a cluster of weak HSs that was only detected in Rxβ and that mapped to a less conserved sequence between CNS1 and CNS2. HSVI, corresponding to CNS3, was detected using probe 3 (Fig. 2C, lower left panel). Probe 4 revealed weak HSs VII and VIII that corresponded to CNS4 (Fig. 2C, lower right panel). Hence these experiments identified eight distinct DNase I hypersensitive sites (HSs) mapping predominantly to the defined CNSs. $CD69^+$ and $CD69^-$ cells presented similar patterns of DNase I sensitivity.

CD69 locus histone modifications

One mechanism by which chromatin structure participates in the regulation of gene expression, is through the modification of histone tails (Berger, 2007). Histone H3 acetylation and lysine 4 dimethylation are associated with active and poised chromatin, respectively(Schneider et al., 2004). We assessed their levels by chromatin immunoprecipitation at conserved genomic regions (CD69 proximal promoter, CNS1, CNS2, CNS3, and CNS4) as well as at several nonconserved regions (-3.4kb, -20kb and -30kb). The β_2-microglobulin gene was used as positive control.

Mononucleosomes were prepared from Rxβ and HA tg thymocytes and were then immunoprecipitated with anti-acetylated histone H3, anti-dimethylated histone H3 K4 or control IgG antibodies. Coprecipitated DNA was purified and subjected to real time PCR to quantify the recovery of regions of interest (Fig. 3A). Results are expressed as the ratio of immunoprecipitated and input DNA normalized to the constitutively active control gene CAD. The promoter demonstrated high levels of H3 acetylation and H3 K4 dimethylation in both $CD69^-$ Rxβ thymocytes and $CD69^+$ HA tg thymocytes. $CD69^-$ Rxβ thymocytes demonstrated moderate acetylation at CNS1 and CNS2, and very low levels at CNS3 and CNS4. $CD69^+$ HA tg thymocytes displayed increases in H3 acetylation at all of these sites. A similar overall pattern was observed for H3 K4 dimethylation, with the exception that this modification was only modestly increased at CNS2 in $CD69^+$ cells. With one exception (H3 K4 dimethyation at position -3.4 kb in $CD69^+$ thymocytes), the two modifications were very low at all nonconserved sites tested.

To investigate CD69 locus chromatin structure in peripheral lymphocytes, lymph node T cells and splenic B cells were treated *in vitro* with PMA and ionomycin for 6h to upregulated CD69 expression or were left untreated. Chromatin was then cross-linked with paraformaldehyde, fragmented by sonication, and immunoprecipitated as above. As in thymocytes, acetylation of H3 and dimethylation of H3 K4 were enriched at the promoter in resting and activated T and B cells (Figs. 3B, C). Also as in thymocytes, H3 acetylation and H3 K4 dimethylation increased substantially at CNS1 upon activation of both peripheral T and B cells (Figs. 3B, C). However, unlike in thymocytes, we detected only

very low H3 acetylation at CNS2, CNS3 and CNS4 in both resting and activated peripheral T cells. Moreover H3 K4 dimethylation at these sites, although mildly elevated in resting T cells, was not inducible. B cells displayed increased amounts of both modifications at CNS2, CNS3 and CNS4, but only CNS3 showed inducible modification. Moreover, in striking contrast to thymocytes, H3 acetylation and H3 K4 dimethylation were constitutively high at CNS4 in B cells.

The above experiments revealed three important aspects of CD69 gene chromatin structure. First, the CD69 promoter is constitutively associated with active chromatin modifications in all three cell types. Second, CNS1 becomes hyperacetylated and hypermethylated upon CD69 induction in all three cell types. Third, histone modifications at CNS2, CNS3 and CNS4 undergoes dynamic changes during T cell development but are differentially modified in peripheral T and B cells. This suggests the possibility of distinct mechanisms of CD69 gene regulation in the various cell types.

Analysis of promoter and CNS1-4 function

The correlation between DNA conservation and the presence of HSs and positive histone marks at the promoter and CNS1-4 prompted us to test their regulatory properties. We prepared luciferase reporter constructs under the control of the CD69 promoter linked to CNS fragments (Fig. 4A). Plasmids were transiently transfected into Jurkat cells, cultured for 24hr, and then stimulated or not with PMA/Io for 24hr more before cells extracts were harvested for luciferase activity. Stimulation conferred a 4-fold increase in the activity of the CD69 promoter. Inducible activity of the CD69 promoter, however, was greatly

enhanced in the presence of CNS2. This fragment conferred a 2-fold increase in basal activity and 20-fold increase over basal activity under conditions of stimulation. CNS4 conferred an approximately 5-fold increase in basal transcription activity and a further 2-fold increase under conditions of stimulation. In contrast, reporter activity was not altered when CNS1 or CNS3 was linked to the CD69 promoter. Luciferase activity of these constructs was also determined upon stimulation with immobilized antibodies against CD3 and CD28 (Supplementary Fig.1). CNS2 was found to confer inducibility to this stimulus, although the magnitude (3-fold) was lower than with PMA/Io. These results provide initial evidence that CNS2 and CNS4 may be enhancers elements for CD69 gene transcription.

To assess their *in vivo* functional properties, transgenic mice were generated using a hCD2 expression construct as a reporter. Transgenic constructs containing the mCD69 promoter alone upstream of hCD2 (construct 1) or together with all four CNS (construct 3) were generated (Fig. 4B). Due to the strong enhancer activity observed for CNS2 element in luciferase assays, transgenic mice containing CNS1 and CNS2 upstream of the promoter (construct 3) were also generated. Two transgenic lines were obtained for construct 1, three for construct 2 and five for construct 3 (Fig. 4C). Copy numbers were determined by quantitative real time PCR (Fig. 4C).

We assessed the magnitude and fidelity of hCD2 reporter expression on gated $CD69^{+}$ and $CD69^{-}$ thymocytes and on purified resting or activated mature lymphocyte populations by flow cytometry (Fig. 4D). Tg lines 1A-23 and 1B-8

(containing 23 and 8 copies of the transgene, respectively) that contained only the CD69 promoter, expressed hCD2 in both thymocytes and peripheral lymphocyte populations (Fig. 5A and supplementary Figs. 2A, 3A). hCD2 expression in DP and SP thymocyte populations was strictly correlated with endogenous CD69 expression, suggesting that the promoter was sufficient to confer specificity to CD69 expression during positive selection (Fig. 5A). However, aberrant expression of hCD2 was observed in resting peripheral T (Fig. 5A) and B (Fig. 6A) lymphocytes, suggesting the need for additional elements.

We tested for inducibility of the mCD69 promoter by overnight activation of isolated peripheral T and B lymphocytes with PMA and ionomycin. Because even resting cells expressed hCD2, we used the mean fluorescence intensity (MFI) of the whole population to measure changes in hCD2 expression. Inducibility was calculated as the ratio of the MFI for the hCD2 staining between activated and resting cells. We detected 1.5- to 2.5-fold hCD2 inducibility in activated T cells (Figs. 5B, 5C) and 1.5- to 2-fold inducibility in activated B cells (Figs. 6B), suggesting that promoter elements support some inducibility *in vivo*.

Unexpectedly, inclusion of CNS1 and CNS2 upstream of the CD69 promoter (tg lines 2A-40, 2B-15 and 2C-15) suppressed hCD2 expression in both thymocytes and peripheral T and B cell populations (Figs. 5A, 6A and supplementary Figs. 2B, 3B). hCD2 expression was substantially reduced in $CD69^+$ DP and SP thymocytes, and was essentially eliminated in resting and activated peripheral $CD4^+$ and $CD8^+$ T cells (Fig. 5A). Expression was also

eliminated in resting and activated peripheral B cells in two of the three tg lines (Fig. 6A supplementary figure 3B). These data suggest that CD69 gene expression in lymphocytes is regulated by silencer elements mapping to CNS1 or CNS2.

In contrast to the results obtained with construct 2, transgenic lines carrying construct 3, including the CD69 promoter and all four CNSs (tg lines 3A-134, 3B-80, 3C-45, 3D-9 and 3E-6) revealed a recovery of hCD2 expression (Figs.5A, 6A and supplementary Figs. 2C, 3C). Expression of the hCD2 reporter correlated well with CD69 expression in all thymocyte populations, ranging from 92% to 22% in DP thymocytes and 89% to 15% in CD4SP and CD8SP thymocytes (supplementary Fig. 2C). Within the SP compartments, hCD2 expression also correlated with mCD69 expression, since downregulation of CD69 and HSA in more mature SP thymocytes was associated with downregulation of reporter expression (Supplementary Fig. 4). Notably, high copy tg lines 3C-45, 3B-80 and 3A-134, but not low copy lines 3E-6 and 3D-9, displayed variegated hCD2 expression indicative of genomic position effects. In the low copy lines hCD2 expression faithfully mimicked mCD69 expression, and expression levels were greater than those observed with construct 1, containing the promoter alone (compare tg line 1B-8 and 3E-6 in Fig. 5A).

Analysis of construct 3 expression in mature $CD4^+$ and $CD8^+$ T cells stimulated with PMA/Io revealed hCD2 expression to be restricted to activated cells in four of five tg lines (3E-6, 3C-45, 3B-80 and 3A-134) with percentages ranging from 99% to 10% (supplementary Fig. 2C). However, hCD2 expression was

inducible in all five lines (range 23-fold to 3.5-fold) and inducibility was substantially higher than for tg lines containing construct 1 with the promoter alone. Tg line 3E-6, with the lowest copy number, was the most tightly regulated, with negligible hCD2 expression in resting $CD4^+$ and $CD8^+$ T cells, and abundant hCD2 expression in the vast majority of activated $CD4^+$ and $CD8^+$ T cells (Figs. 5A). Inducibility was 14-fold and 15-fold in $CD4^+$ and $CD8^+$ T cells, respectively (Figs. 5B, 5C). As in thymocytes, expression was highly variegated in the three tg lines with the highest copy numbers (3C-45, 3B-80 and 3A-134) (supplementary Fig. 2C).

Expression of construct 3 in peripheral T cells was also inducible following direct engagement of TCR with anti-CD3 plus anti-CD28 antibodies (Fig. 5D). Moreover, the kinetics hCD2 expression paralleled mCD69 expression, since both were detected 2h after stimulation, were maximally detected at 12-24h and expression was reduced thereafter (supplementary Figs. 5A, B). Since CD69 expression can be induced *in vivo* by infectious agents that cause in the production of type I interferons, we also analyzed hCD2 induction following *in vivo* administration of poly I:C (supplementary Fig. 6). The hCD2 expression was induced after poly I:C treatment indicating that CNSs can also respond to stimuli distinct from the TCR pathway. These results suggest that the combination of CNS1-4 contributes to regulated CD69 expression in both thymocytes and peripheral T cells under a variety of activation conditions.

We further examined the expression of hCD2 in B cells. The combination of CNS1-4 clearly enhanced hCD2 expression as compared to the promoter alone

(Figs. 6A and supplementary Fig. 3C). However, hCD2 expression was not concordant with mCD69 expression, since substantial numbers of resting B cells were $hCD2^{+}$ in all five transgenic lines (range 22% to 79%)(Supplementary Fig. 2C). This may reflect the constitutive and abundant H3 acetylation and H3 K4 dimethylation detected at CNS4 in B cells (Fig. 3C). Stimulation with PMA and ionomycin resulted in 2-2.5-fold inducibility in the various lines (Fig. 6B). However, the failure to appropriately suppress expression in resting B cells suggests that there are different requirements for regulated CD69 gene expression in B and T cells.

As dysregulated expression of hCD2 was observed in mature B cells, we evaluated whether CNSs and the promoter may have an effect on hCD2 expression at different stages of B cell development (supplementary Fig. 7). Results indicate that hCD2 expression was already present in $B220^{int}IgM^{+}$ immature B cells in transgenic lines containing the promoter alone or in combination with CNS1-4 elements and suggests that specific signals received at this stage of development induce the disregulated expression of hCD2 that is also observed in mature B cells.

Collectively, these experiments indicate that the CD69 promoter has strong activity and by itself can faithfully direct CD69 transcription in developing thymocytes. CNS1 and CNS2 appear to repress promoter activity whereas CNS3 and CNS4 appear to counteract this repression, resulting in more tightly regulated expression in peripheral T lymphocytes and enhanced expression in both thymocytes and peripheral T lymphocytes. The same elements are

insufficient to faithfully direct CD69 expression in the B cell lineage.

Discussion

In this study we have provided insights into the mechanism that regulate CD69 gene expression *in vivo* by the identification of new cis-regulatory elements and analysis of their chromatin structure and function. We identified four conserved noncoding sequences upstream of the CD69 promoter. Chromatin and functional analyses indicated that the CD69 promoter adopts an open chromatin conformation and can direct reporter gene transcription in transgenic mice. However, the reporter, unlike mCD69, was expressed in unstimulated peripheral lymphocytes, suggesting that other elements must participate to achieve appropriate specificity. We detected several DNase I hypersensitive sites that mapped to conserved noncoding sequences (CNSs) and that displayed epigenetic profiles that were distinct in T and B cells and dynamic during T cell development. Analysis of CNS function in transient transfection assays revealed constitutive and inducible enhancer activity for both CNS2 and CNS4, but no apparent activity for CNS1 and CNS3. However, we found that transgenic mice bearing CNS1 and CNS2 reduced reporter expression in T and B cells and that this inhibition was only overcome by inclusion of CNS3 and CNS4. The combination of all four CNSs allowed for high level reporter expression that was appropriately regulated in T cells but not B cells.

DNA sequence comparison has become a useful tool to identify specific remote elements that participate in the regulation of gene expression (Ansel et al.,

2004; Hatton et al., 2006; Shnyreva et al., 2004). We observed discrete islands of conservation in a 45 kb region upstream of the CD69 gene and found them to correspond to sites displaying hypersensitivity to DNase I digestion. It is noteworthy that these HSs were equally sensitive to DNase I digestion in $CD69^-$ and $CD69^+$ lymphocytes. CD69 is one of the earliest antigens expressed upon activation and, therefore, it is possible that these regions are occupied by proteins even before activation. In accord with our results, the first genome-wide map of DNase I HSs in human $CD4^+$ T cells identified HSs lying within the CD69 promoter, CNS2, CNS3 and CNS4 in the human CD69 locus (Boyle et al., 2008).

Previous work showed that the CD69 promoter can support rapid induction of reporter gene expression in transient transfection assays. Here we found that the CD69 promoter is always associated with an “active” chromatin configuration, even in situations where CD69 is not expressed. The promoter displayed high level histone H3 acetylation and H3 K4 dimethylation in all thymocyte and peripheral lymphocyte populations. These two modifications have been shown to mark transcriptional competence of the IL-4 and IL-2 loci, respectively, in T cells (Adachi and Rothenberg, 2005; Avni et al., 2002) and may be important to maintain transcriptional competence of the CD69 gene as well. Interestingly, genome-wide analysis of chromatin modifications in unstimulated human $CD4^+$ T cells demonstrated enrichment of both histone H3 K4 trimethylation and RNA polymerase II at the promoter, exon I and intron I of mCD69 (Barski et al., 2007). These data suggest that, as for other genes that require rapid induction, the CD69 promoter may constitutively harbor a

promoter-paused RNA polymerase II (Cui et al., 2004; Muse et al., 2007). CNS1 is the distal region of the C69 promoter and one notable result is that this region showed a peak of permissive histone marks that correlated with CD69 expression in all cell populations analysed. However, the promoter alone showed inducible activity in both transient and transgenic reporters, and at least in the case of transient assays, CNS1 had no influence on basal or inducible promoter activity. This suggests that inducible histone modifications at this site may depend on other elements.

Tissue- and developmental stage-specific gene expression depends on both activators and silencers. A well-studied case is that of CD4, in which a silencer represses the CD4 promoter and enhancer in DN and CD8 SP thymocytes (Sawada et al., 1994; Zou et al., 2001). Similarly, regulated IL-4 transcription requires the action of a 3' silencer element that can suppress reporter gene expression in transgenic mice (Lee et al., 2001) and whose germline deletion results in the aberrant expression of IL-4 in Th1 cells (Ansel et al., 2004). Moreover, there are examples of bifunctional elements, which function as both enhancers and silencers in a developmental-stage specific way (Bilic et al., 2006; He et al., 2008). Our results from transgenic mice indicates that CNS1, CNS2, or the combination of the two elements can play a repressive role in CD69 gene expression in thymocytes, as well as stimulated T and B cells. Nevertheless, CNS2 displayed strong, inducible enhancer activity when tested in isolation by transient transfection into Jurkat. Notably, the detected enhancer activity is consistent with the inducible histone H3 acetylation detected at this site in thymocytes. We suggest that CNS2 is an inducible thymocyte enhancer

whose activity in the context of chromatin requires the activity of other CD69 regulatory elements. Remarkably, inclusion of CNS3 and CNS4 in transgenic reporter constructs led to both a recovery of reporter gene expression and tightly regulated reporter gene expression in thymocytes and peripheral T cells. Based on its inducible enhancer activity in Jurkat and inducible histone acetylation in thymocytes, CNS4 appears to function as a thymocyte enhancer as well. Thus we suggest that CNS4 may not only function as an enhancer in the context of the endogenous CD69 locus, but may, perhaps in conjunction with CNS3, function as an anti-silencer that switches CNS2 from silencer to enhancer activity. We propose that all four CNSs may converge to interact physically and functionally in the form of an active chromatin hub, as initially described for the β-globin locus (Tolhuis et al., 2002). Perhaps consistent with this, a previous study suggested that CD69 may be regulated by the architectural protein SATB1(Alvarez et al., 2000).

We note that our conclusions about the activities of CNS1-4 *in vivo* assume that these elements function normally in the context of our relatively compact transgenic reporter. Prior studies have validated this approach in other systems (Decker et al., 2009; Lee et al., 2001). However, we cannot rule out that our transgenic constructs lack relevant elements from DNA segments that normally separate the CNSs, or cannot adopt important three dimensional chromatin configurations that are important for physiological regulation. To further study mechanisms of CD69 regulation *in vivo* we will analyze mice transgenic for a bacterial artificial chromosome containing the mCD69 locus.

As compared to thymocytes, the mechanism of induction of CD69 gene expression in mature T cells appears distinct, as only CNS1 displayed inducible H3 histone acetylation and H3 K4 dimethylation in the latter cell population. This was true despite the fact that, as for thymocytes, CNS2-4 were needed for tightly regulated, inducible expression in peripheral T cells of transgenic mice. We suggest that CNS2-4 activity during T cell development may establish a specific chromatin context that is required for proper regulation of the CD69 locus in mature T cells.

Although the combination of all four CNSs was capable of promoting high level and tightly regulated reporter gene expression in thymocytes and peripheral T lymphocyte populations, it did not do so in all transgenic lines. One explanation for this is that additional elements may be required to reconstitute a CD69 locus control region that can consistently overcome chromosomal position effects. However, we note that hCD2 expression was both low and variegated in those transgenic lines containing particularly high copy reporter arrays. Thus it is possible that RNA interference may cause transgene silencing in these lines(Pal-Bhadra et al., 2002).

The unexpected finding that transgenic mice with all CNSs mis-expressed the hCD2 reporter in unstimulated B cells indicates that CD69 gene transcription is differentially controlled in B and T cells. Thus it seems likely that the construct lacked an element required to repress the expression in unstimulated B cells. Further evidence for differential regulation in T and B cells was obtained from the epigenetic profile of CD69, which revealed distinct patterns of histone

modifications at CNS2, CNS3 and CNS4 between T and B cells. In this regard, IL-2 transcription is thought to be regulated by different mechanisms in CD4 and CD8 lymphocytes and IL-4 and IL-13 transcription is reduced in Th2 cells but not in mast cells in mice deficient for the CNS1 region (Mohrs et al., 2001; Yui et al., 2001). Interestingly, transgenic mice bearing a 30kb genomic fragment containing Ly49A, another gene in the NK complex that encodes a C-type lectin, also showed aberrant expression in B cells. Perhaps similar mechanisms are used to suppress CD69 and Ly49A expression in B cells (Tanamachi et al., 2004).

Based on our findings, we propose that the CD69 promoter and upstream elements display an accessible chromatin structure prior to CD69 transcription to support rapid gene induction upon stimulation. Gene induction requires the combined activity of multiple upstream CNSs, two of which display classical enhancer activity in T cells. The interdependence of these CNSs may also allow for physiological repression of CD69 expression by disruption CNS3 and CNS4 interactions with CNS1 and CNS2. Finally, an as yet uncharacterized B cell specific element may be required to suppress CNS activity in unstimulated B cells. The above results indicate CD69 gene regulation is complex and likely differs in different cell types. Future studies are required to elucidate the nature of CD69 regulatory elements and the mechanism through which they regulate CD69 expression.

Figures

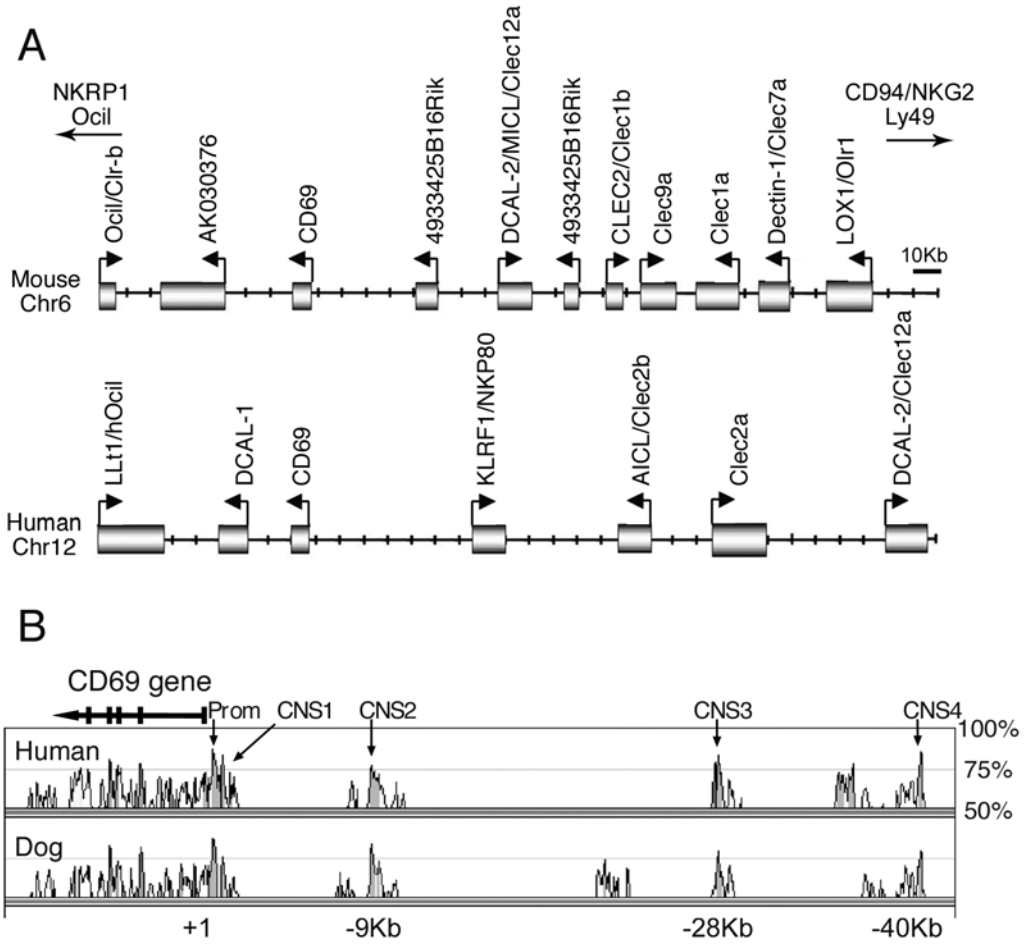


Figure 1. **Genomic organization and conservation of the CD69 locus.** (A) Genomic organization of the human and mouse CD69 gene and neighbouring genes in the natural killer complex. The diagram is drawn to scale according to the most recent gene annotations (July 2007 for mice and March 2006 for human) at the University of California Santa Cruz web site (http://genome.ucsc.edu). Boxes are genes and arrows indicated transcriptional initiation sites and orientation. (B) VISTA Browser diagram identifying conserved noncoding sequences upstream of the CD69 gene. The mouse sequence is shown on the X axis and percentage of similarity to the human and dog genomes on the Y axis. Non-coding sequences of at least 100-bp long with more than 70% sequence identity are shown are indicated with arrows.

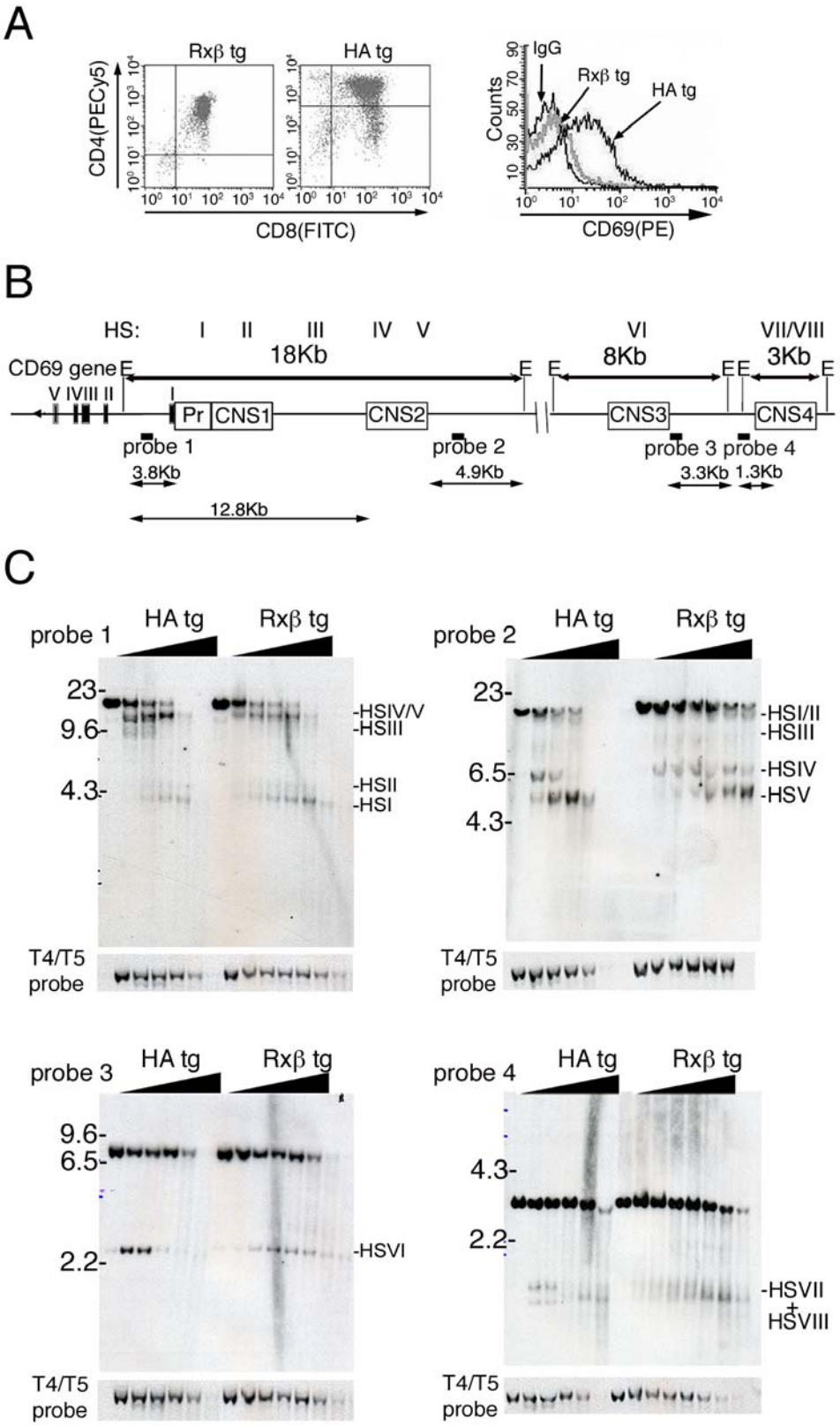


Figure 2. **Conserved noncoding sequences are constitutive hypesensitive sites**.

(A) Flow cytometry analysis showing CD69 expression in Rxβ and HA transgenic thymi. (B) Map of the CD69 locus showing locations of CNSs and the genomic fragments expected from EcoRI digestion. Probes used for southern blot are shown and HSs are indicated. (C) Representative DNase I analyses using the indicated probes. Total thymocytes from Rxβ and HA thymocytes were treated with increasing amounts of DNase I. EcoRI digested DNA was examined by Southern Blot. Size markers and relevant bands are denoted.

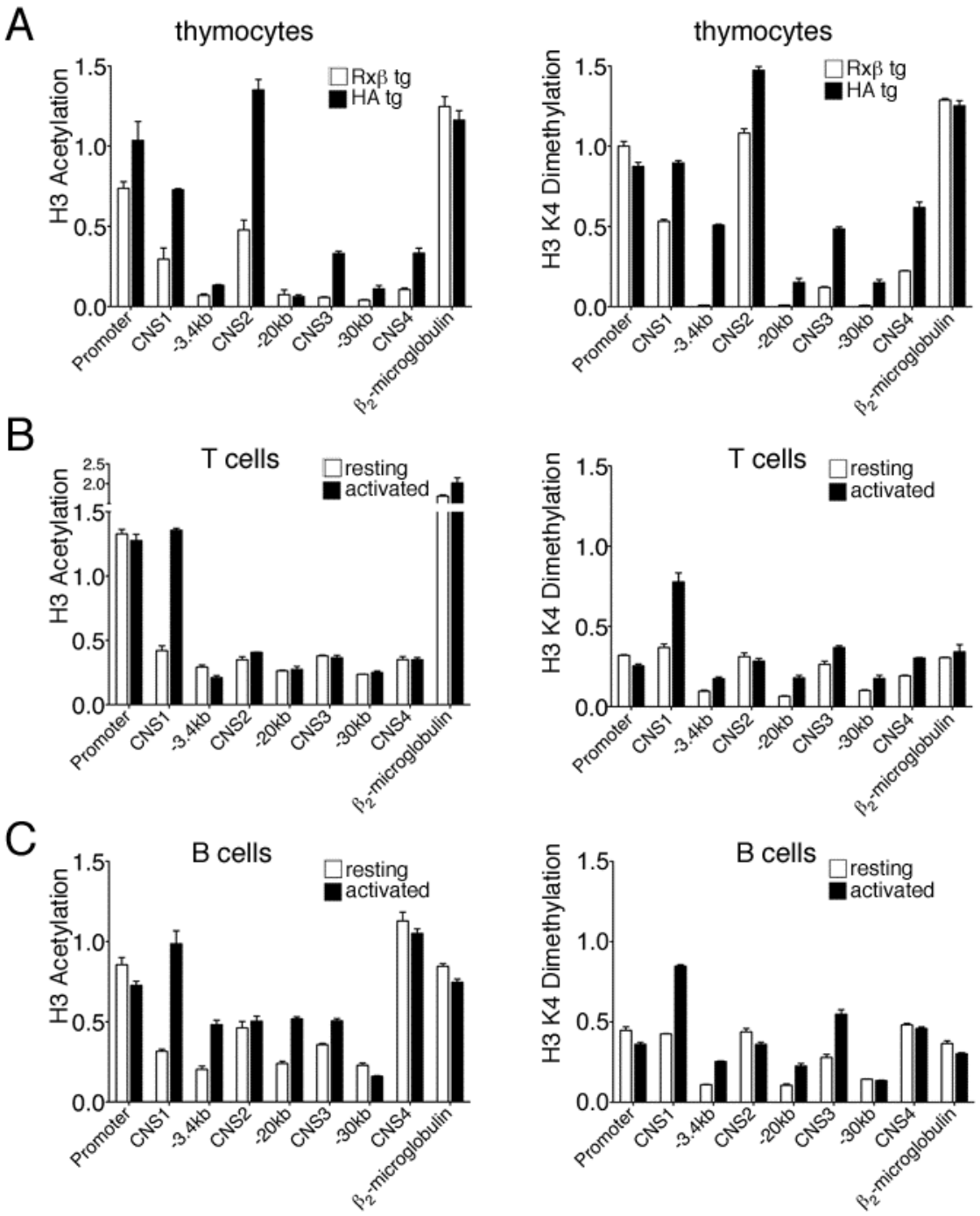
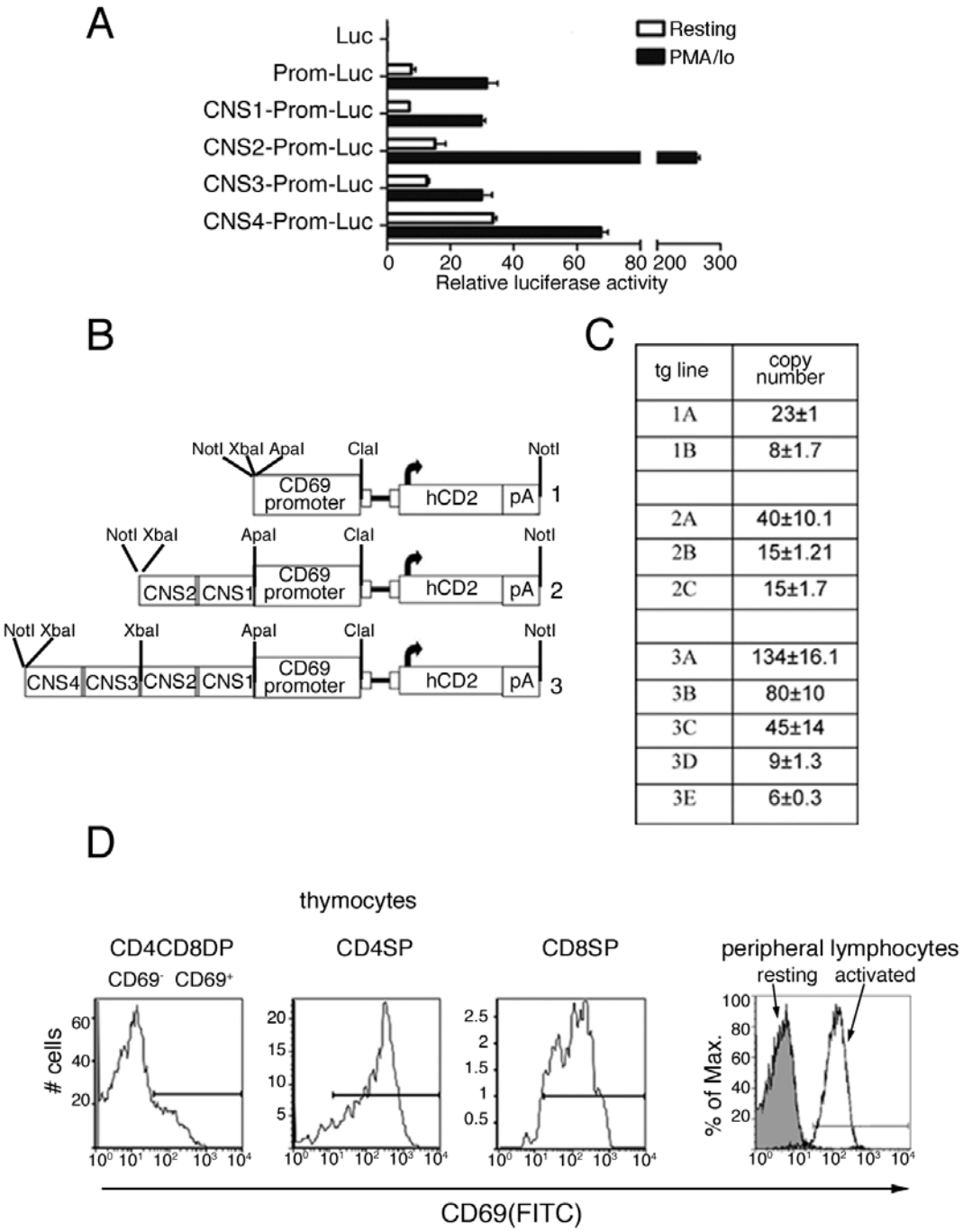


Figure 3. **Epigenetic profile of the CD69 locus in T and B cells.** Chromatin immunoprecipitation with antisera against acetylated H3 and dimethylated H3 K4. (A) ChIP performed on purified mononucleosomes obtained from total thymocytes of Rxβ and HA transgenic mice. (B) ChIP performed on sonicated chromatin from purified T cells either unstimulated or stimulated with 10ng/ml PMA plus 0.5μM Io for 6h. Purity

as determined by flow cytometry was greater than 85%. (C) ChIP performed on sonicated chromatin from B cells isolated from TCR$\delta^{-/-}\beta^{-/-}$ mice, either unstimulated or stimulated with 10ng/ml PMA plus 0.5μM Io for 6h. Flow cytometry analysis showed that more than 95% of spleen cells were B220^{+}. Bars represents the abundance of indicated DNA sequences in immunoprecipitated samples and are expressed as the ratio of immunoprecipitated and input DNA normalized to the abundance of the constitutively active control CAD gene. The data are representative of two experiments and are expressed as the mean ± SEM of triplicate PCRs.



tg line	copy number
1A	23±1
1B	8±1.7
2A	40±10.1
2B	15±1.21
2C	15±1.7
3A	134±16.1
3B	80±10
3C	45±14
3D	9±1.3
3E	6±0.3

Figure 4. **Characterization of CNS function in transfected cells and transgenic mice.** (A) Luciferase activity of reporter constructs in unstimulated or stimulated Jurkat cells. Results are expressed as the mean ±SEM of duplicate transfections and are representative of three experiments. (B) Representation of hCD2 reporter constructs used to generate transgenic mice. (C) Copy numbers of transgenic lines were determined by real time PCR. Values for construct 1 represent the average

amplification using hCD2 primers. Values for construct 2 represent the average amplification using CNS1 and hCD2 primers, and values for construct 3 represent the average amplification using CNS4, CNS3, CNS1 and hCD2 primers. (D) Flow cytometric analysis of CD69 expression in thymus and resting and activated peripheral lymphocytes, indicating gating for $CD69^{+}$ and $CD69^{-}$ populations.

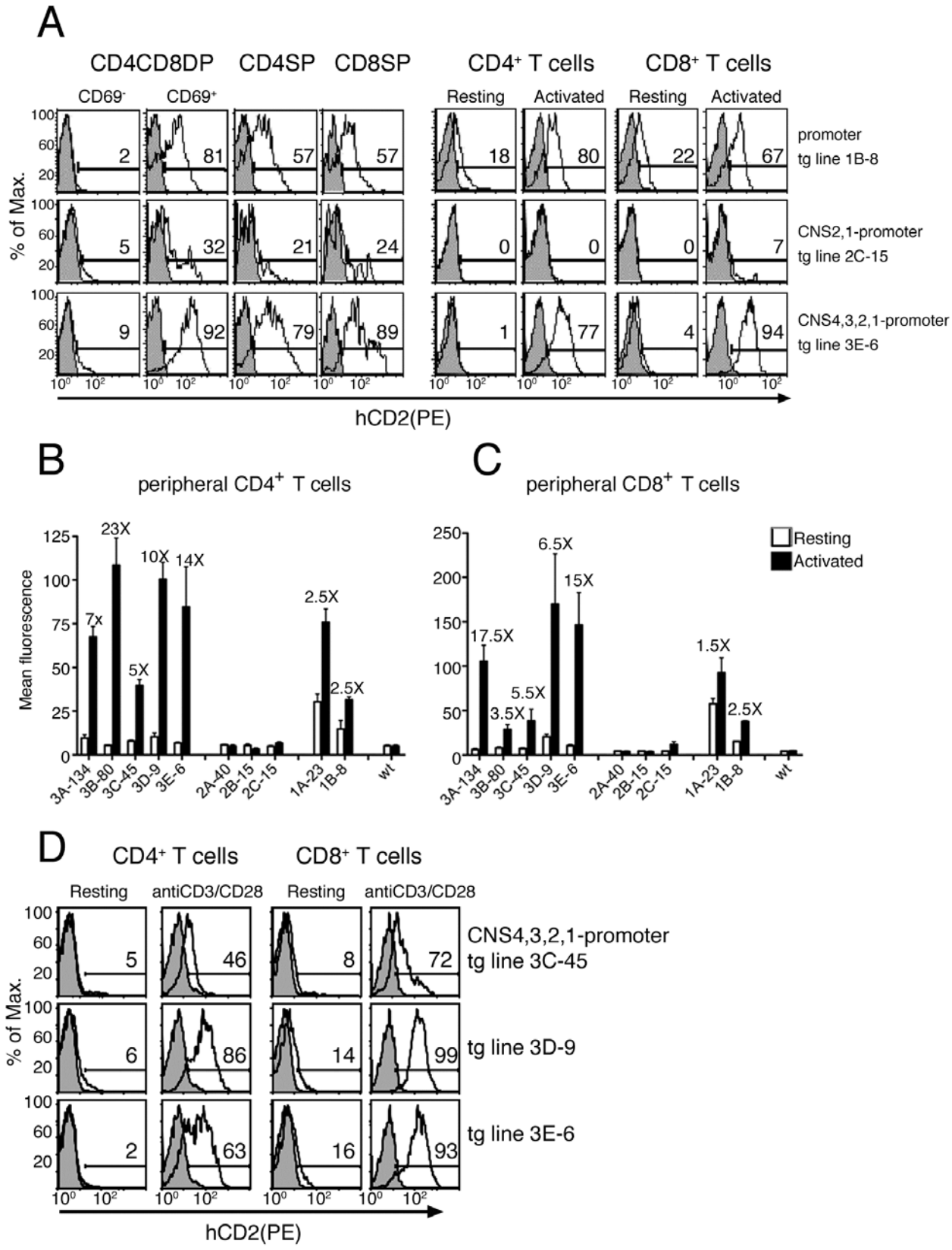


Figure 5. **Reporter expression in thymocytes and peripheral T cells of transgenic mice**.

(A) Cytometric analysis of hCD2 expression in transgenic lines 1B-8, 2C-15 and 3E-6 from thymocytes ($CD69^+$ and $CD69^-$ CD4CD8DP, CD4SP and CD8SP) and splenocytes (resting or activated with PMA/Io, $CD4^+$ and $CD8^+$) (open histograms). Analyses of control non-transgenic mice are also shown (shaded histograms). (B) and (C) Mean fluorescence intensity of the hCD2 expression in resting and activated peripheral $CD4^+$T cells and $CD8^+$ T cells. Values reflect the mean ± SEM of 5 to 10 mice. For each transgenic line, fold-increase in MFI in response to activation is indicated above the bars. (D) Cytometric analysis of hCD2 expresion in transgenic lines 3C-45, 3D-9 and 3E-6 activated or not with plate bound anti CD3/CD28 antibodies. Analyses of control non-transgenic mice are also shown (shaded histograms).

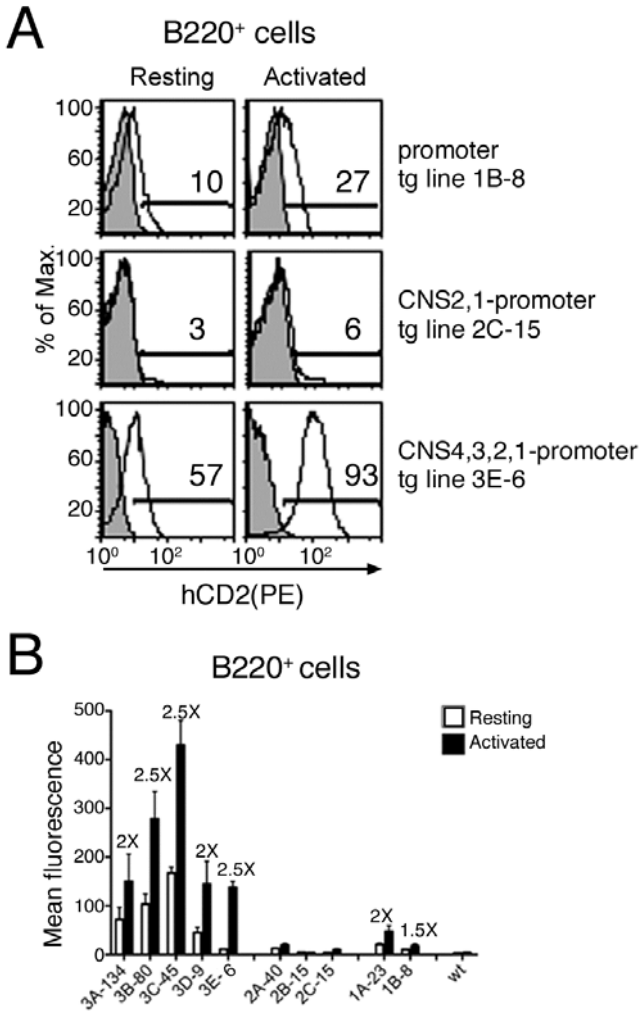
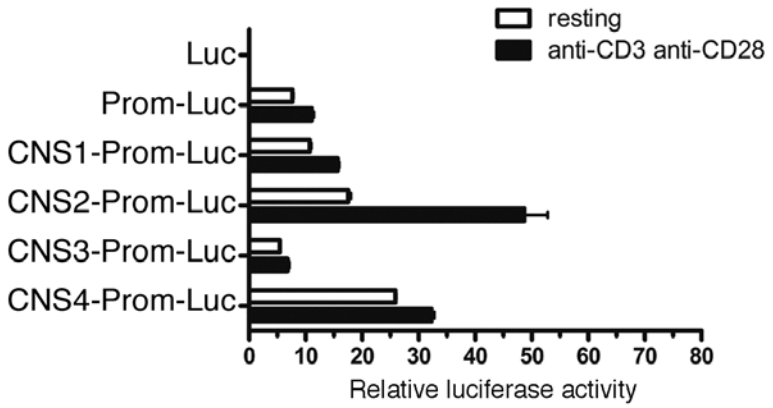
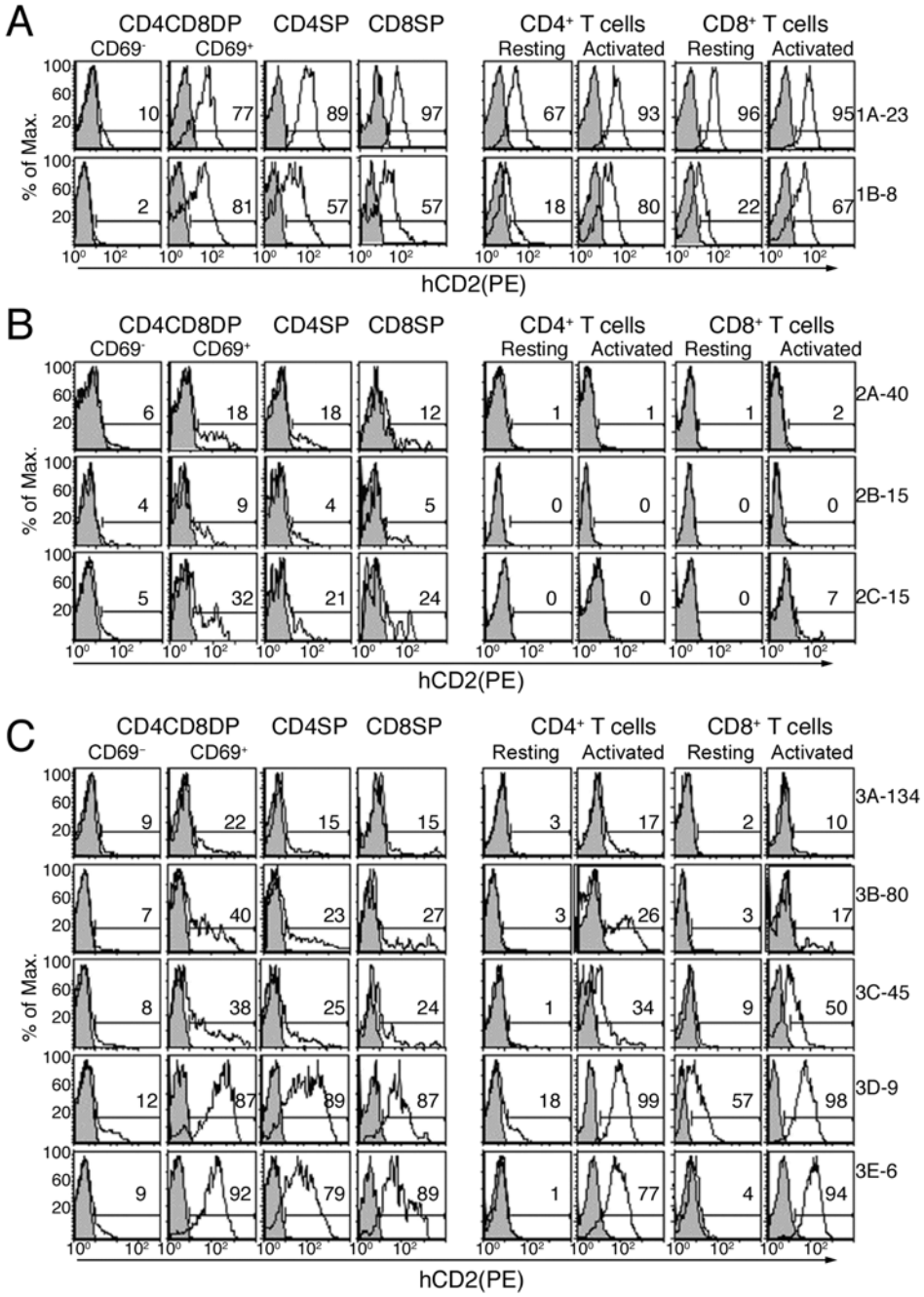


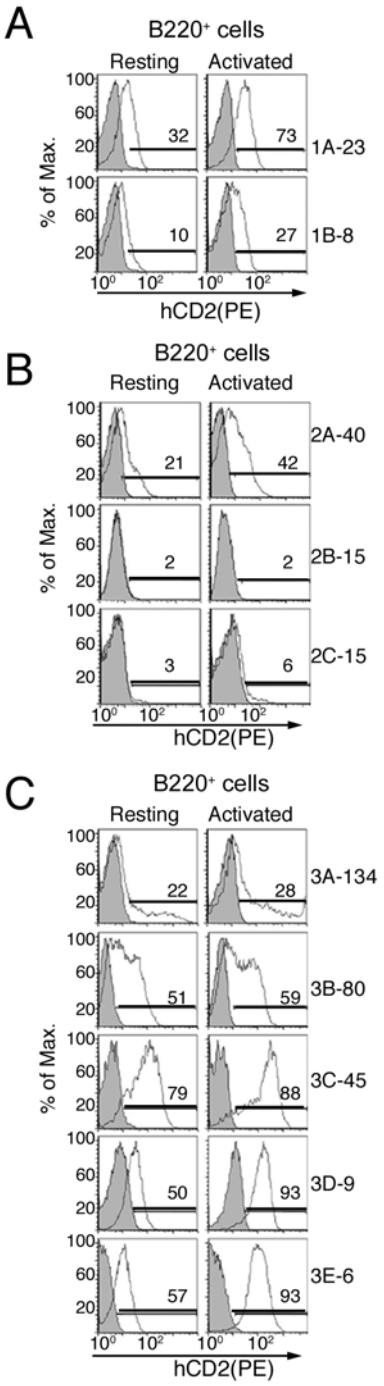
Figure 6. **Reporter expression in resting and activated peripheral B cells of transgenic mice.** (A) Cytometric analysis of hCD2 expression in transgenic lines 1B-8, 2C-15 from resting and activated with PMA/Io B220 splenocytes (open histograms). Analyses of control non-transgenic mice are also shown (shaded histograms). (B) Mean fluorescence intensity of the hCD2 expression in resting and activated peripheral $B220^+$ cells. Values reflect the mean ± SEM of 5 to 10 mice. For each transgenic line, fold-increase in MFI in response to activation is indicated above the bars.



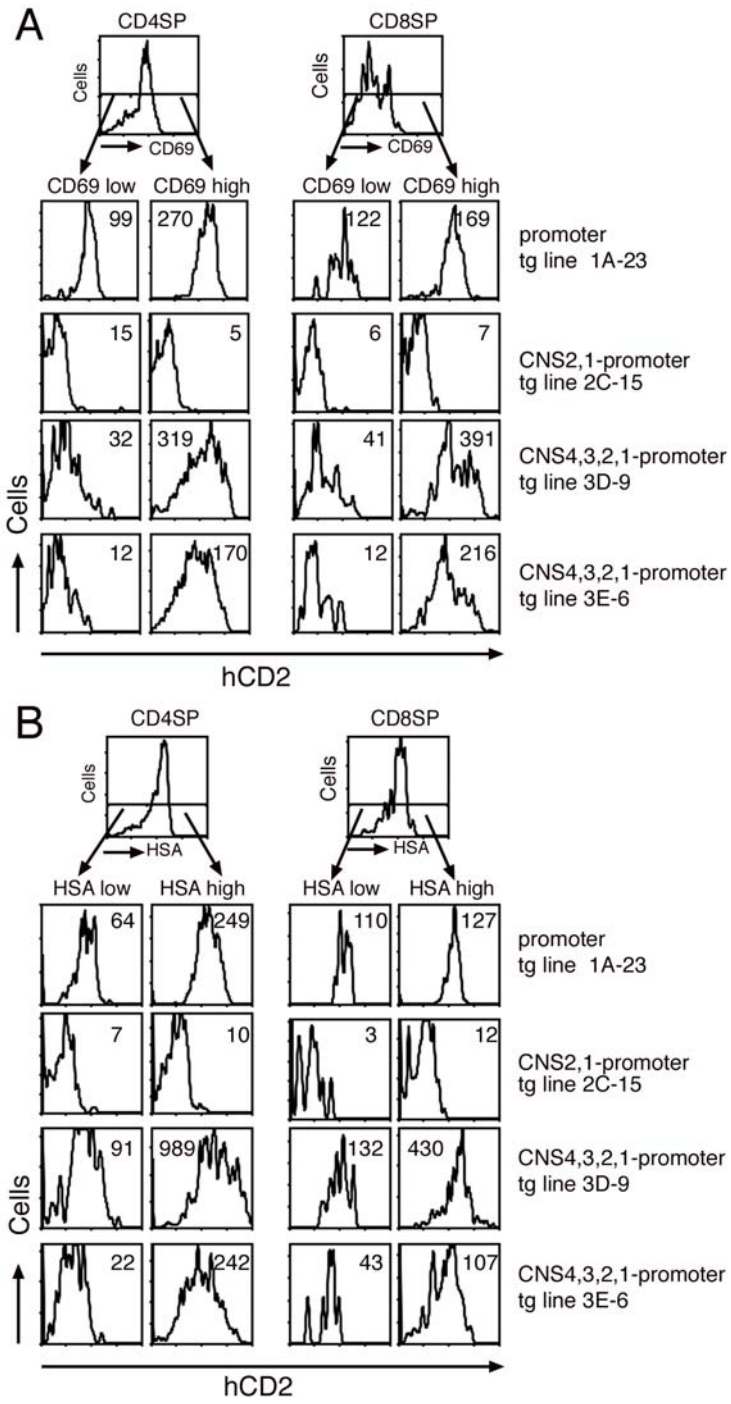
Supplementary figure 1. **Luciferase activity of reporter constructs in Jurkat cells after TCR engagement.** Jurkat cells were transfected with different DNA constructs and were stimulated or not with plate-bound anti-CD3/CD28 antibodies. Luciferase activity was measured 24h after stimulation. Results are expressed as the mean ±SEM of duplicate transfections and are representative of three experiments.



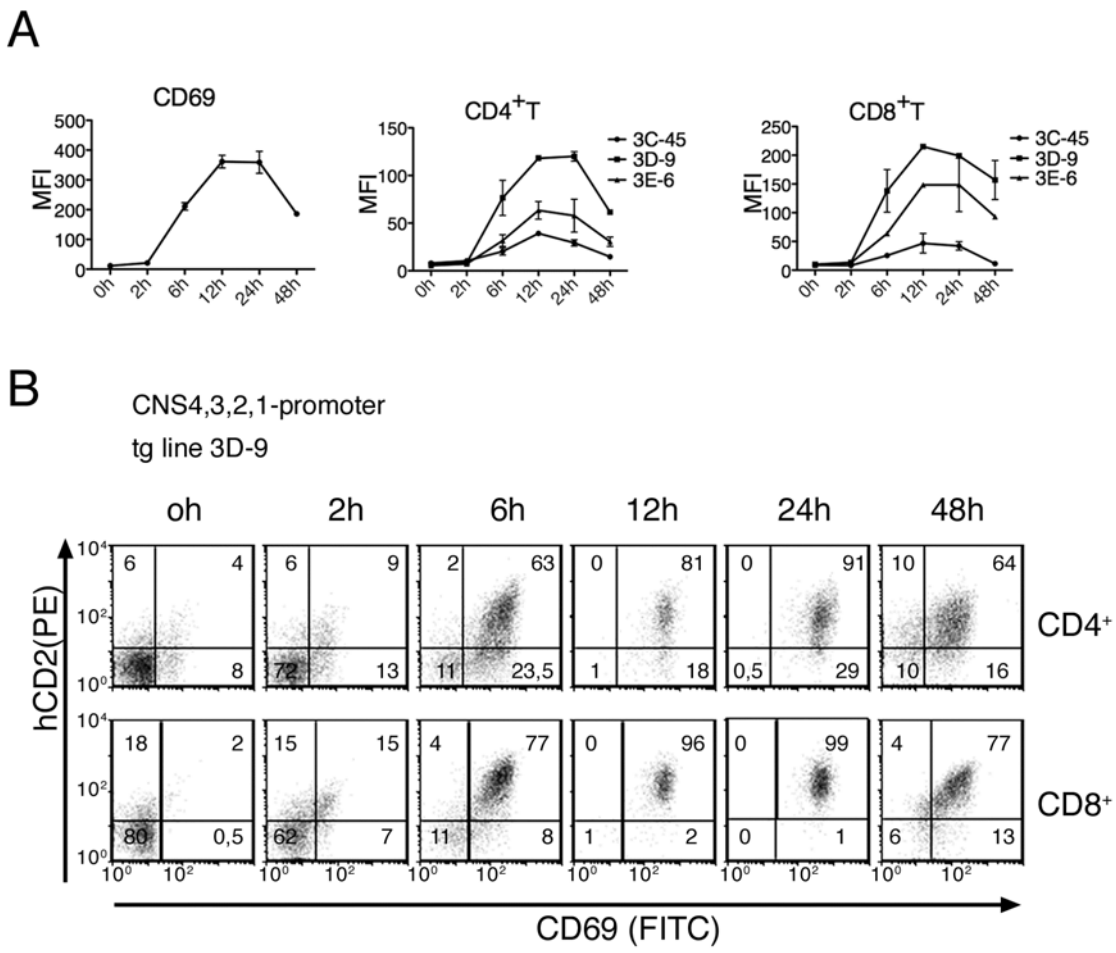
Supplementary figure 2. **Reporter expression in thymocytes and peripheral T cells of transgenic mice**. Thymocytes ($CD69^+$ and $CD69^-$ CD4CD8DP, CD4SP and CD8SP) and splenocytes (resting and PMA/Io activated, $CD4^+$ and $CD8^+$) from transgenic mice were analyzed for hCD2 expression by flow cytometry (open histograms). Analyses of control non-transgenic mice are also shown (shaded histograms). (A) hCD2 expression in transgenic lines 1A-23 and 1B-8. (B) hCD2 expression in transgenic lines 2A-40, 2B-15 and 2C-15. (C) hCD2 expression in transgenic lines 3A-134, 3B-80, 3C-45, 3D-9 and 3E-6.



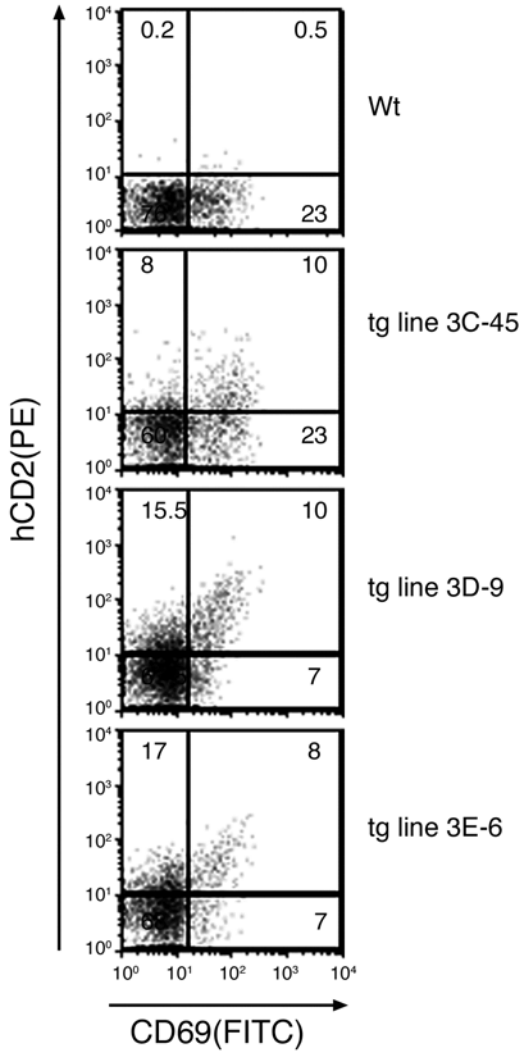
Supplementary figure 3. **Reporter expression in resting and activated peripheral B cells of transgenic mice.** Resting and PMA/Io activated B220$^+$ splenocytes were analyzed for hCD2 expression by flow cytometry (open histograms). Analyses of control non-transgenic mice are also shown (shaded histograms). (A) hCD2 expression in transgenic lines 1A-23 and 1B-8. (B) hCD2 expression in transgenic lines 2A-40, 2B-15 and 2C-15. (C) hCD2 expression in transgenic lines 3A-134, 3B-80, 3C-45, 3D-9 and 3E-6.



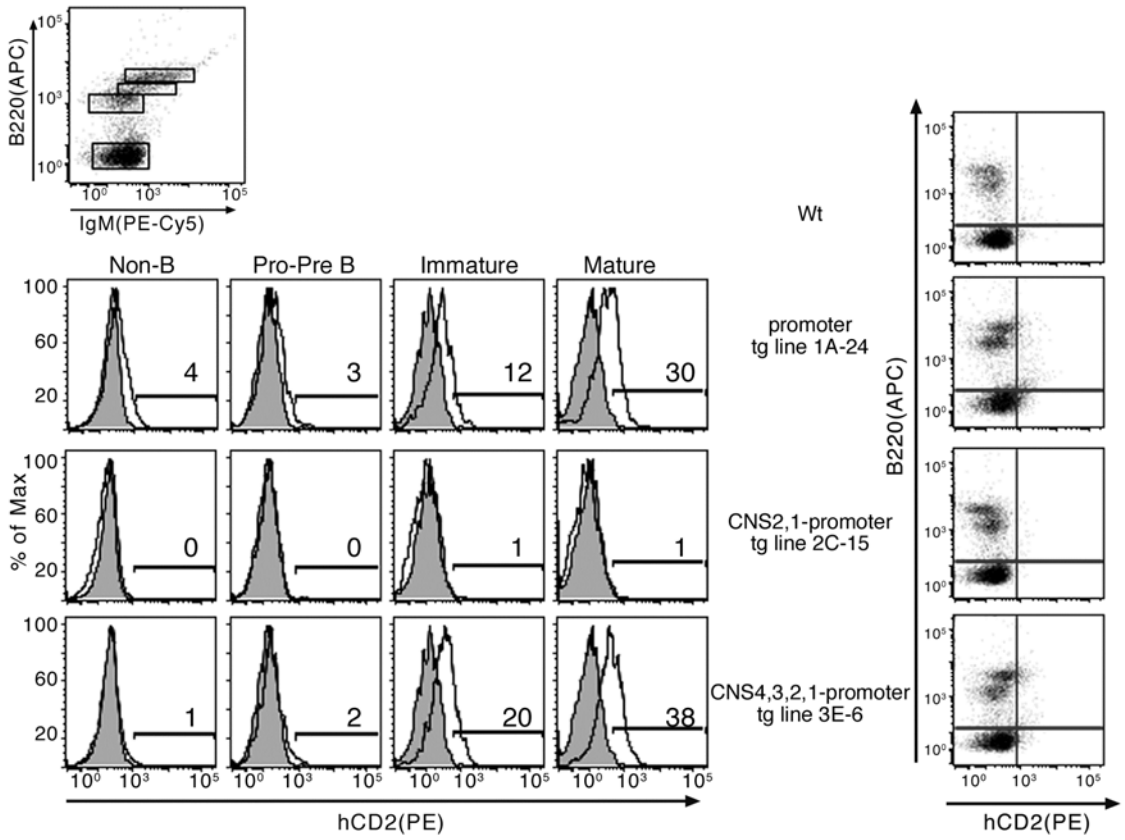
Supplementary figure 4. **Reporter expression in CD4 and CD8 single positive thymocytes in transgenic mice.** Thymocytes from trasngenic lines 1A-24, 2C-15, 3D-9 and 3D-6 were stained for CD4, CD8, hCD2 and either CD69 (A) or HAS (B) . hCD2 expression was then analysed by flow cytometry in CD4SP and CD8SP classified into $CD69^{low}$, $CD69^{high}$, $CD24^{low}$ or $CD24^{high}$ populations. Numbers in histograms represent the mean fluorescence intensity for the hCD2 (PE) stainnig.



Supplementary figure 5: **Expression kinetics of hCD2 in transgenic mice containnig CNS4, 3, 2, 1 elements and the promoter.** Transgenic splenocytes were stimulated with plate-bound CD3 and CD28 specific antibodies for 2, 6, 12, 24 and 48 hours. At indicated times, hCD2 and CD69 was evaluated by flow cytometric analysis. (A) Mean fluorescence intensity (MFI) of CD69 (FITC) and hCD2 (PE) in transgenic lines 3C-45, 3D-9 and 3E-6. Mean values ±SEM of two mice per point are shown. (B) Dot blots showing the expression profiles of hCD2 and CD69 in transgenic line 3D-9.



Supplementary figure 6: **Reporter expression in transgenic mice containnig CNS4, 3, 2, 1 elements and the promoter after poly (I:C) treatment.** (A) hCD2 and CD69 cytometric analysis in T cells from transgenic lines 3C-45, 3D-9 and 3E-9 after 18h of i.p. poly (I:C) injection at a dose of 500μgr.



Supplementary figure 7. **Reporter expression during B cell development in transgenic mice.** (A) hCD2 expression in transgenic lines 1A-24, 2C-15, 3E-6 in non-B cells ($B220^{-}IgM^{-}$) pro-pre B cells ($B220^{+}IgM^{-}$), immature B cells ($B220^{+}IgM^{+}$) and mature B cells ($B220^{high}$ IgM^{+}) from bone marrow samples (open histograms). Analyses of control wild type mice are also shown (shaded histograms). Dots plots of B220 expresion versus hCD2 expression are also shown (right panels).

Supplementary table 1

Table1: Primers used for different applications

Southern blot probes	
P1-CD69 F	5’-CCAACTATCTTGGCGGTGTTG-3’
P1-CD69 R	5’-ATAAATTGCCTGGCTTGACC-3’
P2-CD69 F	5’-GGTAAAATTGTGAAGTTCCTGTGC-3’
P2-CD69 R	5’-GTTAGGTGAAGTGGGCTTGG-3
P3-CD69 F	5’-GCTTTTCTGGTAGTATGAGTTTGTTGG-3’
P3-CD69 R	5’-TCTGTCTATCCAGACACATTTG-3’
P4-CD69 F	5’-ATTACCAAAGGCTGCCACAG –3’
P4-CD69 R	5’-GTGGGAGGCTCATCTCTCAG-3’
hCD2 construct	
Prom F (ClaI)	5’-TATTAGCTAGGAGCTGTCTTGTTCAGTGTC-3’
Prom R (ApaI)	5’-TATGGGCCCAAGCTTTCTGTTTCCTGCACT-3'
CNS1 F (ApaI)	5'-TATGGGCCCGTGGTCTGAAACTTCCTCGGTG-3'
CNS1 R (MluI)	5'-TATACGCGTTGGAAAAAGAACCGCTAAGAC-3’
CNS2 F (MluI)	5’-TATACGCGTATTTCATGGGTTTCATTCC-3'
CNS2 R (EcoRI)	5’-TATGAATTCCACCACTTTCTCTGAGGGTC-3'
CNS3 F (EcoRI)	5'-TATGAATTCCTCCAGCCCTGAAAGTTTTTTC-3’
CNS3 R (EcoRI)	5’-TATGAATTCAGTGACTTCCCTAAGCTCATGAAT-3’
CNS4 F (EcoRI)	5’-TATGAATTCGGGGAAATTTTCTTAATCTGAC-3’
CNS4 R (XbaI)	5’-TATTCTAGAGACCTCCAAAAGTACCCTTC-3’

Detection of hCD2 transgenic mice

hCD2 F	5'-TTACAATGCCTTGGAAACC-3'
hCD2 R	5'-TGACTTTGTTCCCTGCTGTG-3'

hCD2 transgene copy number and chromatin immunoprecipitation

CD2.3 F	5'-ACCACAACCCTGACCTGTG-3'
CD2.4 R	5'-TGGATTCTGAGGGGTTGAAG-3'
Exon III F	5'-CAACTCTTGGTTGTGGTGG-3'
Exon III R	5'-CAGGCTTGTACGAGAAGTTGG-3'
β_2-Microglobulin F	5'-AGGCTGAACGACCAGATACAC-3'
β_2-Microglobulin R	5'-AGGTTACAAAGGGACTTTCCC-3'
CAD F	5'-TTCTAACTTGACCGGCTGGTT-3'
CAD R	5'-GGACCATAGGATGGTTCACAG-3'
PROM F	5'-TGTACTCACTCAACTCAGCC-3'
PROM R	5'-AGCACATTTCAGACAGCAG-3'
IR (-3.4kb) F	5'-TTTAAAGCTGTGGGCTCCTC-3'
IR (-3.4kb) R	5'-AACTCCCCAGAAAAGCCTTG-3'
CNS1 F	5'-GGCACAGACAACTGAGGTGA-3'
CNS1 R	5'-CCAGCTGCTGTGCAGGAAAG-3'
CNS2 F	5'-CGGTGACAACCTGCTCATC-3'
CNS2 R	5'-TGCAAATGGAAACTCTGATCC-3'
IR (-20kb) F	5'-GCCTCTAGAATACCCGCTTG-3'
IR (-20kb) R	5'-GGAGGACTGAGAGGGAGGAA-3'
CNS3 F	5'-AGAAGTGGGTGGGGAATAGC-3'
CNS3 R	5'-AAGAGATCTGTTGGGTCTTTTC-3'

IR (-30kb) F	5’-CCTTCTTCCTTCCTTTGTGC-3’
IR (-30kb) R	5’-GTGCATGTACACTCACACATTTATC-3’
CNS4 F	5’-TGTGGCTGTTTCCTCTTCAG-3’
CNS4 R	5’-ACCCTTCAGTTTTGTGACTTC-3’

CHAPTER 2

Caracterització de l'estructura de la cromatina de l'intró I del gen de CD69 a limfòcits

Resum

CD69 és una de les primeres proteïnes expressades després de l'activació dels leucòcits i el resultat és la regulació del trànsit de limfòcits i la secreció de citocines. L'expressió de CD69 és induïble i està fortament controlada a nivell transcripcional. Les bases moleculars per a una regulació específica de teixit, però, continua sent bastant desconeguda. L'anàlisi *in silico* de regions intragèniques del gen de CD69 va revelar una estructura de la cromatina específica entre les seqüències codificants I i II (Intró I) associada amb nucleosomes posicionats. Hem identificat un lloc nou d'hipersensibilitat al intró I del locus CD69 humà que és induïble per estimulació. Una anàlisi semblant en el locus murí ha revelat una regió hipersensible a la DNasa I, que sembla implicar almenys les primeres 2kb de l'intró I. L'assaig d'immunoprecipitació de cromatina en les diferents etapes del desenvolupament de cèl·lules T va mostrar nivells baixos i intermedis de l'acetilació i dimetilacio de la lisina 4 (K4) de la histona H3 respectivament a timocits $CD69^-$. L'expressió de CD69 a timocits es va associar amb una inducció clara de l'acetilació de la histona H3 a l'intró I. Curiosament, els limfòcits T perifèrics presenten alts nivells d'aquestes marques de forma independent a l'expressió CD69. Per tant, l'intró I pot jugar un paper crucial en el control de l'expressió del gen de CD69.

Chromatin structure characterization of the CD69 gene intron I in lymphocytes

Summary

CD69 is a one of the earliest proteins expressed after leukocyte activation and its engagement results in the regulation of lymphocyte trafficking and cytokine secretion. CD69 inducible expression is strongly controlled at the transcriptional level. The molecular basis for developmental and stage-specific regulation in T cells is beginning to be elucidated while it remains largely unkown in the rest of immune cells. *In silico* analysis of intragenic regions of CD69 gene revealed a specific chromatin structure between coding sequence I and II (intron I) associated with positioned nuclesomes. We have identified a novel hypersensitive site (HS) within intron I of the human CD69 locus that is inducible upon stimulation. Similar analysis in the murine locus has revealed a DNase I hypersensitive region that appears to involve at least the first 2kb of intron I. Chromatin immunoprecipitation assay (Chip) at different stages of T cell development revealed low and intermediate levels of histone H3 acetylation and K4 dimethylation respectively in $CD69^-$ thymocytes. Expression of CD69 in thymocytes was associated with a clear induction of H3 acetylation. Interestingly, peripheral T lymphocytes presented high levels of these activation histone marks independently of CD69 expression. Thus, chromatin structure of the CD69 gene suggests a role for intron I in the control of CD69 expression.

Introduction

CD69 is a transmembrane C-type lectin with type II orientation involved in the control of immune reactivity through the production of a variety of cytokines and lymphocyte migration. CD69 null mice present an enhanced NK cell-dependent antitumor response, an exacerbated collagen-induced arthitis and attenuated eosinophils-dependent induced airway inflamation that supports a role in the balance of Th1/Th2 responses (Esplugues et al., 2003; Miki-Hosokawa et al., 2009; Sancho et al., 2003). Importantly, CD69 has also been described to physically interact with $S1P_1$ receptor in T and B lymphocytes, and CD69 upregulation results in the down modulation of $S1P_1$ receptor at the cell surface resulting in the inhibition of lymphocyte egress from thymus and peripheral lymphoid organs (Shiow et al., 2006).

In the thymus, specific MHC-TCR interactions (Le Bon et al., 1996) induce CD69 expression in $CD4^+CD8^+$ double positive (DP) thymocytes undergoing positive selection and in transitional single positive (SP) thymocytes (Swat et al., 1993; Yamashita et al., 1993). CD69 is broadly expressed in bone marrow-derived cells and its expression is inducible in many immune cells upon stimulation. In this regard, CD69 expression in B-cell lymphocytic leukemia is associated with poor disease prognosis (D'Arena et al., 2001) and a recent work has reported diminished CD69 protein levels in T regulatory lymphocytes in patients with multiple sclerosis that correlates with reduced suppressive activity (Radstake et al., 2009). Importantly, several reports support the therapeutic effect of anti-CD69 monoclonal antibodies in the treatment of

different animal models of cancer, infection, autoimmunity and asthma (Esplugues et al., 2005; Miki-Hosokawa et al., 2009; Sancho et al., 2006; Vega-Ramos et al., 2010).

CD69 gene is located in the NK complex in mouse chromosome 6 and human chromosome 12 (Lopez-Cabrera et al., 1993; Ziegler et al., 1994). CD69 induced expression is transient and it is strongly controlled at the level of transcription (Cebrian et al., 1989; Sutcliffe et al., 2009; Ziegler et al., 1994). The cis- and trans-acting elements involved in the basal and inducible promoter activity have been described in transient transfection assays (Castellanos et al., 1997; Castellanos Mdel et al., 2002; Lopez-Cabrera et al., 1995). In the past decade, increasing evidence for a role of epigenetics in the development of human disease has highlighted the importance of understanding epigenetic mechanisms involved in gene expression (Egger et al., 2004). Transcriptional regulation of the CD69 gene in a genomic context has been addressed by our research group (Vazquez et al., 2009). Despite CD69 promoter presented an open constitutive chromatin configuration and conferred developmentally regulated expression during positive selection of thymocytes, DNA distal elements, identified by its sequence conservation between species and specific chromatin modification, were required for developmental-stage and lineage-specific regulation in T cells. The study, however, failed to identify those elements important to regulate CD69 expression in B-lymphocytes. The nucleosome content at the CD69 promoter was recently reported (Sutcliffe et al., 2009). Transcription start site (TSS) was shown to be flanked by H2A.Z and H3 histones in unstimulated lymphocytes and transcription activation resulted in

their depletion and in a concomitant deposition of H3.3. Importantly, it is thought that H2AZ plays a role in maintaining genes in a poised state while H3.3 is associated with active transcription.

The present study aims to uncover new cis-acting elements that may be involved in CD69 gene expression. We describe the chromatin structure of the intron I of the mouse and human CD69 gene locus. Intriguingly, *in silico* studies in unstimulated cells showed features characteristic of actively transcribed genes in the body of the gene that included intron I and promoter. DNase I digestion analysis also revealed an inducible HS site region at the intron I. Analysis of histone post-translational modifications in this region indicated that acetylation and lysine 4 (K4) dimethylation of histone H3 were dynamically regulated during T cell development and were constitutively high in resting and stimulated mature T lymphocytes. These studies point to intron I as an important regulatory element of CD69 expression.

Material and Methods

DNase I Hypersensitivity assay

Human peripheral blood lymphocytes (PBLs) and Rag-$1^{-/-}$, OT-I TCR transgenic splenocytes were used for the experiments. In both cases, red blood cells were lysed in 0.15M NH_4Cl, 1mM $KHCO_3$, 1mM Na_2-EDTA, pH 7.4 for 3min at 4ºC, subsequently washed in 30ml of cold PBS and resuspended at a concentration of 2.10^6/ml in complete media. Prior to DNase I digestion, human lymphocytes were stimulated *in vitro* with 10ng/ml PMA (Sigma Biochemical) and 0.5µM

Calcium Ionophore (Sigma) and murine lymphocytes with 5µg/ml plate-bound anti-CD3 (clone 145-2C11, eBiosciences) and anti-CD28 (clone 37.51, eBiosciences) monoclonal antibodies for 6h at 37ºC. After this time, cells (10^7/ml) were permeabilized with 0.067mg/ml lysolecithicin (Sigma) in buffer C (0.15M Sucrose, 80mM KCl, 30mM Hepes pH7.4, 5mM $MgCl_2$, 5m $CaCl_2$) for 5 minutes and were left untreated or were treated with DNase I for 10min on ice. Reactions were stopped by the addition of EDTA, SDS and Proteinase K to final concentrations of 10mM, 0.4% (w/vol) and 0.4mg/ml, respectively, and were incubated overnight at 37ºC. DNA was purified by phenol, phenol:chloroform and chloroform extractions and ethanol precipitation. For southern blot, genomic DNA was resuspended in conventional TE buffer (10mM Tris-HCl pH8, 1mM EDTA) and for quantitative PCR analysis in modified TE buffer (10mM Tris-HCl pH8, 0.1mM EDTA).

Measurement of chromatin accessibility by southern blot

For human samples, purified DNA (10µg) was incubated overnight at 37ºC with an excess of NdeI restriction enzyme and was separated by 0.7% (w/vol) agarose gel electrophoresis. DNA was denatured by soaking the gel in 0.5M NaOH/1.5M NaCl for 45min and neutralizing in 1M Tris-HCl pH8, 1.5M NaCl for 45min. DNA was transferred to nitrocellulose membranes by capillary action in 20X SSC overnight, followed by cross linking with UV light (Stratalinker). Blots were prehybridized for 3h at 42ºC with hybridization solution (50% formamide, 5X Denhardt's solution, 0.5% (w/vol) SDS, 50mM Hepes pH7) containing 100 µg/ml denatured salmon sperm DNA and were probed with denatured ^{32}P-labeled DNA fragments (Invitrogen Random primer kit). Primers used to

generate probe S1-H were S1 (F): 5'-CCCTCTGTACAATGGTGAAACA-3' and S2 (R): 5'-GCAACTTCTCTTGGTCCAGTTT-3'. Blots were washed twice in 1X SSC, 0.5% (w/vol) SDS for 15min at room temperature, twice in 0.1X SSC, 0.1% (w/vol) SDS for 15min at 60º C and once in 0.1XSSC, 0.1% (w/vol) SDS, 0.1mg/ml proteinase K for 30 min at 37ºC. DNA bands were visualized by autoradiography.

Measurement of chromatin accessibility by real-time PCR

For mouse CD69 intron I, accessibilty to DNase I digestion was analysed by quantitative PCR as described previously with only minor alterations (Schoenborn et al., 2007). Twenty nanograms of purified genomic DNA were used to perform SYBR Green real time PCR according to manufacturer's instruccions (Roche). The PCR cycling program included a touchdown phase in which the annealing temperature was progressively lowered from 65^0C to 60^0C by 1^0C every two cycles followed by 30 cycles at 60^0C. After amplification, a melting curve and peak profile was obtained to verify the specificity of each primer pair. A standard curve using serial dilutions of genomic DNA was generated to quantify cycle threshold (Ct) values of each amplicon. Each PCR was performed in triplicates and DNase I sensitivity was expressed as the percentage of accessibility [=100*(1-DNase I-treated sample/untreated DNA)]. Primer sequences are:

Set 1 F 5'-TGATAAATTGCCTGGCTTGAC-3',

Set 1 R 5'-TGGATAAGGAAAGAGCAAGAAC-3',

Set 2 F 5'- TTGAGTCCAAGCAATGTGAC-3',

Set 2 R 5'- TAGGTCTGTTTTCCCCTGC-3',

Set 3 F 5'- TCTGAGCATTCCATCTCCC-3',

Set 3 R 5'- TTTAAGACTTCTAAGCACCCAC-3'.

Chromatin Immunoprecipitation assay

Chromatin immunoprecipitation was performed as described previously (Vazquez et al., 2009) with total thymocytes of Rxβ and HA tg cells and resting or activated purified T cells. Antibodies used for Chip were anti-diacetylated H3, anti-dimethylated H3 K4 and control rabbit-IgG antibodies (Upstate Biotechnology). Bound and input fractions were quantified using SYBR green real-time PCR (Roche). Analysis of the constitutively active carbamoyl transferase dihydrorotase (CAD) gene was used to normalize values of different samples. Primer sequences are:

Intron I F 5'-GCACAGCAAACATACCCAC-3',

Intron I R 5'-CACGTGGGAGTTGGTCTAC-3',

Promoter F 5'- TGTACTCACTCAACTCAGCC-3', P

Promoter R 5'- AGCACATTTCAGACAGCAG-3',

Exon III F 5'- CAACTCTTGGTTGTGGTGG-3',

Exon III R 5'-CAGGTTGTACGAGAAGTTGG-3',

CAD F 5'-TTCTAACTTGACCGGCTGGTT-3',

CAD R 5'-GGACCATAGGATGGTTCACAG-3'.

***In silico* analysis of ENCODE data**

Chromatin immunoprecipitation data for RNA Polymerase II and histone methylations was obtained from:

http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.html

Data is displayed on the UCSC human genome browser.

Results

In silico analysis

As an attempt to identify chromatin marks that reveal new regulatory regions in the CD69 gene, we analysed open chromatin regions, nucleosome occupancy and chromatin immunoprecipitation data for RNA Polymerase II (PolII) and histone methylations avaible at the UCSC genome browser and http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.html (fig.1).

Analysis of open chromatin in the CD69 gene in the lymphoblastoid GM12878 cell line showed a marked open chromatin region in the intron I in addition to the promoter (fig. 1A). Similar results, although less pronounced, were observed in erithroleukemic K562 cells. Moreover, nucleosome occupancy studies in the CD69 gene showed certain assignment of nucleosome position at intron I in melanoma and breast cancer cells (fig. 1A).

Methylation marks in the human CD69 locus in primary non-stimulated $CD4^+$ T cells showed the presence of activation marks not only at the intron I but also the promoter and within the gene body (Barski et al., 2007) (fig.1B). H3K4 tri-, di- and monomethylation were enriched sorrounding the TSS, being the trimethylated mark the most pronounced one. H3K4me2 and me1 were also observed in the coding region. Curiously, H3K36me3 and H4K20me1, both associated with actively transcription, was elevated at the gene body. In addition, PolII binding was observed at the promoter and within the coding region. In agreement with these analysis, a recent genome-wide report showed

the association of multiple histone acetylases and deacetylases in the CD69 promoter and gene body in unstimulated primary $CD4^+$ T cells (Wang et al., 2009).

Therefore, by these studies it is predicted the existence of an important elements for CD69 gene regulation in the intron I at the middle region.

Accessibility of CD69 intron I

Hypersensitive sites (HSs) are genomic regions that present increased sensitivity to cleavage by DNase I as a result of altered nuclesomal structure and commonly correspond to cis-acting regulatory elements (Gross and Garrard, 1988). The results from genome wide DNase I HS mapping, prompted us to conduct hypersensitivity experiments in the 4Kb intron I (Fig. 2). For the human CD69 locus, PBLs were *in vitro* cultured with PMA or were left untreated and were subsequently treated with increasing amounts of DNase I. Genomic DNA was purified, digested with NdeI enzyme and analysed by sourthen blot. This assay identified multiple bands in resting and activated PBLs (Fig. 2A). Remarkably, a hypersensitive site was clearly located in intron I (HSa) in unstimulated and stimulated cells, being more prominent after stimulation. Despite this technique does not precisely localize hypersensitive sites, the size of the band suggested its localization in the middle of the intron sequence coincidental with predicted open and active chromatin.

As several HSs were previously identified in the murine CD69 promoter and upstream regulatory regions, we then investigated whether the novel DNase I

HS in human intron I was also present in the murine one. Splenic $Rag^{-/-}$, OT-I T lymphocytes were stimulated or not with antiCD3/CD28 antibodies and were subsequently treated or not with DNase I. After genomic DNA purification, chromatin accesibility was then measured by real-time PCR (fig. 2C). In the basal state, the proximal region of intron I was accessible to nuclease digestion (primer set 1and 2) while the distal one (primer set 3), the closest to exon II, exhibited low level of nuclease cutting. CD69 gene displays rapid transcriptional induction after activation and, consistent with this, stimulation of cells resulted in a substancial increase in the chromatin accessibility across the entire intron I. Hence, our experimental data confirm the existence of HSs located in intron I in the human and mouse CD69 locus.

Chromatin structure of CD69 intron I

CD69 is expressed in the surface of approximately 10-15% of immature $CD4^{+}CD8^{+}$ double positive (DP) thymocytes that are undergoing positive selection and in transitional SP thymocytes (Swat et al., 1993; Yamashita et al., 1993). Only a minor fraction of unstimulated lymphocytes express CD69 but it is transcriptionally induced by many stimuli. We examined the amount of acetylated H3 (AcH3) and K4 dimethylated H3 associated with intron I at different stages of T development and in resting and activated mature T cells. We used thymocytes from B10.D2 $H\text{-}2K^{d}$ mice expressing a transgenic TCR specific for a hemagglutinin peptide presented by $H\text{-}2K^{d}$ (HA) to isolate thymocytes with high CD69 expression and thymocytes from $Rag2^{-/-}$mice that express a *Tcrb* transgene (Rxβ) to isolate thymocytes with low CD69 expression. We recently described that the CD69 promoter is always associated

with an open chromatin configuration. Here, we observed low and moderate levels of AcH3 and H3K4dim in the intron I in CD69$^-$ Rxβ cells (Fig. 3A and 3B). Remarkably, there was a 5-fold increase in the amounts of AcH3 and nearly a 2-fold increase in the amounts of H3K4dim in CD69$^+$HA transgenic cells (Fig. 3A and 3B). In peripheral T cells, regardless the expression of CD69, the chromatin of intron I always presented high levels of AcH3 and H3K4dim, exceeding the amounts observed at the promoter (Fig. 3C and 3D). Thus, this experimental analysis indicates that CD69 intron I is differentially remodelled at different stages of T cell maturation.

Discussion

DNA regulatory elements required for tissue-specific gene transcription may be widely dispersed in the genome or may be located in introns. Indeed, relevant immune genes have been shown to contain potent cis-acting elements within intronic sites (Decker et al., 2009; Sawada et al., 1994; Soutto et al., 2002). In this study we provide evidence for the existence of a new cis-acting element within CD69 intron I by means of DNase I hypersensitivity, chromatin immunoprecipitation assays and *in silico* bioinformatics analysis.

We have identified an inducible HS present in the intron I of CD69 (HSa). Our previous paper identified multiple HSs in the murine CD69 promoter and associated regulatory regions CNS1, CNS2, CNS3 and CNS4 (Vazquez et al., 2009). These HSs sites, however, were constitutive and presented similar pattern in CD69$^-$ and CD69$^+$ cells. HSa was also present in unstimulated human

PBLs and, similarly to the other regulatory regions, suggests the existence of pre-bound transcription factors to this site. The strong inducibility of HSa, however, may also indicate that it is a site of transcription factor recruitment dependent on stimulation. Results from DNase I digestion assays coupled to quantitative PCR in the murine locus are in concordance with data obtained from human samples and database analysis that clearly identified the same intronic HS. Genome wide nucleosome maps have provided information about the organization of nucleosomes around protein-coding genes (Jiang and Pugh, 2009; Ozsolak et al., 2007; Schones et al., 2008). In human $CD4^+$ T cells, nucleosomes near the TSS of expressed or poised genes are well positioned and positively correlates with polymerase binding while there is a tendency for random nucleosome positions in unexpressed genes (Ozsolak et al., 2007; Schones et al., 2008). The binding of transcription factors and other regulatory proteins is thought to be the cause of this nucleosome phasing. Examination of the nucleosome profile at the CD69 locus in different human cells lines reveals both well-positioned and delocalised nucleosomes. Notably, more localized nucleosomes are observed in the HS region in intron I and, thus, supports the potencial existence of regulatory elements in this region. The fact that positioned nucleosomes can be detected in non-lymphoid cell types perhaps suggests that the permissive state occurs prior to lineage differentiation as described for other human genes that already present a permissive structure in embryonic stem cells (Guenther et al., 2007).

Our data for intron I from chip experiments suggests that CD69 gene is differentially regulated at a chromatin level during T cell maturation. In

thymocytes, it is possible that specific MHC:TCR interactions results in signal-induced acetylation and methylation events at the intron I despite detectable levels of these histone marks are observed in $CD69^-$ cells. On the other hand, CD69 expression in mature T cells may be regulated at post-initiation transcriptional steps in a similar manner described for some inflammatory genes (Hargreaves et al., 2009). Indeed, high levels of H3K4me3, H3Ac and prebound Pol II mark many transcriptionally silent primary response genes (Hargreaves et al., 2009) and PolII and H3K4m3 islands have also been described in many inactive human genes (Barski et al., 2007).

In conclusion, although functional assays will be conducted to evaluate the contribution of intron I to the tissue-specific CD69 expression in a chromatin environment, our studies predict the existence of a cis-acting element within this region.

Figures

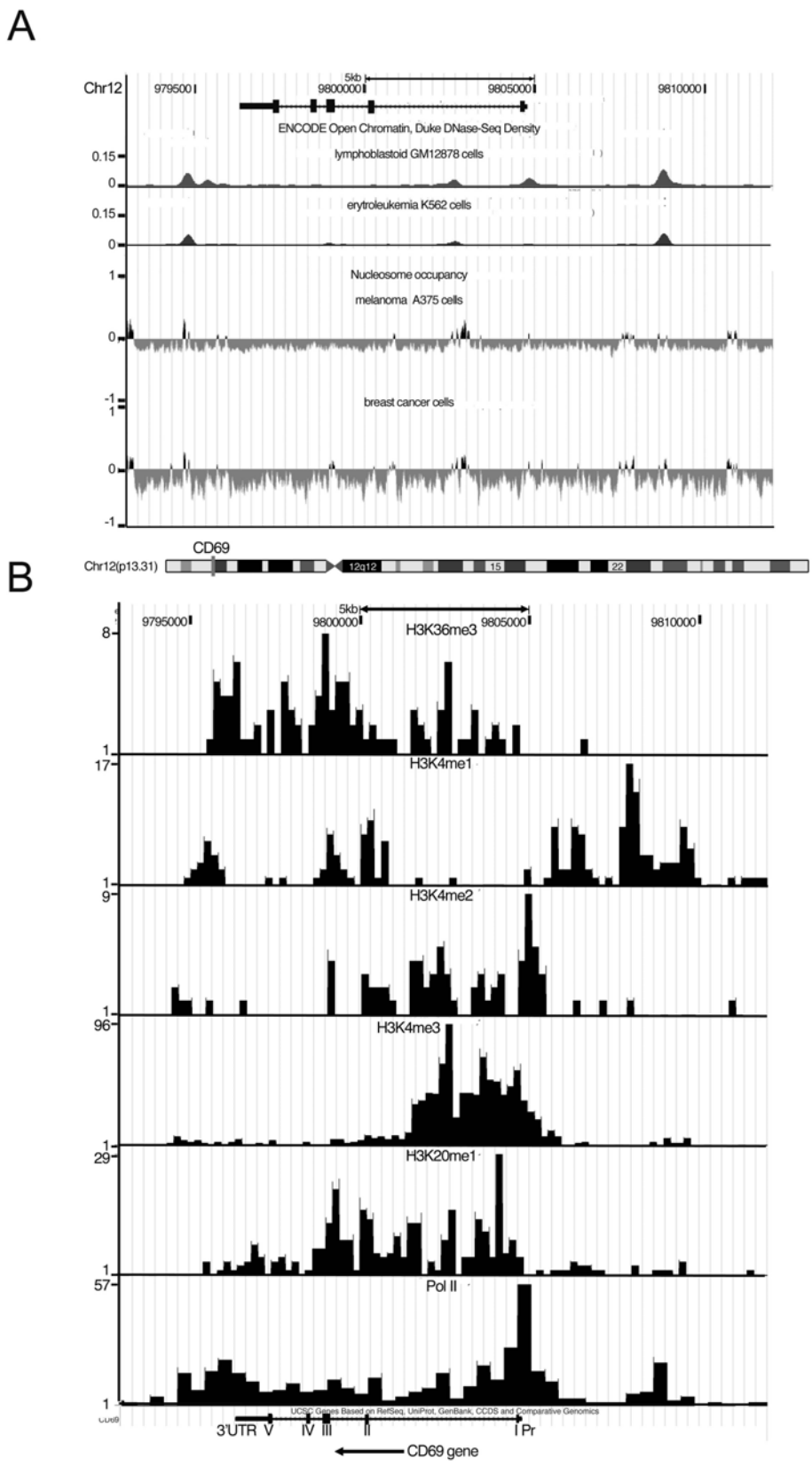


Figure 1: **UCSC genome browser display on human CD69 locus.**

(A) Open chromatin region track in GM12878 and K562 cells and nucleosome occupancy track in melanoma and breast cancer cells. For nucleosome position, a score of 0 indicates an uncertain assignment; a score of 1.0 corresponds to a position of frequent nucleosome occupancy and a score of -1.0 corresponds to a confident prediction for being in the negative class. (B) Chromatin modification features of the CD69 gene showing H3K36me, H3K4me, -me2 and me3 and RNA PolII profiles in primary unstimulated $CD4^+$ T cells.

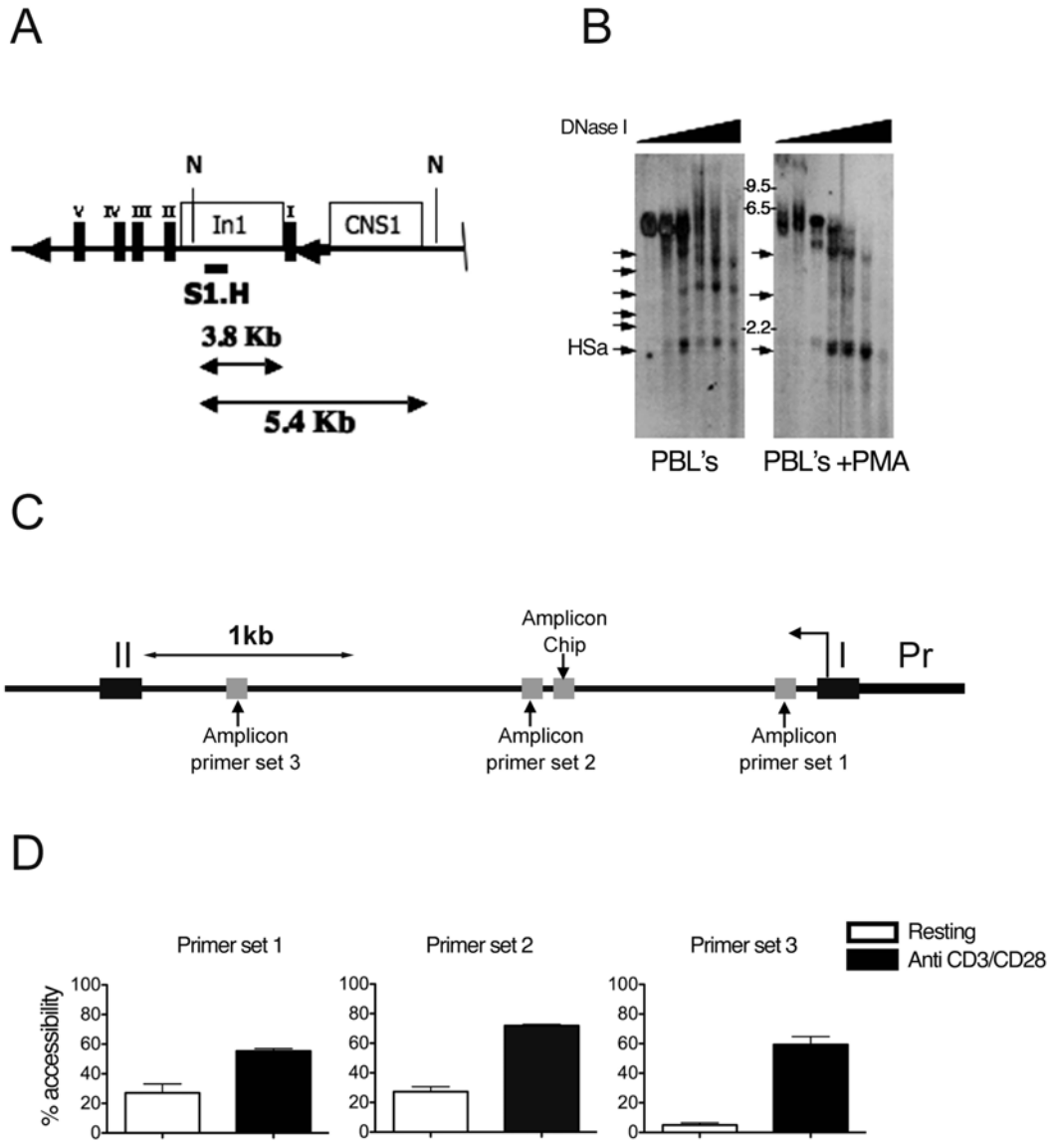


Figure 2: **Chromatin accessibility in the intron I of the CD69 gene.** (A) Map of the human CD69 locus showing locations of significant features (In1: Intron 1; CNS1: Conserved non-coding sequence 1) and the genomic fragment expected from NdeI digestion. Probe used for Southern blot (S1.H) is also shown. (B) DNase I analyses using S1.H probe. Human peripheral blood lymphocytes were activated with 10ng/ml PMA or were left untreated and were subsequently treated with increasing amounts of DNase I. NdeI digested DNA was examined by Southern blot. Size markers and relevant bands (HS sites) are denoted. (C) Schematic diagram showing murine intron I and PCR amplified regions in DNase I and Chip experiments. (D), Chromatin accessibility in murine CD69 intron I. T lymphocytes were either stimulated with

antiCD3/CD28 mAb or not and were incubated with or without 30u/ml of DNase I enzyme. Twenty nanograms of purified genomic DNA from each treatment were subjected to quantitative PCR. Results are plotted as the percentage of digested DNA in relation to the undigested sample. The data are representative of two experiments and are expressed as the mean ± SEM of triplicate PCRs.

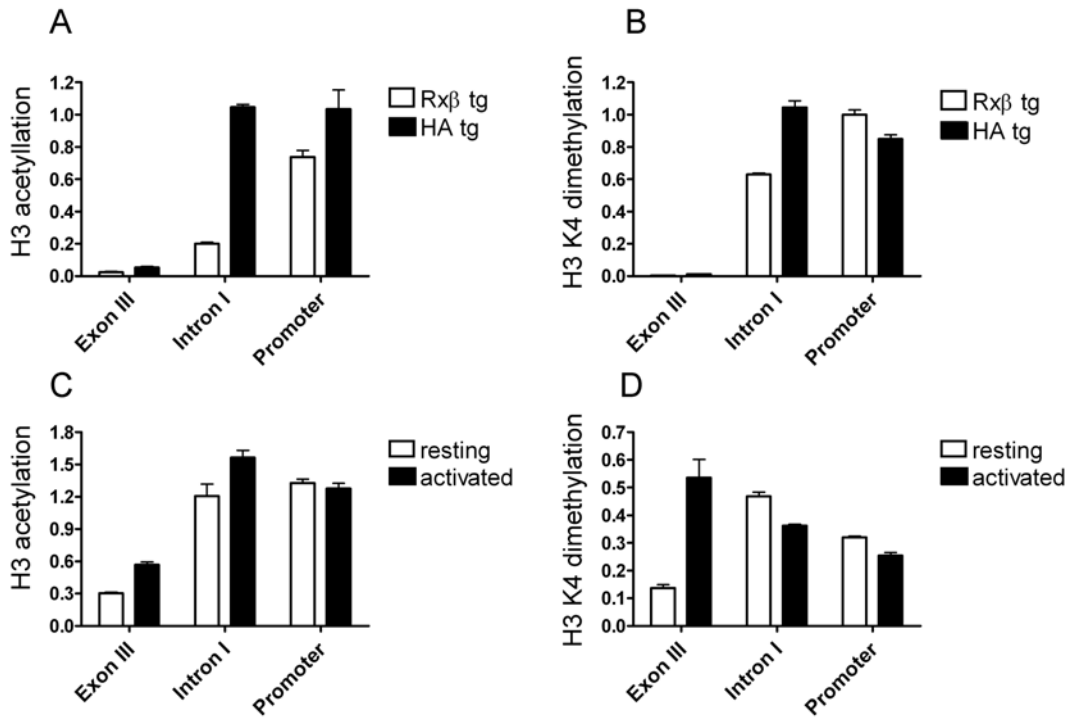


Figure 3: **Chromatin structure at the intron I of the CD69 gene.** (A) and (B) ChIP performed on purified mononucleosomes obtained from total thymocytes of Rxβ and HA transgenic mice using anti acetylated H3 (A) and dimethylated H3 K4 (B). (C) and (D) ChIP performed on sonicated chromatin from purified T cells either unstimulated or stimulated with 10ng/ml PMA plus 0.5μM Io for 6h using acetylated H3 (C) and dimethylated H3 K4 (D).

IV. DISCUSSIÓ

En aquesta tesi s'han proporcionat mecanismes que regulen l'expressió del gen CD69 *in vivo* partir de la identificació de nous elements en *cis* i l'anàlisi de la seva l'estructura de la cromatina i funció. S'han identificat quatre seqüències no codificants conservades a sobre del promotor de CD69. Anàlisis de la cromatina i funcionals indiquen que el promotor de CD69 adopta una conformació oberta i pot dirigir la transcripció del gen reporter en ratolins transgènics. No obstant això, el reporter, a diferència de mCD69 endògen, s'expressava en limfòcits perifèrics no estimulats, la qual cosa suggereix que uns altres elements han de participar per aconseguir una especificitat adequada. Hem detectat diversos llocs d'hipersensibilitat a la DNasa I, que es localitzen en les seqüències conservades no codificants i que mostren un perfil epigenètic diferent entre cèl·lules T i B i dinàmic durant el desenvolupament de cèl·lules T. L'anàlisi de la funció del CNSs en assajos de transfecció transitòria va revelar una activitat enhancer constitutiva i induïble de CNS2 i CNS4, però sense activitat aparent per CNS1 i CNS3. No obstant això, trobem que els ratolins transgènics amb CNS1 i CNS2 presenten una expressió reduïda del reporter en les cèl·lules T i B i que aquesta inhibició només és superada per la inclusió de CNS3 i CNS4. La combinació dels quatre CNSs ha permès nivells alts d'expressió del reporter, degudament regulat, en les cèl·lules T, però no en les B.

La comparació de seqüències d'ADN s'ha convertit en una eina útil per identificar elements específics de control remot que participen en la regulació de l'expressió gènica (Ansel et al., 2004; Hatton et al., 2006). Hem observat

illes discretes de conservació en una regió de 45 kb per sobre del gen CD69 i vam trobar que corresponen a llocs que exhibeixen hipersensibilitat a la digestió per DNasa I. Cal assenyalar que aquestes regions eren igualment sensibles a la digestió per DNasa I en limfòcits CD69- i $CD69^+$. CD69 és un dels primers antígens expressats per activació i, per tant, és possible que aquestes regions estiguin ocupades per proteïnes, fins i tot abans de l'activació. D'acord amb els nostres resultats, el primer mapa genòmic de regions hipersensibles a la DNasa I en cèl·lules T $CD4^+$ humanes identifiquen llocs d'hipersensibilitat situades al promotor de CD69, CNS2, CNS3 i CNS4 al locus CD69 humà (Boyle et al., 2008).

Treballs anteriors han demostrat que el promotor de CD69 pot donar suport a una ràpida inducció de l'expressió de gens reporter en assajos de transfecció transitòria. Nosaltres ens trobem que el promotor de CD69 s'associa sempre amb una configuració "activa" de la cromatina, fins i tot en situacions en què no expressa CD69. El promotor mostra alts nivells d'acetilació i dimetilació K4 de les histones H3 en poblacions de timocits i limfòcits perifèrics. S'ha demostrat que aquestes dues modificacions marquen la competència de la transcripció dels loci IL-4 i IL-2 a les cèl·lules T (Adachi and Rothenberg, 2005; Avni et al., 2002) i pot ser important per mantenir també la competència de la transcripció del gen CD69. Curiosament, l'anàlisi de modificacions de la cromatina a escala genòmica en limfòcits T $CD4^+$ humans no estimulats va mostrar l'enriquiment de H3K4m3 i de l'ARN polimerasa II al promotor, l'exó I i l'intró I de mCD69

(Barski et al., 2007). Aquestes dades suggereixen que, de la mateixa manera que altres gens que requereixen una ràpida inducció, el promotor de CD69 pot albergar l'ARN polimerasa II pausada (Cui et al., 2004; Muse et al., 2007). CNS1 és la regió distal del promotor de C69 i un resultat notable és que aquesta regió va presentar un pic de marques d'histones permissiva que es correlaciona amb l'expressió CD69 en totes les poblacions de cèl·lules analitzades. No obstant això, el promotor per si sol va mostrar activitat induïble en transfeccions transitòries i ratolins transgènics, i almenys en el cas d'assajos transitoris, CNS1 no tenia cap influència sobre l'activitat basal o induïble del promotor. Això suggereix que les modificacions d'histones induïbles en aquest lloc poden dependre d'altres elements.

L'expressió específica de teixit dels gens depèn tant d'activadors com dels silenciadors. Un cas ben estudiat és el de CD4, en el qual un silenciador reprimeix el promotor de CD4 a timocits dobles negatius i $CD4^{-}CD8+$ i un *enhancer* (Sawada et al., 1994; Zou et al., 2001). Així mateix, la regulació transcripcional de l'IL-4 requereix l'acció d'un element silenciador a 3' del gen que s'ha descrit que reprimeix l'expressió del gen reporter en ratolins transgènics (Lee et al., 2001) i la seva deleció de la línia germinal provoca una expressió aberrant d'IL-4 a les cèl·lules Th1 (Ansel et al., 2004). D'altra banda, hi ha exemples d'elements bifuncionals, que funcionen com a enhancers i silenciadors en funció de l'estadi de desenvolupament (Bilic et al., 2006; He et al., 2008). Els resultats en ratolins transgènics indica que CNS1, CNS2, o la combinació dels dos elements poden tenir un paper

repressiu en l'expressió del gen CD69 en timocits, així com en cèl·lules T i B estimulades. No obstant això, CNS2 va mostrar una forta activitat enhancer induïble, quan es va provar aïllat per transfecció transitòria en cèl·lules Jurkat. En particular, l'activitat enhancer detectada és coherent amb l'acetilació induïble de les histones H3 en timòcits. Suggerim que CNS2 és un enhancer induïble específic de timòcits que requereix l'activitat d'altres elements reguladors en un context de cromatina. Sorprenentment, la inclusió de CNS3 i CNS4 en la construcció transgènica va conduir a la recuperació de l'expressió del gen reporter i a una ferma regulació de la seva expressió en timòcits i cèl·lules T perifèriques. Sobre la base de la seva activitat *enhancer* induïble a cèl·lules Jurkat i l'acetilació induïble de les histones, CNS4 també sembla funcionar com un enhnacer específic de timocits. A més, creiem que CNS4, no només pot funcionar com un *enhancer* en el context del locus de CD69 endogen, sinó que també, i en conjunció amb CNS3, funciona com un anti-silenciador que canvia l'activitat de CNS2 de silenciador a enhancer. Proposem que els quatre CNSs poden convergir per interactuar físicament i funcionalment en forma d'un centre de cromatina activa, com el descrit inicialment per al locus de la β-globina (Tolhuis et al., 2002). Potser, en concordança amb això, un estudi anterior suggereix que CD69 pot ser regulat per la proteïna arquitectural SATB1 (Alvarez et al., 2000).

Prenem nota que les nostres conclusions sobre les activitats de CNS1-4 *in vivo* suposen que aquests elements funcionen amb normalitat dins el context del nostre reporter transgènic que és relativament compacte. Estudis

anteriors han validat aquest mètode en altres sistemes (Decker et al., 2009; Lee et al., 2001). Tanmateix, no podem descartar que a les nostres construccions transgèniques manquin elements rellevants dels segments d'ADN que normalment separen els CNSs, o que no puguin adoptar configuracions tridimensionals importants per a la regulació fisiològica. Per estudiar més a fons els mecanismes de regulació de CD69 *in vivo*, analitzarem ratolins transgènics per un cromosoma artificial bacterià que conté el locus murí de CD69.

En comparació amb els timocits, el mecanisme d'inducció de l'expressió del gen CD69 a les cèl·lules T madures sembla diferent ja que només CNS1 mostra acetilació i dimetilacio a la lisina 4 de les histones H3 induïble. Això és cert, malgrat el fet que, com en els timocits, CNS2-4 són necessaris per a una expressió regulada induïble en cèl·lules T perifèriques transgèniques. Suggerim que CNS2-4 poden establir un context específic de la cromatina durant el desenvolupament de cèl·lules T, que es requereix per a una adequada regulació del locus CD69 a les cèl·lules T madures.

Encara que la combinació dels quatre CNS1-4 és capaç de promoure alts nivell ben regulats d'expressió gènica del reporter a timocits i limfòcits T perifèrics, no ho va fer així en totes les línies transgèniques. Una explicació d'aquest fet és que encara calen els elements addicionals per reconstituir un *locus control region* que pugui superar els efectes de posició cromosòmica. No obstant això, observem que l'expressió de hCD2 era baixa i variegada en

les línies transgèniques que contenen gran nombre de còpies del reporter. Així, és possible que mecanismes d'ARN d'interferència puguin causar el silenciament del transgèn en aquestes línies (Pal-Bhadra et al., 2002).

La troballa inesperada que els ratolins transgènics amb tots els CNSs expressaven desreguladament el reporter hCD2 en cèl·lules B no estimulades indica que el control de la transcripció del gen CD69 és diferent a les cèl·lules B i T. Així, sembla probable que en la construcció manca un element necessari per reprimir l'expressió en les cèl·lules B en estat basal. Una altra prova per a la regulació diferencial en cèl·lules T i B es va obtenir pel perfil epigenètic de CD69, que va revelar diferents patrons de modificacions de les histones en CNS2, CNS3 i CNS4 entre cèl·lules T i B. En aquest sentit, la transcripció d'IL-2 es creu que és regulada per diferents mecanismes a les cèl·lules CD4+ i CD8+ i ratolins deficients per la regió CNS1 presenten una expressió reduïda de l'IL-4 i IL-13 en cèl·lules Th2, però no en mastòcits(Yui et al., 2001) (Mohrs et al., 2001). Curiosament, ratolins transgènics que porten un fragment genòmic de 30kb, que conté Ly49A, un altre gen del complex NK, que codifica una lectina tipus C, també va mostrar expressió aberrant en les cèl·lules B. Potser, s'utilitzen mecanismes similars per Ly49A i CD69 per suprimir l'expressió a les cèl·lules B (Tanamachi et al., 2004).

En base als resultats, proposem que el promotor de CD69 i els elements no codificants conservats mostren una estructura de la cromatina accessible

abans de la transcripció CD69 per donar suport a una inducció del gens ràpida, després de processos d'estimulació. La inducció del gen requereix l'activitat combinada de múltiples CNSs, dos dels quals mostren activitat enhancer clàssica a les cèl·lules T. La interdependència d'aquests CNSs també pot permetre que una repressió fisiològica de l'expressió CD69, per disrupció de les interaccions de CNS4 i CNS3 amb CNS1 I CNS2. Finalment, un element de cèl·lules B encara no caracteritzat pot ser necessari per suprimir l'activitat del CNSs en cèl·lules B no estimulades.

Els elements d'ADN reguladors necessaris per a una expressió específica de teixit poden estar dispersos en el genoma o poden estar situats als introns. De fet, molts gens immunològics contenen potents elements en *cis* en llocs intrònics (Sawada, Scarborough et al. 1994; Pasca Soutter, Zhou et al. 2002; Decker, di Magliano et al. 2009) En aquesta tesi es proporciona evidència de l'existència d'un nou element en *cis* localitzat a l'intró I de CD69 mitjançant assajos d' hipersensibilitat a la DNasa, d'immunoprecipitació de cromatina i anàlisis bioinformàtics.

S'ha identificat un lloc d'hipersensibilitat induïble, HSa, present a l'intró I de CD69. HSa també é present en limfòcits de sang perifèrica humana en estat basal i, de la mateixa manera que les altres regions reguladores, suggereix l'existència de factors de transcripció constitutivament units a aquest lloc. La seva inducció forta, però, també pot indicar que és un lloc de reclutament de factors de transcripció depenents d'estimulació. Els resultats dels assajos a

la digestió a la DNase I mesurats per PCR quantitativa en el locus de CD69, es troben en concordança amb les dades obtingudes de mostres d'humans. A més, l'anàlisi de bases de dades identifica també clarament la mateixa HSa. És important destacar que HSa és present en diferents tipus de cèl·lules immunes. Potser aquest estat permissiu es produeix abans de la diferenciació de les cèl·lules immunes com a resultat de les propietats intrínseques del gen. Els elements necessaris en cis, necessaris per regular espacialment i temporalment l'expressió del gen CD69 semblen ser diferent en els subtipus de limfòcits (Vázquez, Laguna et al. 2009). Es duran a terme assajos funcionals per avaluar la contribució del intró I en la regulació de l'expressió especifica de teixit de CD69.

Els mapes genòmics de la posició dels nucleosomes han proporcionat informació sobre l'organització dels nucleosomes al voltant dels gens codificadors de proteïnes (Barski, Cuddapah et al. 2007; Ozsolak, Song, et al. 2007; Jiang i Pugh 2009). En cèl·lules T $CD4^+$ humanes, els nucleosomes a prop de l'inici de transcripció de gens expressats o preparats per ser expressats estan ben posicionats i es correlacionen positivament amb els nivells d'ARN polimerasa II unida, mentre que hi ha una tendència a que les posicions dels nucleosomes siguin a l'atzar als gens no expressats (Barski, Cuddapah et al. 2007; Ozsolak, Song, et al. 2007). La unió de factors de transcripció o seqüències d'ADN especifiques es creu que és la causa d'aquest posicionament. L'examen del perfil de nucleosomes al locus CD69 en diferents línies cel·lulars humanes revela tant nucleosomes ben

posicionats com d'altres deslocalitzats. En particular, els nucleosomes més ben localitzats s'observen a la regió intrònica I, i es superposen al lloc HSa. Això dóna suport a l'existència potencial dels elements reguladors en aquesta regió.

Les nostres dades dels experiments d'immunoprecipitació de la cromatina suggereixen que l'intró I de CD69 és regulat de manera diferent a nivell de la cromatina en els diferents estadis de maduració de les cèl·lules T. A timocits, és possible que interaccions específiques MHC:TCR provoquin una acetilació i metilació induïdes per estímul, tot i que, a les cèl·lules $CD69^-$ s'observen nivells detectables d'aquestes marques d'histones. D'altra banda, a les cèl·lules T madures, les marques permissives d'acetilació i metilació dins les histones són constitutivament altes i suggereixen que CD69 podria estar regulat en etapes de post-iniciació de la transcripció d'una manera similar a la descrita recentment per alguns gens inflamatoris (Hargreaves, Horng et al. 2009). L'anàlisi *in silico*, que mostra alts nivells de H3K4me3, H3Ac i PolII preunida en cèl·lules $CD4^+$ no estimulades, no està renyit amb ser un gen transcripcionalment inactiu, tal i com s'ha demostrat per a gens de resposta primària (Hargreaves, Horng et al. 2009) A més a més, illes de PolII i H3K4m3 han estat descrits també en molts gens inactius humans (Barski, Cuddapah et al. 2.007).

Els nostres resultats indiquen que la regulació del gen CD69 és complexa i probablement és diferent en diferents tipus cel·lulars. Cal continuar realitzant

estudis per aclarir la naturalesa dels elements reguladors i el mecanisme pel qual regulen l'expressió de CD69

V.- CONCLUSIONS

1. Comparative genomics has been useful to identify four conserved non coding sequences (CNS) upstream of the CD69 gene that we called CNS1, CNS2, CNS3 and CNS4. CNSs are conserved in DNA sequence between human dog and mouse genomes.

2. DNase I hypersensitivity site mapping shows that CNSs are constitutive HS in $CD69^-$ and $CD69^+$ cells.

3. Analysis of histone H3 acetylation and K4 dimethylation levels by chromatin immunoprecipitation in $CD69^-$ and $CD69^+$ thymocytes and resting and stimulated T and B cells has showed specific epigenetic marks. The promoter is always associated with high levels of acetylation and methylation. CNS1 presents high levels of these marks in resting peripheral lymphocytes and inducible acetylation and methylation in all populations. CNS2, CNS3 and CNS4 present inducible marks in thymocytes.

4. We have linked individual CNSs to the CD69 promoter and we have tested their function in luciferase experiments. CD69 basal and inducible activity was enhanced when coupled to CNS2 and CNS4 elements. No apparent activity was detected for CNS1 and CNS3 in these assays.

5. We have tested the function of CNSs *in vivo* by generation of transgenic mice. The CD69 promoter conferred developmentally regulated

expression during positive selection of thymocytes but could not support regulated expression in mature lymphocytes. Inclusion of CNS1 and CNS2 caused suppression of CD69 expression whereas further addition of CNS3 and CNS4 supported developmental-stage and lineage-specific regulation in T cells but not in B cells.

6. *In silico* analysis show that CD69 promoter and gene body is associated with permissive histone marks even in the basal state. Genome-wide analysis of chromatin modifications demonstrated enrichment of histone H3 K4 methylation at the promoter, exon I and intron I and enrichment of H3K36me3 and H4K20me1 in the body of the gene. RNA polymerase II binding was observed at the promoter and the gene body as well.

7. We have identified a potential regulatory element within intron I of CD69 gene. DNase I digestion experiments identified a novel HS called HSa located in the intron I of human and mouse CD69 gene. Bioinformatic analysis confirmed the existence of HSa in different immune cells and also revealed positioned nucleosomes.

8. Analysis of the chromatin structure shows that intron I is differentially remodelled in thymocytes and mature T cells. The amounts of H3 acetylation and K4 dimethylation increased upon CD69 expression in thymocytes, while they were constitutive high in peripheral lymphocytes.

9. The control of CD69 expression *in vivo* involves the action of distal DNA elements together with the promoter and appears to be different in lymphocyte subtypes

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