INVESTIGATIONS OF ANTIGEN PRESENTING CELLS INVOLVED IN THYMIC NEGATIVE SELECTION

The influence of dendritic cells on tolerance induction to circulating and extrathymic proteins.

submitted by

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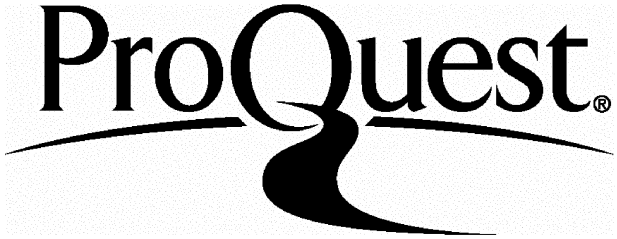

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ABSTRACT

This thesis examines the role of antigen presentation in negative selection of MHC class II restricted T cells specific for the complement component C5. C5 is an extracellular self protein, which only reaches the thymus via the blood circulation. Thymic antigen presenting cells were introduced into fetal thymic reaggregation culture with thymocytes from C5 specific T cell receptor transgenic mice, to follow the development of T cells in the presence or absence of self antigen presented by different antigen presenting cells. In order to mimic the physiological distribution of C5 peptide/MHC class II complexes on the antigen presenting cells as closely as possible, they were isolated from thymi of C5 sufficient mice, so that the amount of C5 peptide bound to MHC class II on their surface would reflect the amount of self antigen they have access to and process normally in vivo. The results showed that not only thymic dendritic cells, but also cortical and medullary epithelial cells were able to induce negative selection of C5 specific thymocytes with similar efficiency. In contrast, thymic macrophages were unable to influence the development of C5 specific T cells, which suggests that the main function of macrophages in the thymus is the disposal of dying thymocytes.

In the second part, a conditionally immortalized dendritic cell line (tsDC) was established from bone marrow of mice transgenic for a temperature sensitive mutant of the simian virus 40 large T antigen under the control of the MHC class I (K^b) promoter. At the permissive temperature of 33-37^oC tsDC divide in the absence of growth factors. They share a number of cell surface markers with bone marrow macrophages, but unlike macrophages constitutively express MHC class II, are negative for non-specific esterase and are unable to phagocytose sheep red blood cells. TsDC show characteristic dendrites, an abundance of acidic vesicles and are highly active in endocytosis. If maintained at 33^oC, tsDC process and present exogenous protein to MHC class II restricted T cell hybridomas and act as potent mixed leukocyte

reaction stimulators, but fail to activate naive T cells. Transfer to 39^{o}C arrests growth and results in upregulation of surface markers such as B7.1, CD40 and ICAM-1. Further upregulation of cell surface markers and acquisition of functional maturity occur following contact with T cells and their cognate antigen or in culture with a cytokine mixture derived from activated T cells.

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Für Oma Paula

ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell(s)
β-hex	Beta-hexosaminidase
BM	Bone marrow
C5	Fifth component of complement
CD	Cluster of differentiation
cEC	Cortical epithelial cell(s)
ConA	Concanavalin A
ConA sup	Concanavalin A activated T cell supernatant
cpm	Counts per minute
CTLA-4	Cytolytic T lymphocyte associated antigen
d	Day(s)
DC	Dendritic cell(s)
DMSO	Dimethylsulfoxide
EDTA	Ethylene-diamine-tetraacetat
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FTOC	Fetal thymic organ culture
GM-CSF	Granulocyte/macrophage-colony stimulating factor
h	Hour(s)
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
IFNγ	Gamma interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
i.p.	Intraperitoneal
i.u.	International units
LN	Lymph node(s)
LPS	Lipopolysaccharide
M	Molar
mAb	Monoclonal antibody
MACS	Magnetic cell sorter

M-CSF	Macrophage-colony stimulating factor
mEC	Medullary epithelial cell(s)
MHC	Major Histocompatibility Complex
min	Minute(s)
ml	Milliliter
MLR	Mixed leucocyte reaction
mM	Millimolar
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PE	Phycoerythrin
Pgp-1	Phagocytic glycoprotein-1
RT	Room temperature
SAg	Superantigen(s)
SDS	sodium dodecyl sulfate
SRBC	Sheep red blood cell(s)
SV40	Simian virus 40
TAg	Large tumour antigen of SV40
TCA	Trichloroacetic acid
TCR	T cell receptor
T_h	T helper cell
TNFα	Tumour necrosis factor alpha
tg $C5^{-}$, $^{+/-}$, $^{+}$	TCR transgenic mice (C5 deficient or sufficient)
tsDC	Temperature sensitive dendritic cell(s)
VCAM-1	Vascular adhesion molecule 1
vol	Volume

1. INTRODUCTION

1.1 Function of the Immune System

The mammalian immune system is responsible for host defence against pathogens like bacteria, viruses, fungi and parasites. Furthermore, it has to eliminate immortalized or virus infected cells of the body. Three main characteristics of the immune system are important to fulfil its function: recognition of pathogens (foreign antigens), the ability to distinguish between "self" and "non-self" and the generation of an adaptive immunity, which is the highly specific immune response to a previously encountered "memorized" antigen (Roitt et al., 1993).

Immune responses are produced by different types of bone marrow derived leukocytes: firstly myeloid cells such as macrophages, dendritic cells and neutrophils, secondly lymphocytes such as T cells and B cells. The former play an important role in the immediate host defence (innate immunity). They ingest and destroy extracellular micro-organisms such as bacteria, whereby the internalized material can be processed to peptides and presented on molecules of the major histocompatibility complex (MHC) to T cells, resulting in the induction of the adaptive immune response (Janeway and Travers, 1994). In addition, factors are secreted which attract and activate other cells of the immune system. Some of those factors, IL-12 and interferon α and β, can activate natural killer (NK) cells. These are lymphoid cells which recognize and destroy cells that have either lost expression of MHC class I molecules, such as some transformed or virus infected cells, or express foreign MHC alleles, such as transplants (reviewed by Colonna, 1996). Another mechanism of the innate immune defence is the activation of complement, a system of several proteins present in the blood, acting against micro-organisms by forming pores in their membranes and by facilitating their uptake by phagocytic cells which bind complement components with specific receptors.

T cells and B cells recognize antigen specifically via their antigen receptors. Binding of protein by the B cell receptor (antibody, surface immunoglobulin) leads to activation of the B cell and, with appropriate T cell help, to antibody secretion, which is the main function of B cells. Recognition of antigen by T cells causes lysis of e.g. virus infected cells if the reacting T cell belongs to the subpopulation of cytotoxic T cells, or it leads to cytokine production by the helper T cell population.

1.2 T cells

Mature T cells are subdivided into two major populations, distinguishable by their function and by expression of certain surface markers:
70% of the peripheral murine T cells express CD4, a single glycoprotein containing four immunoglobulin-like domains. These T cells are further divided into IL-4, IL-5 and IL-10 producing T-helper type 2 (T_h2) cells, which stimulate antigen binding B cells to produce antibodies, and T_h1 cells, which activate macrophages by secreting IFNγ. T_h1 cells can also mediate cell death by interaction of Fas-ligand with Fas, the latter bein expressed on target cells (reviewed by Mosmann and Coffman, 1989). Fas, a member of the tumour necrosis factor receptor (TNF-R) family, is known for its transduction of signals which induce programmed cell death (Itoh et al., 1991; Oehm et al., 1992). Activation of CD4 cells occurs when they recognize antigen in context with MHC class II molecules on the surface of professional antigen presenting cells such as dendritic cells, B cells and macrophages.

In contrast, cytotoxic T cells are MHC class I restricted and express the surface molecule CD8, a heterodimer formed by an α and β chain each containing one immunoglobulin-like domain. CD8 T cells lyse infected cells which carry pathogens such as viruses and which present peptides from those pathogens on their MHC class I molecules. The mechanism of killing can be Fas mediated as in CD4 T_h1 cells, but can also be induced by secretion of perforin and granzymes. Perforin will integrate

into the surface membrane of target cells and form pores, whereas granzymes enter target cells to induce apoptosis.

Both CD4 and CD8 cells bind antigen specifically via their T cell receptor (TCR), a transmembrane glycoprotein consisting of two polypeptides called the α and β chains (Allison et al., 1982; Haskins et al., 1983; Meuer et al., 1983). The genes encoding the TCR α chain are organized in multiple variable (V), joining (J) and constant (C) segments; the β chain genes have diversity (D) segments in addition. During T cell development any V gene segment can be joined to any J or DJ gene segment by somatic recombination, resulting in an enormous diversity of TCR specificities ($\approx 10^{11}$), which is essential for antigen recognition (Davis and Bjorkman, 1988; Kronenberg et al., 1986). To increase the number of T cells responding to a given antigen, a naive T cell starts to divide in response to the growth hormone interleukin-2 (IL-2), which it produces in an autocrine fashion when activated. It thereby gives rise to a clone of T cells bearing TCR with identical antigen specificity as the cell from which they were derived (clonal selection, Burnet, 1959).

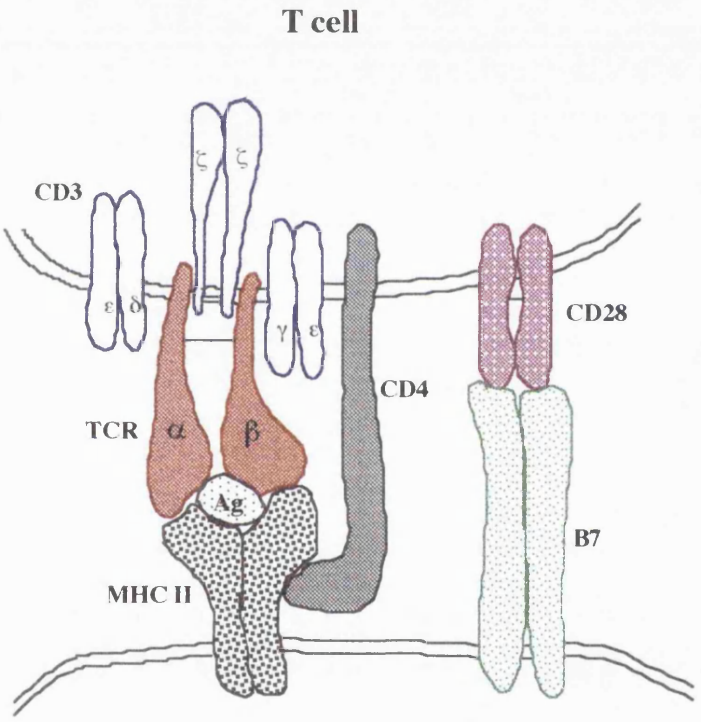
Binding of antigen and MHC by the TCR signals into the T cell through a TCR associated complex of proteins, known as CD3. It consists of six polypeptide chains which form an εγ heterodimer, an εδ heterodimer and a ζζ homodimer, whereby one of the ζ chains is replaced by η in 20% of TCR/CD3 complexes. All proteins of the CD3 complex contain large cytoplasmic domains that allow them to interact with signal transducing proteins within the cell. At the same time, CD4 or CD8, which binds to the non-polymorphic part of the same MHC molecule bound by the TCR, signals into the cell by means of interaction of its cytoplasmic tail with the tyrosine kinase $p56^{lck}$. For this help in signalling CD4 and CD8 are termed co-receptors.

In addition to the signal via the TCR/CD3 complex, activation of resting T cells requires a second signal, which is known as the costimulatory signal (two-signal

model, first proposed by Bretscher and Cohn, 1970). Once activated, triggering of the TCR/CD3 complex alone is sufficient to induce effector function of the T cell. Several receptor-ligand pairs can provide costimulation, the most important being the interaction of CD28 on the T cell side with B7.1 and/or B7.2 on the antigen presenting cell (APC) (Harding and Allison, 1993; Lenschow et al., 1993). CD28 is constitutively expressed on virtually all T cells, whereas the cytolytic T lymphocyte associated antigen (CTLA-4), another T cell molecule binding to B7.1 (CD80) and B7.2 (CD86), is expressed following activation. The two molecules are thought to have distinct functions: CD28 engagement facilitates the initiation of T cell responses (King et al., 1995; Seder et al., 1994), CTLA-4 on the other hand inhibits anti-CD3-mediated T cell activation in the presence of CD28 costimulation (Krummel and Allison, 1995; Walunas et al., 1994). In fact, CTLA-4 has been implicated in induction of programmed cell death (Gribben et al., 1995), suggesting that it promotes downregulation of the immune response. Expression of molecules of the B7 family are restricted to bone marrow derived APC (Lenschow et al., 1996). B7.1 provides sufficient costimulation for T cell activation. However, anti-B7.1 mAb inhibit T cell responses only partially, a finding which led to the identification of B7.2 as a second ligand for CD28/CTLA-4. B7.2 is expressed at higher levels than B7.1 and induced earlier after stimulation. The issue whether B7.1 and B7.2 may differentially control immune responses, in particular the induction of T_h1 versus T_h2, is still controversial (reviewed by Lenschow et al., 1996). Further costimulatory interactions can occur between the leukocyte function-associated antigen-1 (LFA-1) on T cells with intercellular adhesion molecules 1 and 2 (ICAM-1/ICAM-2) on APC (Damle et al., 1992; van Seventer et al., 1990), the very late antigen (VLA-4) with vascular cell adhesion molecule-1 (VCAM-1) (Damle and Aruffo, 1991), or CD2 with CD48 (Chavin et al., 1994).

The absence of costimulation leads to T cell anergy, a state characterized by unresponsiveness due to an inability to produce IL-2, even when rechallenged in the

presence of costimulatory molecules. Anergy can be induced in vitro and in vivo with APC, lacking costimulatory molecules (Jenkins and Schwartz, 1987; Lamb et al., 1983). It provides the immune system with a mechanism to inactivate potentially autoreactive peripheral T cells that might have escaped intrathymic deletion (described in a later section), because of their specificity for a peptide not present in the thymus (reviewed by Mueller and Jenkins, 1995).



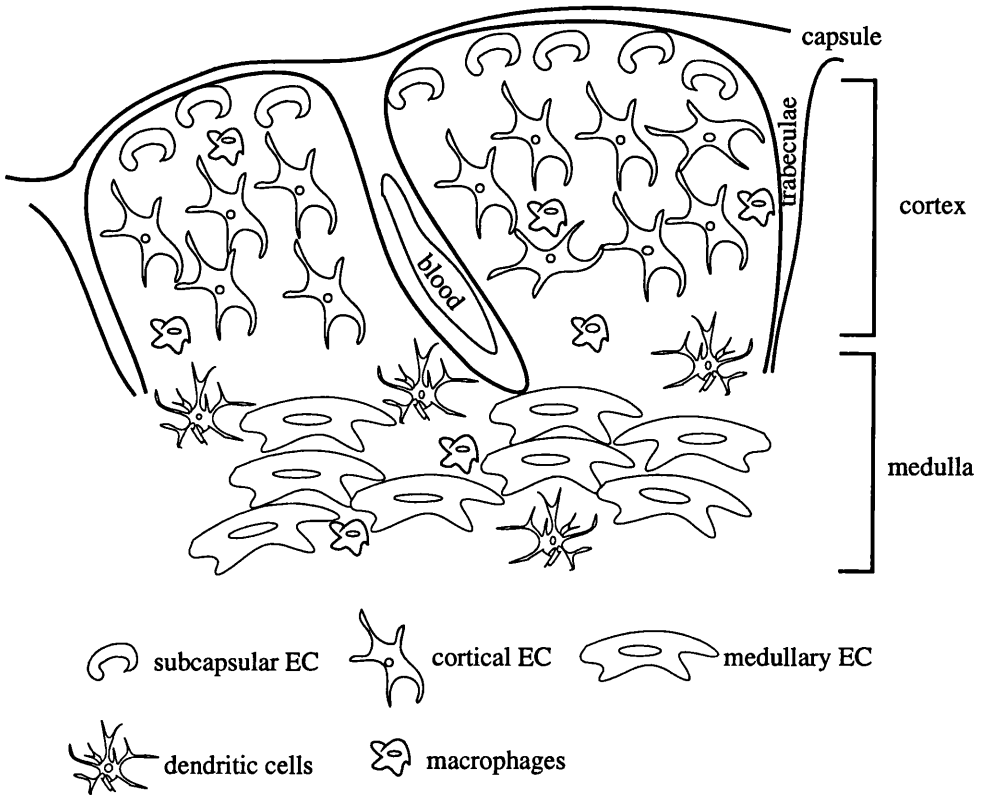
Whereas the mechanism of anergy induction mentioned above relies on the lack of a signal, another system has been described recently using an altered peptide ligand for the interaction with the T cell receptor, which can result in T cell anergy in the presence of costimulation (Sloan-Lancaster et al., 1993). Here, T cells are stimulated with MHC presenting a synthetic peptide analogue of the cognate antigen,

that contains an amino acid substitution at a residue critical for TCR binding. As a consequence, qualitative differences in the signalling cascade can be observed, such as a decrease in CD3ζ phosphorylation and the failure of phosphorylation of the downstream situated ZAP-70 tyrosine kinase (Sloan-Lancaster et al., 1994). This form of anergy induction might have a different application in vivo. Naturally occurring altered peptide ligands designed by pathogens could act as antagonists for viral epitopes, thereby preventing CD8 T cells to respond (Bertoletti et al., 1994; Klenerman et al., 1994).

1.3 Thymic architecture and microenvironment

The thymus, a lymphoid organ situated in the upper thorax, provides a unique environment for T cell proliferation and differentiation. It consists of two lobes, which are separate in early fetal development and held together by a connective tissue capsule from day 16-18 of gestation onwards. Both lobes are supplied by several blood vessels travelling in trabeculae. These trabeculae organize each lobe into numerous lobules, whereby the separation is incomplete or lacking in the centre of the organ. The lobules of an adult murine thymus consist of a network of epithelial cells interspersed with differentiating T cells (thymocytes). Three main classes of epithelium have been identified. Subcapsular epithelium is located at the most outer part of the thymus. It initiates thymopoiesis and produces most of the thymic hormones. In the cortex, another type of epithelial cells creates a loose network connected by desmosomes. Some of those cortical epithelial cells, termed thymic nurse cells (TNC), form complexes with mostly immature thymocytes (Wekerle et al., 1980). The inner part of the thymus, the medulla, contains a third type of epithelial cells which have shorter cytoplasmic processes and are more tightly packed. A unique structure mainly in the human medulla but visible after staining with specific anti-keratin antibodies in mouse (Ferrick et al., 1990) are Hassall's corpuscles, concentric arranged epithelium implied in the removal of dying cells. Cortical and medullary

epithelial cells are derived from different embryological sources and represent the predominant cell type of the thymic stroma.



Other components of the stroma are fibroblasts, eosinophils, neutrophils, mast cells and granulocytes, which are found in the trabeculae. Antigen presenting cells such as dendritic cells and B cells are either restricted to the medulla and the cortico-medullary junction, or distributed throughout the thymus such as macrophages. The localization of different stroma cells in distinct areas of the thymus, where they come in contact with thymocytes of different maturation stages, suggests special functions of stroma cells in their communication with developing thymocytes. Soluble molecules and cell-cell interaction are involved in this communication (Boyd et al., 1993; Janeway and Travers, 1994; Kendall, 1991; van Ewijk, 1991; van Ewijk et al., 1994).

1.4 T cell development and selection

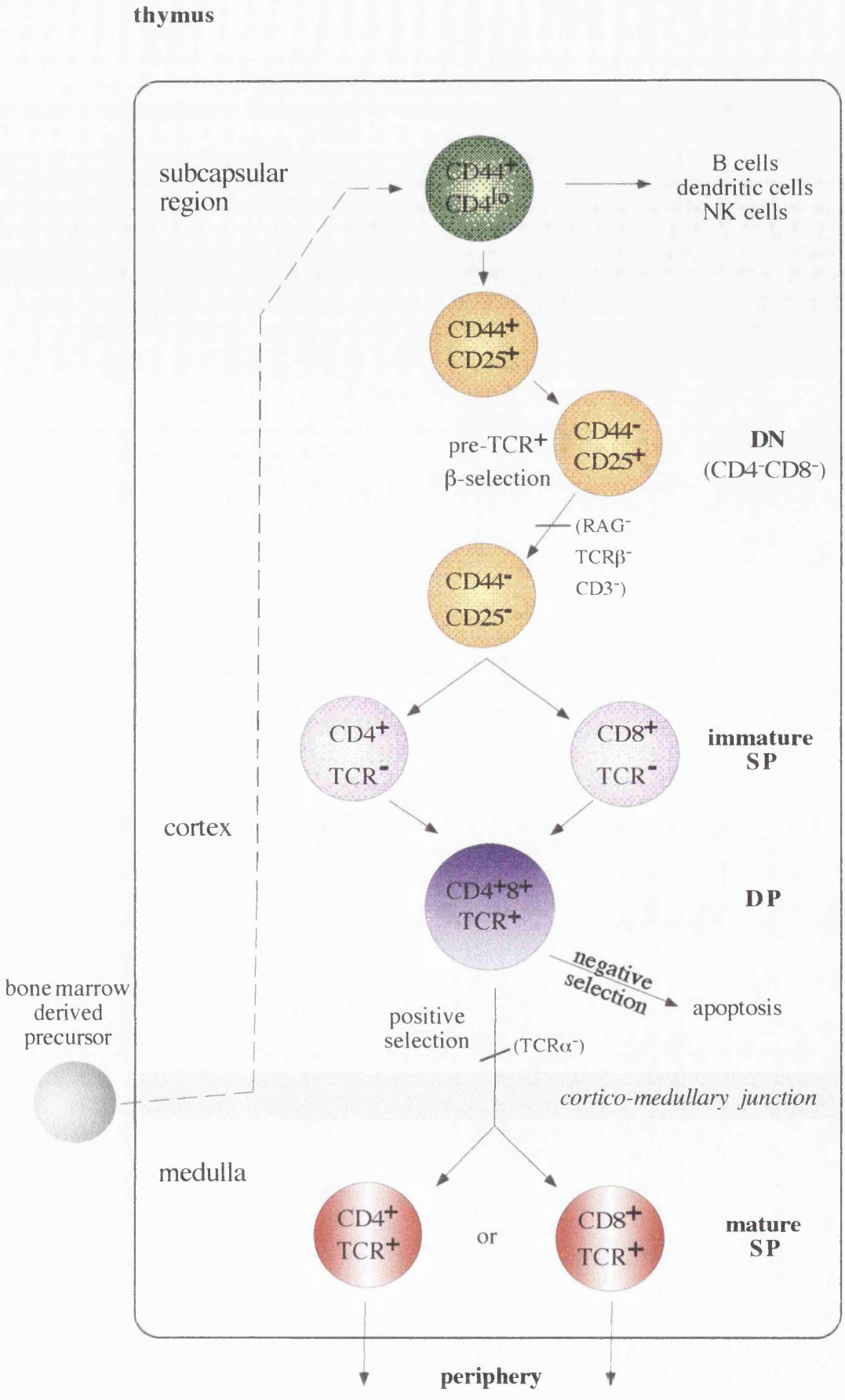
All T cells have their origin in the thymus, where bone marrow derived precursor T cells differentiate via several immature stages into mature T cells, which leave the thymus, enter the bloodstream and migrate to the peripheral lymphoid organs (lymph nodes and spleen) from which they continually recirculate to the blood via the lymphatics and back to lymphoid organs. The first precursor T cells start populating the thymus on day 11 of embryonic development (Owen and Ritter, 1969), coming from fetal liver and bone marrow, but the adult thymus too is continuously supplied with bone marrow derived precursors although in lower frequencies (Fowlkes and Pardoll, 1989). Terminal deoxynucleotidyl transferase (an enzyme adding nucleotides during receptor gene rearrangement independent of the template and thereby increasing the diversity of TCR) and the adhesion molecule CD44 (Pgp-1) are the first markers to identify those precursor cells, which enter the thymus at the cortico-medullary junction and migrate to the subcapsular region. At that stage, the cells express low levels of the CD4 co-receptor, but neither CD8 nor the TCR (Wu et al., 1991). It has been suggested that these cells are still pluripotent and can differentiate into T cells, B cells, dendritic cells or natural killer cells (reviewed by Godfrey and Zlotnik, 1993).

The final commitment to T cells takes place when CD25 (IL-2 receptor) appears on the surface, whereas CD4 disappears. Those so called $CD4^-CD8^-$ double negative thymocytes (DN) gradually loose CD44. From here, a second group of T cells diverges, γδ T cells, which differ in their function and usage of TCR chains (namely γδ instead of αβ) from the αβ T cells discussed earlier. The nature of their antigens as well as the mechanisms of recognition are still not fully understood and the following description will concentrate on αβ T cells only. The stage of $CD44^-CD25^+$ DN represents an important early control point in T cell development, the successful expression of a pre-TCR. TCR β gene rearrangement starts and cells expressing TCR β paired with the pre TCR α chain on the surface receive signals to proceed to the

$CD44^-CD25^-$ stage (Fehling et al., 1995; Groettrup et al., 1993). Prevention of successful TCR β gene rearrangement, e.g. by inactivation of V(D)J recombination activation genes (RAG-1 or RAG-2) by gene targetting (gene knock-out technology) (Mombaerts et al., 1992; Shinkai et al., 1992) leads to a developmental block at the $CD44^-CD25^+$ stage. The differentiation to the next step can be restored by introducing a fully rearranged TCR β transgene (Shinkai et al., 1993), or by treatment with anti-CD3 mAb (Levelt et al., 1993), indicating that a signal through the pre-TCR/CD3 complex mediates early T cell development.

Expression of one of the co-receptors (CD4 or CD8) characterizes an intermediate population between DN and $CD4^+CD8^+$ double positive cells (DP). These $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes are found in various ratios dependent on the inbred mouse strain (Hugo et al., 1991) and differ from mature $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes in that they lack αβ TCR expression.

Development into $CD4^+CD8^+$ cells follows, accompanied by migration into the thymic cortex. Approximately 85% of all thymocytes are DP, a stage where a functional αβ TCR necessary for positive and negative selection is expressed at increasing levels. As mentioned above, the TCR specificity is formed randomly by somatic recombination of TCR gene segments. Therefore, recognition of self-MHC molecules is not self-evident, but nevertheless a requirement for T cells in order to recognize antigen and hence be functional. Only T cells expressing a 'useful', that is a self-MHC recognizing TCR, bind to MHC on thymic stromal cells and receive the signal for positive selection which allows them to survive (Benoist and Mathis, 1989). Non-MHC-binding thymocytes undergo apoptosis, an active process of programmed cell death. A second result of randomly developing TCR specificities is the appearance of potentially self reactive T cells which could cause autoimmune diseases. These bind not only to self MHC molecules, but with high affinity to self peptide presented on MHC, resulting in a negative selection signal causing apoptosis (Smith et al., 1989).


thymus
subcapsular region
CD44+ CD4lo
B cells
dendritic cells
NK cells
CD44+ CD25+
pre-TCR+ β-selection
CD44- CD25+
DN (CD4-CD8-)
(RAG- TCRβ- CD3-)
CD44- CD25-
CD4+ TCR-
CD8+ TCR-
immature SP
cortex
CD4+8+ TCR+
DP
negative selection
apoptosis
bone marrow derived precursor
positive selection
(TCRα-)
cortico-medullary junction
medulla
CD4+ TCR+
or
CD8+ TCR+
mature SP
periphery

Two main models exist to explain how interaction of the TCR with MHC can result in positive selection on one hand, but in negative selection on the other. The 'affinity' model proposes that thymocytes whose TCR bind MHC/peptide with high affinity are eliminated by negative selection, whereas low affinity interaction causes positive selection (Schwartz, 1989; Sprent et al., 1988). An alternative model is called the 'microenvironment' model (van Ewijk, 1991). The basis of this model is the interaction of thymocytes with different cell types in the various compartments within the thymus. Cortical epithelial cells are reported to mediate positive selection (Anderson et al., 1994; Bill and Palmer, 1989), whereas dendritic cells, restricted to the medulla, are potent mediators of negative selection (Matzinger and Guerder, 1989). However, other cell types may constitute to negative selection (reviewed by Anderson et al., 1996). The involvement of cell specific costimulatory molecules in negative selection is still a point of controversy (Jones et al., 1993; Page et al., 1993; Punt et al., 1994; Tan et al., 1992).

Only 3% of DP are successfully selected (Egerton et al., 1990) and differentiate into mature MHC class II restricted $CD4^+CD8^-$, or MHC class I restricted $CD4^-CD8^+$ single positive T cells (SP). Co-engagement of the same MHC molecule by the TCR and either CD4 or CD8 determines the pathway chosen, suggested by the dramatic reduction in CD4-lineage and CD8-lineage T cells in mice lacking the respective co-receptors (Fung-Leung et al., 1991; Rahemtulla et al., 1991). Two main models have been proposed to explain the mechanism by which the two lineages are formed. The 'instruction' model postulates that TCR / CD8 ligation with MHC class I instructs the cell to downregulate CD4 and that TCR / CD4 ligation with MHC class II leads to loss of CD8, respectively (Borgulya et al., 1991; Robey et al., 1991). In the 'stochastic' model, CD4 or CD8 expression is switched off randomly, but only those cells with matched TCR and co-receptor specificity are rescued from apoptosis (Chan et al., 1993; Davis et al., 1993). More recent experimental evidence provides support for a model including both, instructive events for the CD8 lineage and stochastic

elements for CD4 T cells (Benveniste et al., 1996; Lucas et al., 1995; Suzuki et al., 1995). After being positively selected, mature T cells migrate from the thymic medulla into the periphery and populate lymphoid organs.

1.5 Antigen processing and presentation

T cells recognize antigen only in the context of self MHC molecules (MHC restriction, Swain, 1983; Zinkernagel and Doherty, 1979) in form of peptides, cleavage products of the antigen. Peptides derived from intracellular antigens are presented to $CD8^+$ T cells by MHC class I molecules, which are expressed on all nucleated cells, while extracellular antigen-derived peptides are presented to $CD4^+$ T cells by the MHC class II molecules found on specialized antigen presenting cells such as dendritic cells, B cells and macrophages.

The MHC class I molecule consists of a polymorphic transmembrane glycoprotein (α chain, also termed heavy chain), non-covalently associated with the extracellular β_2-microglobulin polypeptide. Two extracellular domains of the α chain form a groove, accommodating peptides with a length of 8-10 amino acids (crystal structure by Bjorkman et al., 1987), which they encounter within the endoplasmic reticulum (ER). Here, the α chain and β_2-microglobulin are synthesized and assemble (Ljunggren et al., 1990), stabilized by a chaperone called calnexin (p88), which is released upon binding of peptide. Peptides that bind to MHC class I are mainly derived from viruses infecting the cell or from cytosolic or nuclear proteins (Brodsky and Guagliardi, 1991). They are degraded in the cytoplasm by ATP-dependent proteasomes which generate peptides that are translocated to the lumen of the ER by a heterodimeric transporter consisting of two multimembrane proteins, TAP1 and TAP2 (reviewed by Lehner and Cresswell, 1996). Once the peptide-MHC class I complex has formed in the ER, it is transported through the Golgi complex to the cell surface (reviewed by Monaco, 1992; Neefjes and Momburg, 1993).

The structure of MHC class II molecules is very similar to that of MHC class I molecules (Brown et al., 1993; Stern et al., 1994). However, class II consists of two non-covalently associated transmembrane glycoproteins, the heavy α chain and the light β chain. Their exposed extracellular domains (α1 and β1) form a peptide binding groove with open ends. This enables peptides of an increased length of 12-25 amino acids to bind to MHC class II molecules. Most of these peptides derive from an exogenous source, e.g. extracellular pathogens, which have to be internalized for presentation. Macrophages phagocytose those pathogens very efficiently, whereas B cells take up antigen preferentially via their antigen receptor. Once within phagosomes or vesicles, the endosomal / lysosomal pathway is entered, where progressively decreasing pH in combination with proteolytic enzymes such as cathepsin B and D lead to antigen degradation. The resulting peptides bind to MHC class II molecules in the recently described MHC class II compartment (MIIC) (Peters et al., 1991; Tulp et al., 1994). Class II molecules are synthesized in the ER, where each αβ heterodimer associates with a subunit of an invariant chain (Ii) trimer forming a nonameric structure (Lamb and Cresswell, 1992). The function of the invariant chain is to prevent binding of intracellular peptides to MHC class II in the ER (Roche and Cresswell, 1990) and to direct MHC class II export through the Golgi and trans Golgi reticulum, where its route diverges from the MHC class I route, to the compartment containing engulfed peptides derived from extracellular proteins (Bakke and Dobberstein, 1990; Elliott et al., 1994). Targetting to this compartment can occur either by direct delivery from the trans-Golgi network or by transport to the cell surface and rapid internalization (Benaroch et al., 1995; Roche et al., 1993). Here, Ii undergoes sequential proteolysis due to protease activity (Maric et al., 1994), leaving a nested set of class II-associated Ii peptides (CLIP) bound in the MHC class II groove (Avva and Cresswell, 1994). CLIP is removed by the MHC-encoded heterodimeric glycoprotein H2-M (HLA-DM in human) to allow peptide binding to class II. In mutants lacking DM, the majority of MHC class II molecules is associated with CLIP, resulting in defective antigen presentation (Denzin et al., 1994). Once loaded with

antigenic peptides, MHC class II is transported directly to the cell surface (R. Wubbolt, pers. communication).

1.6 Professional antigen presenting cells

B cells, macrophages and dendritic cells belong to the group of professional antigen presenting cells (APC). These are the few cell types expressing MHC class II as well as costimulatory molecules required for activation of resting T cells. Macrophages express those molecules only when activated and B cells have to be antigen specific in order to function as potent APC. Activation of naive T cells, so called priming, is thought to be a unique feature of dendritic cells (Inaba et al., 1990). Localization in lymphoid or other organs, the mechanisms of antigen uptake and expression of costimulatory molecules are different in these three types of APC and might therefore determine the outcome of T cell responses.

1.6.1 B cells

B lymphocytes originate and differentiate in the bone marrow, before they migrate into spleen and lymph nodes and recirculate in blood and lymph. The main function of B cells is to become plasma cells, which produce and secrete high amounts of antibodies.

B cells are restricted in their uptake of extracellular antigen, as they are not phagocytic and inefficient in fluid phase endocytosis, the non-specific uptake of soluble proteins. Although B cells express Fc receptors, these fail to mediate antigen uptake since they are not internalized efficiently (Snider and Segal, 1989). Therefore, B cells depend on internalization of cognate antigen via specific membrane antibodies (Lanzavecchia, 1990). This process not only ensures uptake of large amounts of protein but also activates the B cell through its receptors.

Engagement of the receptor leads to increased expression of the costimulatory molecule B7.2 on B cells (Hathcock et al., 1994; Lenschow et al., 1994), which constitutively express high levels of MHC class II. It also directs them towards the T cell area within the spleen (reviewed in Hodgkin and Basten, 1995). B7.1 is not expressed on resting B cells or those which have been activated through their receptors, and its expression is not essential for T cell costimulation once B7.2 is present (Lenschow et al., 1994). However, B7.1 can be induced by signalling through CD40 via CD40 ligand on activated T cells (Roy et al., 1995). Another inducer of B7.1 and, to a greater extent, B7.2 is interleukin 4 (IL-4) (Stack et al., 1994).

1.6.2 Macrophages

Macrophages differentiate from immature bone marrow derived monocytes, which circulate in the blood. Macrophages are present in lymphoid organs and most non-lymphoid tissues and can differ in phenotype as well as activation state.

Activation is required for the expression of sufficient levels of MHC class II and costimulatory molecules for T cell activation. Unstimulated macrophages do not stimulate alloantigen-specific resting CD4 T cells (Inaba and Steinman, 1984) and are unable to prime specific T cells when pulsed with protein (Inaba et al., 1990). Activation of macrophages is induced by gamma interferon (IFNγ), resulting in expression of B7.2 and upregulation of MHC class II (Freeman et al., 1991). Once activated, macrophages can produce cytokines like IL-1, IL-6, IL-12 and tumour necrosis factor (TNF), which are involved in T cell activation.

Macrophages are the main cell type able to internalize particulate antigens, due to their efficient phagocytic capacity. Phagocytosis is the basis for their major role in the clearance of particles such as micro-organisms, apoptotic thymocytes and senescent red blood cells. Another means of antigen uptake is through receptors which

mediate endocytosis, such as the mannose receptor binds mannosylated or fucosylated antigens (Sallusto et al., 1995), Fc receptors bind the Fc portion of antibodies -thereby inducing uptake of antigen/antibody complexes (Ukkonen et al., 1986)- and the complement receptors CR1 and CR3 bind activated complement factor 3 that is associated with immune complexes and pathogens (Arvieux et al., 1988).

1.6.3 Dendritic cells

Dendritic cells (DC) are bone marrow derived cells distributed in all lymphoid organs as well as in some non-lymphoid organs. They are also known as veiled cells in the blood and afferent lymph, interdigitating cells in lymph nodes, spleen and thymus, Langerhans cells in the skin and interstitial dendritic cells in other non-lymphoid organs. Langerhans cells represent an immature stage of DC, since they are reported to migrate into lymph nodes out of grafted skin or leave in vitro cultured skin fragments, whereby they undergo phenotypic changes after which they fully resemble lymphoid DC (Larsen et al., 1990[b]; Teunissen et al., 1990). DC progenitors have been found in blood (Inaba et al., 1992[b]) and bone marrow, the latter giving rise to DC when cultured with granulocyte/macrophage colony-stimulating factor (GM-CSF) in vitro (Inaba et al., 1992[a]; Scheicher et al., 1992). However, their maturation and functional specification might occur differently in particular organs; e.g. in the thymus a DC precursor identical with the progenitor for T cells has been proposed (Ardavin et al., 1993). Characteristic of all DC are long dendrites, supposedly to increase their surface area for interaction with T cells. High levels of MHC class I and class II are typical for DC, another marker is CD11c (mAb N418, Metlay et al., 1990), which can also be present on macrophages. Other DC surface molecules are restricted to DC in particular organs, e.g. NLDC145 on DC in skin and several lymphoid organs (Kraal et al., 1986), 33D1 on DC in spleen and Peyer's patches (Crowley et al., 1989). A DC specific marker has not yet been found. Instead, an association between expression of the relB subunit of the NF-κB transcription factor

complex and DC in spleen, LN and thymus has been shown (Carrasco et al., 1993). Mice carrying an insertional mutation in the relB gene lack mature DC but not Langerhans cells, indicating its requirement for DC differentiation (Burkly et al., 1995). Antigen presenting function in these mice is impaired. Salomon et al. (1994) have generated transgenic mice in which they claim that DC can be selectively destroyed in spleen and thymus, leading to e.g. a reduced stimulating potential of splenocytes in mixed leukocyte reactions (MLR). Surprisingly, this correlates with major thymic atrophy and the disappearance of DP thymocytes, which seems difficult to relate to the lack of DC.

Antigen capture. Langerhans cells, DC generated from bone marrow and other immature DC are phagocytic to a certain extent and express Fc receptors (FcγRII, FcεRI) and C3bi receptors (CD11b) on their surface which enable them to internalize antigen through receptor mediated uptake. In order to facilitate uptake of bacterial products, DC express the mannose receptor which binds mannosylated or fucosylated antigens. Recently, the C-type lectin DEC-205, a receptor found on DC and thymic cortical epithelial cells, has been implicated in antigen internalization (Jiang et al., 1995). In addition, DC are reported to be very efficient in macropinocytosis (Sallusto et al., 1995), a process by which extracellular fluid is internalized by invagination of the plasma membrane forming a large early endosome (1-3 μm). Macropinocytosis is mediated by cytoskeleton driven membrane ruffling and constitutive only in DC, whereas macrophages and epithelial cells can be induced by growth factors (Swanson and Watts, 1995). Mature DC are not phagocytic and express little or no FcγR, C3R and mannose receptor. Their macropinocytic capacity is decreased, too, indicating that antigen capture is highly regulated in dendritic cells and that several mechanisms of uptake are confined to immature stages (Steinman and Swanson, 1995).

Migration. What are the physiological reasons for antigen capture regulations dependent on the differentiation stage of dendritic cells? The current view describes

immature dendritic cells, in particular Langerhans cells (LC), as sentinel cells resident in non-lymphoid organs, where they continuously internalize and process antigen. Maturation signals induced by injury or infection result in migration of LC via afferent lymph to the T cell areas of the draining lymph nodes, whereby expression of CD44 and β_2 integrins might support homing. During migration, LC downregulate MHC class II synthesis and antigen uptake, perhaps to avoid displacement of e.g. bacterial antigens acquired at the place of infection. Instead, surface expression of class II and costimulatory molecules such as B7 increase and the cells become potent stimulators of naive T cells (Steinman, 1991). This migration can be experimentally induced by skin grafting, skin contact sensitization with fluorescein isothiocyanate (FITC), or in vitro culture of skin explants (Larsen and Austyn, 1991; Larsen et al., 1990[b]; Macatonia et al., 1987). The factors that stimulate and direct the migration and maturation of dendritic cells in vivo are not clear. In mice, GM-CSF and IL-1 have been reported to mediate maturation of LC (Heufler et al., 1987), whereas TNFα was implied in maintenance of LC in culture without induction of maturation (Koch et al., 1990). However, another in vitro culture system uses GM-CSF and IL-4 to generate immature DC from human peripheral blood mononuclear cells (PBMC), which can be driven into maturity by TNFα or LPS (Sallusto and Lanzavecchia, 1994).

Distinct migration patterns as those for LC have also been shown for blood dendritic cells, which migrate into the T cell area of the spleen, but not into lymph nodes when injected intravenously or when introduced in cardiac allografts, and subcutaneously injected DC traffic into the draining nodes but not further (Austyn et al., 1988; Kupiec-Weglinski et al., 1988; Larsen et al., 1990[a]).

Stimulatory molecules. DC constitutively express the adhesion molecules ICAM-1 and CD48 (LFA-3 in human), allowing the first contact with LFA-1 and CD2 on T cells and providing time for the interaction of TCR and MHC molecules in the presence of specific peptide. Costimulatory molecules, such as B7-1, B7-2 and CD40

are also constitutively expressed by DC. However, immature DC show low surface expression levels, which are remarkably increased in mature DC.

Taken together, DC possess all important features which make a potent APC for T cell activation, and possibly tolerance induction, since DC are also implicated in negative selection in the thymus (Kyewski et al., 1986), as mentioned above.

1.7 C5 as model antigen

C5, the fifth component of complement, is used as a model self antigen in this project. It is produced by hepatocytes (Patel and Minta, 1979) which are the main source of circulating C5 protein. Lung epithelial cells (Strunk et al., 1978) and macrophages (Ooi and Colten, 1979) have been shown to synthesize C5 as well, but do not contribute to serum C5 levels (Stockinger et al., 1993). Serum of adult male mice contains about 50 µg C5 protein per ml, whereas female mice contain lower levels of C5 (Nilsson and Müller-Eberhard, 1967). The C5 production starts on day 10 in mouse gestation at a low level (Tachibana and Rosenberg, 1966), reaching the final amount around 4 weeks after birth. A 2 base-pair deletion in the C5 gene, occurring in 38% of inbred mouse strains results in the absence of C5 protein in the circulation (Cinader et al., 1964; Nilsson and Müller-Eberhard, 1967; Wetsel et al., 1990). Mice heterozygous for the mutation contain half the C5 level of normal mice. C5 deficient mice ($C5^-$), when immunized with C5 protein, generate a $CD4^+$ T cell response, whereas normal mice ($C5^+$) are fully tolerant to C5 (Lin and Stockinger, 1989).

1.8 T cell receptor transgenic mice

In an unmanipulated animal, the repertoire of T cell receptor specificities is enormous. The TCR transgenic mouse system provides a useful tool for the investigation of the development of single T cells with defined receptor specificity. In particular, positive

and negative selection of T cells, as well as allelic exclusion, have been the main issues of interest which were amenable for studies in TCR transgenic mice.

When a functionally rearranged TCR β gene was first introduced into the germ-line of mice (Uematsu et al., 1988, for review about transgenic mice, see Palmiter and Brinster, 1986) it completely suppressed rearrangements of endogenous TCR β genes at the V to DJ level, leading to surface expression of the transgenic TCR β chain only. This process, termed allelic exclusion, was previously identified in B cells, where only one allele of the heavy and light immunoglobulin chain is rearranged (reviewed by Storb, 1987). With the appearance of TCR transgenic mice allelic exclusion was found to be regulated in T cells also. Suppression of endogenous Vβ genes depends on the amount of transgenic TCR β chain expressed: a seeming exception to the rule of allelic exclusion was found in another TCR β transgenic system with low expression of the transgene, where both transgenic and endogenous TCR β chains were detected on the surface of the same cell (Pircher et al., 1990). Two TCR β chains are rarely seen in T cell clones from normal mice (Matis et al., 1988; Schittek et al., 1989) and are present in only about 1% of human T cells (Padovan et al., 1995), whereas several T cell clones were isolated with two productively rearranged α chains (Malissen et al., 1988; Marolleau et al., 1988) and up to one-third of human T cells express two different α chains (Padovan et al., 1993). Moreover, in mice carrying functionally rearranged TCR α and β transgenes, the transgenic β chain pairs with endogenous α chains to some extent (Zal et al., 1994), especially when T cells bearing the transgenic receptor are subject to negative selection (Blüthmann et al., 1988). Petrie et al. (1993) suggest continuous TCRα chain gene rearrangement in DP until the occurrence of positive selection, rather than until surface expression of a rearranged TCRα chain, to maximize the selection chances. Taken together, allelic exclusion seems to be rather complete for the TCR β locus, leading to surface expression of the transgenic TCR β chain in almost all T cells from αβ TCR transgenic mice, whereas it is less strictly controlled for TCR α genes, resulting in

skewing to the CD4 lineage when the transgenic TCR is MHC class II restricted (Berg et al., 1989[b]; Kaye et al., 1989; Murphy et al., 1990; Zal et al., 1994) and to CD8 cells in case of MHC class I restriction (Kisielow et al., 1988; Mamalaki et al., 1992; Pircher et al., 1989; Sha et al., 1988), but also in the appearance of few cells of the other lineage (CD8 or CD4, respectively) due to expression of endogenous α chains.

TCR transgenic models established several principles of positive selection, such as the requirement for expression of restricting MHC molecules without the cognate peptide on thymic radioresistant tissue for the maturation of CD4 and CD8 single positive thymocytes from immature DP precursors (Berg et al., 1989[b]; Scott et al., 1989; Teh et al., 1988). Furthermore, it was shown that the specificity of the TCR for MHC class I directs differentiation to CD8 cells (Kisielow et al., 1988; Pircher et al., 1989; Sha et al., 1988; Teh et al., 1988), whereas DP expressing a MHC class II restricted TCR differentiate into CD4 cells (Berg et al., 1989[b]; Kaye et al., 1989). Experiments with TAP1 deficient mice (Ashton-Rickardt et al., 1993) or β_2M deficient mice (Hogquist et al., 1993), which are impaired in positive selection of CD8 cells due to the lack of MHC class I expression, demonstrated that peptides serve a greater function in positive selection than simply stabilizing MHC at the surface, because a complex mixture of peptides was better at selecting the repertoire than single peptides. These results could be extended with the use of TCR transgenic mice, by characterizing the positively selecting peptides as variants (antagonists) of the cognate peptide and thereby showing that the process is peptide dependent (Hogquist et al., 1994).

Studies of negative selection in the thymus using TCR transgenic mice provided the first direct evidence for tolerance induction by means of clonal deletion of antigen specific thymocytes (Kisielow et al., 1988; Pircher et al., 1989; Sha et al., 1988), thereby confirming experiments employing superantigens in normal mice (Blackman et al., 1989; Kappler et al., 1987; MacDonald et al., 1988).

Negative selection to intracellular antigen, expressed on MHC class I, has been investigated using TCR transgenic mice expressing a class I restricted TCR, specific for either allogeneic MHC (Sha et al., 1988), the male (HY) antigen (Douek et al., 1996; Kisielow et al., 1988) or a virus derived peptide (Pircher et al., 1989; Mamalaki et al., 1992). In all cases, the presence of cognate antigen in the thymus, either due to crossing with mice of the allogeneic haplotype, natural occurrence in male mice, virus infection or injection of the antigenic peptide, respectively, led to thymic tolerance induction by deletion of DP. Some class I restricted TCR transgenic mouse systems dealt with the question of peripheral tolerance, that is tolerance induced to antigens which are not present in the thymus. Using tissue specific promoters, antigen (K^b) expression was directed to the appropriate peripheral site and tolerance was achieved by downregulation of the transgenic TCR or CD8 rather than thymic deletion (Morahan et al., 1991; Schönrich et al., 1991). When K^b expression was mainly in the periphery but partly in the thymus, these low levels were nevertheless sufficient to induce thymic deletion (Sponaas et al., 1994). Only a late onset of extrathymic antigen expression, such as reported for the large T antigen (TAg) of SV40 under the control of the pancreatic elastase promoter (expression starts on day 4-25 postnatally), resulted in autoimmunity (Geiger et al., 1992).

On the side of TCR transgenic mice with MHC class II restricted receptors, several transgenic models were established using a class II restricted TCR specific for antigens which are not normally expressed in mice. To study tolerance induction, antigen was introduced in form of peptide, which can efficiently bind to class II molecules at the cell surface of all class II^+ APC. As a result, deletion of DP was observed (Murphy et al., 1990; Spain and Berg, 1992; Vasquez et al., 1992). Alternatively, the antigen was expressed as a transgene in another mouse strain which then was crossed with the TCR transgenic strain (Degermann et al., 1994[a]; Scott et al., 1994; Förster et al., 1995). Those models were intended to answer questions about peripheral tolerance and autoimmunity. A promoter was used which directed

antigen expression to β-islet cells of the pancreas with no access to the thymus; the antigen chosen (influenza hemagglutinin and SV40 TAg) was intracellularly expressed and not secreted and therefore mainly presented in the context of MHC class I. Thus, it is perhaps not surprising that spontaneous autoimmunity developed in most cases.

The tolerance inducing capacity of a few true self antigens has been studied in class II restricted TCR transgenic systems. One of those TCR transgenic mice is specific for I-A^k, presenting an unknown endogenous peptide (Kubo et al., 1994). The TCR used originates from an autoreactive T cell clone established from a normal, non-autoimmune mouse. Transgenic T cells are positively selected on I-A^k and are tolerant in vivo, but self-reactive in vitro. Although the CD4 SP cells in these mice exhibit an aberrant phenotype in being $CD69^{lo}HSA^{+}CD2^{lo}$, the reason why they are not deleted in the thymus, but are kept in a non-responsive state in the periphery, is not known. Using the nonobese diabetic (NOD) mice which spontaneously develop diabetes, a TCR transgenic mouse has been generated bearing a H-2^{g7} restricted, islet antigen specific TCR (Katz et al., 1993). In these mice, T cells are not deleted or anergized, but infiltrate the site of antigen expression (pancreas) and finally cause disease. Autoimmunity in TCR transgenic and non-transgenic NOD mice is influenced by at least five non-MHC-linked genes (Garchon, 1992) and the H-2^{g7} MHC haplotype, thereby representing a very special case of "failure" of tolerance induction. In fact, it is probably not as much a failure of negative selection in the thymus as an unexpected activation of T cells in the periphery, because the antigen is not expressed in the thymus, but on peripheral cells which are normally unable to activate naive T cells. A possible explanation for the sudden activation of potentially autoreactive T cells comes from Goverman et al. (1993), who found spontaneous autoimmunity (experimental allergic encephalomyelitis, EAE) in TCR transgenic mice specific for the self antigen MBP (myelin basic protein) only when the mice were kept under non-sterile conditions but not when they were maintained in a specific pathogen-free facility, suggesting that the T cells were activated by antigens derived from infectious

organisms and presented by professional APC, e.g. through cross-reactivity to a viral antigen or by recognition of foreign antigen via a second TCR. The latter possibility could be excluded when Lafaille et al. (1994) demonstrated autoimmunity in MBP specific TCR transgenic mice which were RAG-1 deficient and could therefore express the transgenic receptor only. In that case, even mice kept in a specific pathogen-free facility responded to MBP, arguing against infections being responsible for the induction of the disease, although infections could not be completely excluded, since the facility was not germ-free. A genetic contribution to autoimmunity in the EAE model is made by the H-2^u MHC haplotype, placing it in the same category of special cases of "failure" of tolerance induction as NOD mice.

How is CD4 T cell tolerance induced to a circulating antigen which has access to the thymus, in mice which are not genetically or environmentally predisposed to autoimmunity? Two attempts have been made to address tolerance induction to a circulating self antigen. In one of the transgenic models, anti-idiotype (λ_2 immunoglobulin light chain) specific T cells, I-E^d restricted, were deleted in the thymus only if the idiotype was expressed within the thymus as a transgene, not if it reached the thymus via the circulation only. Alternatively, non-physiological amounts of Ig (>180 µg/ml) had to be injected (Bogen et al., 1993). Another transgenic mouse, expressing a TCR specific for Ig (IgG2a^b) presented on I-A^d, was generated by Granucci et al. (1996). Injection of peptide led to thymic deletion of DP as described for other systems, but IgG2a^b serum levels of 10 µg/ml achieved by immunization with bacteria did not, probably due to inefficient processing and presentation of Ig, which is thought to be mainly restricted to self-synthesized Ig by B cells (Rudensky and Yurin, 1989).

The particular interest of this thesis was the mechanism of tolerance induction to a circulating antigen which is present in normal mice, so that it does not require non-physiological introduction, either by protein/peptide injection, or by bringing in a transgene. Furthermore, the antigen should represent an extracellular

protein which can be normally processed and presented in the context of MHC class II in order to induce tolerance. The TCR chosen for this project is MHC class II (I-E^k) restricted and recognizes peptide 107-121 derived from the serum protein C5. Mice carry the TCR transgene under the control of the human CD2 promoter. CD2 is a surface molecule characteristic for B cells, natural killer cells and T cells with an onset of expression at the immature double negative thymocyte stage. Therefore, transcription of the transgenic TCR is directed to the cell types listed, whereby B cells do not express CD3, which is required for surface expression of the TCR. Consequently, the mice express the transgenic TCR on the surface of all their T cells. Its variable region belongs to the Vα11.1^a family of α-chains and the Vβ8.3 family of β-chains, respectively. The transgenic TCR can be recognized by a mAb against Vβ8.3 (7G8.2), whereas no antibody is available for Vα11.1^a. The transgene has been introduced originally into $C5^+$ mice, which were subsequently crossed to $C5^-$ mice, enabling the comparison of T cell development in the presence or absence of cognate antigen. It could be shown that C5 reactive T cells are generated in $C5^-$ TCR transgenic mice (tg $C5^-$), whereas complete tolerance to C5 in vivo is induced in $C5^+$ TCR transgenic mice (tg $C5^+$) (Zal et al., 1994).

1.9 Aims of the project

The first aim of this project was to define the role of different antigen presenting cells in thymic tolerance induction to an extracellular protein. For this purpose, mice transgenic for a C5 specific TCR were used. Several parameters have to be taken into account for presentation of a circulating antigen in the thymus:

a) C5 has to be presented on MHC class II. Therefore, only a few cell types in the thymus are candidates as mediators for negative selection, namely the MHC class II expressing macrophages, dendritic cells, epithelial cells (and a minor subpopulation of B cells which was not considered here).

b) The APC need to be capable of internalizing and processing the protein in order to present it for negative selection. It is known, that macrophages and dendritic cells are efficient in endocytosis (Lanzavecchia, 1996). The ability to internalize protein is less characterized for epithelial cells, although cortical epithelial cells were shown to present murine haemoglobin in vivo (Lorenz and Allen, 1989[a]).

c) Another factor is the access of C5 to different compartments in the thymus. Circulating proteins can permeate the vessels of the medulla (Kyewski et al., 1986), whereas the cortex seems to be protected from those proteins by the blood-thymus barrier (Raviola and Karnovsky, 1972), or is at least less accessible by means of the transcapsular route (Nieuwenhuis et al., 1988). Thus, preferential access of C5 to the medulla might result in preferential presentation by dendritic cells and medullary epithelial cells.

d) It is a question of debate, whether costimulation is involved in the process of negative selection (Jones et al., 1993; Page et al., 1993; Punt et al., 1994; Tan et al., 1992). Thus, expression of costimulatory molecules such as B7 on dendritic cells (Larsen et al., 1994) and medullary epithelial cells (Degermann et al., 1994[b]) could play a role in antigen presentation for negative selection. Furthermore, the adhesion molecule ICAM-1 has been indicated to influence negative selection in vitro (Carlow et al., 1992[b]; Pircher et al., 1993).

Most of the parameters mentioned differ from presentation in context with ubiquitously expressed MHC class I, suggesting that antigen presentation to MHC class II restricted T cells might be under more constraint. The TCR transgenic system using C5 as a model antigen allows the investigation of cellular requirements for tolerance induction to a true extracellular self protein under conditions close to the physiological situation.

The second part of the project deals with the generation and characterization of a dendritic cell line. The establishment of such a cell line was intended to provide a steady source of sufficient numbers of DC for in vitro and in vivo studies, since isolation of DC from mice in high numbers and purity is difficult, if not impossible. For the generation of the line, mice transgenic for a temperature sensitive mutant of the large tumour antigen (TAg) of simian virus 40 (SV40) have been used. The advantage of the temperature sensitive TAg is the possible reversion of a conditionally immortalized cell into a normal cell at non-permissive temperatures. This should allow to address maturation steps in DC development, which are presently not well defined.

In particular, one aim was to characterize the conditions for maturation of DC. In human, GM-CSF and IL-4 were described to cause differentiation of peripheral blood mononuclear cells into immature DC. TNFα or LPS could induce those DC to mature further (Sallusto and Lanzavecchia, 1994). In mice, immature LC have been reported to differentiate under the influence of GM-CSF and IL-1; TNFα in this case mediated maintenance but not maturation of LC (Heufler et al., 1987; Koch et al., 1990). Secondly, an immature DC line provides high numbers of a homogeneous cell population to study changes in phenotype as well as functional antigen presentation capacity during maturation. Moreover, biochemical analyses of DC are practicable with the availability of a DC line.

2. MATERIALS AND METHODS

2.1 Monoclonal antibodies

SPECIFICITY	ANTIBODY	ISOTYPE	REFERENCE
B7	CTLA-4	human IgG1 fusion protein	(Linsley et al., 1991)
B7.1, CD80	1G10	rat IgG2a	Pharmingen, CA
B7.2, CD86	GL-1	rat IgG2a	Pharmingen, CA
CD2	AT37	rat IgG2a	gift from Dr. M. Glennie
CD2, human	OKT11	mouse IgG2a	(Verbi et al., 1982)
CD3	145-2C11	hamster Ig	(Leo et al., 1987)
CD3	KT3	rat IgG2b	(Tomonari, 1988)
CD4 (-PE)	H129.19	rat IgG2a	Boehringer Mannheim
CD4 (-FITC)	GK1.5	rat IgG2b	(Dialynas et al., 1983)
CD8α	YTS169.4	rat IgG2b	(Cobbold et al., 1984)
CD8β	53.5.8	rat IgG1	Pharmingen, CA
CD11b, C3biR	M1/70 (Mac-1)	rat IgG2b	(Springer et al., 1979)
CD11c	N418	hamster Ig	(Metlay et al., 1990)
CD25 (IL-2Rα)	7D4	rat IgM	(Ortega et al., 1984)
CD40	FGK-145	human γ1 fusion protein	gift from Dr. D. Gray
CD44 (Pgp-1)	IM7.8.1	rat IgG2b	(Trowsbridge et al., 1982)
cortical EC	CDR-1	rat IgG2a	(Rouse et al., 1988)
DEC-205	NLDC-145	rat IgG2a	(Kraal et al., 1986)
FcRγII	2.4.G2	rat IgG2b	(Unkeless, 1979)
Gr-1	RB6-8C5	rat IgG2b	Pharmingen, CA
ICAM-1, CD54	YNI/1.7.4	rat IgG2b	ECCAC, UK
medullary EC	G8.8	rat IgG	(Farr et al., 1991)
MHC II I-E$\alpha^{k,d}$	14.4.4S	mouse IgG2a	ATCC (HB32)
mouse IgG+IgM	goatαmouse Ig	goat	Jackson/Stratech
macrophages	F4/80	rat IgG2b	(Austyn and Gordon, 1981)
rat IgG (H+L)	mouse α rat Ig	mouse	Jackson/Stratech
Sca-2	E381-2.4	rat IgG2a	(Aihara et al., 1986)
TCR-Vβ8 (1,2,3)	F23.1	mouse IgG2a	(Staerz et al., 1985)

TCR-Vβ8.3	7G8.2	mouse IgG2a	gift from Dr. I. Förster
Thy-1.2	6-68	mouse IgM	(Hämmerling et al., 1978)
Thy-1	YTS 109	rat IgM	gift from Dr. S. Cobbold
VCAM-1, CD106	M/K-2.7	rat IgG1	ATCC

2.2 Animals

The inbred mouse strains A/J ($C5^-$), CBA/Ca ($C5^+$) and B10 were obtained from the SPF (specific pathogen free) unit at the National Institute for Medical Research. The original SV40 large T transgenic mouse strain H-$2K^b$-tsA58 (Jat et al., 1991) was back-crossed to CBA (H-2^k) at the NIMR. T cell receptor transgenic mice (A18.A), carrying the I-E^k restricted TCR (Vα11.1^a, Vβ8.3) recognizing peptide 107-121 derived from the fifth complement component C5 (Zal et al., 1994), are bred in conventional animal facilities.

2.3 Media

Culture medium was Iscove's modified Dulbecco's medium (IMDM) (Gibco BRL, Paisley, Scotland) supplemented with 5% (10% for FTOC and reaggregation cultures) heat inactivated fetal calf serum (FCS) (Gibco BRL), 2 x 10^{-3} M L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 x 10^{-5} M mercaptoethanol (all Sigma, Poole, GB).

Medium for washing cells was air buffered IMDM (Gibco BRL) supplemented with 0.21% NaCl, 100 U/ml penicillin and 100 µg/ml streptomycin (AB medium).

2.4 Determination of cell viability and number

Trypan blue (Sigma) at a final concentration of 0.08% in phosphate buffered saline (PBS, 10.1g NaCl, 0.362g KCl, O.362g KH_2PO_4, 1.449g Na_2HPO_4, in 1l H_2O)

was used to determine the viability of cells. Cells were counted in a 1:1 mixture of Trypan blue using a Neubauer counting chamber (BDH Ltd, UK) and light microscopy. Dead cells (stained blue) were excluded from counting.

2.5 FACS analysis

Expression of cell surface antigens was determined by cytofluorimetric analysis (Parks and Herzenberg, 1984). $1x10^6$ cells were stained with biotinylated, FITC- or PE-conjugated or unconjugated monoclonal antibodies (mAb) in PBS containing 0.1% sodium azide and 1% FCS for 30 min at 4°C, except when stained with F23.1 mAb which took place at RT. Staining with unconjugated mAb was followed by a second staining step with goat anti mouse Ig-FITC or mouse anti rat Ig-FITC (both from Jackson ImmunoResearch Lab.), in case of a biotinylated first mAb the cells were incubated with streptavidin-PE (Biogenesis, UK) or streptavidin-RED670 (Gibco BRL) for 30 min at 4°C. Stainings of tsDC were controlled with isotype matched mAb unless otherwise stated. Aquisition was performed on a FACScan (Becton Dickinson, CA) using forward- and side-scatter characteristics to exclude dead cells. Data were analysed using CellQuest (Becton Dickinson).

2.6 Magnetic cell sorting

Positive selection of thymic stromal cells was performed by magnetic cell sorting (Miltenyi et al., 1990) with the Vario-MACS (Miltenyi, Biotech, Gergisch Gladbach, Germany) using as first labelling step biotinylated N418 to select dendritic cells (DC), biotinylated F4/80 to select macrophages and biotinylated G8.8 to select medullary epithelial cells (mEC), in all cases followed by labelling with streptavidin-magnetic beads. Unconjugated anti CDR-1 was used to select cortical epithelial cells (cEC), followed by an incubation step with anti-rat Ig magnetic beads. The procedure recommended in the manual was performed. Positively selected cells were passed over

the selection column twice to increase purity and the depleted cells from the previous selection were always used as a source for the next selection to avoid any contamination, especially with DC.

2.7 Bone marrow cultures

Bone marrow derived dendritic cells (DC) were generated as described (Stockinger and Hausmann, 1994), with some modifications. Briefly, femurs from A/J ($C5^-$) mice were rinsed with AB medium using a 1 ml syringe (Sherwood Medical) with a 0.5 x 16 mm needle (25G, Sherwood Medical). Red blood cells were lysed with distilled water, remaining cells were washed and $5x10^6$ cells/dish were cultured in 9 cm diameter petri dishes (Nunc, Denmark) in 10 ml culture medium containing 10% supernatant (≈ 25 U/ml) of Ag8653 myeloma cells transfected with murine granulocyte macrophage colony stimulating factor (GM-CSF) cDNA. On day 4 of culture non-adherent granulocytes were removed. Loosely adherent cells were transferred onto a second dish on day 6 of culture. From day 6 to day 12 these transferred loosely adherent cells were used as a source of dendritic cells. Their purity, assessed by MHC class II staining, was about 80%.

2.8 Generation of a dendritic cell line

Bone marrow cultures from a 4 week old SV40 temperature sensitive TAg transgenic female were prepared as described above and cultured for several weeks at 33°C in the presence of 200 U/ml IFNγ (supernatant of Ag8653 myeloma cells, IFNγ transfected) in addition to GM-CSF. Nonadherent cells were passaged weekly. After three months, GM-CSF and IFNγ were removed from the medium and cells were cloned by limiting dilution. MHC class II and N418 expressing clones were characterized further.

2.9 Antigen presentation assays

5 x 10^4 A18 T cell hybridoma cells (Lin and Stockinger, 1989) per well were cultured with different numbers of APC or 2 x 10^4 DC without additional antigen, or either 5µg/ml C5 protein, or 1 µM C5 peptide 107-121 unless otherwise stated for 24 h in flat bottom 96-well microtiter plates (Costar). After 24h, 100 µl aliquots of supernatant were transferred to fresh microtiter plates together with 5000 IL-2 dependent CTLL (ATCC HB 98) per well. [^{3}H]thymidine incorporation of CTLL was measured 24 h later, whereby 1 µCi thymidine (Amersham)/well was added for the last 9 h of culture.

1-5 x 10^5 $C5^-$ tg thymocytes or splenocytes were cultured with APC for 48 h in round bottom 96-well microtiter plates (Falcon, Becton Dickinson) but otherwise treated like T cell hybridoma cells. When antigen was present in form of C5 peptide or protein during the culture, thymocytes or splenocytes were depleted of endogenous APC by magnetic cell sorting (MACS, Miltenyi, UK) using N418 and 14.4.4 mAb.

2.10 Fetal thymic organ culture (FTOC)

Fetal thymic lobes were cultured according to procedures previously described (Smith et al., 1989). Briefly, thymic lobes were taken from heterozygous transgenic or non-transgenic embryos on day 14 or day 15 of gestation as indicated. The heterozygous embryos were obtained by time-mating homozygous transgenic males with superovulated (5 i.u./mouse serum gonadotrophin (Folligon) i.p., followed after 46 h by 5 i.u./mouse chorionic gonadotrophin (Chorulon) i.p., both Intervet, Cambridge, UK) non-transgenic females. Lobes were placed on a nucleopore filter (0.8µm pore size, Costar) floating on culture medium. The filter was supported by a gelatin foam sponge (Gelfoam, Upjohn Co.) when lobes were cultured for 10 days or longer.

2.11 Thymic reaggregation culture

Reaggregate organ cultures were performed according to Anderson et al. (1993) with some modifications. Single cell suspensions were prepared by digesting fetal thymi from day 15 $C5^-$ TCR transgenic embryos with a cocktail of 1.6 mg/ml collagenase (CLS4, Worthington Biochemical Corp., NJ), 0.1% deoxyribonuclease (DNase I, Fraction IX, Sigma), 0.2 mg/ml dispase (grade I, #241750, Boehringer Mannheim) for 30 min at 37°C. Thymus cell suspensions (3 x 10^5 cells) were mixed with 1 x 10^5 stromal cells from $C5^-$ day 15 embryos, prepared by organ culture in 1.35 mM dGuo (2' deoxyguanosine, #D0901, Sigma) containing medium for 4-5 days, and different ratios of thymic APC from $C5^+$ adult mice isolated by magnetic cell sorting. This mixture was pelleted by centrifugation, resuspended in 3 µl culture medium containing 10% FCS and placed on a nucleopore filter in organ culture. Cell yields after 4 days of culture were about 40 % of the input if a successful reaggregate had formed.

2.12 Nonspecific esterase staining

2.5 x 10^3 cells in 120µl AB medium were spun onto glass slides for 6 min at 500 rpm in a cytocentrifuge. The slides were air-dried and subsequently stained for nonspecific esterase according to the manual using the Sigma Diagnostic kit (#91-A, Sigma).

2.13 Phagocytosis assay

2 x 10^6 cells per well were plated in 4 ml medium containing 1 µl blue latex beads (0.8 µm diameter, 10% solids content, Sigma) or 1% sheep red blood cells (SRBC) in 6-well plates. Cover slips were placed on the bottom of each well for the cells to adhere onto. After a 2 h incubation period with latex beads the cover slips were removed and placed on slides for microscopical analysis using a 100x objective. Cover slips with

cells incubated with SRBC were removed after 4 h, rinsed shortly in distilled water to lyse non-phagocytosed SRBC, then washed in AB medium and placed on slides.

2.14 Mixed leukocyte reaction (MLR)

2.5 x 10^5 responder splenocytes from H-2^b (B10) mice per well were cultured with 1 x 10^3 to 1 x 10^5 stimulator cells (M-CSF cultured macrophages or GM-CSF cultured DC from H-2^k bone marrow, or H-2^k tsDC) for 3 days in 96-well U-bottom microtiter plates (Falcon, Becton Dickinson). Proliferation of the responding T cells was measured as incorporation of [^{3}H] thymidine present for the last 18 h of culture.

2.15 Cytokine treatment of tsDC

1 x 10^6 tsDC were cultured for 3 days at 39°C in a 50 ml tissue culture flask (Nunc) containing 5 ml culture medium supplemented with 25 U/ml GM-CSF and one additional cytokine. The cytokine concentrations were: 100 U/ml TNFα (gift from Dr. M. Turner), 100 U/ml IL-1 (Upjohn), 200 U/ml IFNγ, 1% supernatant of IL-4 or IL-5 transfected cells, 0.2% supernatant of IL-6 transfected cells (all transfected cells were Ag8653 myeloma (Karasuyama and Melchers, 1988), percentage of supernatant used was tested on cell lines). Alternatively, culture medium contained 20% supernatant of concanavalin A stimulated T cells (ConA sup) and was used with tsDC for 2 days at 39°C. ConA sup was obtained by culture of mouse spleen cells at 5 x 10^6/ml with 2.5 μg/ml ConA for 48h.

2.16 Preparation of skin cells

Ears were obtained from B10 mice by cutting them off near the base. They were rinsed with 70% ethanol, washed in PBS and placed in AB medium. Dorsal and ventral halves were separated and incubated for 45 min at 37°C in IMDM medium,

containing 0.25% trypsin. The trypsin solution was then removed and a single cell suspension was prepared by mechanical disaggregation of the sheets on a steel mesh. The cells were washed twice in culture medium.
2 x 10^6 cells were co-cultured with 1 x 10^6 tsDC in 5 ml culture medium in a 6-well plate for 2 days at 37°C.

2.17 Confocal microscopy

Cover slips were coated with 0.1 mg/ml poly-L-lysine (Sigma) for 15 min at RT, washed thoroughly with PBS and placed in 6-well plates. 2 x 10^6 tsDC/well or bone marrow DC from cultures with GM-CSF were added and cultured for 1-2 days before confocal microscopy. Living tsDC or bone marrow DC were analysed in culture medium in a tissue culture device at 37°C. The temperature of the medium was continuously checked during analysis. In order to stain acidic vesicles (lysosomes), cells were incubated for 1 min with LysoTracker Red DND99 (Molecular Probes) at a concentration of 300 nM prior analysis. LysoTracker Red diffuses through membranes and becomes fluorescent under acidic pH conditions. Endocytosis was assessed by internalization of sulforhodamine 101 (SR101, Molecular Probes) for 2 min at a concentration of 25 µg/ml. Confocal analysis was performed using a BioRad-600MRC with x 60 objective, red fluorochromes were excited at 586 nm and emission was detected at 585 nm.

2.18 Electron microscopy

TsDC were fixed for 1 h in PBS containing 4% formaldehyde and 0.5% glutaraldehyde. Ultrathin cryosections (≈ 100 nm) were subsequently incubated with anti-MHC class II mAb (14.4.4), rabbit anti-mouse Ig and goat anti-rabbit Ig linked to 10 nm gold particles (Amersham, The Netherlands); or they were stained with rabbit anti-class I serum (provided by Dr. J. Neefjes), followed by gold labelled goat anti-

rabbit Ig. Alternatively, tsDC pulsed with 2 mg/ml horseradish peroxidase (HRP, type VIA, Sigma) for 30 min at 37°C were fixed and stained with rabbit anti-HRP serum followed by gold labelled goat anti-rabbit Ig. The sections were embedded in a mixture of methylcellulose and uranyl acetate and analysed by electron microscopy (CM 10, Philips Electronic Instruments, The Netherlands).

2.19 Treatment with brefeldin A and cycloheximide

TsDC were cultured for 48 h at 37°C in the presence of 20% ConA sup. For the last 8 h, cycloheximide (Sigma) was added at a final concentration of 200 µM. Alternatively, 5 µg/ml brefeldin A (BFA, Sigma) was included during the last 14 h of culture.

2.20 Biochemical analysis

2.20.1 Subcellular fractionation by density gradient electrophoresis

5 x 10^7 tsDC cultured at 33°C or tsDC cultured for 2 days at 37°C in the presence of 20% ConA sup were incubated for 30 min at 37°C with HRP (type VIA, Sigma) at a concentration of 2 mg/ml. Alternatively, they were incubated with HRP for 5 min at 4°C, washed and incubated for further 30 min at 37°C. Cells were washed two times with ice cold PBS and once with homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, 0.25 M sucrose, pH 7.4). They were then homogenized by passing 20 times through a cell cracker (EMBL) and post nuclear supernatant (PNS) was collected by centrifugation at 2500 rpm for 15 min at 4°C. PNS was trypsinized (25 µg/mg total protein) for 5 min at 37°C, trypsin inactivated with 100 µg soybean (Merck) / mg total protein and protease inhibitors were added (PMSF 1 mM, aprotinin 10 µg/ml, leupeptin 10 µg/ml (all Sigma)). Vesicles were collected by centrifugation for 1 h at 100,000 g at 4°C and separated by a density gradient electrophoresis (DGE) device with an extended electrophoretic pathway of 7

cm as described (Tulp et al., 1996). Electrophoresis was performed at 10.30 mA constant current for 80 min. 250 µl fractions were collected starting at the anodic site. Proteins were TCA precipitated and analysed by Western blotting after separation on a 10% SDS-PAGE. HRP-activity was determined spectrophotometrically at 455 nm using O-dianisidine as substrate, as described (Gruenberg et al., 1989). β-hexosaminidase activity was measured at 405 nm using p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) as substrate. Protein concentration was determined using a protein assay kit (BioRad, Richard, CA).

2.20.2 SDS-PAGE and Western blotting

The protein content in the subcellular fractions was precipitated in 10% (v/v) trichloroacetic acid (TCA) for 30 min on ice. Proteins were pelleted by centrifugation for 15 min at 13,000 rpm. Pellets were resuspended in SDS sample buffer, boiled for 5 min at 95°C and proteins were separated on a 10% SDS-PAGE (Laemmli, 1970) followed by transfer to nitrocellulose membranes (Schleicher & Schuell, Germany) for 2 h at 150 mA in 4°C cold blot buffer (20% (v/v) methanol, 12.5 mM Tris, 96 mM glycine). Blots were blocked in 5% (w/v) milk powder in buffer (0.05% Tween 20, 10 mM Tris-HCl, pH 8.0, 150 mM NaCl). Blots were incubated with a polyclonal rabbit anti mouse MHC class II serum, a polyclonal rabbit anti MHC class I K^b serum, or a rabbit anti human cathepsin D serum (all provided by Dr. J. Neefjes) overnight at 4°C. They were then washed more than 5 times in buffer followed by incubation with swine anti-rabbit HRP-conjugated secondary antibody (Dako, Glostrup, Denmark) for 1 h. After washing several times in buffer, proteins were detected using an enhanced chemiluminescent system (ECL, Amersham, UK) and exposure to a Kodak X-Omat S film. For reprobing, blots were stripped in 67 mM Tris pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol for 30 min at 50°C, followed by blocking and staining as described.

2.20.3 Biochemical analysis of MHC class II surface molecules

3 x 10^7 tsDC cultured at 33°C and 1 x 10^7 tsDC cultured for 2 days at 37°C in the presence of 20% ConA sup were labelled at 4°C using lactoperoxidase-catalysed iodination with 400 µCi $Na^{125}I$ (Amersham). Cells were washed 4 times with ice cold PBS and lysed in NP-40 containing lysis mixture (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 0.5% (v/v) NP40, all Sigma) supplemented with 1 µg/ml trypsin inhibitor, 0.2 mM phenylmethyl-sulfonyl fluoride (PMSF) and 0.1% aprotinin (all Sigma). Nuclei were removed by centrifugation at 13,000 rpm for 5 min and lysates were precleared twice with normal mouse serum coupled to Protein A-Sepharose beads (Pharmacia Biotech, Uppsala, Sweden) to prevent nonspecific binding of proteins. The lysates were immunoprecipitated with anti-class II mAb (14.4.4), coupled to Protein A-Sepharose beads. Precipitates were washed several times in lysis mixture and separated by 12% SDS-PAGE over night at 120 V after boiling in reducing sample buffer. Gels were fixed in 10% ethanol, 5% acetic acid for 30 min RT, washed twice in water for 15 min and dried under vacuum for 2 h at 80°C. Dry gels were exposed to a phosphoimager screen for 4 days at 4°C before analysis on the phosphoimager.

3. RESULTS

3.1 Tolerance induction in early T cell development

3.1.1 T cell development in fetal thymic organ culture

To investigate the mechanisms of tolerance induction to an extracellular self protein, the early development of T cells from TCR transgenic mice was followed in the presence or absence of their antigen in fetal thymic organ culture (FTOC). This in vitro system mimics the in vivo T cell development in both, differentiation events and kinetics (Jenkinson and Owen, 1990).

The transgenic TCR is MHC class II restricted and recognizes a peptide derived from the circulating self protein C5 (fifth component of complement). Transgenic mice carrying a natural mutation in their C5 gene (tg $C5^-$) do not contain the protein and therefore are not tolerant to C5, whereas adult transgenic mice containing C5 (tg $C5^+$) are fully tolerant in vivo. The first question to address was whether this tolerance is complete in early ontogeny, when the C5 level in blood is known to be lower than in adult mice. Thymic lobes from day 14 transgenic $C5^-$ and $C5^+$ embryos, tg $C5^{+/-}$ embryos (from $C5^+$ females mated with tg $C5^-$ males to halve the C5 level) and non-transgenic control embryos were set up in organ culture for 4, 6 or 10 days, after which the cells were harvested and tested for surface marker expression as well as for functional reactivity.

On day 14 of embryonic development a thymus lobe contains on average 10,000 thymocytes. Proliferation within 10 days of organ culture resulted in an 30 to 50 fold increase in cell numbers (Fig. 1A). Cell numbers of individual lobes are subject to variations, presumably due to the litter size, which partly determines the growth rate of the embryos and thereby their thymus lobes. Nevertheless, thymocytes in lobes from non-transgenic mice might have a disadvantage in early stages of

development because, in contrast to cells from transgenic mice, they need time to rearrange genes encoding the TCR α and β chains. Later on, the diverse TCR repertoire of T cells from non-transgenic mice might be useful to ensure broad selection. However, in some experiments more cells were recovered from day 10 lobes from tg $C5^-$ and tg $C5^{+/-}$ embryos than from non-transgenics or tg $C5^+$, questioning the significance of the observed trend shown in Fig. 1A.

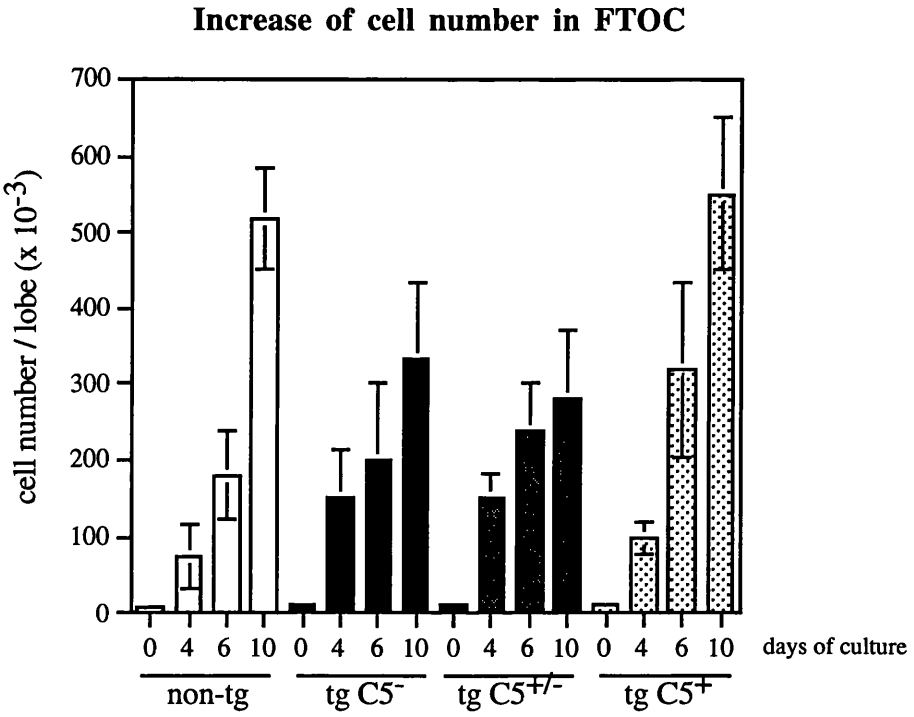


Figure 1A

Total cell numbers of thymus lobes from non-transgenic mice (non-tg, white columns), TCR transgenic $C5^-$ mice (tg $C5^-$, black columns), TCR transgenic $C5^{+/-}$ mice (tg $C5^{+/-}$, dark grey columns) and TCR transgenic $C5^+$ mice (tg $C5^+$, dotted columns), recovered on day 14 of gestation without culture or after organ culture for the number of days indicated. The mean values of 4-9 independent experiments with 2-6 pooled thymus lobes are shown. Bars represent standard deviations.

The vast majority of thymocytes expressed neither CD4 nor CD8 on day 14 of gestation, only 2-4% showed low levels of CD4, representing an immature stage

of single positive cells (SP), demonstrated by the lack of TCR expression (Fig. 1B-E). Non-transgenic lobes gave rise to immature $CD4^+TCR^-$ cells and $CD4^+CD8^+$ double positive thymocytes (DP) after 4 days in culture. Mature T cells appeared after 10 days, as this was the first time point when TCR expression on gated CD4 SP became visible (Fig. 1B). The antibody used recognizes only those TCR containing Vβ8, the variable region used by all C5 specific, transgenic T cells and 20% of non-transgenic T cells. Therefore, the actual percentage of $CD4^+TCR^+$ cells in lobes from non-transgenic mice is higher than evident from the Vβ8 staining. Lobes from tg $C5^-$ embryos contained $CD4^+TCR^+$ cells already on day 4 of organ culture, indicating an accelerated development to mature cells (Fig. 1C). This is presumably due to the presence of an already functionally rearranged transgenic TCR. Thymocytes in tg $C5^{+/-}$ lobes (Fig. 1D) developed indistinguishably from those in tg $C5^-$ lobes, even though $C5^{+/-}$ mice must contain some C5 on day 14 of gestation (see section 1.7). The situation looked different in lobes from tg $C5^+$ embryos, in which DP but no $CD4^+TCR^+$ cells were detectable on day 4 and day 6 of organ culture. However, mature thymocytes appeared on day 10 (Fig. 1E). These $CD4^+$ cells express the TCR at the same level as those from tg $C5^-$ and tg $C5^{+/-}$ embryos. Transgenic Vβ8 TCR levels are consistently lower than the endogenous level of Vβ8 expression in non-transgenics embryos (compare with day 10 in Fig. 1B). In the absence of a clonotype specific antibody, the level of Vβ8 expression can be used to distinguish the transgenic receptor from endogenous TCR.

The appearance of $CD4^+TCR^+$ cells in tg $C5^+$ lobes on day 10 of culture led to the question, whether tolerance induction was incomplete or whether the cells were tolerized by other means than deletion and would not be reactive against C5. To address this, thymocytes from tg $C5^+$, tg $C5^{+/-}$, tg $C5^-$ and non-transgenic lobes cultured for 6 and 10 days were set up in vitro with dendritic cells (DC) as antigen presenting cells (APC) in the presence of C5 peptide.

Figure 1B-E

Thymocytes from lobes of 14 days old non-transgenic (B), tg $C5^-$ (C), tg $C5^{+/-}$ (D), or tg $C5^+$ embryos (E) were triple stained with PE-conjugated mAb against CD4, FITC-conjugated anti-CD8 and biotinylated anti-Vβ8, followed by streptavidin-RED670. Expression of CD4 and CD8 on day 0 ex vivo, or after 4, 6 and 10 days of organ culture is shown in dot plots. The percentages of subpopulations are given within the quadrants, staining of Vβ8 on gated CD4 SP and on gated DP is shown in histograms.

Fig. 1B

Non-transgenic thymocytes in FTOC

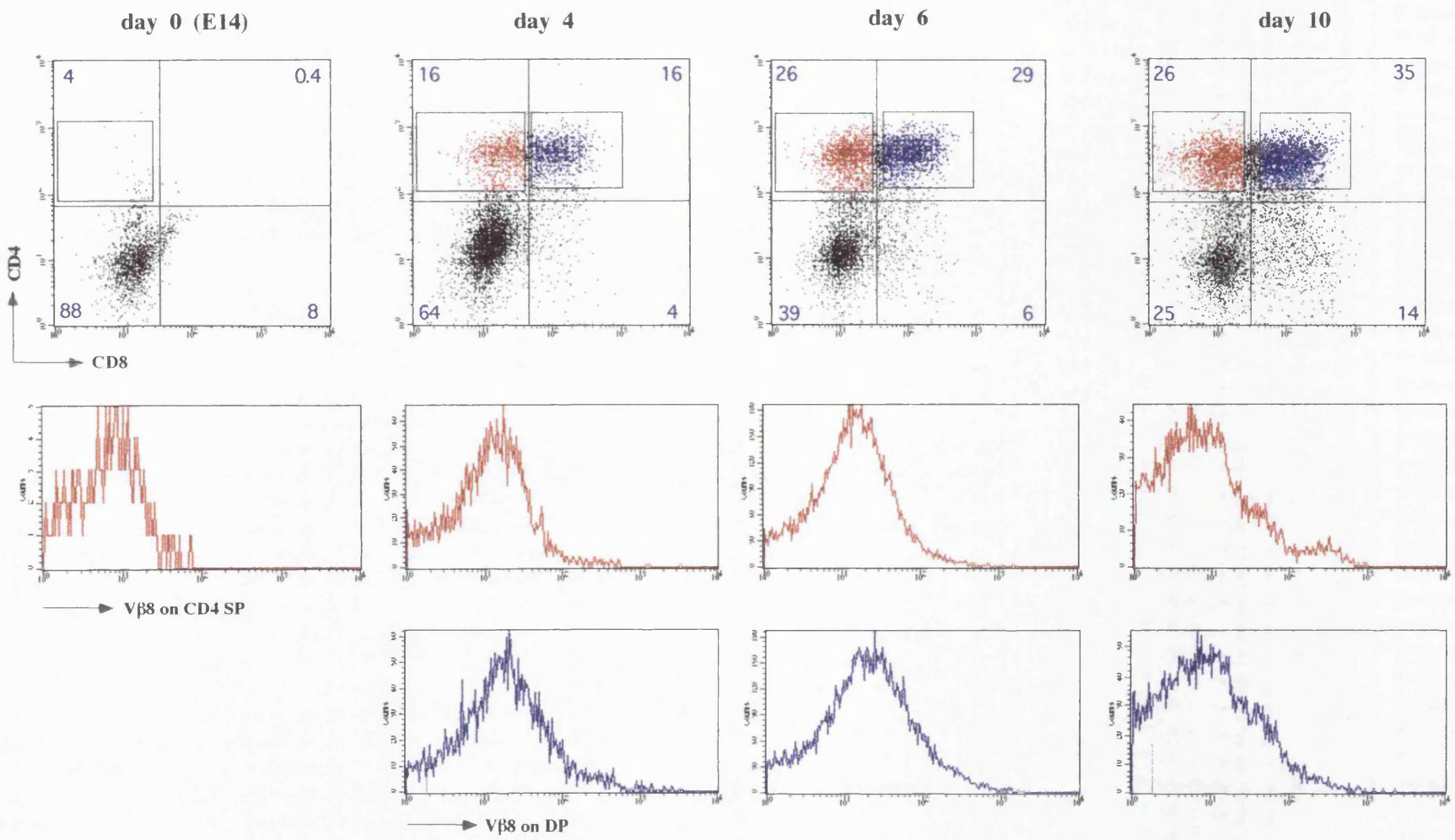


Fig. 1C

C5^{-} TCR transgenic thymocytes in FTOC

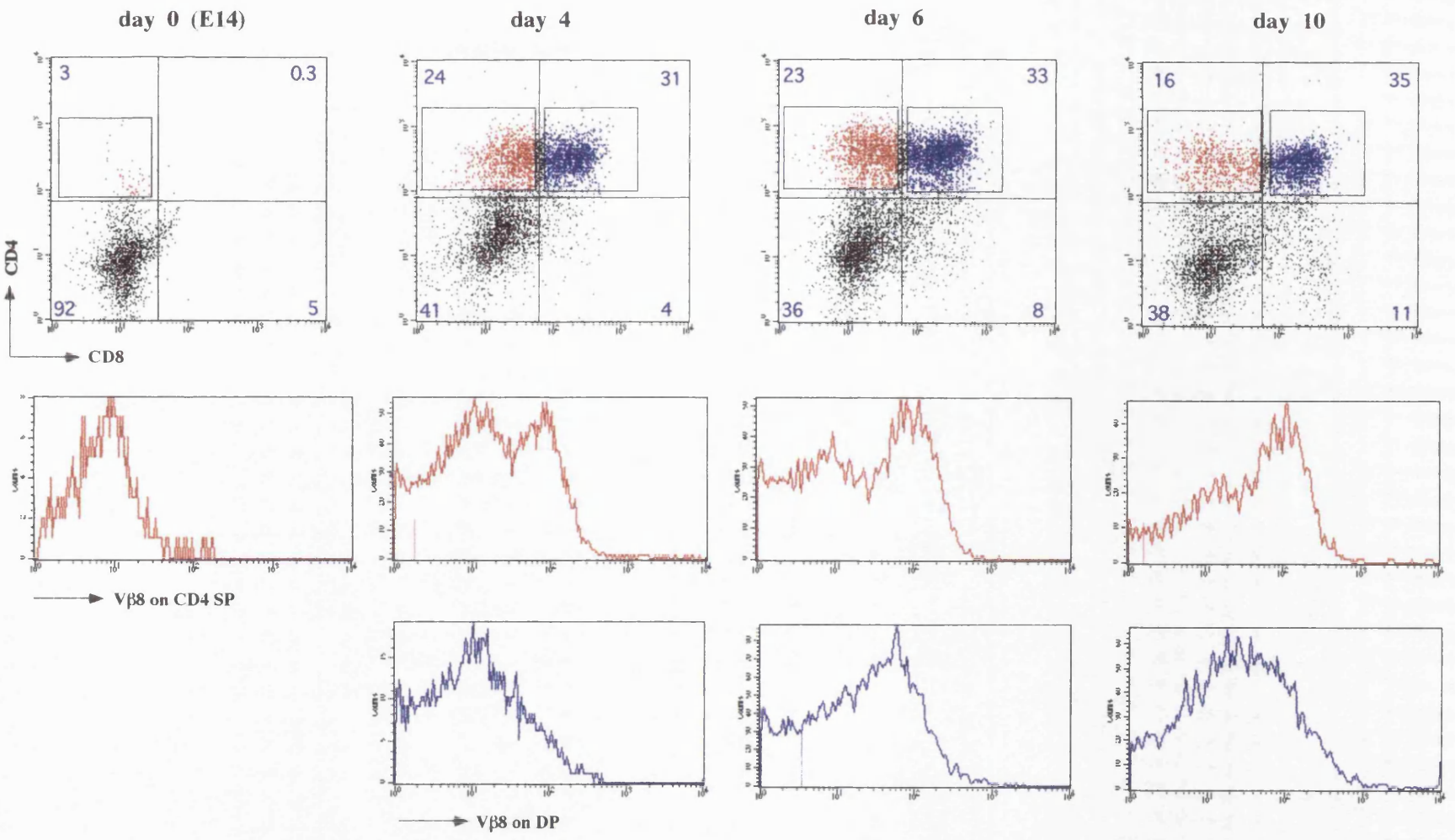


Fig. 1D

C5$^{+/-}$ TCR transgenic thymocytes in FTOC

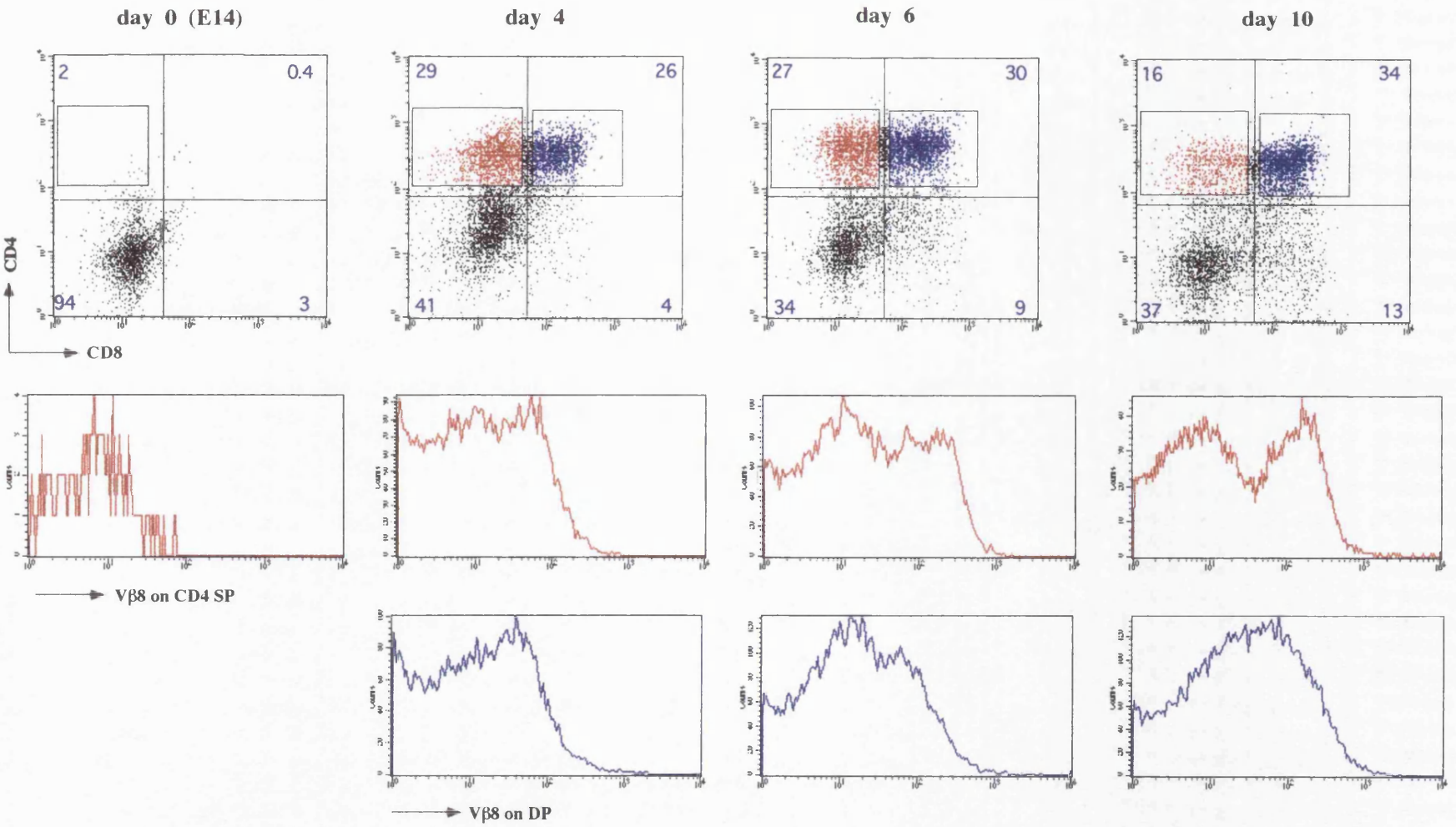
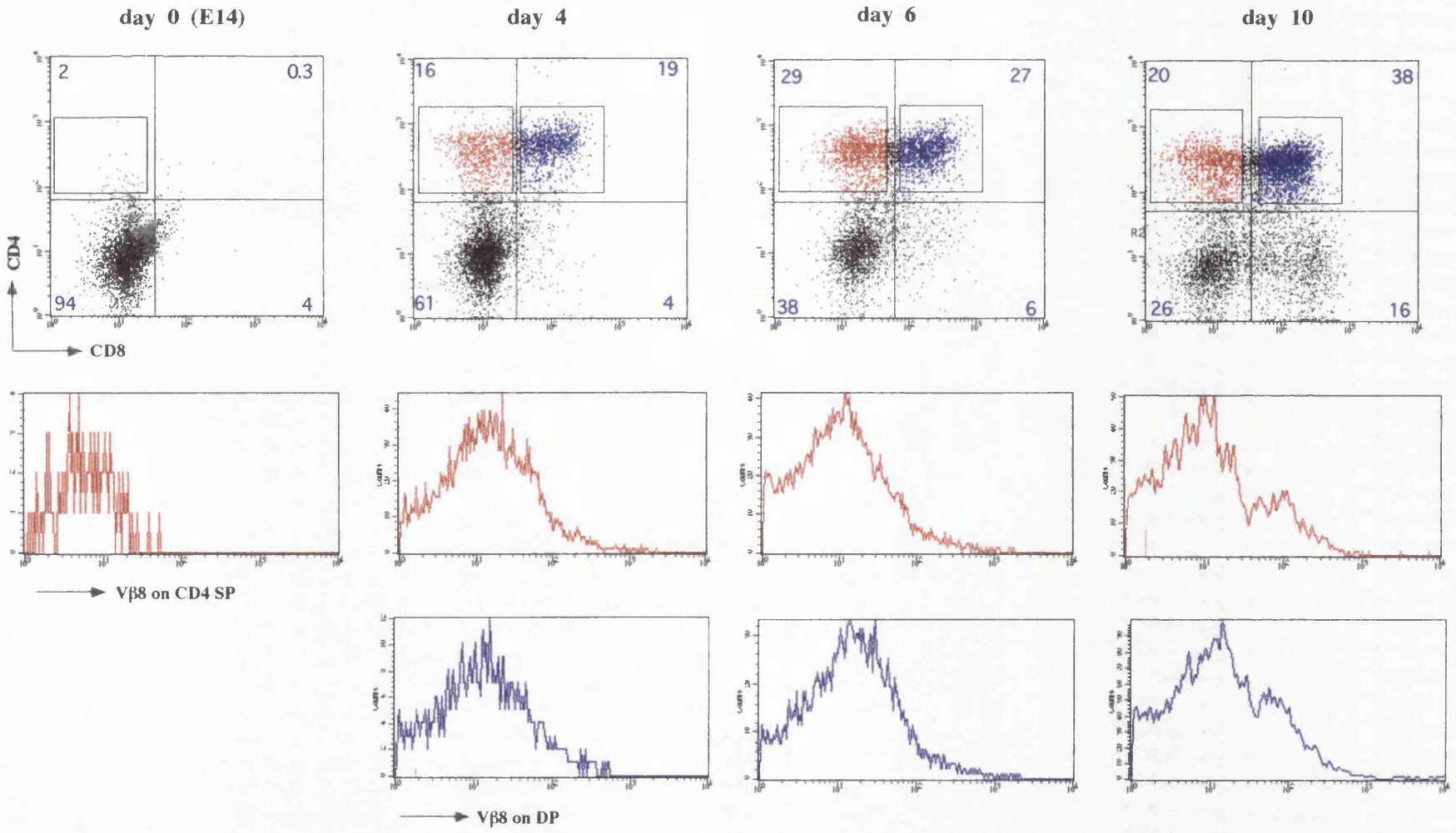


Fig. 1E

C5+ TCR transgenic thymocytes in FTOC



Activation of thymocytes was then tested by their IL-2 production, which was measured as proliferation of an IL-2 dependent T cell line (CTLL), read out as ^{3}H-thymidine incorporation (Fig. 2). Thymocytes from non-transgenic lobes did not respond to C5 at either time point, because the frequency of C5 specific T cells in non-transgenic mice is very low. After 6 days of culture, C5 reactive thymocytes were absent from lobes of tg $C5^{+}$ embryos, in agreement with the absence of $CD4^{+}TCR^{+}$ cells from those lobes. In contrast, $C5^{-}$ and $C5^{+/-}$ transgenic lobes gave rise to C5 reactive thymocytes after 6 days of culture. However, after 10 days of FTOC thymocytes from all transgenic lobes responded to C5, regardless of their $C5^{+}$, $C5^{+/-}$ or $C5^{-}$ origin. This shows that the $CD4^{+}TCR^{+}$ cells found in tg $C5^{+}$ lobes on day 10 are not anergic and suggests incomplete tolerance in thymus lobes from C5 containing embryos at that stage of organ culture.

3.1.2 Status of prenatal tolerance in vivo

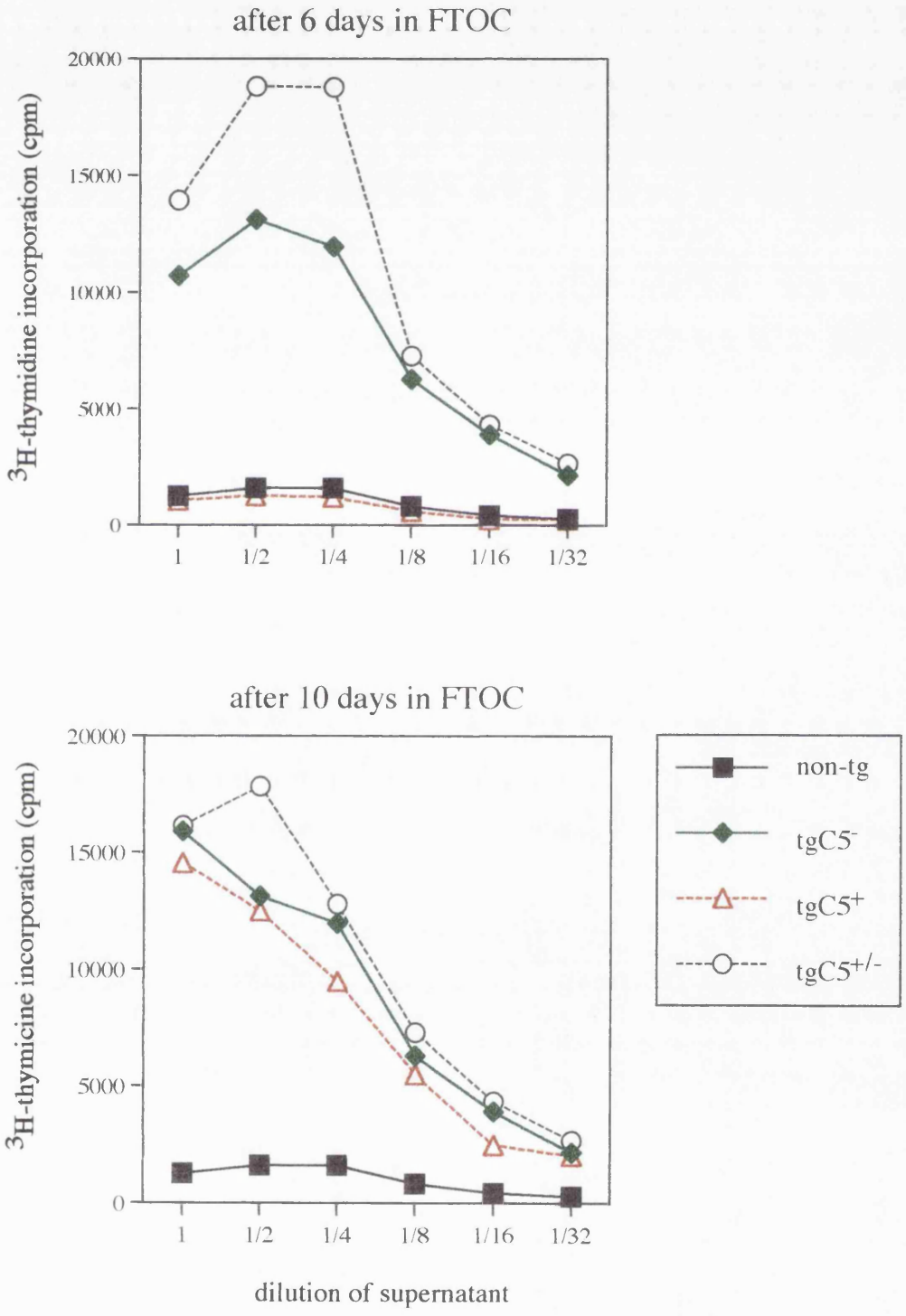
The appearance of C5 reactive mature T cells in FTOC of tg $C5^{+}$ mice could be explained either by low levels of C5 early in ontogeny, or by the particular treatment of thymocytes in organ culture, namely the transfer of lobes from their C5 containing environment in vivo into an in vitro culture without continuous C5 supply. If tolerance induction was incomplete due to low levels of the antigen early in ontogeny, one would expect to find C5 reactive T cells at a corresponding time point in vivo. To test this possibility, thymocytes from 19 days old $C5^{+}$, $C5^{+/-}$ and $C5^{-}$ transgenic embryos, as well as from non-transgenics were analysed for surface marker expression and functional reactivity. The staining pattern for CD4 and CD8 looked very similar in all cases (Fig. 3), as did TCR expression on gated DP (data not shown). In contrast, staining with anti Vβ8 antibody revealed different TCR expression on gated CD4 SP. The majority of CD4 SP from non-transgenic embryos did not express Vβ8, most of the cells are still immature on day 19 of gestation. In the thymus of tg $C5^{-}$ embryos almost all CD4 SP expressed Vβ8, as expected.

Figure 2

Thymocytes from non-transgenic (non-tg), tg $C5^-$, tg $C5^{+/-}$ and tg $C5^+$ lobes were prepared after 6 and 10 days of organ culture. 5 x 10^5 cells were co-cultured in 96-well plates with 2 x 10^4 bone marrow DC in the presence of 1μM C5 peptide 107-121. After 48h, supernatants were removed and tested in serial dilution on the IL-2 dependent CTLL line. Proliferation of CTLL was measured by means of [^{3}H]-thymidine incorporation, added after 24h of culture for the last 9h. Results are expressed as mean cpm of duplicate cultures.

Fig. 2

C5 specific response of thymocytes



In contrast, both tg $C5^{+/-}$ and tg $C5^{+}$ thymi contained only a minority of Vβ8 expressing CD4 SP. The levels of Vβ8 expression on these cells suggests the presence of endogenous receptors, since they are 2-5 fold higher than the transgenic TCR levels. This indicates that in the presence of C5, T cells expressing the transgenic TCR were deleted, and those expressing the Vβ8 chain together with an endogenous α chain to create a specificity different from C5 were allowed to mature.

To further confirm the absence of mature, C5 reactive T cells in C5 sufficient transgenic embryos, thymocytes were tested for their C5 reactivity on day 19 of gestation. When cultured with C5 presenting DC in vitro, only thymocytes from tg $C5^{-}$ embryos responded with IL-2 production (Fig. 4A). Neither transgenic $C5^{+}$, $C5^{+/-}$, nor control thymocytes from an adult tg $C5^{+}$ mouse showed any C5 reactivity. Moreover, cell suspensions of tg $C5^{+/-}$ or tg $C5^{+}$ thymi, when used as a source of APC, were able to activate a C5 specific T cell hybridoma (Fig. 4B). This assay also reflected the various amounts of C5 accessible to thymic APC: adult tg $C5^{+}$ thymi contain the highest C5 levels indicated by their more potent APC function, followed by neonatal tg $C5^{+}$ thymi and neonatal tg $C5^{+/-}$ thymi, whereas tg $C5^{-}$ thymi obviously do not contain any C5 for APC to present.

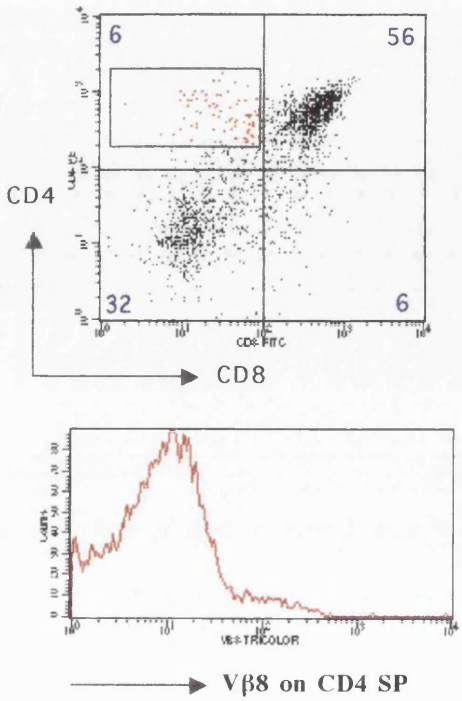
It can therefore be concluded that C5 synthesis between day 14 and day 19 of gestation is sufficient to induce full tolerance in vivo. Thus, the appearance of C5 reactive mature CD4 cells in FTOC of tg $C5^{+}$ lobes must be explained by the lack of continuous C5 supply in organ culture.

Figure 3

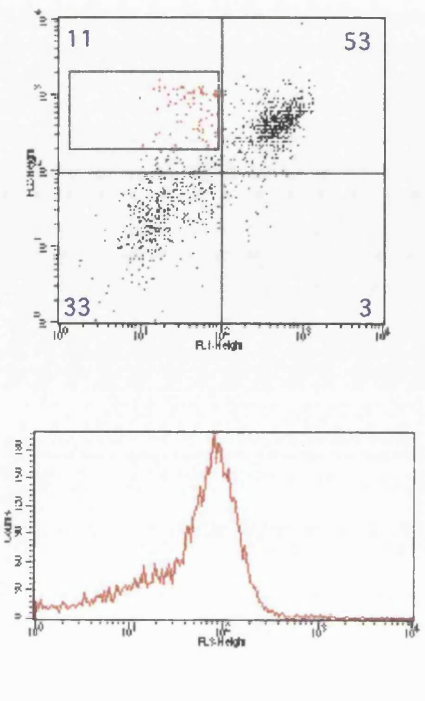
Cells from non-transgenic, tg $C5^-$, tg $C5^{+/-}$, or tg $C5^+$ enzyme digested embryonic thymi on day 19 of gestation were triple stained for CD4, CD8 and Vβ8 expression. Dot plots show CD4 and CD8 expression, with percentages of subpopulations given in the quadrants. Expression of Vβ8 is shown on gated CD4 SP in histograms. Cell numbers per thymus are expressed as mean value ± (standard deviation) of 6 individual embryos in each group.

Fig. 3 **Thymocytes on day 19 of gestation**

nontransgenic



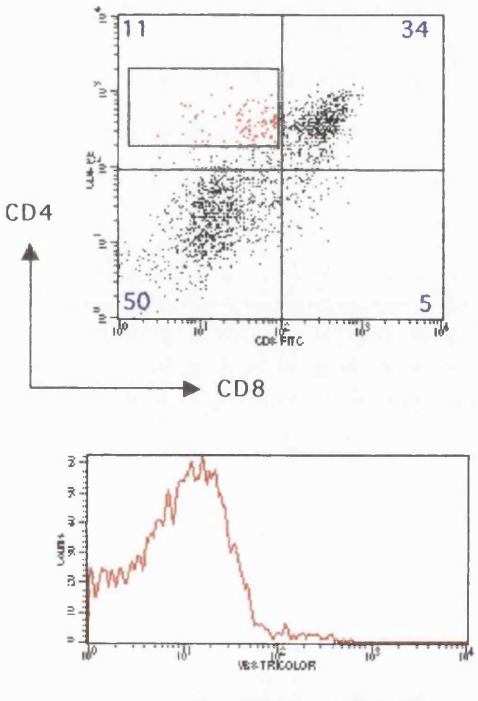
tg C5^{-}



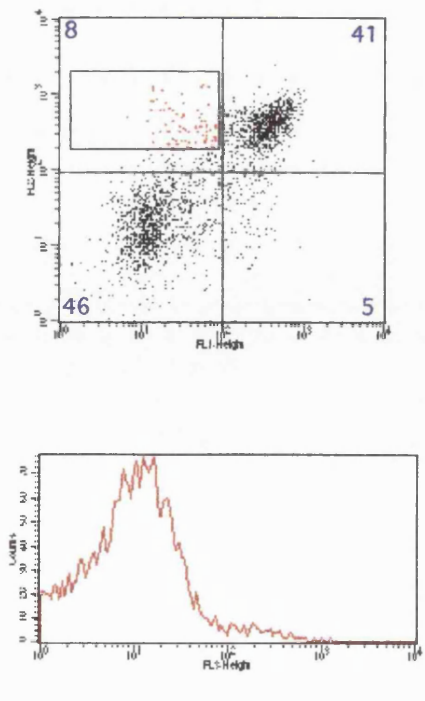
total cell number [x10^{-6}] /thymus: 6 (1.2)

5.5 (2.7)

tg C5$^{+/-}$



tg C5^{+}



4.8 (0.7)

4.7 (2.8)

Figure 4

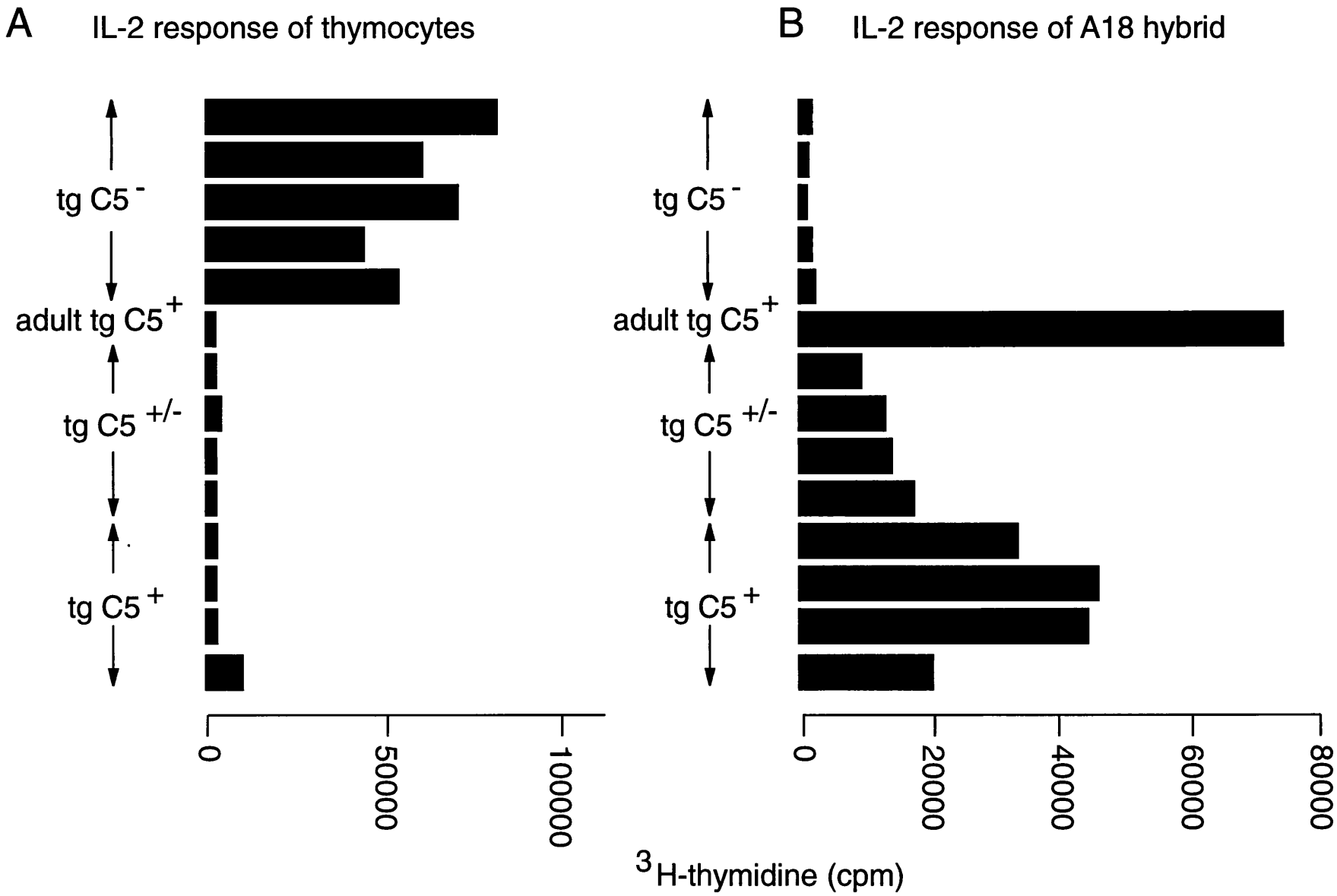
Thymocytes from d19 tg $C5^+$ embryos are not C5 reactive.

Thymus suspensions from individual day 19 embryos (5 x tg $C5^-$, 4 x tg $C5^{+/-}$, 4 x tg $C5^+$) and one adult tg $C5^+$ control were prepared by enzyme digestion.

In **A**, 2 x 10^5 thymocytes/well of a 96-well plate were cultured with 2 x 10^4 DC in the presence of 1 μM C5 peptide for 48h. Supernatant was then removed and tested for IL-2 activity on CTLL.

In **B**, the same thymus cell suspensions were irradiated with 200 Gy and cultured at 2 x 10^5 cells/well with 5 x 10^4 cells/well of the C5 specific A18 T cell hybridoma. 24h later supernatants were transferred to CTLL for assessment of IL-2 activity.

The results are expressed as mean cpm (triplicate cultures) of incorporated [^{3}H]-thymidine.


A
IL-2 response of thymocytes
tg C5 -
adult tg C5 +
tg C5 +/-
tg C5 +
0
50000
100000
B
IL-2 response of A18 hybrid
tg C5 -
adult tg C5 +
tg C5 +/-
tg C5 +
0
20000
40000
60000
80000
3H-thymidine (cpm)

3.1.3 C5 specific double positive thymocytes are susceptible to deletion

Experiments described so far showed a complete lack of mature, C5 reactive T cells in tg $C5^+$ mice in vivo, indicating deletion of those thymocytes. The induction of negative selection by deletion in some other experimental systems takes place at the stage of $CD4^+CD8^+$ double positive cells (Guidos et al., 1990; Shortman et al., 1991) using non-transgenic mice, (Kisielow et al., 1988; Murphy et al., 1990) in transgenic mouse systems). The staining pattern of tg $C5^+$ mice for CD4 and CD8 (Fig. 3), together with thymocyte numbers, imply that tolerance induction to C5 does not affect the majority of immature DP, but rather those at the transition to single positive cells.

Why does tolerance induction occur at a later stage of T cell differentiation in this system? Firstly, the TCR levels might account for this. The majority of DP express low or intermediate levels of TCR, but expression increases at the transition to SP as a result of positive selection (Huesmann et al., 1991; Swat et al., 1992). With regard to the 2-5 fold lower TCR expression level on C5 specific T cells, the level on DP might not be sufficient to receive a negative selection signal until TCR expression increases following positive selection. Secondly, certain antigen presentation requirements might be responsible for late deletion. C5, as a blood circulating self protein, has to be internalized and processed by thymic MHC class II expressing APC for negative selection. Blood circulating proteins are known to have better access to the thymic medulla than to the cortex (Nieuwenhuis et al., 1988), the latter being the place were the bulk of DP are found. Thus, the amount of available C5 might not be high enough for cortical APC to present the antigen efficiently, and the deletion would take place only when DP, at their transition to SP, enter the medulla and encounter medullary APC. In addition, different types of thymic APC are located in either cortex or medulla, presumably with diverse internalization properties. DC have been shown to be the most efficient thymic APC (Kyewski et al., 1986; Matzinger and Guerder, 1989; Stockinger and Hausmann, 1994),

especially when internalization and processing of proteins are involved. The presence of thymic DC is restricted to the medulla and cortico-medullary boundary, which means that, again, the bulk of DP may not have access to sufficient C5 presentation.

Introduction of C5 peptide into organ culture not only increases the amount of available antigen, but avoids the necessity of internalization and processing. Its direct binding onto MHC class II molecules on cell surfaces gives every MHC class II bearing cell the opportunity to present C5. Culturing of lobes from d14 $C5^-$, $C5^{+/-}$ and $C5^+$ transgenic embryos in the presence of various concentrations of C5 peptide led indeed to a loss of DP in all cases (Fig. 5A). The percentages of $CD4^+TCR^+$ cells, DP and DN after 11 days of organ culture in medium alone or in the presence of 10 µM, 100 µM or 500 µM C5 peptide showed no significant changes for non-transgenic lobes, whereas the percentage of $CD4^+TCR^+$ cells and of DP in $C5^-$, $C5^{+/-}$ and $C5^+$ transgenic lobes decreased dramatically. The apparent increase of DN in those lobes reflects over-representation due to massive deletion of DP, because their absolute numbers remained constant. Deletion of transgenic cells was also manifested in low cell recovery after peptide treatment (Table 1).

	tg $C5^-$	tg $C5^{+/-}$	tg $C5^+$	non-tg
untreated	370, 000	310, 000	380, 000	360, 000
in 500µM pep	80, 000	140, 000	120, 000	320, 000

Table 1

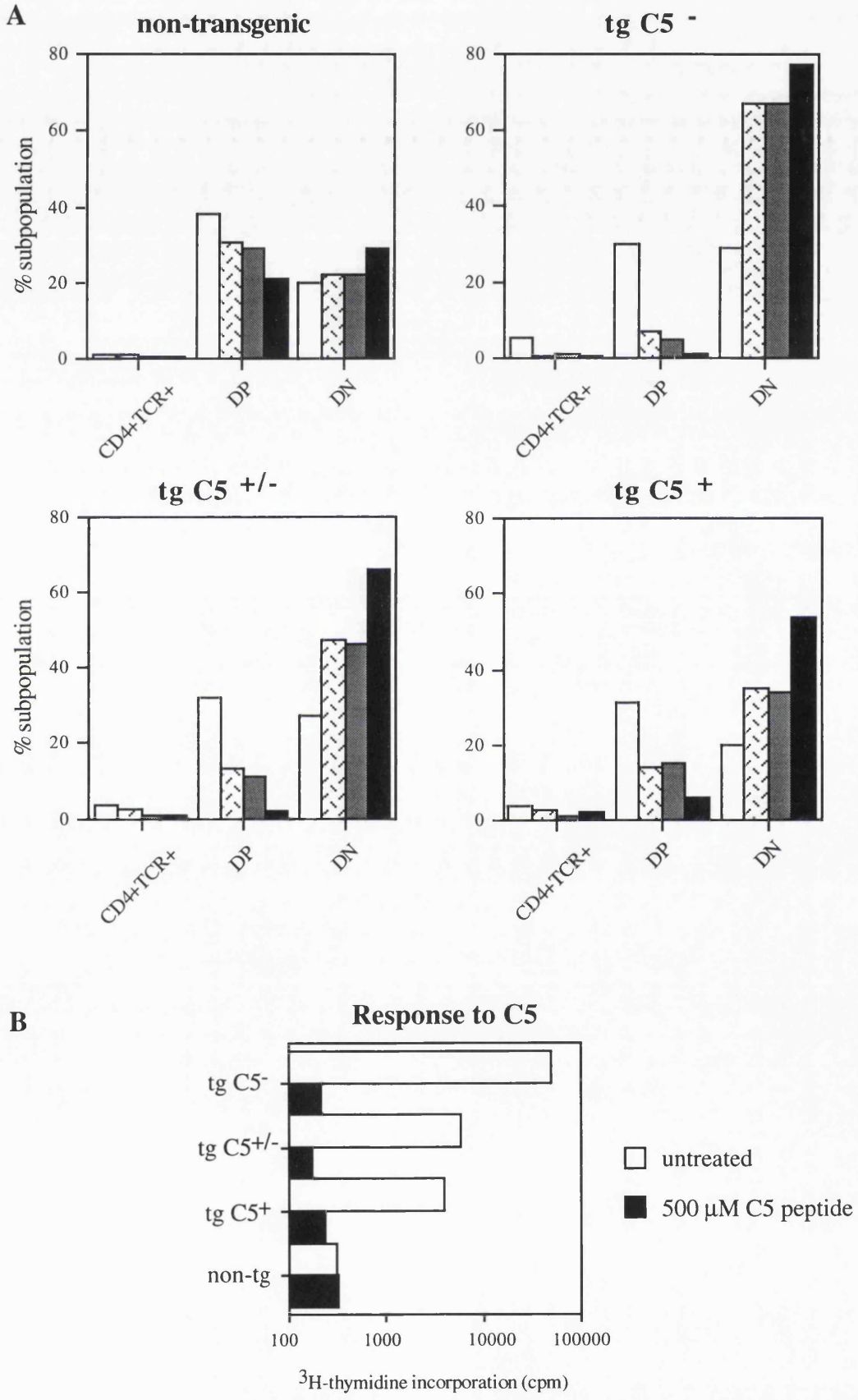
Cell recovery from thymus lobes after culture in medium alone or in the presence of 500 µM C5 peptide for 11 days. The mean values of 3 lobes per point are given.

Figure 5

Thymus lobes from non-transgenic, tg $C5^-$, tg $C5^{+/-}$ and tg $C5^+$ 14 days old embryos were cultured in medium alone (white columns) or in the presence of 10 μM (stippled columns), 100 μM (grey columns) or 500 μM (black columns) C5 peptide for 11 days. Thymocytes obtained from 3 lobes each were triple stained for CD4, CD8 and Vβ8 expression. The percentages of subpopulations are shown in **A**.

In **B**, 1 x 10^5 thymocytes from organ cultures in medium alone or in the presence of 500 μM C5 peptide were co-cultured in 96-well plates with 3 x 10^4 BM DC in the presence of 1μM C5 peptide. After 48h, 100 μl supernatant was removed and tested for IL-2 activity on CTLL cells. The results are expressed as mean cpm (duplicate cultures) of incorporated [^{3}H]-thymidine.

Fig. 5 **C5 peptide induced deletion of DP in FTOC**



The absence of C5 reactive cells in transgenic lobes after 11 days of peptide treatment was further demonstrated by the lack of C5 reactivity when cultured with C5 presenting DC in vitro (Fig. 5B). Thymocytes from untreated $C5^-$, $C5^{+/-}$ and $C5^+$ transgenic lobes responded with IL-2 production to C5 presentation, whereas they did not react when previously treated with 500 µM antigen in organ culture. Treatment with 10 µM or 100 µM peptide resulted in the same lack of response as when treated with 500 µM (data not shown). Thymocytes from non-transgenic lobes did not react to C5 in either case, due to their low frequency (compare with Fig. 2).

It can be concluded, that DP in both tg $C5^+$ and tg $C5^-$ mice are susceptible to deletion when internalization and processing of the antigen is not necessary and the amount of antigen is not limiting.

3.1.4 Efficiency of thymic APC in T cell activation and thymocyte deletion

The following experiments concentrate on the possibility that the location of different thymic APC in cortex and medulla might be responsible for the lack of deletion of the majority of C5 specific DP. The approach takes into account variations in the access to C5 by medullary versus cortical APC, as well as their ability to internalize and process the protein.

It has already been mentioned that only few cell types in the thymus express MHC class II necessary to present C5: APC restricted to the cortex are the cortical epithelial cells (cEC), APC restricted to the medulla are medullary epithelial cells (mEC) and DC, whereas macrophages are distributed throughout the thymus.

The efficiency of these APC to present in vivo internalized and processed C5 was determined by functional assays in vitro, using thymic APC freshly purified from $C5^+$ non-transgenic adult mice. In this way, the amount of C5 obtained in vivo could be assessed without the addition of exogenous C5. The purification procedure

involved several steps of magnetic cell sorting (MACS) as outlined in the scheme in Fig. 6. Cells labelled with a specific monoclonal antibody (mAb) and magnetic beads were selected on a column within a magnet. DC were isolated first, on the basis of labelling with N418, a mAb against the DC marker CD11c. The depleted fraction was then used as a source of macrophages, positively selected with F4/80, which produced a second fraction of depleted cells used for purification of G8.8 positive mEC and CDR-1 positive cEC. This order is important to include a negative selection step for DC in the isolation of other APC, and thus to reduce the likelihood of DC contamination in those fractions. Purity of APC was assessed by staining for the selecting markers and MHC class II (Fig. 6). MHC class II staining revealed high expression levels on DC and both mEC and cEC, but lower levels on macrophages. The majority of cells contaminating the individual APC preparations did not express MHC class II and most likely represented thymocytes. Being class II negative, they could not interfere in the assays performed. On the other hand, cross-contamination with other types of APC had to be excluded. Staining of the isolated APC with markers specific for each of the remaining APC excluded contamination of cEC, mEC and macrophages with any other APC. In contrast, a fraction of DC showed a small shift when stained for the macrophage marker F4/80 (Fig. 6). None of the antibodies available for DC or macrophages are exclusively specific for either cell type. Due to a common precursor, most macrophage-antigens are expressed on DC and vice versa, albeit at levels decreasing with maturation. Therefore, cells in the DC preparation expressing F4/80 might either represent contaminating macrophages, or DC actually expressing F4/80, possibly immature DC developing in the thymus.

The capacity of the isolated thymic APC to present C5 was tested in vitro, using a C5 specific T cell hybridoma or naive T cells from spleens of $C5^-$ transgenic mice. When asked to activate the T cell hybridoma with in vivo internalized and processed C5, thymic DC appeared to be the most efficient APC, followed by macrophages, mEC and cEC (Fig. 7A, left panel).

Figure 6

DC, macrophages (MΦ), mEC and cEC were purified from thymi from adult $C5^+$ mice by MACS separation as illustrated. Cells were stained for MHC class II expression using 14.4.4-FITC and DC, MΦ and mEC were in addition stained for their specific biotinylated marker, using streptavidin-PE. CEC, which were isolated with an unconjugated antibody followed by goat-anti-rat coupled magnetic beads (non crossreactive on mouse Ig), were single stained for MHC class II expression. Histograms show stainings with mAb specific for the non-selected APC, using FITC-conjugated mAb. Background of unstained cells is shown as grey shaded curve.

Fig. 6 **Purification of thymic APC by MACS:**

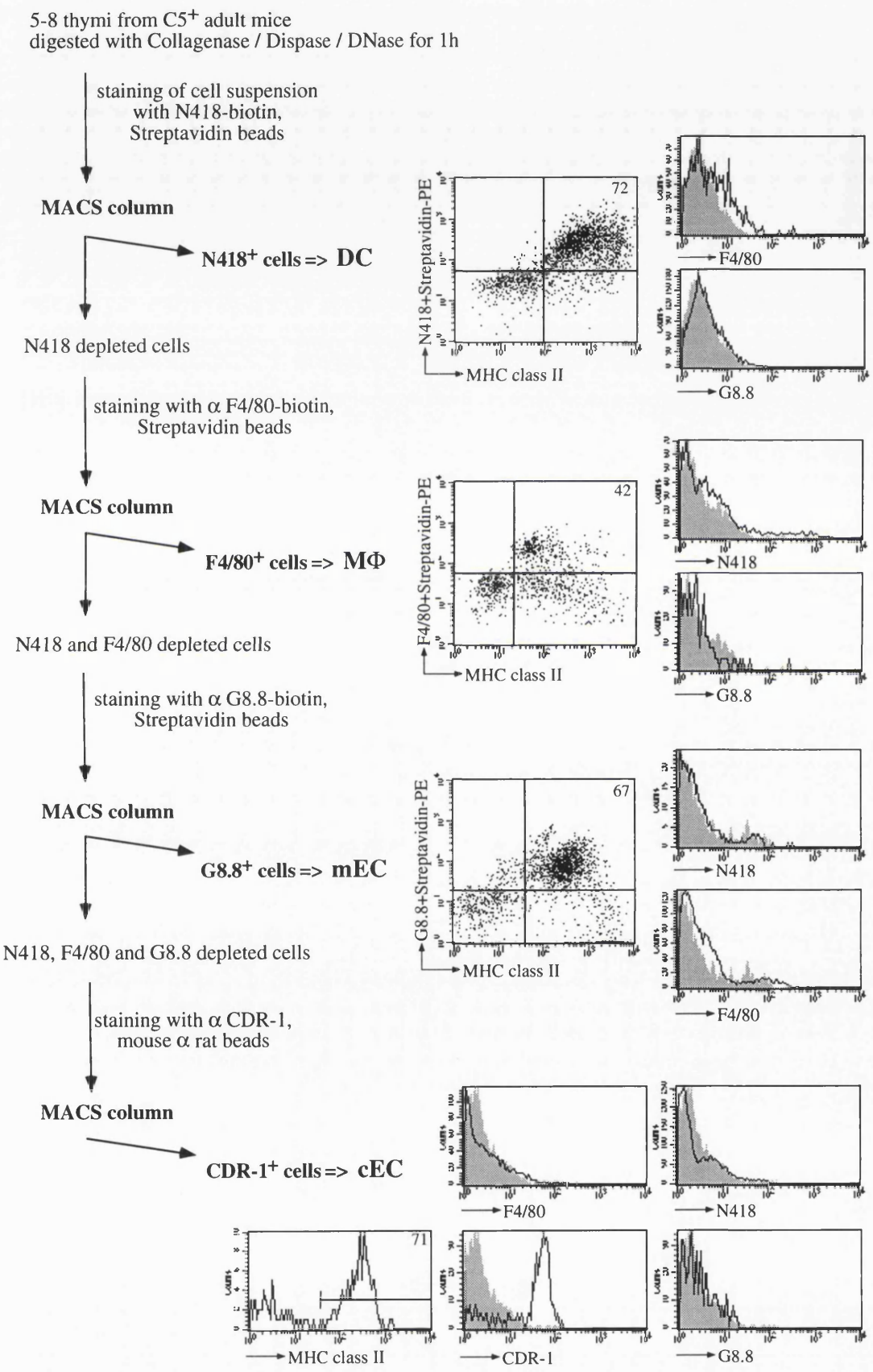
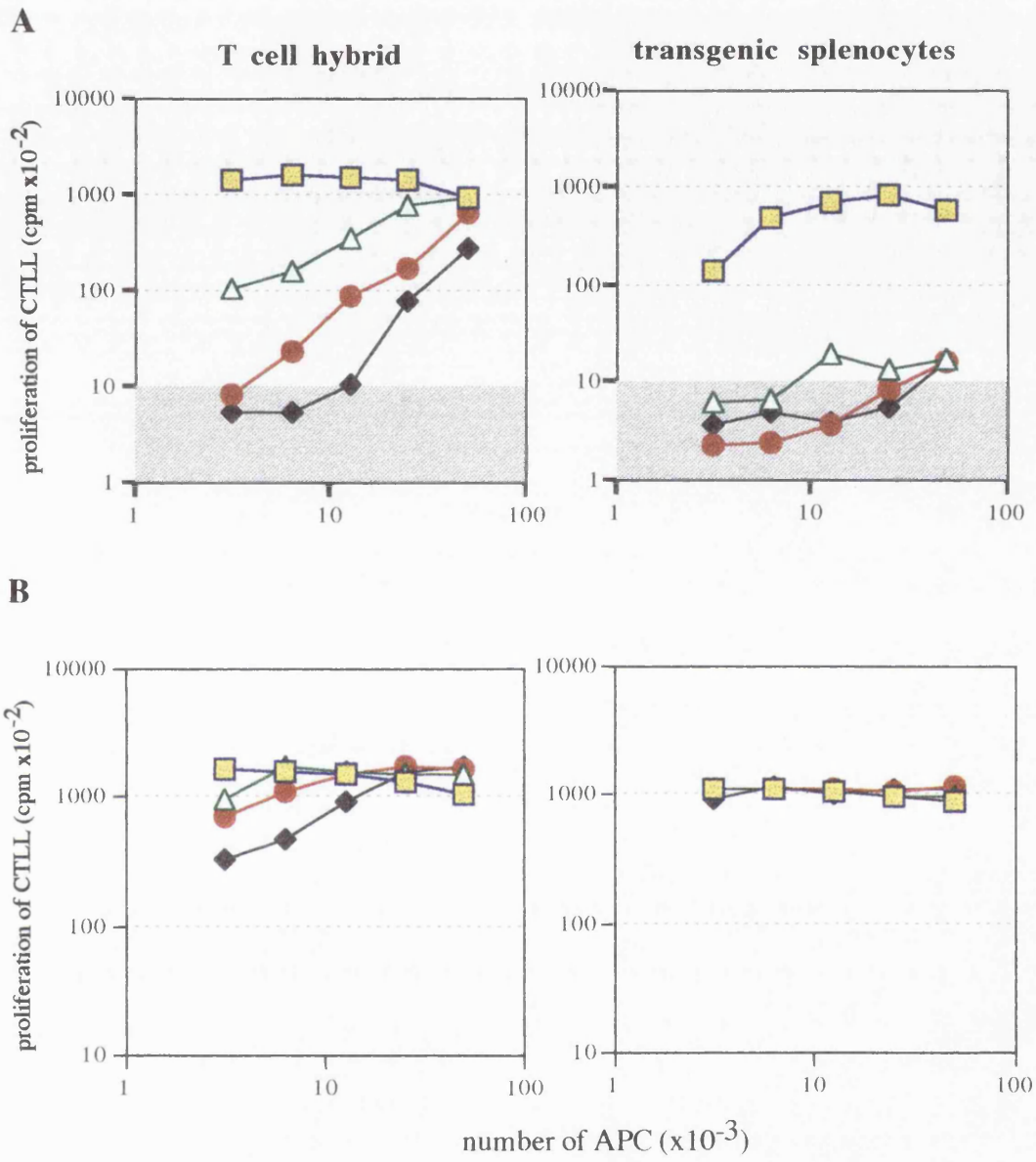


Figure 7

(A) Indicated numbers of thymic DC (yellow squares), macrophages (open green triangles), mEC (red circles) and cEC (black diamonds), purified from $C5^+$ mice, were cultured in a 96-well plate with either 5 x 10^4 cells/well of the C5 specific T cell hybridoma (left panel) or with 2 x 10^5/well splenocytes from $C5^-$ TCR transgenic mice (right panel), depleted of endogenous APC with mAb 14.4.4 and N418 by MACS. After 24 h (T cell hybridoma) or 48 h (transgenic splenocytes) supernatants were removed and cultured with CTLL for assessment of IL-2 activity. The results are expressed as mean cpm (duplicate set up) of incorporated [^{3}H]-thymidine.

In **(B)**, thymic APC and the T cell hybridoma (left panel) or transgenic splenocytes (right panel) were cultured like in A, but in the presence of 1 μM C5 peptide.

Fig. 7 **C5 presentation by thymic APC**



The fact that all thymic APC tested could activate the T cell hybridoma shows that they had access to C5 in vivo and demonstrates their ability to internalize and process the protein. Interestingly, lower expression levels of MHC class II by macrophages did not result in decreased activation capacity when compared with mEC and cEC. Addition of C5 peptide in the in vitro culture, and thereby loading of MHC class II molecules extracellularly, resulted in equally good activation of the T

cell hybridoma by all APC tested (Fig. 7B, left panel). This indicates, that all APC express sufficient amounts of MHC class II for optimal activation of the T cell hybridoma. The right hand panel of Fig. 7A illustrates the unique ability of DC to prime naive T cells (Croft et al., 1992; Levin et al., 1993). It also shows that none of the other APC populations contained DC. Addition of exogenous C5 peptide, however, overwrites the need for professional APC to prime T cells, since with these non-physiologically high amounts of antigen all APC could activate naive T cells (Fig. 7B, right panel).

Does the capability of different thymic APC to activate T cells mimic their potential to induce negative selection? To address this question, thymic APC, prepared in the same way as for activation of T cells, were set up with fetal thymocytes from d15 tg $C5^-$ embryos in reaggregation cultures, and their effect on T cell development was analysed. Single cell suspensions from thymic lobes obtained by enzyme digestion, when placed in 3 µl medium on a membrane filter floating on medium, formed a reaggregate within 24 h only when a minimum of 8 x 10^5 cells was used (data not shown). In order to perform several reaggregation cultures with titrating numbers of APC in parallel, the method was optimized by the addition of 1 x 10^5 stromal cells to 3 x 10^5 cells from d15 tg $C5^-$ thymus lobes. Stromal cells support T cell development in reaggregate cultures and produce the cell density required to form the reaggregate structure with lower numbers of thymocytes. The stromal cells, mainly epithelium, were obtained from d15 $C5^-$ thymus lobes, maintained for 4-5 days in organ culture in the presence of deoxyguanosine (dGuo), which results in elimination of thymocytes (Jenkinson et al., 1982).

Reaggregates were cultured for 4 days, since FTOC experiments described in Fig. 1 had shown that thymocytes mature into CD4 SP within 4 days when no antigen is present. Furthermore, FTOC had shown that APC from $C5^+$ mice retain sufficient amounts of C5 to induce tolerance without further C5 supply during that time.

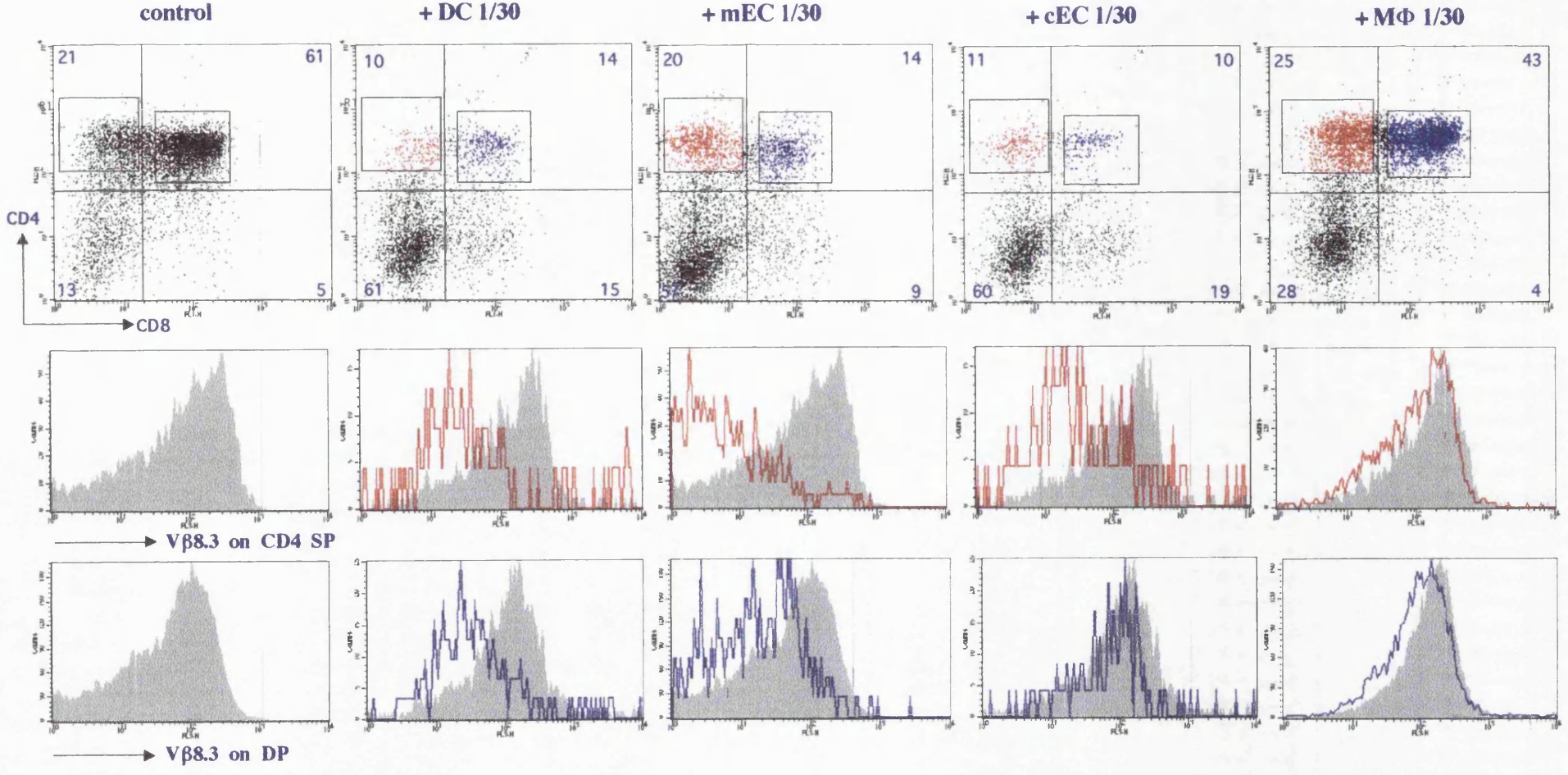
As expected, reaggregates of dGuo stromal cells mixed with cells from tg $C5^-$ thymus lobes, which contain exclusively immature DN on day 15 of gestation as shown for day 14 (Fig. 1C), gave rise to DP and mature CD4 SP within 4 days of reaggregation culture (see control, Fig. 8). In contrast, when thymic DC isolated from $C5^+$ mice were included in the reaggregate at a ratio of 1 DC to 30 thymocytes, only few DP and CD4 SP were detected and the total cell recovery was reduced to about 1/3 of the control reaggregate. Moreover, the CD4 SP lacked Vβ8.3 expression, indicating that no mature C5 reactive cells could develop in the presence of C5 presenting DC. Surprisingly, the same was true for mEC and cEC. In contrast, thymic macrophages did not influence the development of transgenic thymocytes (Fig. 8). Thus, with the exception of macrophages, all thymic APC tested were able to induce negative selection in C5 specific thymocytes. This is supported by the functional in vitro assay shown in Fig. 9. Thymocytes obtained from d4 control reaggregates and from those with C5 presenting thymic macrophages contained fully mature cells which released IL-2 upon stimulation with C5 peptide, whereas thymocytes derived from cultures with DC, mEC or cEC did not respond to the antigen.

Another observation concerns the levels of Vβ8.3 expression on DP from the various reaggregation cultures shown in Fig. 8. Under the influence of DC or mEC, expression of Vβ8.3 by DP is notably decreased, indicating deletion of those DP with higher amounts of the transgenic TCR. In comparison, DP from reaggregates containing C5 presenting macrophages or cEC express levels of Vβ8.3 similar to those from control reaggregates (without C5 presenting APC). This suggests, that although cEC induce negative selection and prevent maturation of C5 specific T cells, they might not effect DP expressing the transgenic TCR to the same degree as DC and mEC do.

Figure 8

Cell suspensions from reaggregates of 3 x 10^5 thymocytes from 15 days old tg $C5^-$ embryos with 1 x 10^5 dGuo stromal cells alone or mixed with 1 x 10^4 MACS separated DC, mEC, cEC or macrophages were prepared by enzyme digestion after 4 days of culture. Cells were triple stained for CD4, CD8 and Vβ8.3 expression. The percentages of subpopulations are given within the quadrants, staining of Vβ8.3 on gated CD4 SP and DP is shown in histograms. Staining for Vβ8.3 on CD4 cells or DP from control reaggregates (without $C5^+$ APC) is shown as filled curve. Cell recoveries are given in % of control values of reaggregates cultured in the absence of $C5^+$ APC.

Fig.8 **DC, mEC and cEC, but not macrophages can induce negative selection in thymus reaggregation culture**



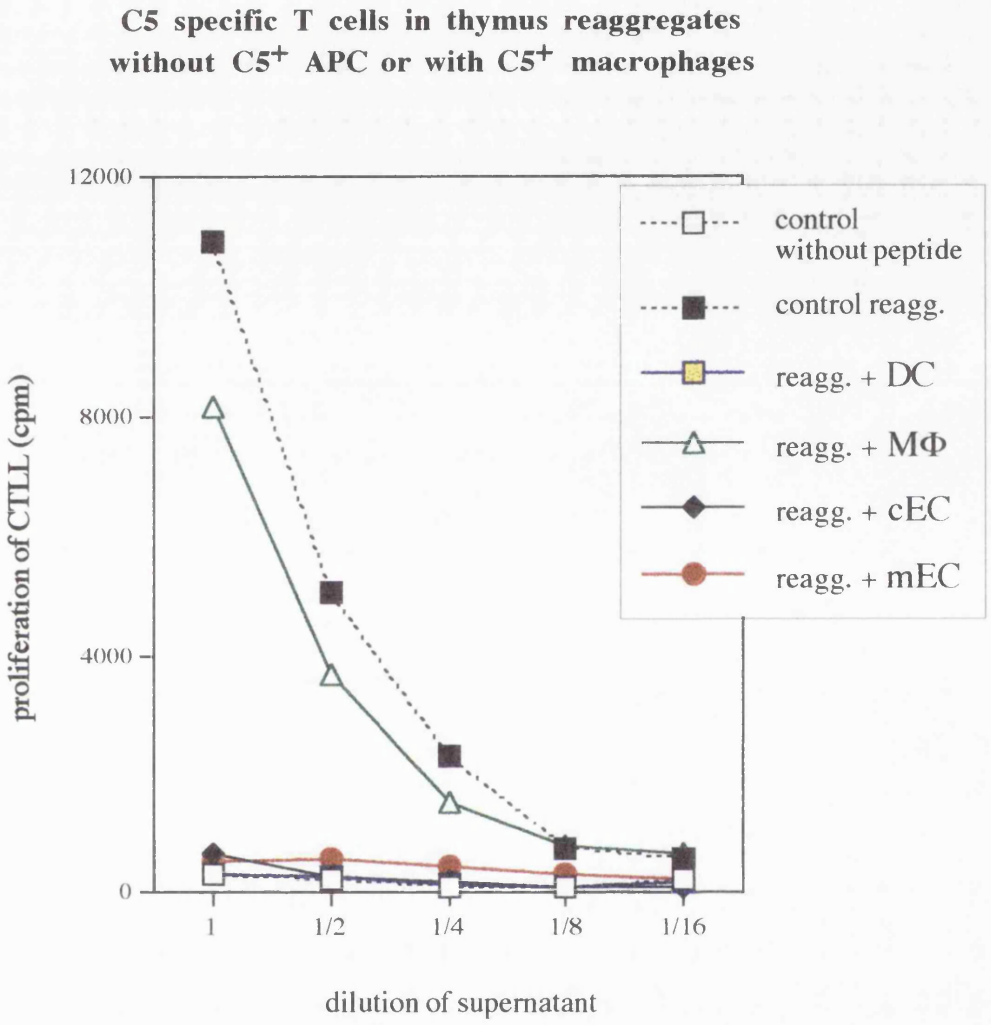


Figure 9

After 4 days of reaggregation culture, single cell suspensions were prepared from reaggregates cultured without $C5^+$ APC or with DC, macrophages, cEC or mEC at the ratio of 1 APC : 30 thymocytes. 5 x 10^4/well thymocytes were cultured with 2 x 10^4/well BM DC in the presence of 1μM C5 peptide 107-121 for 72 h. Supernatants were then removed and assayed for their content of IL-2 as described in the legend to Fig. 2.

In order to compare more precisely the capacity of DC, mEC and cEC to induce negative selection, several reaggregation cultures were performed using different APC to thymocyte ratios ranging from 1:4 to 1:80. A representative example with titrating numbers of DC is shown in Fig. 10A. It is noticeable, that the more DC are present in the reaggregate, the more DP are affected, suggesting deletion of DP at an early stage under the influence of an APC: T cell ratio in favour of DC when compared to the actual ratio *in vivo*, whereas lower numbers of DC seem to cause deletion at a later stage of DP. Tolerance induction is still complete at the ratio of 1 DC: 80 thymocytes, since no mature C5 reactive cells could be detected in a functional assay in vitro (data not shown). Fig.10 also illustrates that not all CD4 SP recovered from reaggregates containing C5 presenting APC lack expression of any TCR. Although negative for the transgenic β-chain (Vβ8.3), a proportion of CD4 SP express CD3. The level of expression is higher than that of CD4 cells from a control reaggregate (filled grey overlay), which is characteristic for endogenous TCR. A very small percentage of cells bearing endogenous β-chains, and more frequently endogenous α-chains, is presumably present in the TCR transgenic mice under non-deleting conditions, but becomes only detectable when extensive deletion takes place.

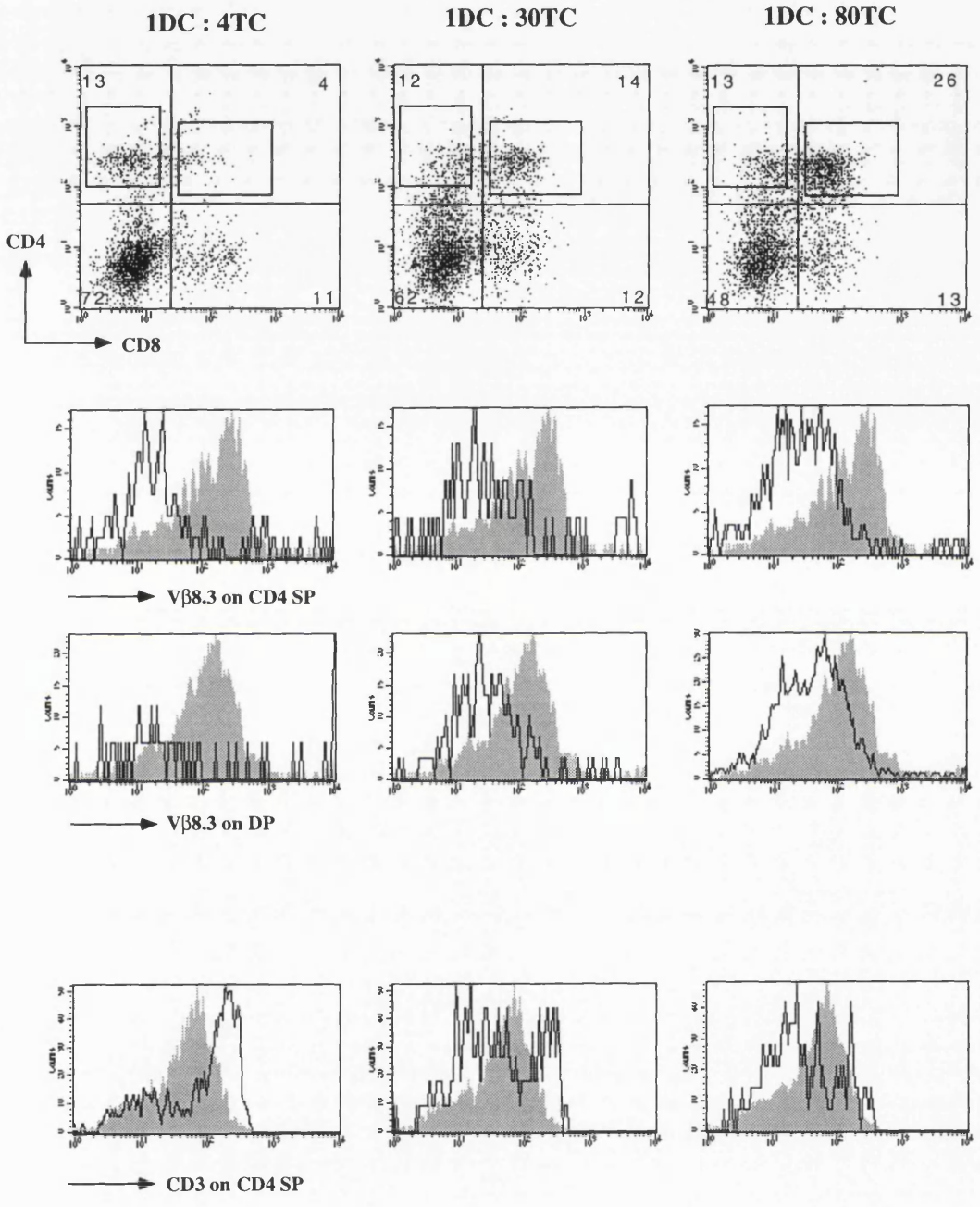
When reaggregates with titrating numbers of different thymic APC were compared, they clearly showed that DC, mEC and cEC could induce negative selection with comparable efficiency. Macrophages did not influence thymocyte development at any ratio, neither did thymic DC isolated from $C5^-$ mice, which demonstrates the antigen specific effect of C5 presenting DC. The results are summarized in Fig. 10B, using the decreased expression level of Vβ8.3 on CD4 SP as a read out for the induction of negative selection.

Figure 10A

Cell suspensions from reaggregates of 3 x 10^5 thymocytes from 15 days old tg $C5^-$ embryos with 1 x 10^5 dGuo stromal cells alone or mixed with MACS separated DC at the ratios indicated, were prepared by enzyme digestion after 4 days of culture. Cells were triple stained for CD4, CD8 and Vβ8.3 expression, or for CD4, CD8 and CD3 expression. The percentages of subpopulations are given within the quadrants, staining of Vβ8.3 on gated CD4 SP and DP and staining of CD3 on gated CD4 SP is shown in histograms. Staining for CD3 and Vβ8.3 on CD4 cells or for Vβ8.3 on DP from control reaggregates (without $C5^+$ APC) is shown as filled curve. Cell recoveries are given in % of control values of reaggregates cultured in the absence of $C5^+$ APC.

Fig. 10A

High ratios of APC : thymocytes in reaggregation culture lead to deletion of DP



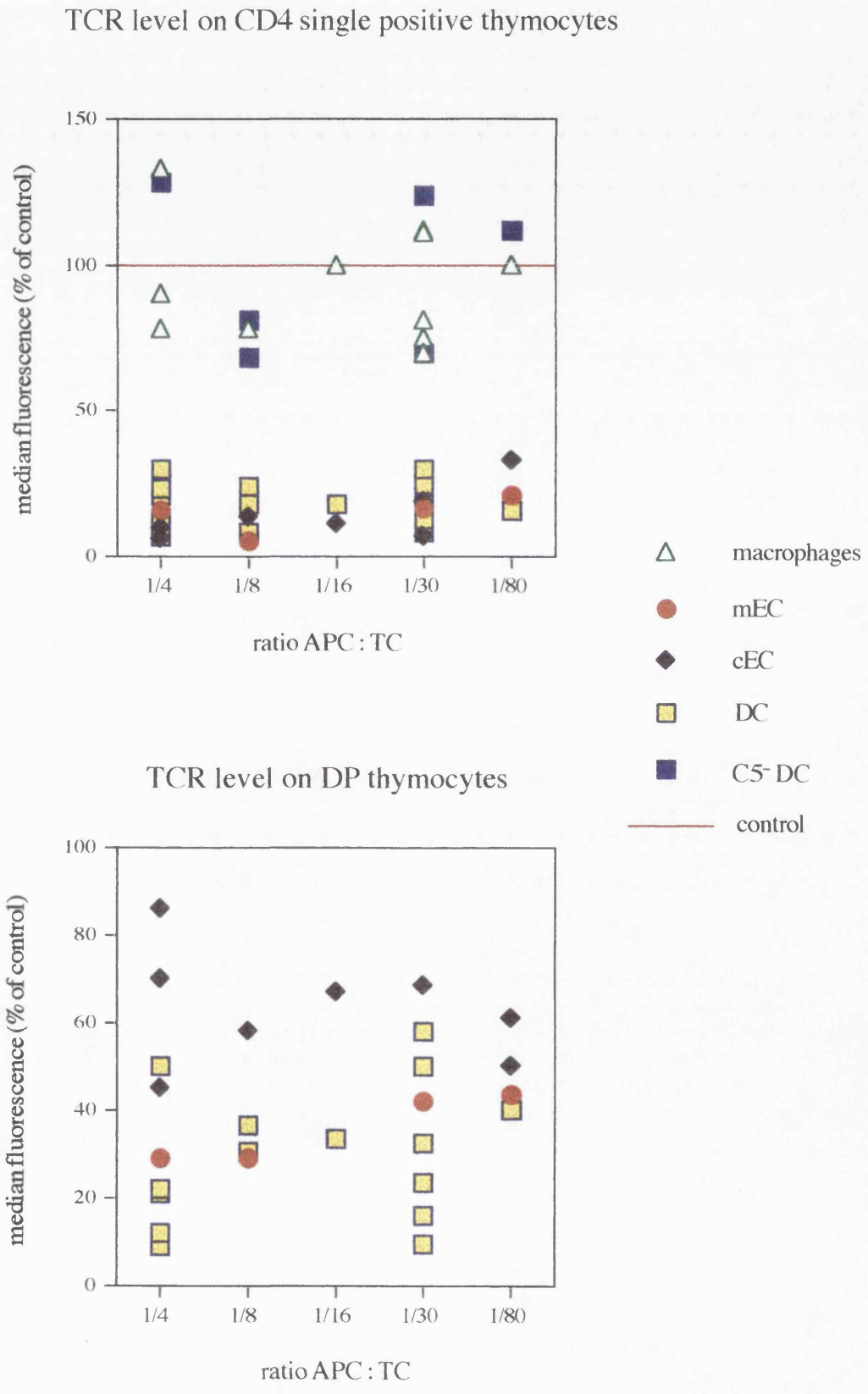
total cell recovery:
(% of control)

1DC : 4TC	1DC : 30TC	1DC : 80TC
23%	33%	45%

Figure 10B

Summary of reaggregation cultures performed with thymic APC at the ratios indicated. Each point represents the result of 1-3 pooled reaggregates. Vβ8.3 levels on CD4 SP (upper panel) or DP (lower panel) are given as median fluorescence levels in percentage of the median fluorescence levels on CD4 SP or DP obtained from control reaggregates.

Fig. 10B


TCR level on CD4 single positive thymocytes
median fluorescence (% of control)
150
100
50
0
1/4
1/8
1/16
1/30
1/80
ratio APC : TC
macrophages
mEC
cEC
DC
C5- DC
control
TCR level on DP thymocytes
median fluorescence (% of control)
100
80
60
40
20
0
1/4
1/8
1/16
1/30
1/80
ratio APC : TC

In order to normalize the results of reaggregation cultures performed on different days, Vβ8.3 levels on CD4 SP (upper panel) or DP (lower panel) are given as median fluorescence levels in percentage of the median fluorescence levels on CD4 SP or DP obtained from control reaggregates. TCR levels on CD4 SP from individual reaggregation cultures containing DC from $C5^-$ mice or C5 presenting macrophages are comparable with those from control reaggregates, whereas TCR levels on CD4 SP from all reaggregates containing C5 presenting DC, mEC or cEC are below 40 % of control levels. The summary of TCR levels on DP shows that reaggregate cultures with cEC had higher levels of TCR on DP than those cultured in the presence of DC or mEC. (see also Fig. 8, ratio 1:30).

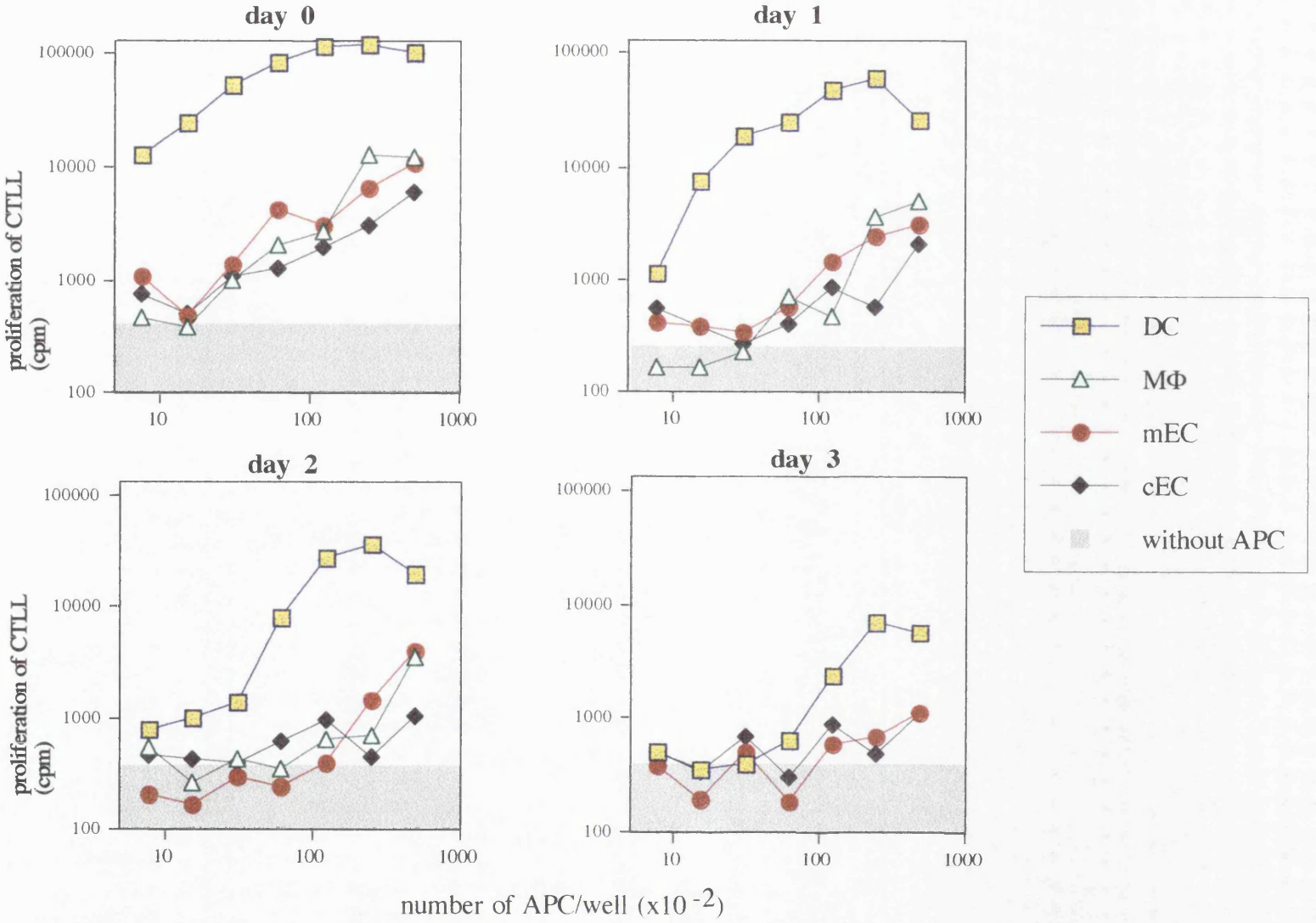
3.1.5 What are the reasons underlying the failure of macrophages to present C5 for negative selection?

The data obtained from FTOC suggested that negatively selecting APC from $C5^+$ mice maintained their C5 peptide / MHC class II complexes for up to 6 days (Fig. 1). However, it is possible that macrophages have a higher turnover rate of the complexes than other APC and that this caused their failure to induce negative selection in reaggregation culture. To address that possibility, the lifespan of C5 peptide / MHC class II complexes on thymic APC was compared in a presentation kinetic in vitro. Purified APC were placed in culture and C5 specific T cell hybridoma cells were added either immediately, or with a delay of 1-3 days. As shown in Fig. 11, all APC gradually lost stimulatory complexes from their surface. This was least prominent in DC, but similar in all other APC. Based on these results it seems unlikely that selective rapid loss of antigen explains the failure of macrophages to induce negative selection.

Figure 11

MACS purified APC from thymi of $C5^+$ non-transgenic mice were set up in serial dilution in flat bottom 96-well plates. C5 specific T cell hybridoma cells (5 x 10^4/well) were added either immediately (day 0) or 1, 2 and 3 days later. 24 h after addition of T cells supernatants were removed and frozen. After the last time point all supernatants were assayed for their IL-2 content as described in the legend to Fig. 2. The experimental background represented by T cells cultured in the absence of APC is shown as shaded area.

Fig. 11 **Maintenance of MHC class II / C5 -complexes on thymic APC in vitro**



Another reason for the inability to induce negative selection might be inefficient MHC class II expression. As shown in Fig. 6, macrophages express a lower level of MHC class II than the other thymic APC tested. Therefore, the density of stimulating ligand was increased by incubation of thymic macrophages with C5 peptide, which binds to MHC class II molecules already expressed on the cell surface. Alternatively, thymic macrophages were treated with γ-interferon (IFNγ) to upregulate MHC class II and were subsequently loaded with peptide. Since IFNγ is released by activated T cells in the periphery, but not in the thymus, splenic macrophages naturally express higher levels of MHC class II. Thus, they were purified from $C5^+$ mice and used unpulsed as a third type of macrophages with increased density of stimulating ligand. Fig. 12 shows that thymic macrophages treated with IFNγ as well as untreated splenic macrophages indeed express higher amounts of MHC class II than untreated thymic macrophages, although the levels are still not nearly as high as on DC.

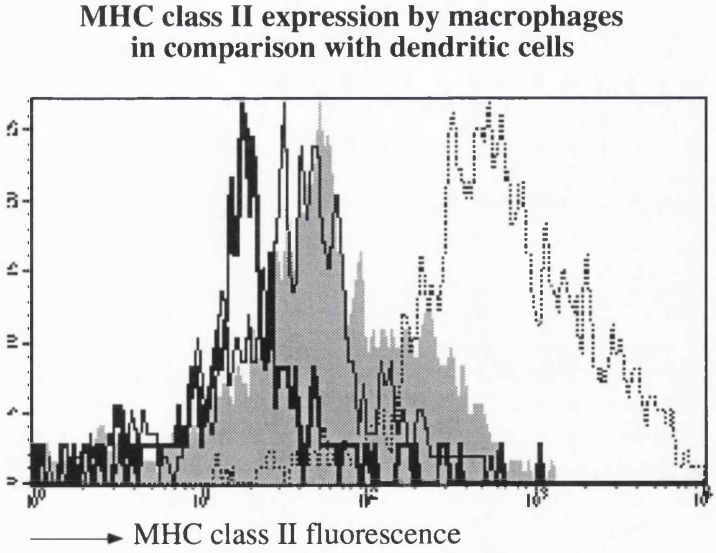


Figure 12

MHC class II expression detected with FITC labelled 14.4.4 mAb. Untreated thymic macrophages are represented by the bold curve, thymic macrophages treated with 200 U/ml IFNγ over night by a plain curve, splenic macrophages by the grey filled curve, and thymic DC are shown in a dotted line.

These macrophages were then introduced into thymus reaggregation culture to investigate whether increased density of stimulating ligand would enable them to induce negative selection. Peptide pulsed thymic macrophages were still unable to delete C5 specific thymocytes (Fig. 13). In comparison, DC from $C5^-$ mice, which on their own were unable to delete due to the absence of C5 peptide / MHC class II complexes on their surface, readily deleted C5 specific thymocytes when pulsed with the same dose of peptide. Given that a dose of 1 μM peptide will saturate far more MHC class II molecules than physiological antigen concentrations encountered in vivo could, it seems that the concentration of antigen per se is not the limiting factor for macrophage presentation. The situation looked different when the actual amount of MHC class II was increased. Both IFNγ treated, peptide pulsed thymic macrophages and untreated splenic macrophages led to a reduction of total cell numbers comparable to deleting cultures, but the loss of TCR expression was not as striking as seen with any of the other thymic APC populations (compare Fig. 13 with Fig. 8). In conclusion, the low level of MHC class II might be partly responsible for the inability of unmanipulated thymic macrophages to induce negative selection in C5 specific thymocytes. The fact that, even after upregulation, MHC class II levels on macrophages are still much lower than on other thymic APC, might explain why CD4 SP with transgenic TCR could be recovered from the reaggregation cultures.

Increased MHC class II expression is not the only effect of IFNγ treatment. It also leads to upregulation of certain adhesion molecules, such as ICAM-1 (Kawakami et al., 1995; Shen et al., 1995). Since in the normal thymus no immune responses take place, which are known to maintain the expression of molecules important in immune interactions such as MHC class II (Dalton et al., 1993; Steeg et al., 1981) and adhesion molecules, it is conceivable that thymic macrophages express low levels not just of MHC class II. In support of this, staining of APC with monoclonal antibodies against ICAM-1 and VCAM-1 revealed high expression by thymic DC and EC, but low or no expression by thymic macrophages. In contrast,

splenic macrophages show higher levels of ICAM-1, but no VCAM-1 (Fig. 14). Therefore, a combination of low amounts of adhesion molecules and low MHC class II levels, rather than the latter alone, might be responsible for the inefficiency of thymic macrophages to present C5 for negative selection.

In conclusion, every MHC class II expressing APC in the thymus, apart from macrophages, is in principle capable of presenting extracellular, circulating self protein for induction of negative selection.

Figure 13

Staining of day 4 reaggregation cultures as described in the legend to Fig. 8. Reaggregates were cultured with different APC from $C5^+$ or $C5^-$ non-transgenic mice at a ratio of 1 APC to 30 thymocytes. From left to right: thymic macrophages from $C5^+$ mice pulsed for 3 h with 1μM C5 peptide, DC from thymi of $C5^-$ mice, the same DC pulsed with 1μM C5 peptide for 3 h, thymic macrophages from $C5^+$ mice treated for 24 h with 200 U/ml recombinant IFNγ and pulsed with 1μM C5 peptide for the last 3 h, and splenic macrophages from $C5^+$ mice untreated. The histograms show expression of Vβ8.3 on gated CD4 SP and DP in comparison to those of control reaggregates without additional APC which are shown as shaded curves. Cell recoveries in % of control reaggregates are given below.

Fig. 13 **Negative selection induced by splenic macrophages and IFNγ treated thymic macrophages**

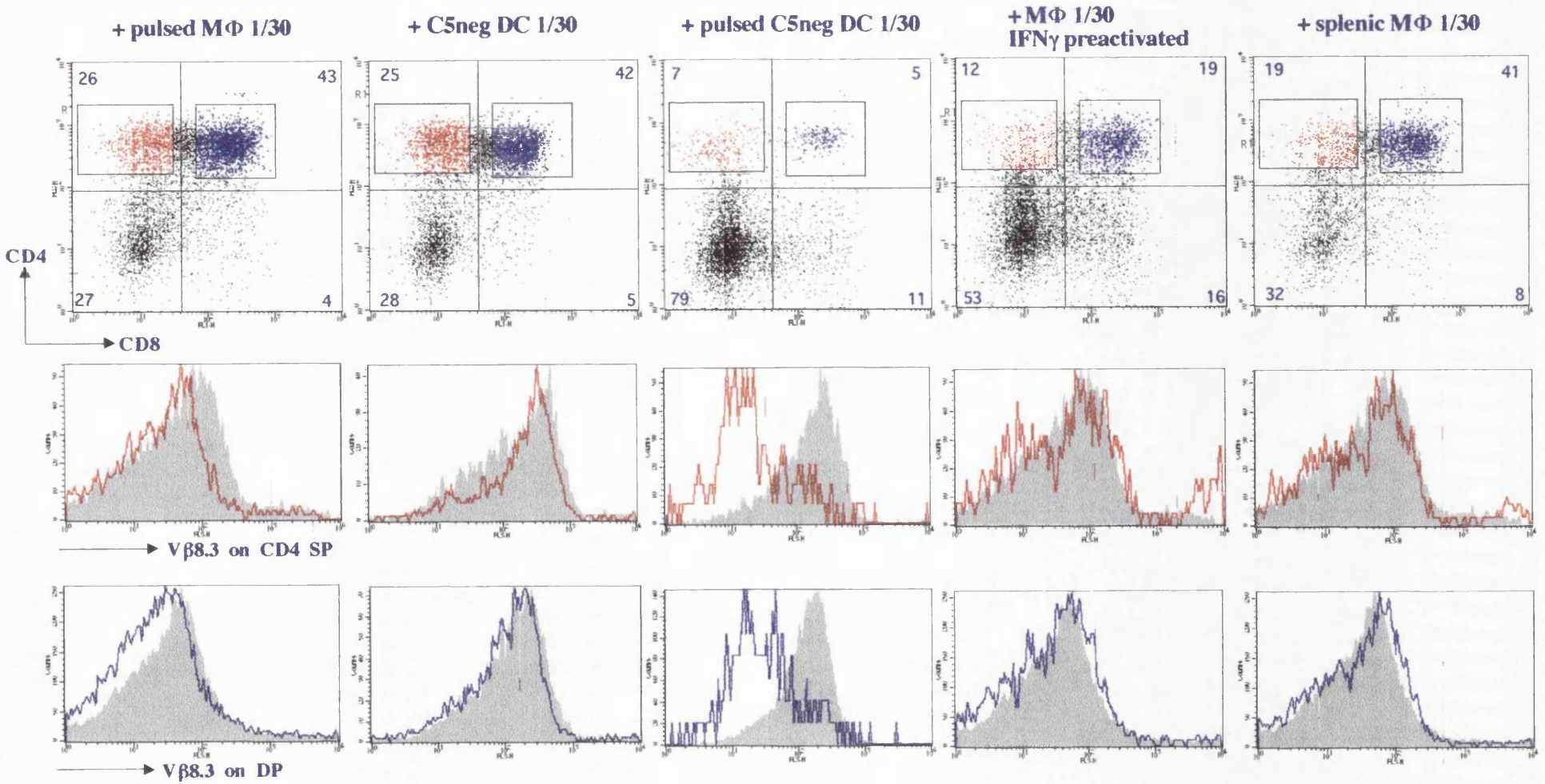


Fig. 14 **Expression of ICAM-1 and VCAM-1 by antigen presenting cells**

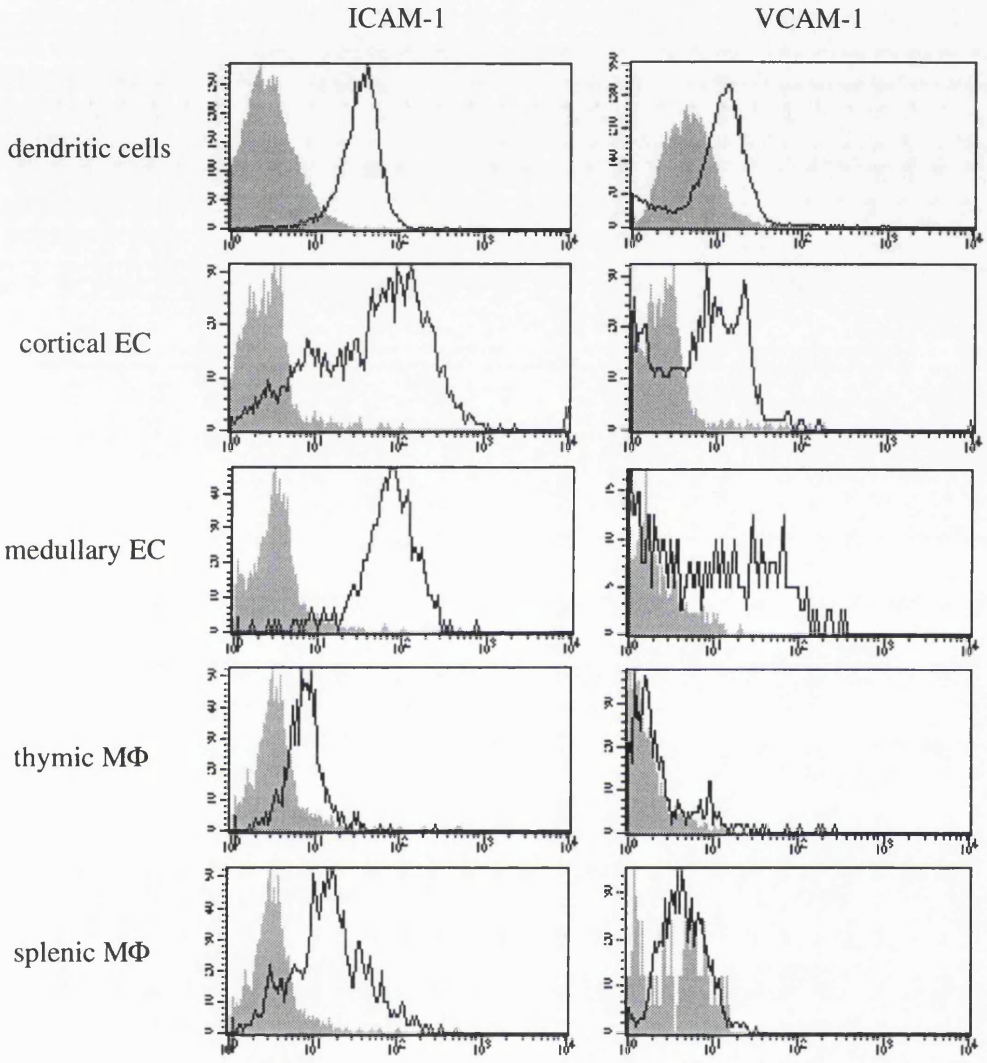


Figure 14

Expression of ICAM-1 and VCAM-1 on MACS purified APC following triple staining with 14.4.4-PE, ICAM-1-FITC or VCAM-1-FITC and Streptavidin-RED 670. The histograms show levels of ICAM-1 and VCAM-1 on cells gated for expression of the selecting marker (detected with Streptavidin-RED binding the biotinylated mAb used for MACS separation) and MHC class II. In case of cEC, gating was done on MHC class II positive cells only.

3.2 Characterization of a conditionally immortalized dendritic cell line

3.2.1 Morphology and surface marker expression

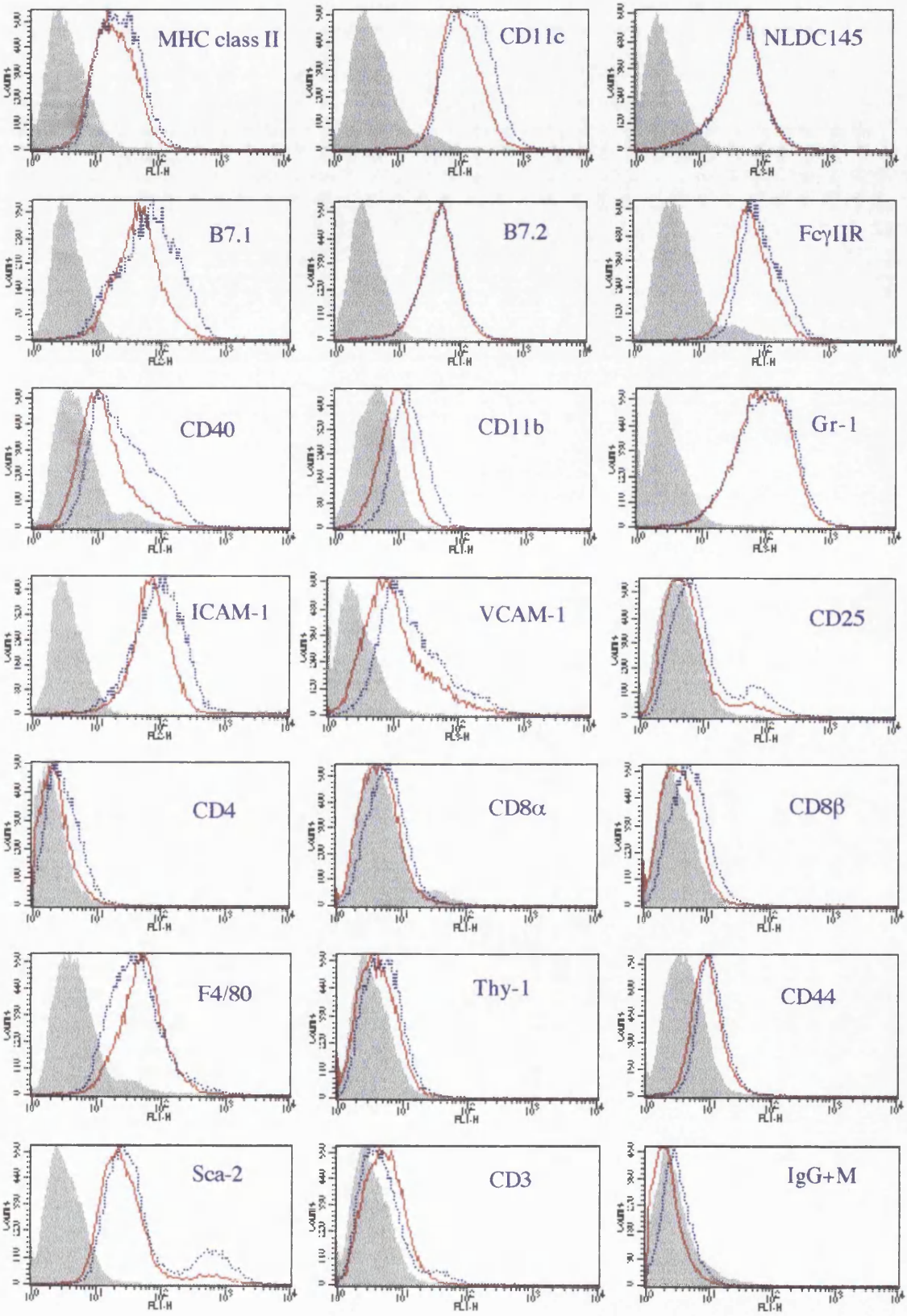
The second part of the project is dealing with the generation and characterization of a dendritic cell line. The establishment of such a cell line was intended to serve as a steady source of sufficient numbers of uncontaminated DC for in vitro and in vivo studies, since the number of dendritic cells in lymphoid organs is low and their purification in sufficiently high amounts difficult.

The line was established using bone marrow from transgenic mice which express a thermolabile form of the large tumour antigen (TAg) of simian virus 40 (SV40), under the control of the MHC class I K^b promoter (Jat et al., 1991). The temperature sensitive (ts) mutant of TAg enables immortalization of cells in vitro at 33°C, which can be reversed at 39°C in vitro, where the TAg protein becomes unstable. Bone marrow (BM) cells from a 4-week old female TAg transgenic mouse were set up in culture with GM-CSF to promote differentiation of DC and in the presence of IFNγ (described in material and methods), which enhances TAg expression. Nonadherent cells were passaged weekly to separate floating DC and granulocytes from adherent macrophages and fibroblasts. After several weeks, the cells grew without GM-CSF and IFNγ. A minor proportion of them stained with N418, a mAb against CD11c, which is found on DC and BM macrophages, and they were positive for MHC class II, expressed constitutively only by DC in the BM. Clones were established by limiting dilution cloning in the presence of irradiated normal spleen cells. Several clones with similar characteristics were obtained and one of them, referred to as tsDC (temperature sensitive dendritic cells) was characterized in detail.

Figure 15

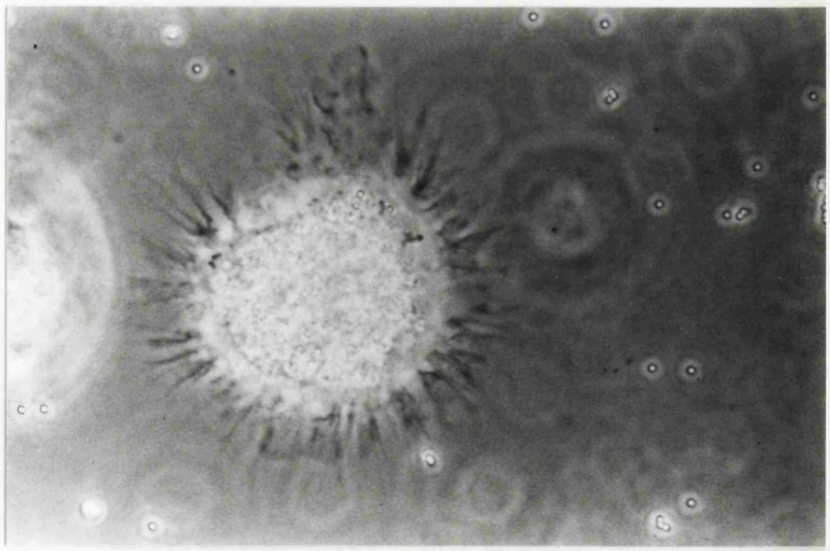
10^6 tsDC per sample were stained when cultured at 33°C and after incubation at 39°C for 2 days. The mAb used were either FITC conjugated or biotinylated. Streptavidin-Red 670 was used as a second layer when stained with biotinylated mAb. Control stainings with isotype matched mAb are shown as filled grey curves, the staining of tsDC cultured at 33°C is illustrated with red curves, expression levels after 2 days of culture at 39°C with blue curves.

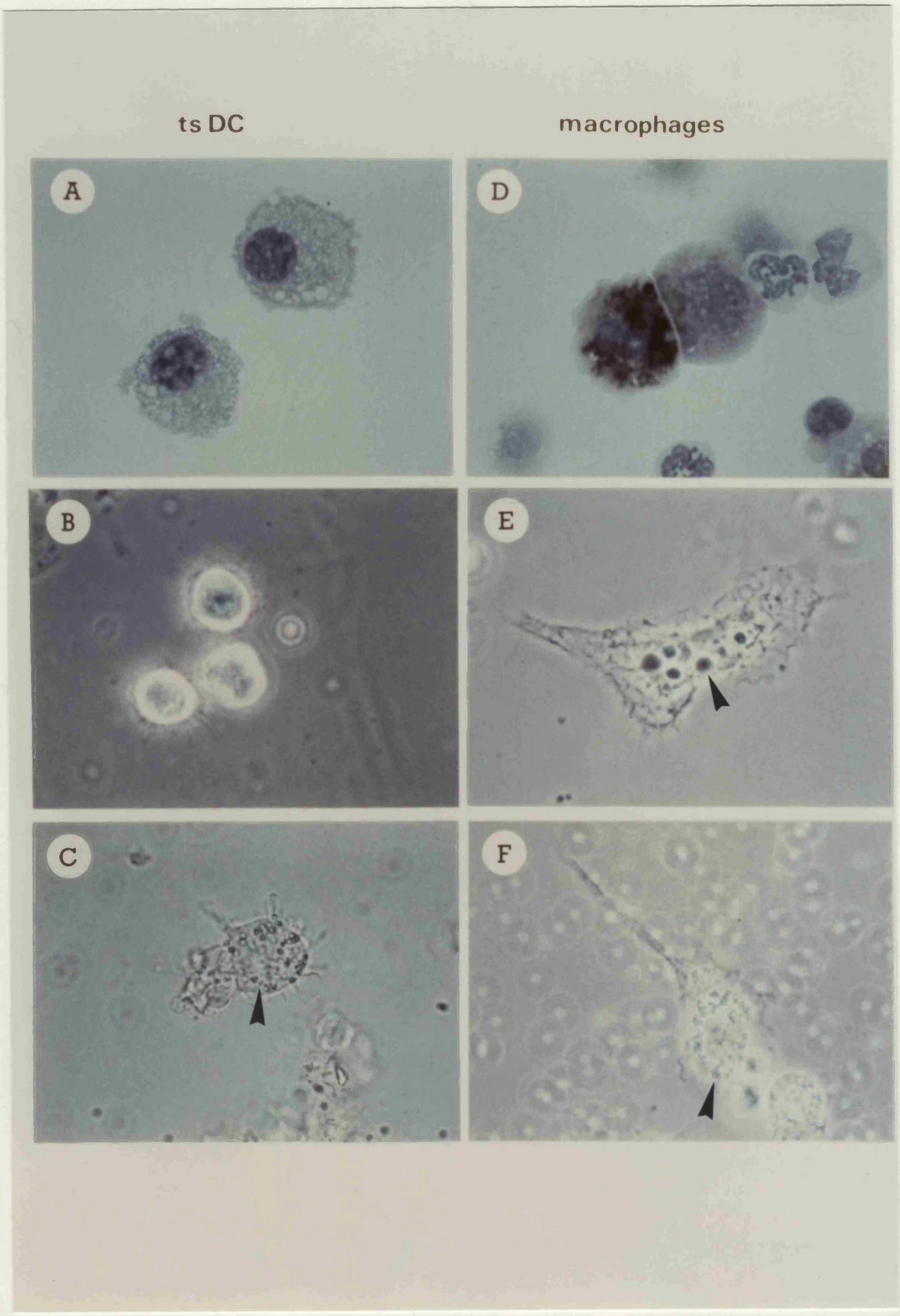
Fig. 15 **Expression of surface antigens at permissive (-) and non permissive (...) temperatures**



TsDC grow indefinitely in semi-adherent fashion at 33°C in normal culture medium and divide about once every 4 days. Transfer to 39°C, but not 37°C which is still a permissive temperature, results in growth arrest (assessed by cell counting) as a consequence of TAg degradation (Jat and Sharp, 1989) and the cells die after 4-5 days. Changes in surface antigen expression is another result of the temperature increase (Fig. 15). Some markers expressed at a higher level at 39°C such as B7.1, CD40 and the adhesion molecules ICAM-1 and VCAM-1, are reported to be upregulated during DC differentiation (Caux et al., 1994; Sallusto and Lanzavecchia, 1994), indicating that a maturation process might occur with the conversion to non-permissive temperature.

At 33°C, some of the cells show typical dendritic morphology, and most of them show this morphology at 39°C:




ts DC
macrophages
A
D
B
E
C
F

3.2.2 TsDC differ from macrophages in their phagocytosis abilities and in their enzyme content

Several markers present on the tsDC are characteristic for immature DC (Inaba et al., 1992; Scheicher et al., 1992; Steinman, 1991), but are also shared by bone marrow macrophages, e.g. F4/80, CD11b and Fc receptors. In contrast to macrophages, tsDC constitutively express MHC class II, B7.1 and B7.2, as well as NLDC 145. In addition, they do not contain the intracellular enzyme 'nonspecific esterase' (Fig. 16A), an enzyme present in macrophages (Fig. 16D and Yam et al., 1971). Another way of distinction are phagocytosis assays. Macrophages are efficient phagocytic cells, whilst mature DC are described as non-phagocytic, or restricted phagocytic when immature (Steinman, 1991). Accordingly, tsDC are unable to phagocytose sheep red blood cells (SRBC) (Fig. 16B) but take up 0.8μm beads (Fig. 16C), whereas macrophages are capable of phagocytosing SRBC (Fig. 16E) as well as beads (Fig. 16F).

3.2.3 Antigen presentation capacity of tsDC

Dendritic cells are the most potent APC, which is often demonstrated by their ability to stimulate a mixed leukocyte reaction (MLR). This in vitro assay is based on the reaction of haplotype incompatible T cells against MHC molecules on DC, which differ from their own. Therefore, the reaction takes place when T cells from one mouse strain are mixed with DC from a MHC haplotype different second mouse strain. TsDC (CBA, H-2^k), when cultured for 3 days with splenocytes from a B10 mouse (H-2^b), caused activation of the splenic T cells, read out by [^{3}H]-thymidine incorporation during T cell proliferation (Fig. 17). DC, generated from BM cultures with GM-CSF, showed comparable MLR stimulating activity, while BM macrophages, generated from M-CSF cultures which promote macrophage differentiation only, could not induce T cell proliferation.

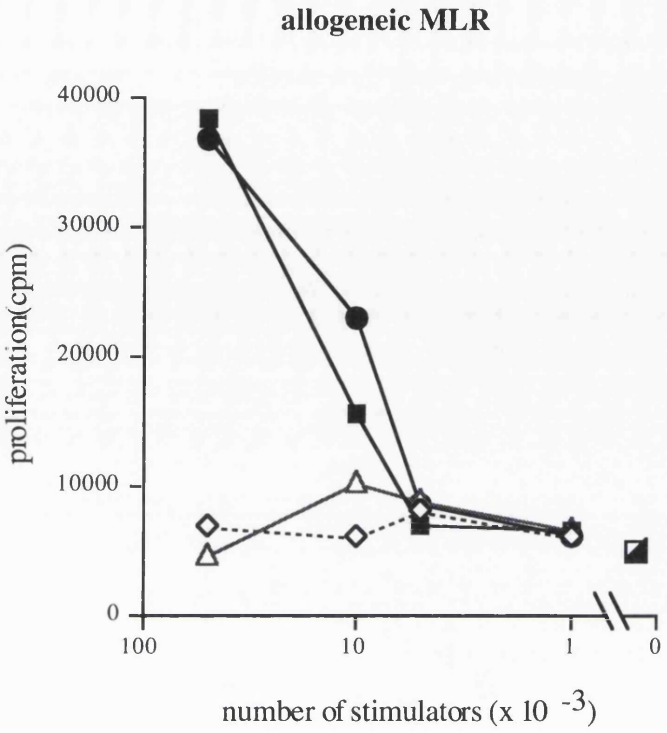


Figure 17
2 x 10^5 responder splenocytes from H-2^b mice per well of a 96-well plate were cultured with titrated numbers of tsDC (filled circles), BM macrophages (open triangles), DC generated in BM cultures with GM-CSF (filled squares) or H-2^b splenocytes (open diamonds) for 3 days. Proliferation of the responding T cells is expressed as mean cpm of incorporated [^{3}H]-thymidine of triplicate cultures. The proliferative response of responder splenocytes in the absence of stimulator cells is shown as a semi-filled square.

Do tsDC fulfil the physiological function of T cell activation, especially priming of naive T cells, in an antigen specific manner? To test this, their capacity to process intact protein and present it to either activated or to naive T cells was assessed. Thymocytes from C5$^-$ TCR transgenic mice were used as a source of truly naive T cells. To avoid presentation by endogenous thymic APC, these were removed by depletion of N418$^+$ and MHC class II$^+$ cells. A C5 specific T cell hybridoma represented pre-activated T cells. As shown in Fig. 18, tsDC grown at 33^{o}C and DC from BM cultures with GM-CSF were able to present C5 to the T cell

hybridoma, indicating that they are capable of internalizing and processing exogenous protein. In contrast, BM DC but not tsDC could activate naive, transgenic T cells. Pre-activated T cells, in contrast to naive ones, can be stimulated through their TCR without costimulatory signal(s). The fact that tsDC are unable to prime T cells might indicate that the expression levels of costimulatory molecules are insufficient, although they were perfectly capable of inducing the primary MLR.

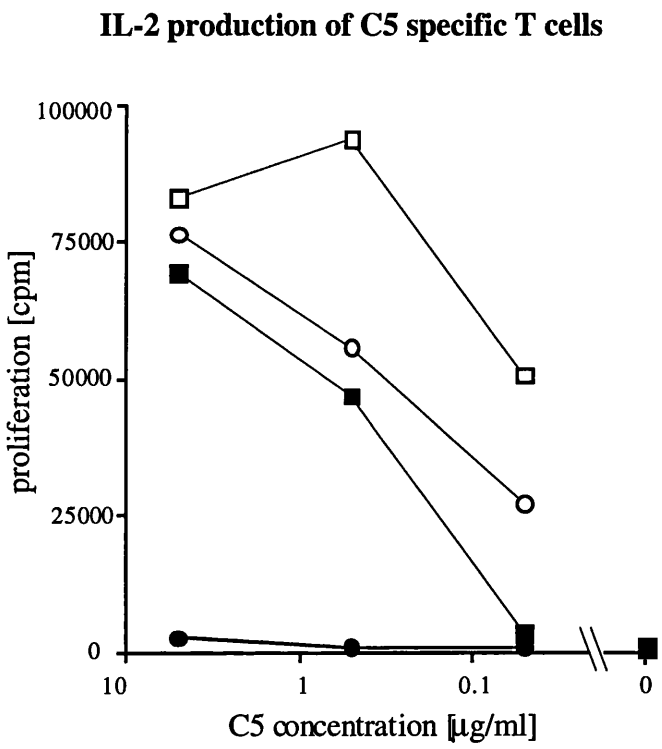


Figure 18

The C5 specific A18 T cell hybridoma (5 x 10^4/well) was cultured with 2 x 10^4/well tsDC (open circles) or DC generated in BM cultures with GM-CSF (open squares) in the presence of titrated amounts of C5 protein. Cultures of naive, transgenic thymocytes (5 x 10^5/well) with BM DC are shown as filled squares and those with tsDC as filled circles. Thymocytes were depleted of endogenous APC with N418 and anti-class II mAb by MACS. Supernatant was tested for IL-2 activity on CTLL after 24 h for cultures with A18 hybridoma cells and 72 h for cultures with transgenic thymocytes. The results are expressed as mean cpm of [^{3}H]-thymidine incorporation of triplicate cultures.

3.2.4 The influence of cytokines on surface molecule expression

Immature DC isolated from human blood have been shown to mature upon culture with cytokines, such as GM-CSF, IL-4 and TNFα. To examine whether cytokines can differentiate tsDC into more mature DC expressing higher levels of costimulatory molecules, cells were incubated for 3 days at the non-permissive temperature of 39°C in cytokine containing medium, prior to analysis of MHC class II expression as well as expression of B7 and CD40. As shown in Fig. 19, the increased temperature on its own had a marginal effect on antigen expression as already seen in Fig. 15, but upregulation of MHC class II, B7 and CD40 was slightly enhanced by TNFα and more by IFNγ. All other cytokines tested did not result in significant changes, when compared to the effect of increased temperature alone. In conclusion, some cytokines seem to promote upregulation of molecules important for functional properties of tsDC, but it is conceivable that a cocktail of several cytokines is ultimately responsible for further differentiation of DC.

Cytokines, produced by the *in vivo* environment of DC might influence their maturation. Keratinocytes in the skin produce e.g. TNFα, IL-6, IL-10 and IL-1 (Chang et al., 1994; Enk and Katz, 1992[b]; Koch et al., 1990), which, in a particular mixture, might induce migration and maturation of Langerhans cells. T cells on the other hand, produce a defined set of cytokines after their activation in spleen and lymph nodes, which could play a role in differentiation of resident DC in those organs. If that was the case, treatment of tsDC with one cytokine alone would not result in full maturation. Therefore, the effect of co-culture with skin cells, obtained by trypsinization of ear skin, or with $C5^-$ TCR transgenic splenocytes and antigen, on cell surface markers on tsDC was tested. Skin cells were taken from I-E negative H-2^b mice to allow assessment of class II I-E levels on tsDC, whereas TCR transgenic splenocytes were depleted of endogenous DC by treatment with N418 and subsequent MACS separation.

Figure 19

10^6 tsDC were cultured for 3 days at 33°C, at 39°C, or at 39°C in the presence of 25 U/ml GM-CSF and either 100 U/ml TNFα, 200 U/ml IFNγ, 100 U/ml IL-1, 1% supernatant of IL-4 or 1% supernatant of IL-5. The cells were then stained with either 14.4.4-FITC (anti-MHC I-E^k), CTLA-4-Biotin (anti-B7) followed by streptavidin-PE, or FGK 145-FITC (anti-CD40) as indicated.

Fig. 19 **The influence of single cytokines on surface molecule expression**

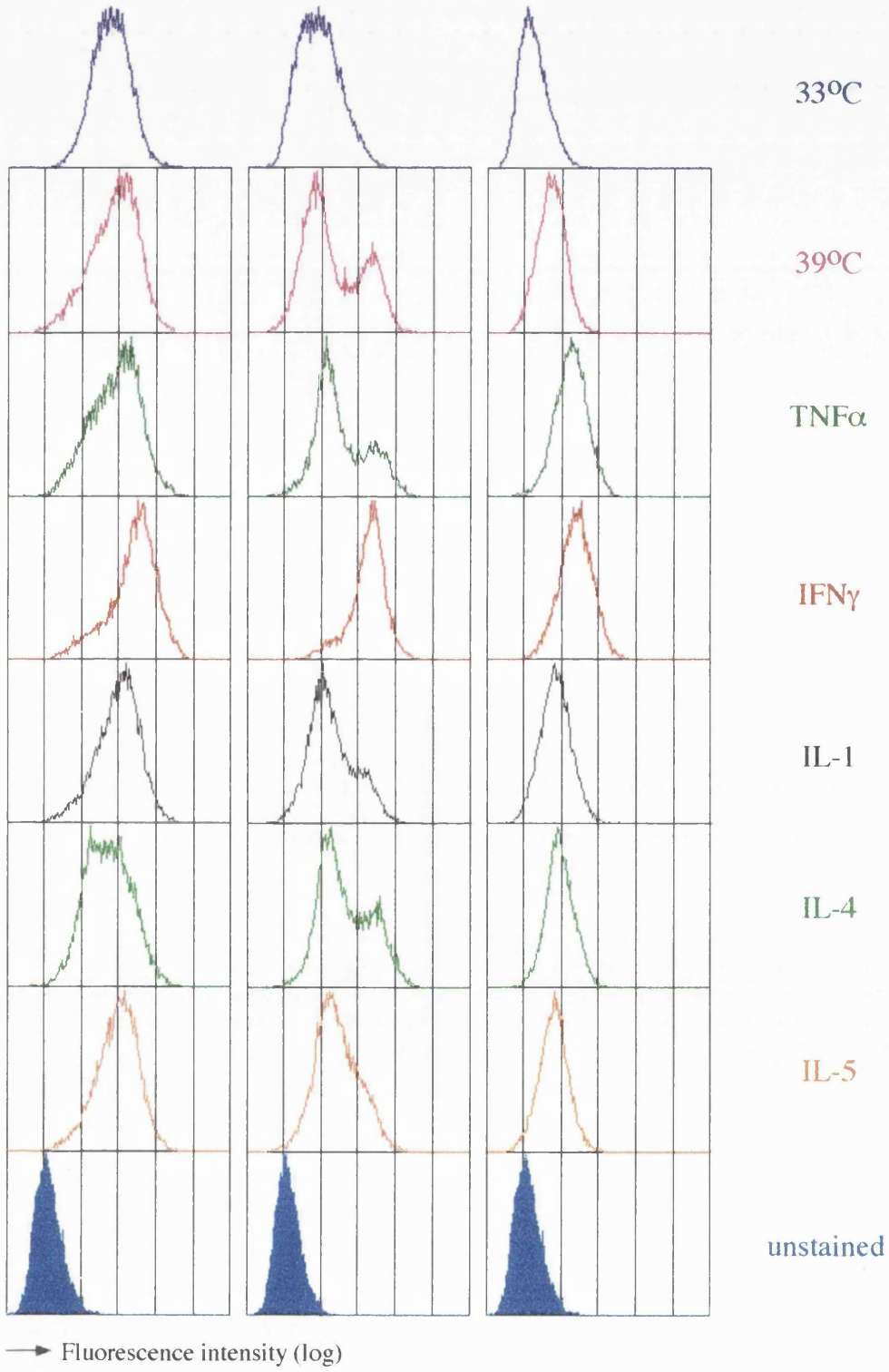
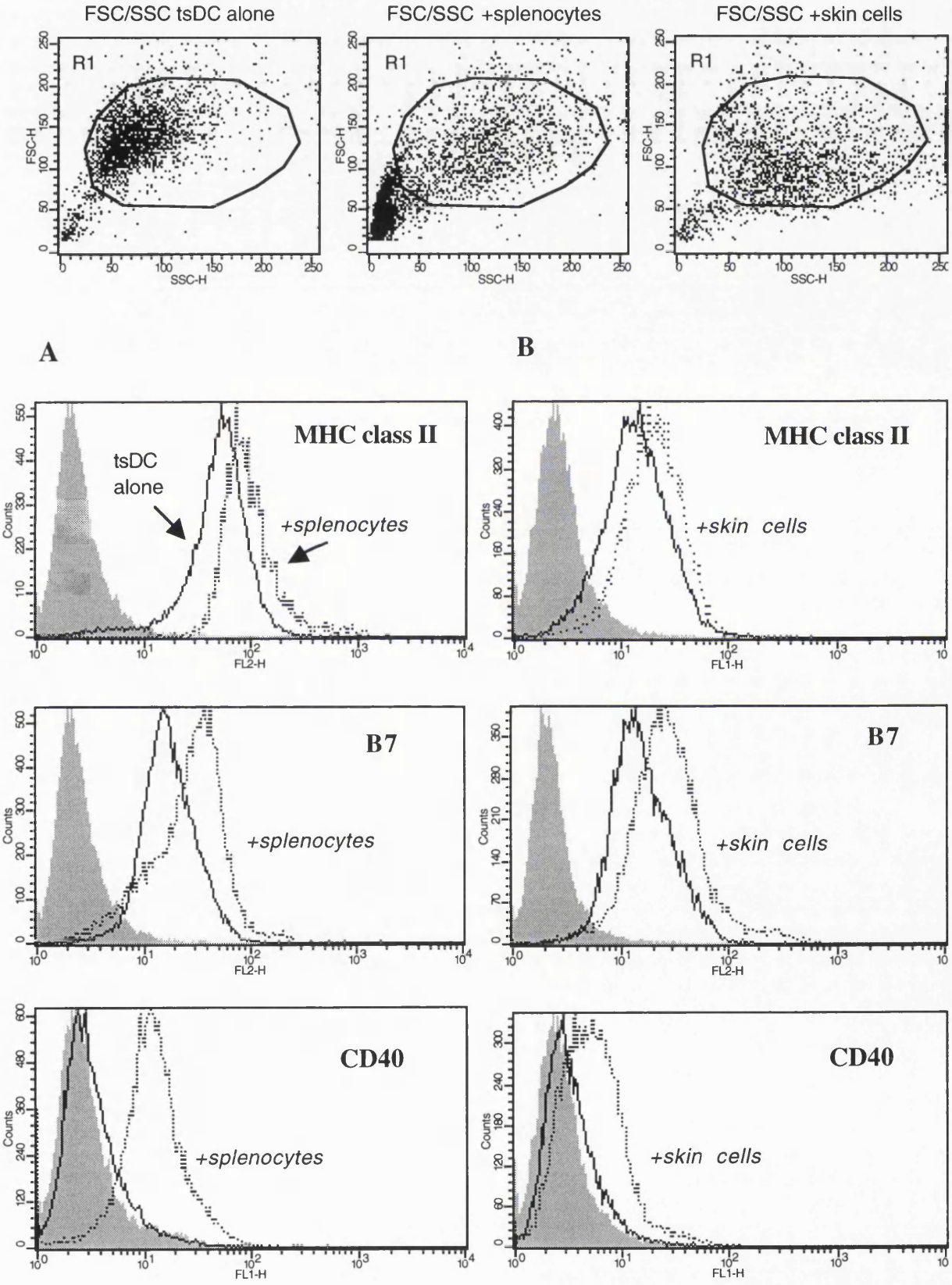


Figure 20

10^6 tsDC were cultured at 37°C for 2 days with 2 x 10^6 $N418^+$ depleted splenocytes from tg $C5^-$ mice in the presence of 1μM C5 peptide or with 2 x 10^6 cells from skin in wells of a 6-well plate. The cells were then stained with either 14.4.4 (anti-I-E^k), CTLA-4-Biotin (anti-B7) followed by Streptavidin-PE, or FGK 145-FITC (anti-CD40) and analysed by FACS for their size (FSC on y-axis) and granularity (SSC on x-axis) shown as dot-plot. Surface molecule expression of cells within R1 is shown in histograms, whereby the filled grey curves represent unstained controls, staining of tsDC cultured at 37°C is illustrated with continuous lines, expression levels after 2 days of co-culture at 37°C are shown with dotted lines. When co-cultured with splenocytes, analysed cells are gated on the $N418^+$ population, when co-cultured with skin cells, analysed cells are gated on the $14.4.4^+$ population.

Fig. 20 **Upregulation of MHC class II, B7 and CD40 on tsDC after presenting Ag to splenocytes (A) or when cultured with skin cells (B)**



The first change apparent after co-culture with either skin cells or splenocytes and antigen was an increased side scatter, reflecting more pronounced granularity (Fig. 20). Co-culture with skin cells enhanced upregulation of MHC class II, B7 and CD40 on tsDC (Fig. 20B), an outcome even more striking after co-culture with splenocytes and antigen (Fig. 20A).

Is the secretion of cytokines sufficient for differentiation into more mature DC or is cell-cell contact an important part of that process? This question was addressed by comparison of expression levels of MHC class II, B7.1, CD40 and ICAM-1 on tsDC with or without treatment with a cocktail of cytokines released by concanavalin A activated T cells (ConA sup, Fig. 21A). Upregulation of cell surface markers was clearly not dependent on cell-cell contact and was in addition not reversible upon transfer back to 33°C (Fig. 21B). The kinetic illustrated in Fig. 21B shows also that it takes 2 days to upregulate the surface molecules. After 1 day of culture with ConA sup at 39°C no changes could be detected, whereas upregulation was seen after 2 days. Most importantly, even without continuing treatment beyond the first day, upregulation was readily detectable after further culture at 33°C, indicating that differentiation signals were induced during the first day of treatment, but that the subsequent changes in tsDC required another day to be visible.

Upregulation of MHC class II expression after treatment with ConA sup could be confirmed biochemically by surface protein iodination (Fig. 22). TsDC cultured at 33°C and tsDC cultured for 2 days at 37°C in the presence of ConA sup were lysed after surface iodination and lysates were precleared 3 times with normal mouse serum (lanes 5-10) prior to immunoprecipitation with polyclonal rabbit anti class II antiserum (lanes 1-4). Precipitates were split and analysed by SDS-PAGE after boiling (lane 3 and 4) or after incubation at room temperature in SDS containing sample buffer (lanes 1 and 2).

Figure 21A

Cell surface expression of MHC class II, B7.1, CD40 and ICAM-1 of tsDC grown for 2 days at 33°C, 39°C or 39°C in the presence of 20% supernatant from ConA stimulated T cells (ConA sup) was analysed by FACS. Filled grey histograms represent negative controls with isotype matched control antibodies.

Fig. 21A **Upregulation of MHC class II and costimulatory molecules on tsDC**

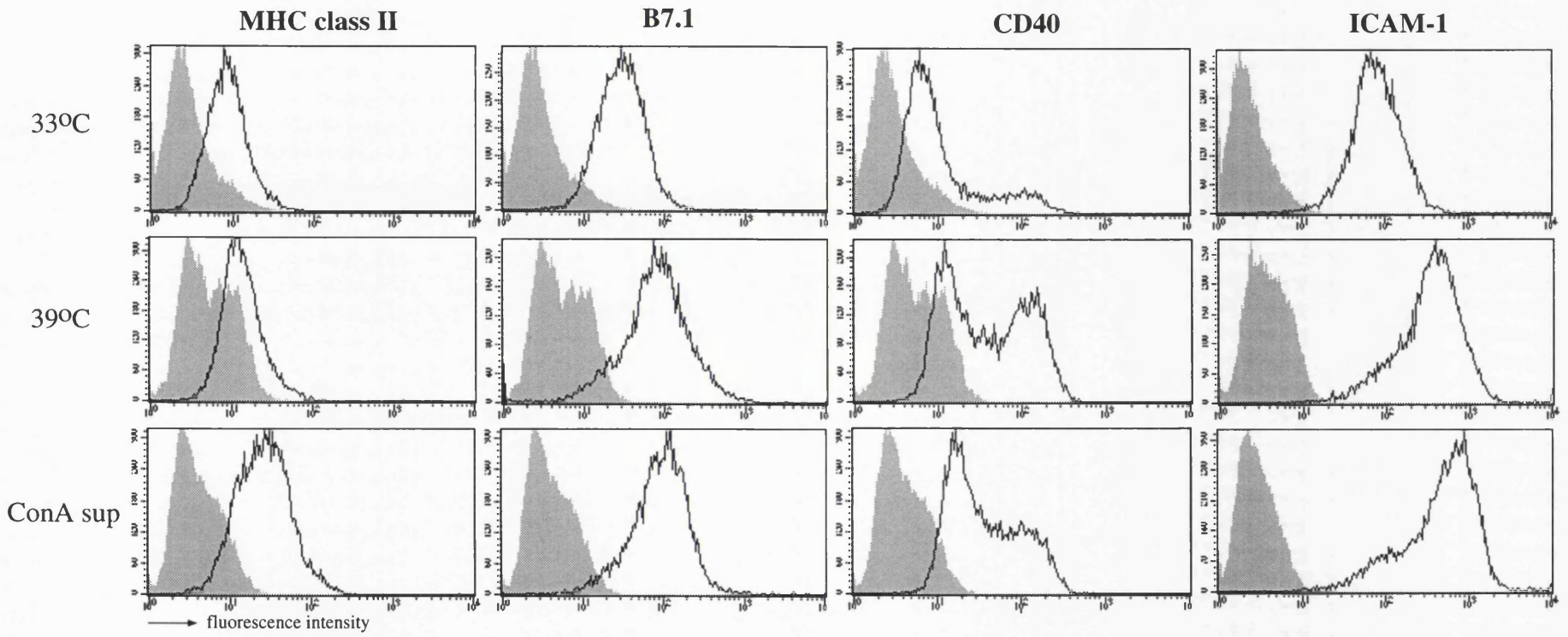
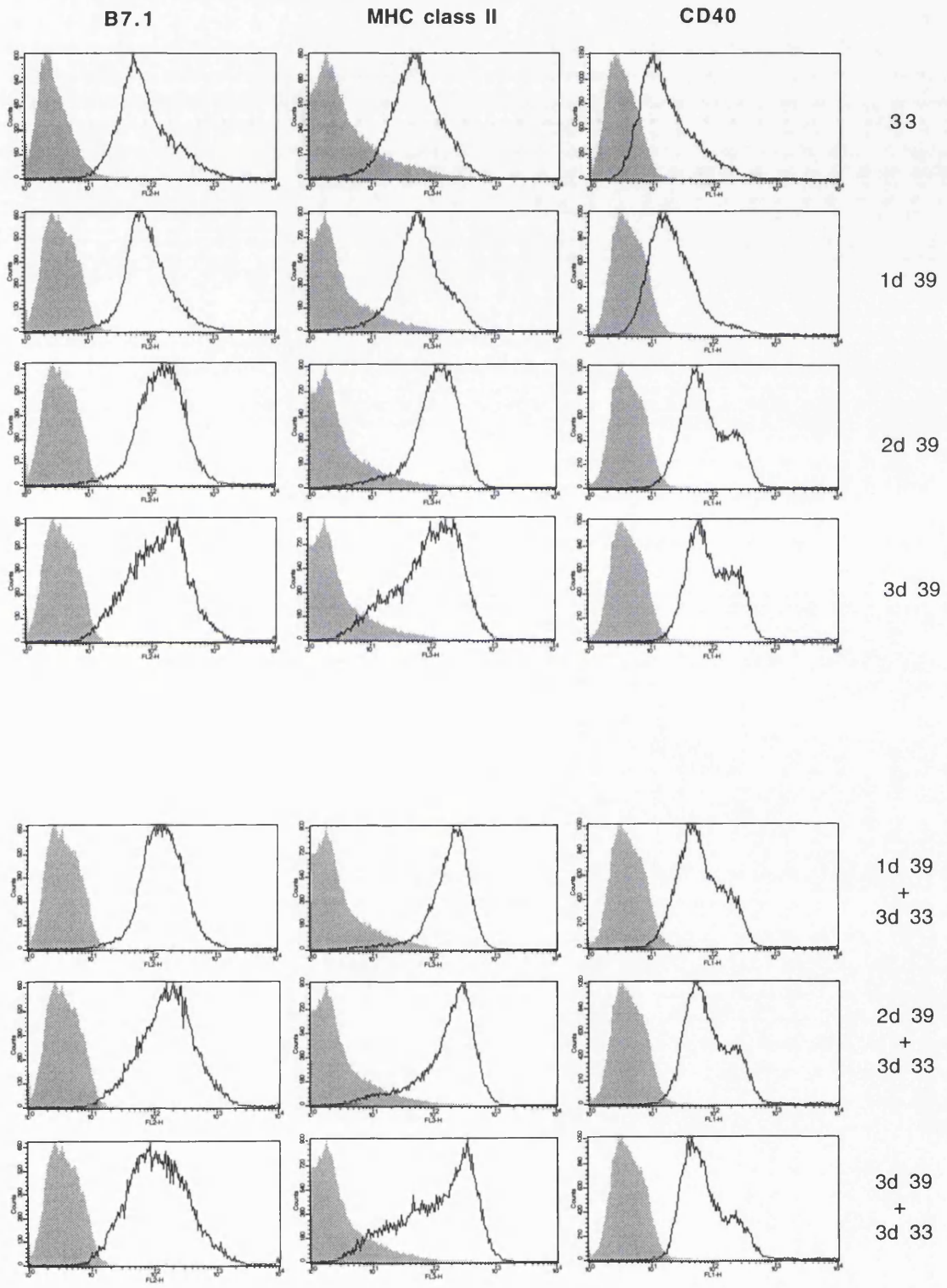


Figure 21B

TsDC were cultured continuously at 33°C, or they were cultured for the time indicated at 39°C in the presence of 20% ConA sup before staining for surface expression of B7.1, MHC class II and CD40 followed by FACS analysis. Alternatively, they were washed extensively after treatment and cultured for further 3 days at 33°C prior to analysis.

Fig. 21B **ConA sup induces irreversible upregulation of MHC class II, B7 and CD40**



Biochemical analysis of MHC class II

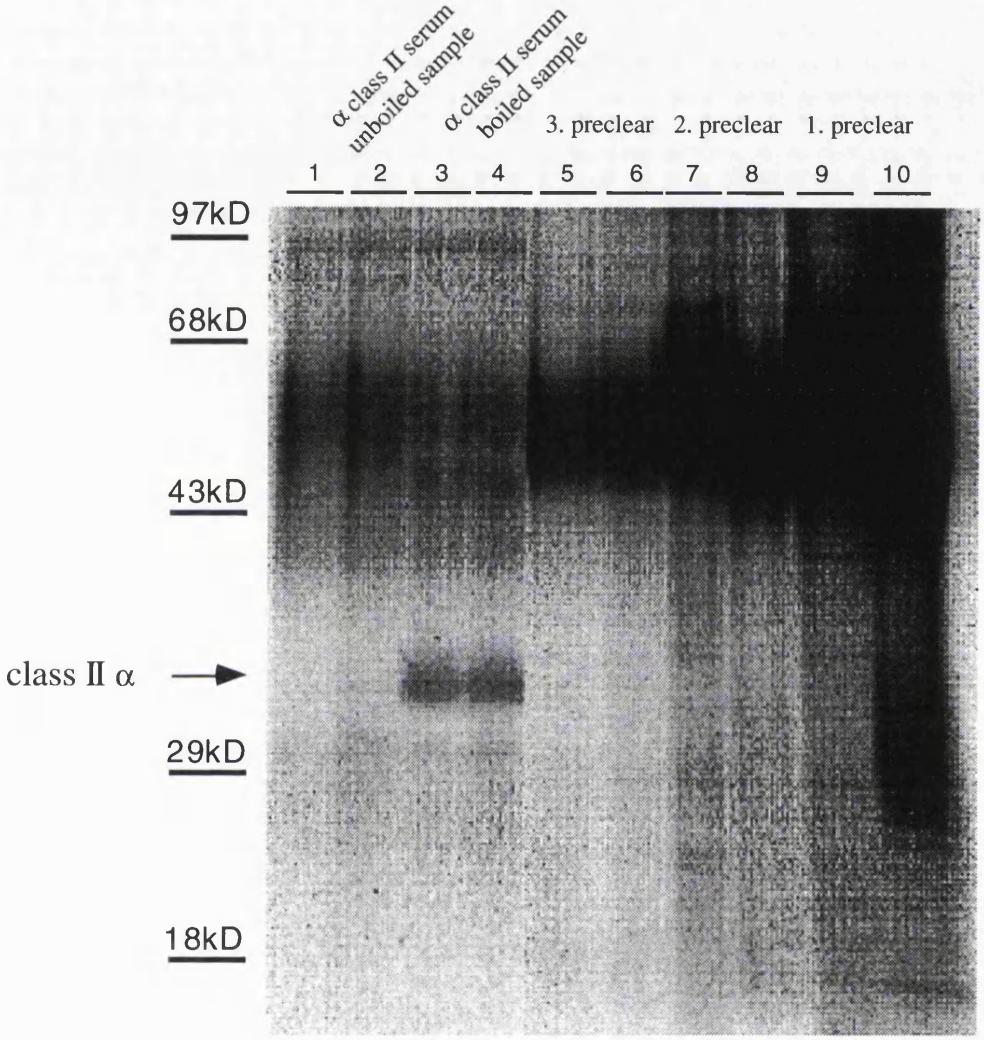


Figure 22

3 x 10^7 tsDC cultured at 33°C (even numbered lanes) or 10^7 tsDC cultured for 2 days at 37°C in the presence of 20% ConA sup (uneven numbered lanes) were surface iodinated and lysed. Lysates were precleared with normal mouse serum 3 times before immunoprecipitating MHC class II with polyclonal rabbit serum. Precipitates were separated by SDS-PAGE after boiling in reducing sample buffer or after incubation at room temperature as indicated. Analysis was performed using the phosphoimager after 4 days of exposure.

A proportion of peptide-loaded class II molecules will not dissociate under the latter conditions and remain as dimers. After boiling, class II α chains (migrating at 35 kD) could be detected in ConA sup treated tsDC (lane 3). No signal was visible in tsDC cultured at 33°C in a previous experiment (data not shown), therefore the cell number was increased to 3 times the number of treated tsDC, allowing detection of low amounts of class II α chains in untreated tsDC (lane 4). In samples incubated at room temperature free class II α chains were not detected (lanes 1 and 2), indicating that MHC class II molecules expressed by tsDC are assembled into a stable peptide-binding class II complex. However, non-specific binding of the antibody resulting in background signal around 50-60 kD prevented direct detection of stable class II complexes (migrating at 54 kD). Taken together, these results indicate the presence of stable MHC class II dimers at the surface of both non-activated tsDC (cultured at 33°C) and tsDC activated with ConA sup, whereby the latter express about 3-fold higher amounts on their cell surface.

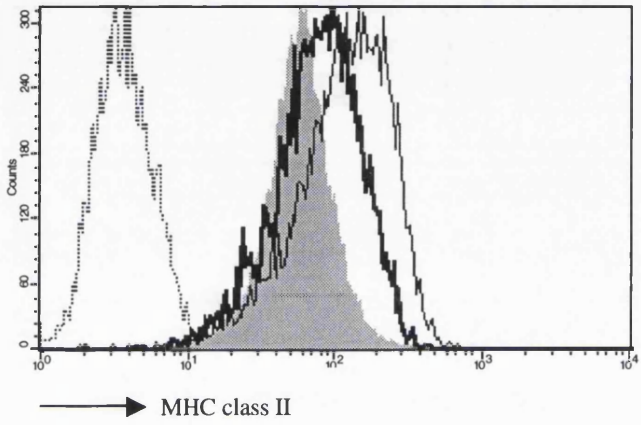
An interesting question was, whether transport of vesicles with stored class II molecules to the plasma membrane or increased biosynthesis of class II is the mechanism responsible for increased class II surface expression by tsDC activated with ConA sup. The presence of cycloheximide, which blocks protein biosynthesis by direct inhibition of ribosomes, or of brefeldin A, which blocks protein transport from the ER to the Golgi, prevented full upregulation of MHC class II (Fig. 23). This indicates increased biosynthesis rather than accumulation or redistribution from a pre-existing source. Both cycloheximide and brefeldin A could only be added for a fraction of the time of ConA sup treatment (of the 48 h period, the last 8 h or 14 h, respectively), without affecting cell viability. This might explain why inhibition of MHC class II synthesis was not complete.

Figure 23

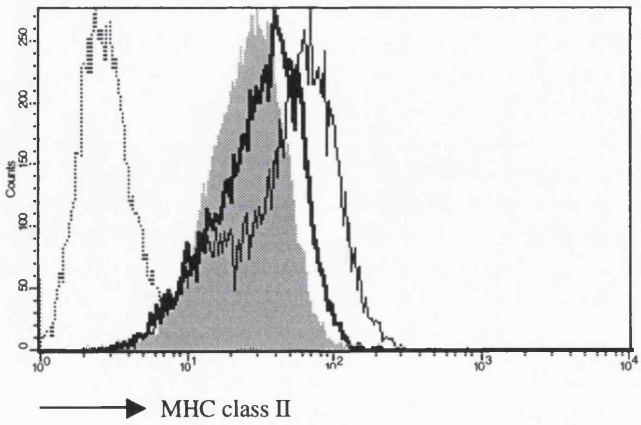
TsDC were activated with 20% ConA sup for 48 h at 37°C in the absence (continuous curve) or presence (bold curve) of cycloheximide or brefeldin A. Cycloheximide (200 μM) was present for the last 8 h of the activation period, brefeldin A (5μg/ml) was present for the last 14 h of activation. MHC class II expression is shown in comparison to non-activated tsDC cultured at 33°C (filled grey curve). The dotted curve represents staining with the isotype matched control antibody OKT11.

Fig. 23

Inhibition of class II upregulation on activated tsDC by cycloheximide



Inhibition of class II upregulation on activated tsDC by brefeldin A



3.2.5 Antigen capture

One of the features important for the function of dendritic cells as efficient antigen presenting cells is the capture of extracellular protein. Primarily immature DC have been reported to efficiently endocytose antigen (Sallusto et al., 1995). To investigate fluid phase endocytosis in tsDC and to test whether activation with ConA sup results in any changes, uptake of the fluorescent dye sulforhodamine was examined in living cells under the confocal microscope (Fig. 24A). Already after a short pulse of 2 min, vesicles in both non-activated and activated tsDC were filled with sulforhodamine and were visibly moving within the cells. Some of the vesicles were bigger than others, possibly representing macropinosomes, vesicles formed by cytoskeleton dependent membrane ruffling. To analyse whether the moving vesicles were acidic, tsDC were incubated with LysoTracker Red, a membrane diffusable red fluorochrome that accumulates in acidic compartments, such as late endosomes and lysosomes (Fig. 24B). Both, non-activated and activated tsDC, showed an abundance of acidic vesicles, but the structures were increased in size when compared to sulforhodamine containing structures and moved with decreased speed. As a comparison, DC generated in BM cultures (from non-transgenic mice) with GM-CSF were treated and analysed in the same way (Fig 24C). Their endocytosis capacity and content of acidic vesicles are comparable to that of tsDC, demonstrating that it is acommon feature of dendritic cells rather than typical for the cell line. In conclusion, tsDC appear to be very active in antigen uptake and processing. This parameter did not change after activation with ConA sup.

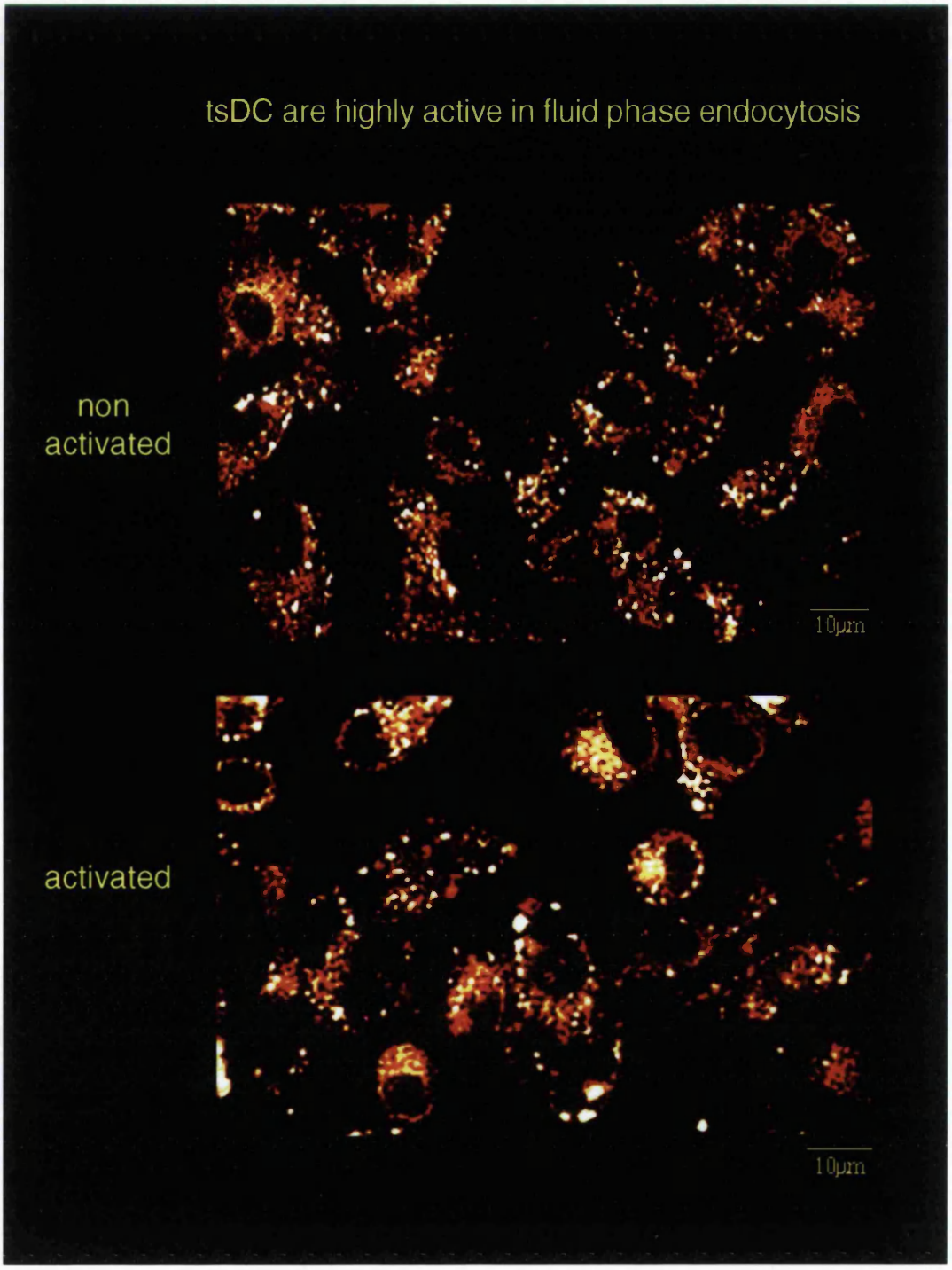
Internalization of proteins such as HRP, as well as intracellular distribution of MHC class I and class II, can be analysed in more detail by electron microscopy (EM). For this purpose, non-activated and activated tsDC were allowed to internalize HRP for 30 min before fixation. They were incubated with antibodies against MHC class I, MHC class II or HRP, followed by staining with the gold labelled second antibody and analysis by EM (Fig. 25).

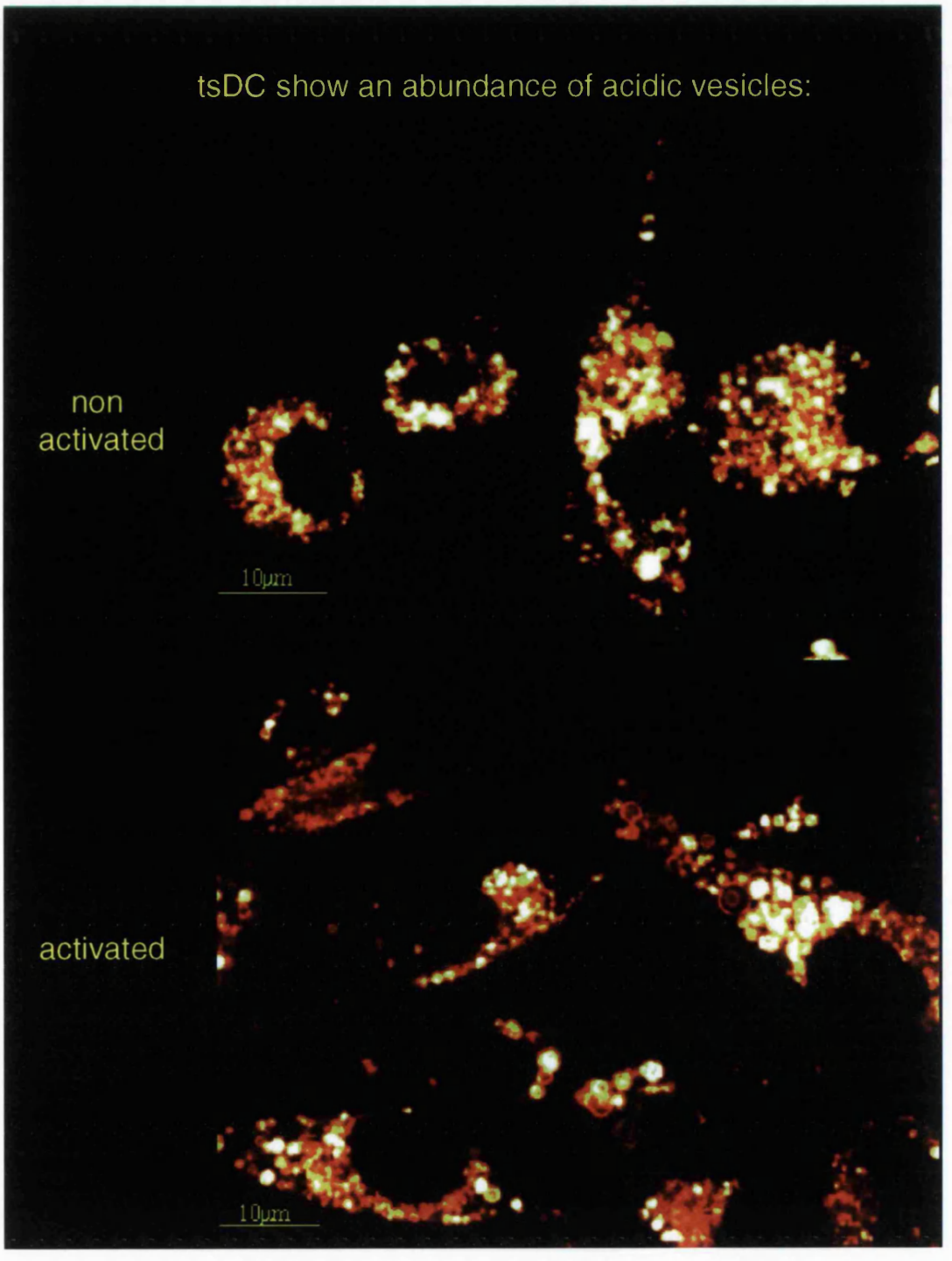
Figure 24

(A) Confocal analysis of fluid phase endocytosis by non-activated tsDC cultured at 33°C and activated tsDC cultured for 2 days at 37°C in the presence of ConA sup. Cells were allowed to internalize sulforhodamine for 2 min, the dye was washed off, and endocytosis was immediately analysed in living cells by confocal microscopy (magnification x 120).

(B) Acidic vesicles were visualized after incubation with LysoTracker Red which diffuses through membranes and becomes fluorescent in an acidic pH milieu. Incubation with the dye was for 1 min followed by confocal imaging (magnification x 120).

(C) Endocytosis and acidic vesicles in BM DC was analysed in the same way. Cells were generated with GM-CSF from bone marrow of non-transgenic mice.


tsDC are highly active in fluid phase endocytosis
non activated
10µm
activated
10µm


tsDC show an abundance of acidic vesicles:
non
activated
10µm
activated
10µm

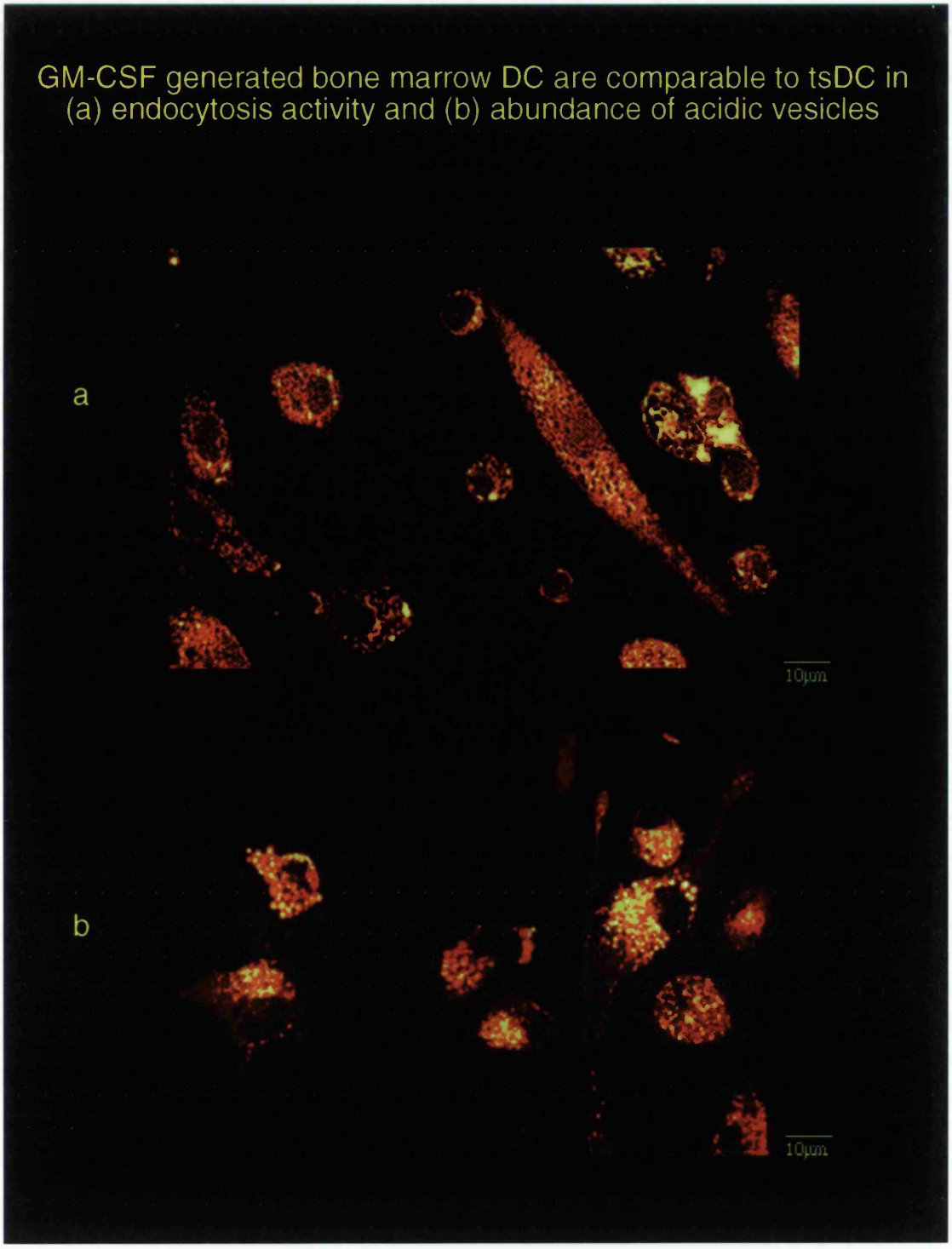
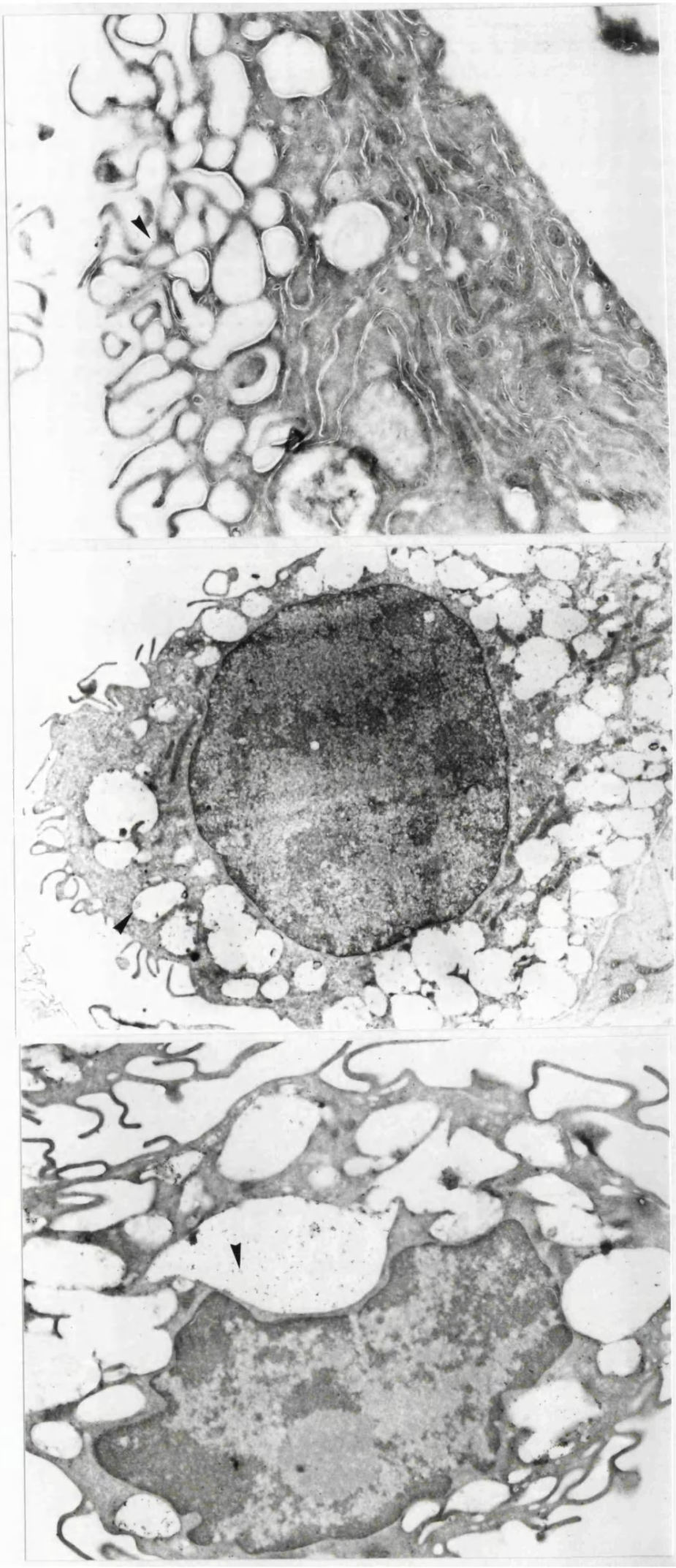

GM-CSF generated bone marrow DC are comparable to tsDC in
(a) endocytosis activity and (b) abundance of acidic vesicles
a
10µm
b
10µm

Figure 25

Electron microscopy analysis.

MHC class I was detected by EM in tsDC cultured for 2 days at 37°C in the presence of ConA sup (**A**). 10 nm gold particles attached to antibodies localized class I on the cell surface (arrow) and in the ER (cells were stained with rabbit anti-mouse class I serum, followed by gold labelled goat anti-rabbit Ig antibody). (**B**) 14.4.4 was used to detect MHC class II, which was found in vesicles (arrow) and the cell surface of tsDC cultured at 33°C (14.4.4 staining was followed by incubation with rabbit anti-mouse Ig and gold labelled goat anti-rabbit Ig antibody). In (**C**), ConA activated tsDC were allowed to internalize HRP for 30 min before staining with rabbit anti-HRP serum, followed by gold labelled goat anti-rabbit Ig antibody. HRP was enriched in large vesicles (arrow).

For further photographs see appendix (back-flap).


A
B
C

Both, non-activated and activated tsDC show an abundance of large vesicles, they contain high numbers of mitochondria, ER and other membranes, pointing to metabolically very active cells. Also apparent are long veils, prolongations of the surface membrane typical for dendritic cells, which are even more extended in activated tsDC. When stained for MHC class I, gold particles are found mainly in ER membranes and to a lesser extent at the plasma membrane. Class II molecules are predominantly associated with membranes which are engulfed by the vesicles. The majority of these vesicles are part of the endosomal / lysosomal system, as many of them are filled with endocytosed HRP.

3.2.6 The intracellular distribution of molecules involved in antigen processing and presentation

A method to characterize the intracellular sites where MHC class I and class II or molecules involved in antigen processing are located, is density gradient electrophoresis (DGE). Hereby, lysosomal and endosomal compartments, as well as ER and other subcellular organelles, are separated according to their surface charge. Early and late endosomes of non-activated and activated tsDC were labelled by incubation of tsDC with horseradish peroxidase (HRP) for 30 min, vesicles were prepared, subjected to DGE, and the distribution of markers for the various subcellular compartments was determined (Fig. 26). First, fractions with uneven numbers were used to determine the positions of endosomal vesicles and lysosomes by measuring the enzymatic activity of the endocytosis marker HRP and of the lysosomal marker β-hexosaminidase (β-hex), respectively (Fig 26A). In non-activated tsDC (top panel), HRP activity was found in fractions 19-35 and β-hex in fractions 11-19. In activated tsDC (bottom panel), HRP activity could be detected in fractions 17-33 and β-hex in fractions 13-23, thus showing a greater overlap of both activities. This might indicate that in activated tsDC endocytosis took place at a

faster rate than in non-activated tsDC, because in the former HRP had already reached compartments with lysosomal characteristics after 30 min.

Fractions with even numbers were precipitated and then analysed for the distribution of MHC class I, MHC class II and cathepsin D by Western blotting (Fig. 26B). The alpha chain of class II is observed in activated tsDC as a diffuse band running at 35 kD predominately in fractions 12 and 14, corresponding to β-hex activity in lysosomes, in fractions 22-38, corresponding to HRP activity in endosomes, and in fractions 44-54, which possibly represents ER and surface membrane. The latter is the least negatively charged membrane and therefore present in the late fractions. Unfortunately, class II could not be detected in any fractions of non-activated tsDC. This indicates that the amount of class II molecules present in non-activated tsDC is not high enough to be visualized by this technique, since the same numbers of both activated and non-activated tsDC were used.

The heavy chain of MHC class I could be detected in fractions 36-48 in non-activated tsDC and in fractions 24-54 in activated tsDC, corresponding to ER and plasma membrane. The lack of class I detection in later fractions of non-activated tsDC reflects its lower expression levels on the cell surface.

Three forms of cathepsin D can be distinguished, the precursor pre-pro form, which is inactive and located in the ER and Golgi, the pro form, which is generated in endosomes by autocatalysis and is already active, and the mature fully active form, the final product after further degradation by various proteases (Gieselmann et al., 1983). All three forms migrated similarly in non-activated and activated tsDC. Pro-cathepsin D was detected in fractions 6-36, mature cathepsin D in fractions 10-30 (non-activated tsDC) or 10-26 (activated tsDC), indicating that pro-cathepsin D is present in endosomes and lysosomes, whereas mature cathepsin D is mainly located in lysosomes. Pre-pro-cathepsin D migrates at the position of the Golgi and ER fractions (30-48), difficult to distinguish from pro-cathepsin D due to similar sizes.

Taken together, qualitatively different distribution of class I, class II or cathepsin D in non-activated and activated tsDC could not be observed. In case of class II no statement can be made due to detection problems in non-activated cells.

Dendritic cells have been reported to take up certain antigens by receptor mediated endocytosis using the mannose receptor (Sallusto et al., 1995). To investigate whether receptor mediated endocytosis plays a role in uptake of HRP, density gradient electrophoresis was repeated after a 5 min pulse of HRP at 4°C, followed by 30 min chase at 37°C after removal of unbound HRP. The short pulse on ice allows binding of HRP to the mannose receptor, but not internalization of the protein by fluid phase endocytosis. Only receptor bound HRP can then be taken up during the following 30 min. Fig. 26C shows that the amount of HRP internalized by non-activated and activated tsDC, as well as the localization in the fractions in relation to β-hex activity and protein content, is comparable to the previous experiment (Fig. 26A), in which continuous uptake of HRP was allowed. This indicates, that receptor mediated endocytosis not only plays a role in the uptake of HRP but that it is the main mechanism by which HRP is internalized. Fig. 26C also shows a greater overlap of the fractions containing HRP activity with fractions containing β-hex activity in activated tsDC when compared to non-activated tsDC. Thus, it reproduces the result shown in Fig. 26A and again indicates that in activated tsDC HRP is delivered to lysosomal compartments at a faster rate than in non-activated tsDC.

Fig. 26A **Subcellular fractionation of tsDC pulsed with HRP for 30 min**

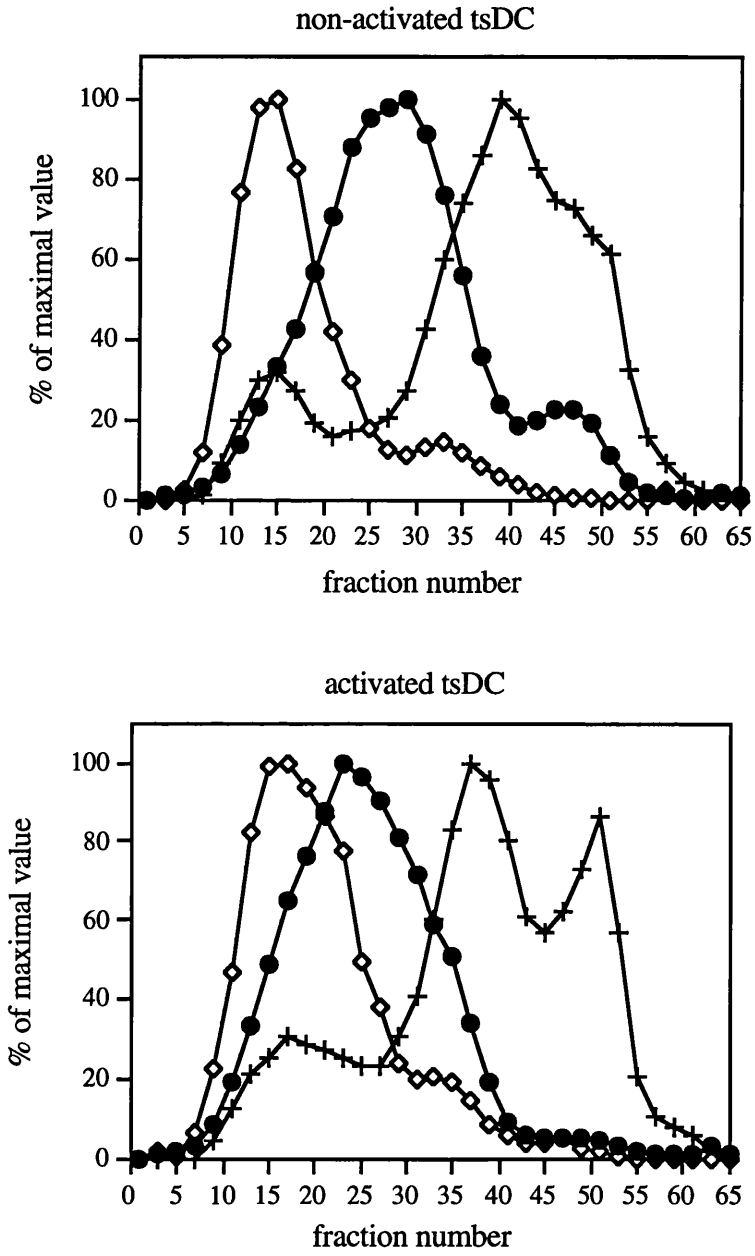


Figure 26A

5 x 10^7 tsDC cultured in the absence (non-activated tsDC) or for 2 days in the presence of ConA sup (activated tsDC) were allowed to internalize HRP for 30 min. Cells were homogenized and vesicles were subjected to density gradient electrophoresis. Uneven numbered fractions were analysed for content of β-hexosaminidase (open diamonds), HRP (filled circles) and total protein (crosses).

Figure 26B

Cells were prepared as described in **(A)**. Protein in even numbered fractions was precipitated, SDS-PAGE separated and analysed by Western blotting using polyclonal rabbit serum against mouse MHC class II, against class I (K^b), or a rabbit anti human cathepsin D serum. Blots were then incubated with HRP labelled swine ant-rabbit secondary antibody before analysis using ECL. Analysis of each marker was followed by removal of antibodies prior to staining for another marker.

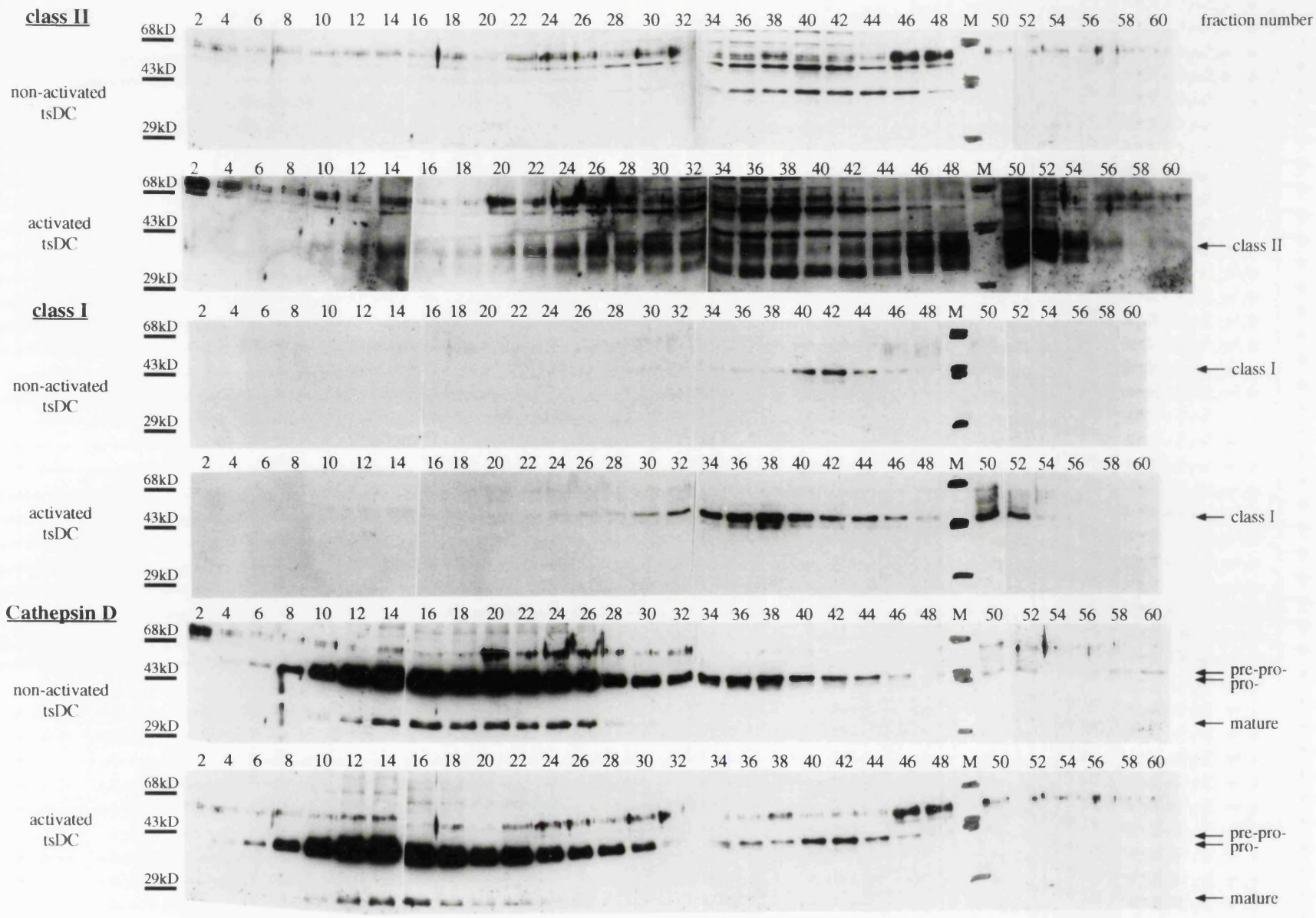

class II
non-activated tsDC
activated tsDC
class I
non-activated tsDC
activated tsDC
Cathepsin D
non-activated tsDC
activated tsDC
68kD
43kD
29kD
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 M 50 52 54 56 58 60
fraction number
class II
class I
class I
pre-pro-
pro-
mature
pre-pro-
pro-
mature

Fig. 26C **Subcellular fractionation of tsDC after receptor mediated uptake of HRP**

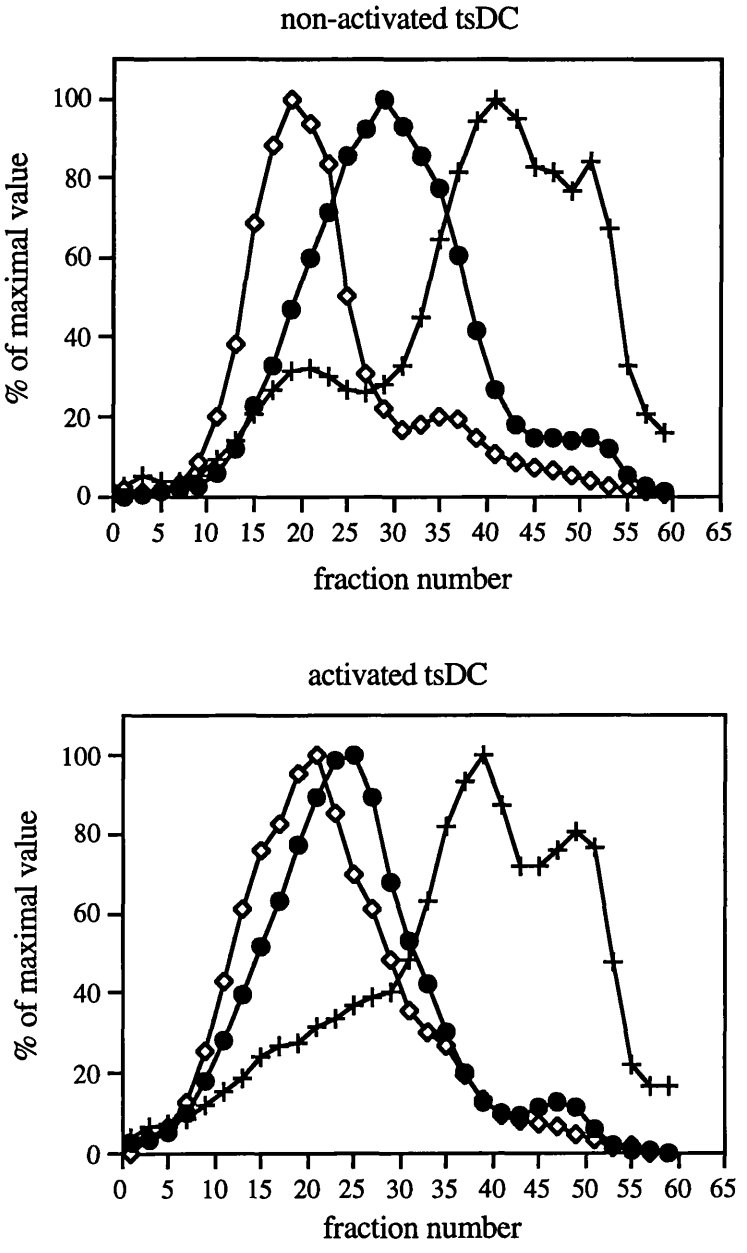


Figure 26C

Cells were incubated with HRP for 5 min on ice, washed and internalization of receptor bound HRP was allowed for 30 min at 37°C. Cells were then treated as described for (A).

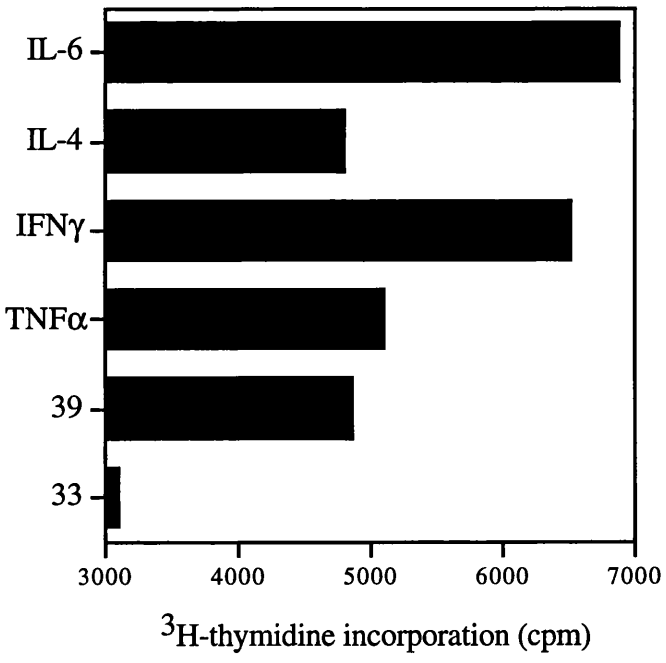
Figure 27A

$N418^+$ and MHC class II^+ depleted thymocytes from tg $C5^-$ mice were cultured at 5 x 10^5 cells/well of a round bottom 96-well plate with 5 μg/ml C5 protein and 2 x 10^4 tsDC cultured at 33°C, at 39°C for 3 days in medium alone or in the presence of 100 U/ml TNFα, 200 U/ml IFNγ, 1% IL-4 supernatant or 0.2% IL-6 supernatant. After 72 h, supernatant was tested for IL-2 activity on CTLL. The results are expressed as mean cpm of [^{3}H]-thymidine incorporation of triplicate cultures. Thymocytes alone cultured with C5 protein resulted in background counts of up to 2000 cpm.

3.2.7 The influence of cytokines on functional activity

It was shown that cytokines, especially a cocktail produced by activated T cells, are able to increase surface expression of those molecules on tsDC, which are crucially important in the initiation of T cell responses. Furthermore, tsDC have demonstrated to be highly active in endocytosis of intact proteins. Consequently, activated tsDC might fulfil the criteria to stimulate naive T cells. When incubated with single cytokines prior to culture with thymocytes from $C5^-$ TCR transgenic mice, tsDC were indeed able to present C5 protein for activation of these naive T cells (Fig. 27A). This ability was partly induced by the temperature increase to 39°C on its own and enhanced under the influence of certain cytokines such as IFNγ and IL-6. Pre-treatment with ConA sup enabled tsDC to prime T cells more effectively (Fig. 27B), an observation supporting the finding that several factors are important for the induction of tsDC differentiation.

Fig. 27A **Activation of naive T cells by tsDC**



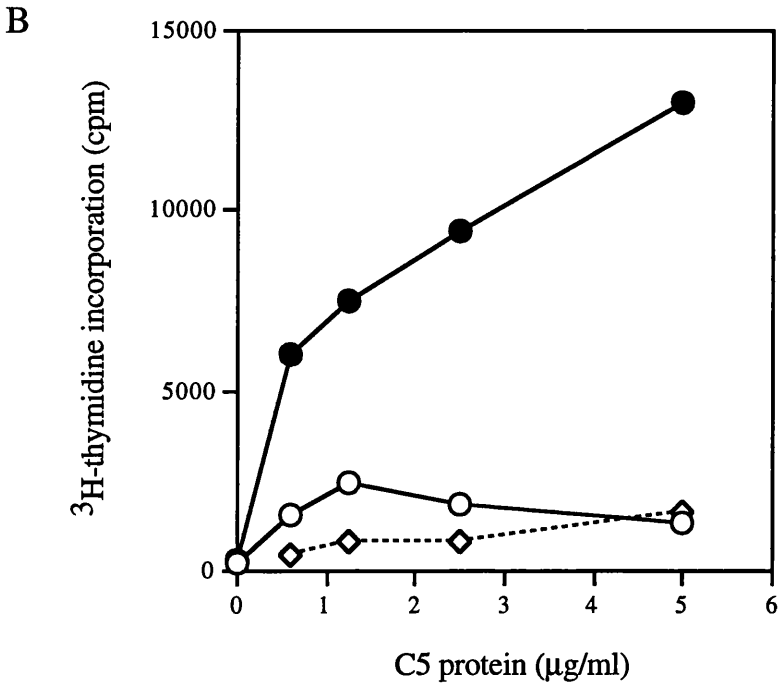


Figure 27B

The same thymocytes as in (A) were cultured with 2 x 10^4/well tsDC (grown at 33°C, open circles, or activated with 20% ConA sup for 48 h at 39°C, filled circles). Thymocytes alone cultured with C5 protein are shown as open diamonds, dotted line, as a control for complete removal of endogenous dendritic cells. Supernatant was tested for IL-2 activity as described for (A).

Most strikingly, the capacity of tsDC to activate naive T cells can be acquired during a co-culture period with naive T cells and cognate antigen, as shown in Fig. 28. TsDC from 33°C cultures were co-cultured with splenocytes from $C5^-$ TCR transgenic mice for two consecutive periods of three days. After the first three days, all cultures were washed extensively to remove cytokines and subsequently irradiated to eliminate responses by the original T cells. For the second three day culture period, fresh naive T cells were added. It was apparent that no T cell response took place when tsDC were added without antigen. Presentation of tsDC which carried antigen only in the secondary culture and were therefore exposed to cytokines for 3 days only is not very efficient. TsDC pulsed with antigen for the first

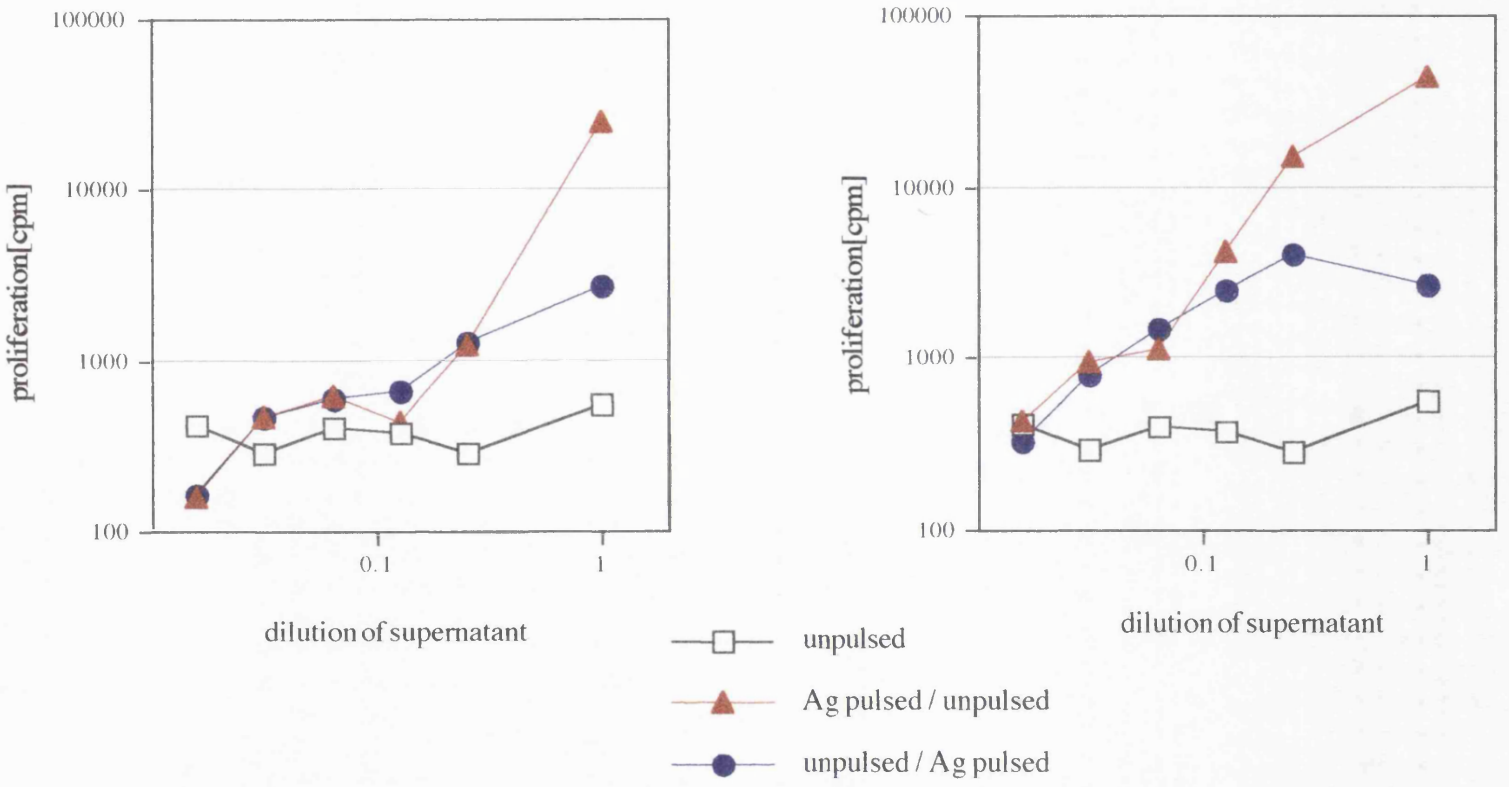
3 day culture showed enhanced presentation capacity to fresh naive T cells in the consecutive culture even without further addition of antigen. This not only confirms that dendritic cells can retain stimulatory peptide/class II complexes for some time (Lin and Stockinger, 1989), but also illustrates functional maturation as a consequence of exposure to cytokines derived from activated T cells. Thus, tsDC and T cells might be mutually responsible for their functional differentiation.

Taken together, the data presented characterize the cell line tsDC as immature dendritic cells highly capable of antigen capture via fluid phase and receptor mediated endocytosis, as expected of immature DC. Furthermore, molecules such as MHC class II, B7, CD40 and ICAM-1, which are important for antigen presentation, are constitutively expressed, albeit at low levels. The temperature sensitive TAg has been proven to conditionally immortalize tsDC, as transfer to the non-permissive temperature of 39°C not only results in growth arrest, but permits differentiation to more mature DC under the influence of T cells and their cognate antigen or a cytokine cocktail provided by activated T cells. This differentiation step, although resulting in upregulation of molecules involved in antigen presentation and the ability of tsDC to activate naive T cells, cannot be considered as final maturation step, since activated tsDC still differ from mature DC, such as splenic DC, in their remaining ability to capture antigen, their lower expression levels of e.g. MHC class II and their less efficient presentation capacity.

Figure 28

TsDC (2 x 10^4/well) were co-cultured with 2 x 10^5 unseparated splenocytes from tg $C5^-$ mice for two consecutive culture periods of 3 days at 37°C. The primary culture for 3 days was either in the absence of antigen (open squares), or with tsDC which had been prepulsed with antigen (5µg/ml C5 protein in the left panel, 1µM C5 peptide in the right panel) before culture (red triangles). Pulsed tsDC were thoroughly washed before addition to T cells to avoid carry-over of antigen to APC present in the splenocyte suspension. Alternatively, unpulsed tsDC were left with splenocytes for 3 days and prepulsed with antigen for the second 3 day culture period (blue circles). In all cases primary cultures were irradiated with 200 Gy after 3 days to eliminate the original T cell population and washed extensively to remove cytokines present in the supernatant. Fresh splenocytes (2 x 10^5/well) were added and IL-2 responses were assayed 48 h later. Supernatants were removed and tested in serial dilution on the IL-2 dependent CTLL line. Proliferation of CTLL was measured by means of [^{3}H]-thymidine incorporation, added after 24h of culture for the last 9h. Results are expressed as mean cpm of triplicate cultures.

Fig. 28 **Mutual activation of tsDC and T cells in co-culture**



4. DISCUSSION

The historical view of antigen presenting cells in tolerance induction

The mechanisms underlying negative selection of potentially self reactive T cells in the thymus have been subject to investigation for some time. While there is a general agreement about the efficiency of dendritic cells in inducing tolerance, the role of macrophages has not been analysed in detail and studies about the involvement of epithelial cells have led to controversial results. Early experiments with haematopoietic chimeras and thymus transplanted mice (Jenkinson et al., 1985; von Boehmer and Schubiger, 1984; Zinkernagel et al., 1980) using e.g. thymus grafts depleted or non-depleted of haematopoietic cells, implied that bone marrow derived cells, rather than epithelial cells, were responsible for tolerance induction. Others (Gao et al., 1990; Webb and Sprent, 1990) found that T cells specific for allo-MHC were deleted by epithelial cells, whereas some reports (Hoffmann et al., 1992; Salaün et al., 1990) described a state of "split tolerance" in which the T cells reacted in vitro but not in vivo. In all these studies the development of a heterogeneous T cell population in an MHC disparate environment was followed.

APC involved in tolerance induction in TCR transgenic mice

With the appearance of T cell receptor transgenic mice it became possible to study the fate of individual T cells bearing receptors with defined antigen specificity. When dGuo treated (to deplete haematopoietic cells) fetal thymus lobes from male mice were implanted into female mice, which were transgenic for a TCR recognizing the male antigen H-Y presented on MHC class I D^b, tolerance was induced (Bonomo and Matzinger, 1993; Carlow et al., 1992[a]). Similarly, radioresistant thymic host cells deleted developing graft TCR transgenic T cells specific for lymphocytic choriomeningitis virus (LCMV) plus H-2D^b in BM chimeras, when the host mice were LCMV infected (Speiser et al., 1992). Using T cells specific for chicken

ovalbumin (OVA) in the context of H-2K^b from TCR transgenic mice, Vukmanovic et al. (1994) demonstrated that an OVA transfected thymic epithelial cell line induced negative selection in vitro. As far as TCR transgenic mice with MHC class II (I-E^k) restricted receptor were concerned, self-tolerance was established in thymus organ culture of TCR transgenic mice specific for pigeon cytochrome c in the presence of antigen, even when I-E^k expression and therefore antigen presentation was restricted to cEC (Spain and Berg, 1994). However, the ability of thymic epithelium to induce tolerance remained a question of debate, when in vitro deletion experiments resulted in failure of epithelial cells to induce negative selection in some cases (Carlow et al., 1992[a]), but in deletion caused in the presence of cognate peptide in other cases (Iwabuchi et al., 1992; Pircher et al., 1993).

The special features in tolerance induction of MHC class II restricted T cells specific for an exogenous antigen

Subject of this thesis was to study the involvement of different thymic APC in presentation of a defined, circulating self antigen for negative selection of MHC class II restricted T cells. First, the status of tolerance had to be established in the particular model system of C5 specific TCR transgenic mice. Several findings in fetal thymus organ culture are of importance: 1. T cell development is accelerated due to the presence of an already fully rearranged TCR in transgenic lobes, leading to functionally mature CD4$^+$ T cells at day 4 of culture, compared with day 10 in cultures of non-transgenic lobes. The differentiation and kinetics are consistent with reports describing T cell development in FTOC from normal mice (Jenkinson and Owen, 1990) and FTOC from transgenic mice with similarly early appearance of TCR$^+$CD4$^+$ cells (Spain and Berg, 1992); 2. MHC class II / C5 peptide complexes are present on APC for up to 6 days of organ culture to ensure negative selection; 3. Tolerance in TCR transgenic C5$^+$ mice is complete, as demonstrated by the absence of CD4 SP thymocytes expressing the transgenic TCR in 19 days old tg C5$^+$ embryos. Due to the

lack of continued C5 supply under culture conditions, tolerance in FTOC of tg $C5^+$ wanes after 6 days; 4. The bulk of DP is not affected in FTOC of tg $C5^+$, consistent with the situation in adult tg $C5^+$ mice; 5. There is no endogenous source of C5 in the thymus, confirming that this protein has access to the thymus only via the blood circulation.

The finding that the majority of DP are not affected in tg $C5^+$ mice initially suggested an inability of cEC to induce tolerance. In other TCR transgenic systems, both MHC class II restricted (Murphy et al., 1990; Spain and Berg, 1992; Vasquez et al., 1992) and MHC class I restricted (Kisielow et al., 1988; Mamalaki et al., 1992; Sha et al., 1988), the presence of antigen was shown to cause deletion of DP. In most cases described, cognate antigen had been introduced in the form of peptide, which avoids protein processing and assures high concentrations of antigen. Under these circumstances, C5 specific DP are deleted as well (section 3.1.3 and Zal et al., 1994). In MHC class I restricted TCR transgenic mice endogenous ligands are presented, which do not require internalization and processing. The possibility remained that cEC might be not be as efficient in negative selection to exogenous proteins, as a deficiency in antigen processing and presentation has been reported (Lorenz and Allen, 1989[b]; Mizuochi et al., 1992; Ransom et al., 1991), although others (Lorenz and Allen, 1989[a]) found them able to present exogenous antigen. Furthermore, Jiang et al. (1995) identified the receptor DEC-205, homologous to the macrophage mannose receptor, as an endocytic receptor mediating Ag uptake in cEC and DC.

To keep the antigen presentation conditions as close to the physiological situation as possible and, at the same time, dissect different types of APC in their capacity to induce tolerance, thymic APC were isolated from $C5^+$ mice and tested for negative selection of C5 specific thymocytes in reaggregation culture. In that way, contact with C5, internalization, processing and expression on the surface in context with MHC class II molecules all had taken place in vivo before isolation of the APC.

Technical remarks on thymic APC isolation

Purification of APC was achieved by positive selection through magnetic cell sorting using mAb against characteristic cell surface markers. CD11c was chosen as the marker for DC. Since CD11c is also expressed by some macrophages, albeit at a low level, it cannot be excluded that the DC preparation might have been contaminated with some macrophages. However, given the known potency of DC in negative selection, such contamination would not have influenced the results. Since thymic DC themselves are not a homogeneous population (Ardavin and Shortman, 1992) and there is no antibody available which stains exclusively DC, N418 (anti-CD11c) was the antibody of choice to target at least all DC subpopulations in the thymus. The use of F4/80 for the isolation of thymic macrophages is justified by its wide range of expression on macrophages from all tissues (Austyn and Gordon, 1981). A note of caution, however, concerns the description of a seemingly F4/80 negative macrophage population in the thymic medulla (Surh and Sprent, 1994). Although this was defined in tissue sections which is not as sensitivity for detection of surface molecules as e.g. FACS analyses, it can not be excluded that this subpopulation is missing from the macrophage preparation obtained by MACS sorting.

Numerous antibodies are available to distinguish cEC from mEC, two thymic EC populations which can be subdivided even further by means of surface markers, since they are both heterogeneous (Boyd et al., 1993; van Ewijk, 1991). G8.8, the mAb chosen for isolation of mEC, recognizes a surface glycoprotein expressed by mEC throughout the medulla as early as at d14 of gestation (Farr et al., 1991). It is also present on subcapsular EC, but neither on cEC, nor on DC or macrophages. Cortical EC were purified using the mAb CDR-1, which is specific for a surface molecule expressed by murine cEC, including thymic nurse cells, from postnatal day 9 onwards (Rouse et al., 1988). Thymic cells other than cEC are completely negative for CDR-1.

Cortical EC are efficient mediators of negative selection

Surprisingly, all thymic APC tested -with the exception of macrophages- induced negative selection in an indistinguishable manner. The fact that medullary as well as cortical EC were capable to induce negative selection of C5 specific thymocytes clearly demonstrated that the amount of C5 accessible to cortical cells was sufficient, even if the cortex contained less C5 than the medulla. It furthermore showed, that cEC are perfectly able to internalize and process exogenous proteins. The possibility remains that serum proteins present in the circulation at lower concentrations than C5 (50 µg/ml) might be presented less efficiently by cEC. Is it possible to interpret the apparent failure of EC to induce tolerance in early experiments? One explanation arises from studies which demonstrated that tolerance induced by thymic epithelium is complete with respect to the thymic stroma itself, resulting in acceptance of thymic grafts, but rejection of other tissues (Bonomo and Matzinger, 1993). These findings suggest that the lack of tolerance observed in some cases was due to differences in the peptides expressed by tolerizing thymic EC and skin grafts, or thymic EC and BM derived cells used as stimulators in in vitro assays. Although peptide elution studies showed that the same peptides are presented in the context of MHC class II molecules on splenocytes, total thymic EC and cEC (Marrack et al., 1993), peptides identified by the elution may well represent the most abundant peptides only, whereas less dominant peptides may not have been included in the study. In fact, few peptides, such as those derived from the Fcε receptor II and a member of the surface Ig complex, were unique to splenocytes. Similarly, the finding that thymic EC were unable to delete thymocytes bearing superantigen-reactive Vβ chains (Bandeira et al., 1992; Marrack et al., 1988; Webb and Sprent, 1990), could be explained by the lack of expression of those superantigens on EC. This has been revealed by the inability of thymic EC to stimulate SAg-reactive T cell hybridomas (Webb and Sprent, 1990) and by the lack of specific mRNA expression (Moore et al., 1994). Finally, in cases of split tolerance (Hoffmann et al., 1992; Salaün et al., 1990), thymic EC could induce

negative selection in vivo. The fact that the T cells showed some reactivity in vitro may be explained by thymic deletion of high avidity T cells, leaving T cells with low avidity for the antigen which can only respond to particularly strong immunogenic stimuli such as antigen in the presence of IL-2 in vitro (Hoffmann et al., 1995).

A very recent report of the inability of cEC to induce negative selection (Laufer et al., 1996) is more difficult to explain. The authors used the MHC class II β chain ($A\beta$) under the control of the keratin 14 promoter as a transgene, which allows re-expression of I-A^b in class II β chain negative ($A\beta^{b-/-}$) mice on cEC only. Whereas positive selection of CD4 cells was restored, negative selection to I-A^b or to superantigen after SEB injection was not observed. Although expression of class II was shown by staining of thymic tissue sections with a mAb, the presence of correctly folded I-A^b molecules was not demonstrated in functional assays using cEC from the transgenic mice as antigen presenting cells. If the transgene used was in any way different from normal I-A^b, the developing CD4 T cells would have been selected for that specific molecule and the reactivity against I-A^b on presenting cells from normal mice would not be surprising. Laufer et al. do not discuss the discrepancy between their results and data reported by Spain et al., who found that when cEC were the only APC expressing the appropriate MHC class II molecule (I-E_α^k transgene in H-2^b mice, which lack endogenous Eα expression), negative selection of T cells from mice transgenic for a cytochrome c specific TCR was as efficient as when the antigen was presented by all thymic APC (Spain and Berg, 1994).

Why are DP mainly unaffected by tolerance induction in $C5^+$ mice?

Tolerance in reaggregation culture induced by cEC leads to the question, why only a portion of double positive thymocytes are affected in vivo in $C5^+$ TCR transgenic mice. The original hypothesis that the more potent APC are localized in the medulla apparently does not hold true. However, one observation suggested that cEC, although capable of tolerance induction in reaggregation culture, were less effective

than mEC and DC: when the level of TCR expressed on DP was compared, thymocytes recovered from reaggregation cultures with mEC or DC showed lower TCR levels than those cultured in the presence of cEC. This could be explained with the requirement of increased TCR expression levels on DP in order to represent a target for deletion by cEC. Clearly, tolerance induction is complete when caused by cEC (as shown in functional assays by the lack of C5 reactive thymocytes recovered from cultures with cEC), but the time point might be delayed to the more maturationally advanced DP.

Why do macrophages fail to induce tolerance and what is their role in the thymus?

The only APC population unable to induce negative selection were macrophages. Early indications for this was the previously reported failure of C5 tolerance induction in bone marrow chimeras, in which C5 production was restricted to intracellular expression in macrophages (Grant et al., 1996; Stockinger et al., 1993). Furthermore, thymic or splenic macrophages injected intrathymically into mice across an Mls-1 superantigen barrier did not mediate negative selection (Inaba et al., 1991). This superantigen is mainly expressed by B cells and it is not known to what extent macrophages have access to it. The present data show that inability of macrophages to induce tolerance extends even to an extracellular source of C5. What are the reasons underlying the apparent failure of macrophages to induce negative selection? Insufficient access to C5 protein could be excluded as explanation, since loading with peptide prior to introduction into reaggregation culture did not enable them to delete C5 reactive thymocytes. Experiments designed to test the possibility that macrophages, in contrast to the other APC, rapidly lose C5 peptide / MHC class II complexes in culture, did not reveal this possibility as the sole reason either. Earlier experiments with thymic macrophages indicated that these cells express virtually no MHC class II (Stockinger and Lin, 1989). However, in these studies macrophages were isolated via an overnight adherence step, rather than by direct positive selection by MACS.

Staining of freshly isolated macrophages revealed MHC class II expression, also evident from their capacity to activate C5 specific T cell hybridomas, but the levels are 5-10 fold lower than on DC and EC.

In addition to low levels of MHC class II, thymic macrophages also express little ICAM-1, an adhesion molecule which has been shown to play a role in tolerance induction (Carlow et al., 1992[b]; Pircher et al., 1993) and which is highly expressed by the other APC (Ardavin and Shortman, 1992; Georgiou and Constantinou, 1994; Nelson et al., 1993). Recently, Kishimoto et al. (1996) reported a synergistic effect of B7.1 and ICAM-1 on the induction of negative selection of thymocytes from TCR transgenic mice in vitro, when they were co-expressed on MHC class I transfected Drosophila cells. Surprisingly, when ICAM-1 was expressed in the absence of B7.1, negative selection was inhibited. Non-activated macrophages have very little B7 and ICAM-1 on their surface, therefore the finding is not contradictory to the failure of macrophages to induce negative selection. DC and mEC express both molecules (Larsen et al., 1994; Nelson et al., 1993; Prieto et al., 1989), in agreement with their effectiveness to induce deletion. The only exception would be cEC, which express ICAM-1 (Prieto et al., 1989) but not B7 (Mizuochi et al., 1992; Nelson et al., 1993). However, the latter express costimulatory molecules apart from B7 which may play a role in negative selection (such as CD40, Foy et al., 1995). Redundancy of costimulatory molecules may also explain why e.g. CD28 deficient mice are not impaired in negative selection (Walunas et al., 1996).

In contrast to thymic macrophages, splenic macrophages express higher levels of ICAM-1 and MHC class II which presumably reflects the presence of IFNγ, released by activated T cells, in the periphery, but not in the thymus. Splenic macrophages, as well as IFNγ pre-treated thymic macrophages, have some ability to induce negative selection, although not nearly as striking as DC and EC, which could be explained by increased levels of ICAM-1 and MHC class II. Taken together, the data suggest that in principle all APC are able to induce negative selection, but that in

vivo thymic macrophages are not equipped for efficient presentation to MHC class II restricted T cells. Their role may lie preferentially in disposal of apoptotic thymocytes (Duvall et al., 1985; Fadok et al., 1992; Surh and Sprent, 1994) rather than involvement in selection processes.

What parameters other than the source of APC can influence the time point of deletion?

The amount and localization of antigen available for presentation can play a role. For instance, Mls^a and the lymphocytic choriomeningitis virus represent antigens which cause late and early deletion, respectively, of the same transgenic TCR expressing T cells (Pircher et al., 1989). In another system, negative selection of αβ TCR transgenic T cells specific for cytochrome c in association with $I\text{-}E^{k,s,b}$ can be varied with antigen. T cells bearing the transgenic TCR can be positively selected by either $I\text{-}E^k$ or $I\text{-}A^b$. In vitro, the majority of DP from these mice are deleted when cytochrome c is recognized in the context with $I\text{-}E^{b/k}$. In contrast, presentation of superantigen by $I\text{-}E^{b/k}$ results in minimal deletion of DP. In TCR transgenic $H\text{-}2^{s/k}$ mice, $I\text{-}A^s$ induced negative selection (caused by cross-reactivity) occurs at an even later stage, resulting in the absence of CD4 SP only (Vasquez et al., 1992).

Mls^a is a superantigen produced by B cells. It can preferentially be found in the medulla and consequently results in late deletion of DP at the transition to SP (Mazda et al., 1991).

With regard to the effect of antigen concentration, a threshold of 10^{-9} - 10^{-10} M has been reported for the induction of T cell tolerance (Mitchison, 1985). F liver protein induces T cell tolerance at the concentration of 10^{-9} M (Robertson et al., 1992), hen egg lysozyme even at levels as low as 10^{-10} M (Adelstein et al., 1991), whereas thyroglobulin, at 10^{-10} M, is unable to induce complete tolerance. Thyroglobulin specific T cells react after immunization of mice, although the response is reduced (Romball and Weigle, 1984).

The concentration of C5 protein in the blood at about 50 μg/ml (10^{-7} M) is relatively high. It may therefore not be surprising that cEC could delete C5 specific T cells, since even 100 fold lower amounts would have been sufficient for negative selection if one takes into account the examples cited above. Thus, C5 concentration is not a limiting factor for tolerance induction.

The expression levels of CD4 or CD8 as co-receptors may play a role, since they increase the overall avidity of TCR - MHC/peptide interaction. Lee et al. (1992) could convert positive selection in MHC class I restricted TCR transgenic mice into negative selection by introducing a CD8 transgene, which resulted in increased expression of the co-receptor on T cells. However, it has not been demonstrated that increasing expression of the co-receptors during thymocyte differentiation results in higher susceptibility to negative selection.

The expression level of MHC, on the other hand, has been shown to influence the time point of deletion. Invariant chain deficient ($Ii^{-/-}$) mice have reduced expression levels of MHC class II on their APC. Comparing negative selection to superantigen in $Ii^{-/-}$ with wild type mice, Huang et al. (1996) found that Vβ-specific deletion occurs in more mature thymocyte populations in $Ii^{-/-}$ mice.

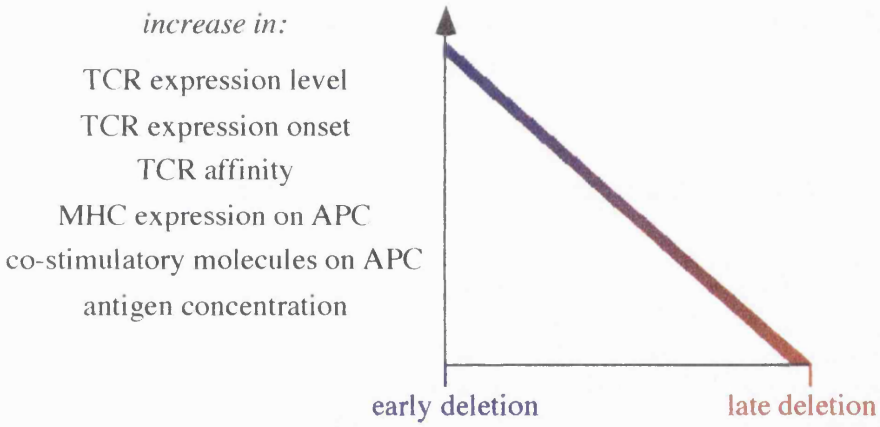
An important parameter influencing the time point of deletion concerns the TCR. Differences in TCR levels and the onset of TCR expression in transgenic mouse models may explain, why e.g. in H-Y specific TCR transgenic mice which express high TCR levels already at the stage of DN, deletion in male mice results in the disappearance of virtually all DP (Kisielow et al., 1988). Likewise, TCR transgenic mice specific for MHC class I L^d show severe depletion of DP in the presence of antigen (Sha et al., 1988). In contrast, C5 specific TCR transgenic mice with 2-3 fold lower TCR levels than in non-transgenic mice and very low to undetectable expression at the DN stage, delete thymocytes at the transition from DP to SP. A similar explanation for differences in timing of deletion has been suggested for another system

by Berg et al. (1989[a]), who compared negative selection to the superantigen Mls $2^a/3^a$ in Vβ3 bearing αβ- with β-TCR transgenic mice. In mice, transgenic for TCR β only, TCR level and onset of expression was comparable to non-transgenic mice and deletion of Vβ3 bearing cells occurred at the transition from DP to SP. In contrast, αβ TCR transgenic mice, with early onset of TCR expression and higher levels on DP, showed severe depletion of DP. In conclusion, it seems most likely that in $C5^+$ TCR transgenic mice thymocytes have to upregulate their levels of TCR to become sensitive to negative selection. Therefore, only a minority of DP, namely those which have undergone positive selection and thereby upregulated their TCR (Penit, 1990; Swat et al., 1992), are likely to be affected by recognition of self antigen, regardless of the type of APC presenting it.

Deletion observed in reaggregation cultures appeared to effect DP, whereas in $C5^+$ TCR transgenic mice only a minor proportion was affected. This discrepancy observed in the extent of deletion of DP in $C5^+$ TCR transgenic mice *in vitro* and *in vivo* is due to the relatively high APC : thymocyte ratios employed in the former. This was suggested by decreasing deletion of DP when the numbers of APC were reduced in reaggregation culture (see Fig. 10A, results).

Thus, the overall avidity of TCR- MHC/antigen interaction, which is thought to determine positive or negative selection as the outcome of TCR engagement in the thymus (Sprent et al., 1988), correlate also with the timing of negative selection. It may in addition determine whether every potential APC capable of inducing negative selection -DC, mEC and cEC- is in fact involved in the process in vivo.

time point of thymic negative selection



The conditionally immortalized line tsDC and its advantages

The second part of this thesis dealt with the generation and phenotypic, as well as functional characterization of a conditionally immortalized bone marrow derived dendritic cell line (tsDC). The first advantage of this DC line is to have a readily available homogeneous source of DC for in vitro and in vivo studies. Another DC line, established from mouse spleen by transformation with recombinant retroviruses, was described by Paglia et al. (1993). This cell line, comparable to tsDC in their expression of surface markers, was able to activate a T cell hybridoma only after treatment with GM-CSF, suggesting that it represents DC at a more immature stage, despite its origin from spleen. It remains unclear whether or not the active v-Myc oncoprotein affects the DC function in these cells. The advantage of the thermolabile SV40 TAg used in this project is its transforming capacity at permissive temperatures, which is reversible at 39°C. Thus, alterations in cellular physiology, which are commonly induced by transforming agents such as viruses, can be avoided. Moreover, conditionally transformed cell lines can be analysed with respect to differentiation requirements by modifying the growth conditions (Jat and Sharp, 1989).

DC heterogeneity and their relation to macrophages

Members of the DC lineage are distributed in non-lymphoid tissues as well as in lymphoid organs. While they share features which are unique for DC, they nevertheless show significant variation in the expression of cell surface markers. In fact, since DC change in phenotype during their lifespan, they do not bear stable markers and even share several surface molecules with macrophages. The exact relationship among DC in various anatomical sites is not fully elucidated, but all DC are derived from a common bone marrow precursor (Metlay et al., 1989), which may mature distinctively under different environmental conditions. This precursor, $CD34^+$ in human, gives rise to both DC and macrophages (Reid et al., 1992), which explains

why some markers are shared by DC and macrophages, especially when they are immature. It is not clear yet, at what stage the differentiation into either DC or macrophages diverges. Development into DC or macrophages can be influenced *in vitro* by growth factors such as GM-CSF, IL-4 and TNFα for the former and M-CSF for the latter. Moreover, the lack of DC differentiation in relB deficient mice has been found to be compensated for by enhanced production of macrophages and neutrophils (Burkly et al., 1995), in line with the assumption of a common lineage. Boehmelt et al. (1995) reported the transformation of chicken BM cells with a hormone inducible v-Rel estrogen receptor fusion protein (v-RelER). Inactivation of v-RelER oncoprotein activity by administration of an estrogen antagonist caused differentiation of the cells into either DC or polymorphonuclear neutrophils, depending on the culture medium conditions. This also argues for a common myeloid progenitor. In the human system, blood monocytes, which were thought to be the immediate precursors for tissue macrophages only, could be differentiated into DC when cultured with certain stimuli such as IL-4 and GM-CSF (reviewed by Peters et al., 1996). Peters suggested that early precursors differentiate into blood monocytes, which can then develop further to become either DC or macrophages. Experiments using macrophage growth factors such as M-CSF intended to drive tsDC into macrophages were unsuccessful (data not shown), demonstrating that tsDC have already diverged from the common differentiation programme to DC.

How is differentiation of DC defined?

Differentiation or maturation of immature DC is characterized by alterations in phenotype and function as a consequence of contact with antigen or culture with different cytokines. One example is the differentiation of LC under the influence of cytokines released by keratinocytes. Emigration of LC into peripheral lymphoid organs is accompanied by differentiation to functionally mature DC capable of activating virgin T cells (Cumberbatch et al., 1991; Larsen et al., 1990[b]; Witmer-Pack

et al., 1987). The phenotypic changes LC undergo upon contact with antigen in vivo (Kraal et al., 1993) are similar to those observed when these cells are placed in tissue culture and include upregulation of MHC class II molecules in concert with termination of class II and invariant chain biosynthesis (Cumberbatch et al., 1991; Kämpgen et al., 1991; Pure et al., 1990) and an increase in adhesion molecules and costimulatory molecules like members of the B7 family (Larsen et al., 1994; Teunissen et al., 1990) or CD40 (Caux et al., 1994). Differentiation of DC has been studied in other systems in vitro, whereby e.g. human peripheral blood mononuclear cells have been differentiated into immature DC under the influence of GM-CSF and IL-4, which could mature further when cultured with TNFα, CD40 ligand, IL-1 or LPS (Sallusto et al., 1995; Sallusto and Lanzavecchia, 1994). Immature DC generated with GM-CSF and TNFα from human cord blood $CD34^+$ progenitor cells could be differentiated further through CD40 cross-linking (Caux et al., 1994).

It furthermore appears that the two key functions of DC, handling of antigen and the interaction with naive T cells, which results in activation, are separated in distinct stages of DC development. The capacity for antigen internalization and processing is highest in immature DC and can be almost absent in the most mature stage of DC isolated from lymphoid organs (Pure et al., 1990; Romani et al., 1989[a]; Romani et al., 1989[b]). Immature DC generated with GM-CSF and IL-4 from human blood mononuclear cells are highly efficient in antigen internalization, but terminate this process in the presence of TNFα (Sallusto et al., 1995; Sallusto and Lanzavecchia, 1994). Conversely, the capacity for activation of naive, resting T cells is low in immature DC and very efficient at the end stage of DC isolated from spleen and lymph nodes (Dai et al., 1993; Streilein and Grammer, 1989). The ability for T cell activation is due to upregulation of adhesion molecules which allow the typical cluster formation between DC and T cells essential for initiation of primary T cell responses (Inaba and Steinman, 1986).

The immature phenotype of non-activated tsDC

Given these criteria for the differentiation and maturation of DC, the data shown characterize tsDC, maintained at 33°C, as immature DC. They are highly endocytic, have low levels of MHC class II, B7 and CD40 and express a number of markers which were reported to be absent or down-regulated on DC in lymphoid organs, such as Fc receptors and the macrophage marker F4/80. Sca-2 and Gr-1, which have been found on myeloid progenitors or granulocytes, are also expressed by tsDC. Interestingly, a DC precursor line from mouse skin does not express Gr-1 (Girolomoni et al., 1995), whereas it is found on the supposedly more mature splenic DC line transformed in the same way (Paglia et al., 1993). This may indicate that the expression pattern of some markers ascribed to either immature or mature cells is not as strictly regulated. Another feature characterizing tsDC as immature DC is their incapability to activate naive T cells in an antigen specific manner, although they already fulfil some functions reported for mature DC, such as allogeneic MLR stimulation and efficient activation of experienced T cells. Antigen presentation to activated T cells is not subject to the same restraints as presentation to naive T cells and can be carried out by every MHC class II/peptide complex bearing cell. In contrast, expression of co-stimulatory molecules such as B7 in addition to adhesion molecules is a prerequisite for stimulators of a primary MLR. Thus, the inability to activate naive T cells from TCR transgenic mice should be accompanied by an inability to initiate a mixed leukocyte reaction. An explanation for the apparently contradictory result might be the presence of some allogeneic T cells which were activated previously due to cross-reactivity to infectious agents. Alternatively, the expression levels of B7, CD40 and ICAM-1 on non-activated tsDC might be sufficient to stimulate an allogeneic response, which is most likely carried out by T cells which have a very high affinity to foreign MHC. Virgin T cells from TCR transgenic mice, on the other hand, might require up-regulated expression levels of those molecules.

In agreement with that, the slight changes in cell surface marker expression which became visible upon culture at the non-permissive temperature and which were more pronounced in the presence of certain cytokines such as IFNγ, were accompanied by the acquisition of T cell priming capacity to some extent. The cytokines could mimic partly the in vivo environment of DC, e.g. the skin, supported by the upregulation of MHC class II, B7 and CD40 observed following culture with skin cells.

The symbiotic interaction with T cells leads to activation of tsDC

The most striking and consistent upregulation of surface markers was observed upon co-culture of tsDC with T cells and their cognate antigen or in the presence of a mixture of cytokines released by activated T cells. These changes were not reversed upon transfer of tsDC back to 33°C, indicating stable differentiation. Most notably the two molecules which are crucially involved in the interaction with T cells, the costimulatory molecules B7.1 and CD40 were highly expressed after contact with T cells. Naive T cells themselves are dependent on co-stimulation through CD28 for activation (Harding et al., 1992; Linsley et al., 1991[a]) and in the course of activation switch on transient expression of CD40-ligand (gp39), an activation marker of T cells (Banchereau et al., 1994; Noelle et al., 1992).

Increased expression of these molecules on tsDC after activation of T cells might reflect an interaction needed by both cell types, leading to the activation of T cells and DC. Such an interesting aspect of cell communication has been reported to take place in the thymus, where thymocytes are unable to develop without epithelial cells, and vice versa (Anderson et al., 1993; Holländer et al., 1995; Ritter and Boyd, 1993). In a physiological environment in vivo, T cell / DC inter-dependence is suggested by a number of findings: migration of DC from blood into the spleen is disturbed in T cell deficient (nude) mice and can be reconstituted by injection of T cells (Kupiec-Weglinski et al., 1988), LC from nude mice have diminished antigen

presentation capacity which is restored by thymus transplantation (Grabbe et al., 1993). Furthermore it appears that T cell development in the thymus proceeds in close interaction with DC, since the thymus is colonized simultaneously by precursor cells which give rise to T cells and DC (Ardavin et al., 1993). An observation in RAG-1 deficient mice, in which T cells are absent, is the reduced expression of MHC class II, CD40 and B7 by DC (B. Stockinger, unpublished observation). On the other hand, the development of thymocytes is dependent on the presence of DC as well, since transgenic mice with conditional ablation of DC show severe thymic atrophy (Salomon et al., 1994).

Are activated tsDC terminally differentiated?

Despite clearcut, irreversible morphological and functional changes observed after contact of tsDC with T cells, they cannot be considered fully differentiated like DC isolated from lymphoid organs. They retain their abundant vesicles and the high rate of endocytosis. As mentioned above, antigen uptake and T cell priming are two functions of DC which seem to be confined to the immature and mature state of DC development, respectively. This separation may not be complete, since DC isolated from spleen, pulsed with protein, can prime T cells in vivo (Inaba et al., 1990). One could argue, that the splenic DC population consists of mature and immature DC, the latter being responsible for antigen uptake. This is supported by the loss of internalization capacity after over night culture (Inaba et al., 1990), in which immature DC presumably mature. However, Levine et al. (1992) reported efficient fluid phase endocytosis by splenic DC even after 24 h in culture. Moreover, cultured DC from spleen were shown to activate a HEL-specific T cell hybridoma with 100-fold less protein than LPS-activated B cells (Kleijmeer et al., 1995). Thus, endocytic capacity alone might not identify activated tsDC as still immature DC, especially because the efficiency of protein internalization in non-activated tsDC is enormously high, so that slight decreases in activated tsDC would be difficult to measure. In line with that, a

comparison of freshly isolated, versus cultured human blood DC resulted in less than a 2-fold decrease of endocytosed BSA-gold particles in the latter, and internalization of the heat shock protein 65 for presentation to a T cell clone was -albeit slightly decreased- still more efficient in cultured DC than in peripheral blood monocytes (Nijman et al., 1995). Based on the finding that internalized protein reached MHC class II containing compartments long before a significant T cell response could be observed, it has been suggested that antigen processing rather than internalization may be diminished in activated DC (Kleijmeer et al., 1995). The group hypothesized further that some class II containing compartments could represent storage vesicles for class II and that the balance between those compartments might be influenced by the activation state of the cell. However, upregulation of class II surface expression after activation was sensitive to cycloheximide and brefeldin A, indicating de novo biosynthesis. Unfortunately, the analysis of the distribution of class II molecules in non-activated versus activated tsDC was restricted to the latter, due to the lack of a more sensitive method of detection than antibody binding on Western blots after vesicle separation by density gradient electrophoresis. Nevertheless, other molecules involved in antigen processing and presentation, such as cathepsin D, were found in similar vesicle-fractions in non-activated tsDC, suggesting that antigen processing might not be influenced by the differentiation process.

Instead of decreasing their ability to internalize protein, activated tsDC seem to accelerate its transport through the endocytic pathway to lysosomes, which should rather optimize antigen presentation. The acceleration is suggested by the detection of internalized HRP in mainly the same fractions as the lysosomal enzyme β-hexosaminidase, in comparison to only partially overlapping fraction of HRP and β-hex in case of non-activated tsDC. Strikingly, this efficient uptake of HRP is not impaired in tsDC under conditions which allow receptor mediated endocytosis only, demonstrating their ability to capture antigen via receptors such as the mannose receptor (Sallusto et al., 1995). Another method of antigen capture, which is

constitutively active in DC, rather than growth factor stimulated as in macrophages, is macropinocytosis (Norbury et al., 1995; Sallusto et al., 1995). Indications for the macropinocytic activity of tsDC are their large vesicles revealed by EM studies as well as their ability to concentrate high amounts of FITC-Dextran within minutes (latter not shown). Again, no decrease in this ability could be observed in EM studies after activation of tsDC, although their more mature phenotype was apparent in the form of more and longer cytoplasmic processes.

The most convincing observation characterizing tsDC as not terminally differentiated is the level of MHC class II expression, which is upregulated due to increased class II biosynthesis similar to the cycloheximide sensitive class II upregulation on LC within the first 12 h of culture (Witmer-Pack et al., 1988), but remains still much lower than that found on mature DC. In fact, the relatively low levels of MHC class II may be limiting antigen presentation, given that antigen is internalized with such high efficiency. It seems possible, therefore, that essential factors required for terminal differentiation of tsDC are limiting or absent in the activation conditions used.

Which additional factors are required for final maturation?

It is conceivable, that immature DC require certain stimuli obtained from surrounding cells which cause maturation to some extent, before they interact with T cells, leading to mutual final activation. Immature DC in vivo are exposed to a complex mixture of cytokines and other growth factors, the composition and concentration of which can be altered by injury or infection. The best known example are LC in the skin, which are in close contact with keratinocytes producing IL-1, IL-3, IL-6, IL-7, IL-10, GM-CSF, M-CSF, TNF-α, platelet-derived growth factor (PDGF), transforming growth factor (TGF-) β and IFN-α and -β (Chang et al., 1994; Cooper et al., 1990; Enk and Katz, 1992[a]; Enk and Katz, 1992[b]; Koch et al., 1990; Xu et al., 1995), together with dendritic epidermal T cells producing IL-2 and IFN-γ upon stimulation (Havran et al.,

1989; Xu et al., 1995). It is not known, which of those factors are needed for the maintenance of LC. Accordingly, Xu et al. (1995) are able to grow murine epidermal DC clones or lines only in the presence of a skin-derived stromal cell line or its supernatant. Therefore, it may not be surprising that different factors have been reported to facilitate maturation of DC, which might have the opposite effect in another system. In mice e.g., TNFα was implied in the maintenance of LC in culture without induction of maturation (Koch et al., 1990), whereas immature DC generated from human peripheral blood mononuclear cells can be driven into maturity by TNFα (Sallusto and Lanzavecchia, 1994). Those differences possibly dependent on the exact differentiation state of the cells or the species (mouse versus man), or both. Other factors demonstrated to cause terminal DC maturation are LPS, IL-1 and CD40 ligand (Caux et al., 1994; Sallusto et al., 1995).

Although all of these stimuli have been used in the attempt to terminally differentiate tsDC, the concentration or sequence of administration might not have been correct. The treatment of tsDC with a mixture of cytokines produced by keratinocytes, followed by culture in the presence of ConA sup might result in further maturation, possibly even with subsequent treatment with LPS and CD40 ligand, since e.g. LPS leads to upregulation of only some markers, a partial activation which other factors might be able to supplement. In that way, the availability of a conditionally immortalized DC line should facilitate further analysis of the signals and molecules required for terminal differentiation.

Usage of tsDC for in vivo studies

A second outlook on the usefulness of the DC line are experiments in vivo, which require high numbers of DC, not varying in stage of maturity or purity. An interesting question which could be addressed with the use of tsDC is whether DC can pick up antigen in the periphery and present it for tolerance induction in the thymus. Although

it has been known for some time that DC in the periphery, such as epidermal LC, can capture antigen and present it in the draining lymph nodes (LN) (Larsen et al., 1990[b]; Macatonia et al., 1987), it remains unclear whether they can reach the thymus.

Migration of DC was reported to be restricted to some extent: blood DC migrated into the T cell area of the spleen, but not into LN when injected intravenously or when introduced in cardiac allografts. Traffic of subcutaneously injected DC into the draining LN was observed, but not to other locations (Austyn et al., 1988; Kupiec-Weglinski et al., 1988; Larsen et al., 1990[a]). These findings were challenged recently, when myeloid cells (monocytes, macrophages, DC) were found among B cell and T cell immigrants in the thymic medulla, which were derived either from congeneic spleen or lung implants, or from the periphery after labelling with bromodeoxyuridine (Westermann et al., 1996). All thymic immigrants were restricted to the medulla and represented up to 10% of all medullary cells. Of those, only 5% belonged to the myeloid lineage, demonstrating that sensitive methods are required for their detection.

TCR transgenic mice offer a sensitive system, in which the presence of few DC can be read out in either activation or deletion of T cells, depending on their location in the periphery or thymus, respectively. Preliminary experiments with C5 peptide pulsed tsDC, injected intrathymically into tg $C5^-$ mice, indicated the ability of tsDC to delete C5 reactive cells within 19 h. The injected cells were labelled with a fluorescent marker and could be visualized in the thymus by FACS. Intravenous injection of the same number of labelled tsDC (2×10^6) led to thymic deletion after 5 days, but no labelled cells were detected. This indicates that the effect on T cells serves as a more sensitive method to monitor DC migration and suggests that DC can indeed present peripheral antigen in the thymus for negative selection. Unfortunately, those preliminary results were not always reproducible, so that the experiments have to be repeated several times before final conclusions can be drawn. In addition, tsDC will be injected intraperitoneally and subcutaneously to investigate their ability to migrate into the thymus when they do not have direct access to the blood circulation. These studies

should answer the question of whether tolerance induction to tissue specific antigens, which per se do not have access to the thymus, can nevertheless be induced in the thymus by means of migrating dendritic cells.

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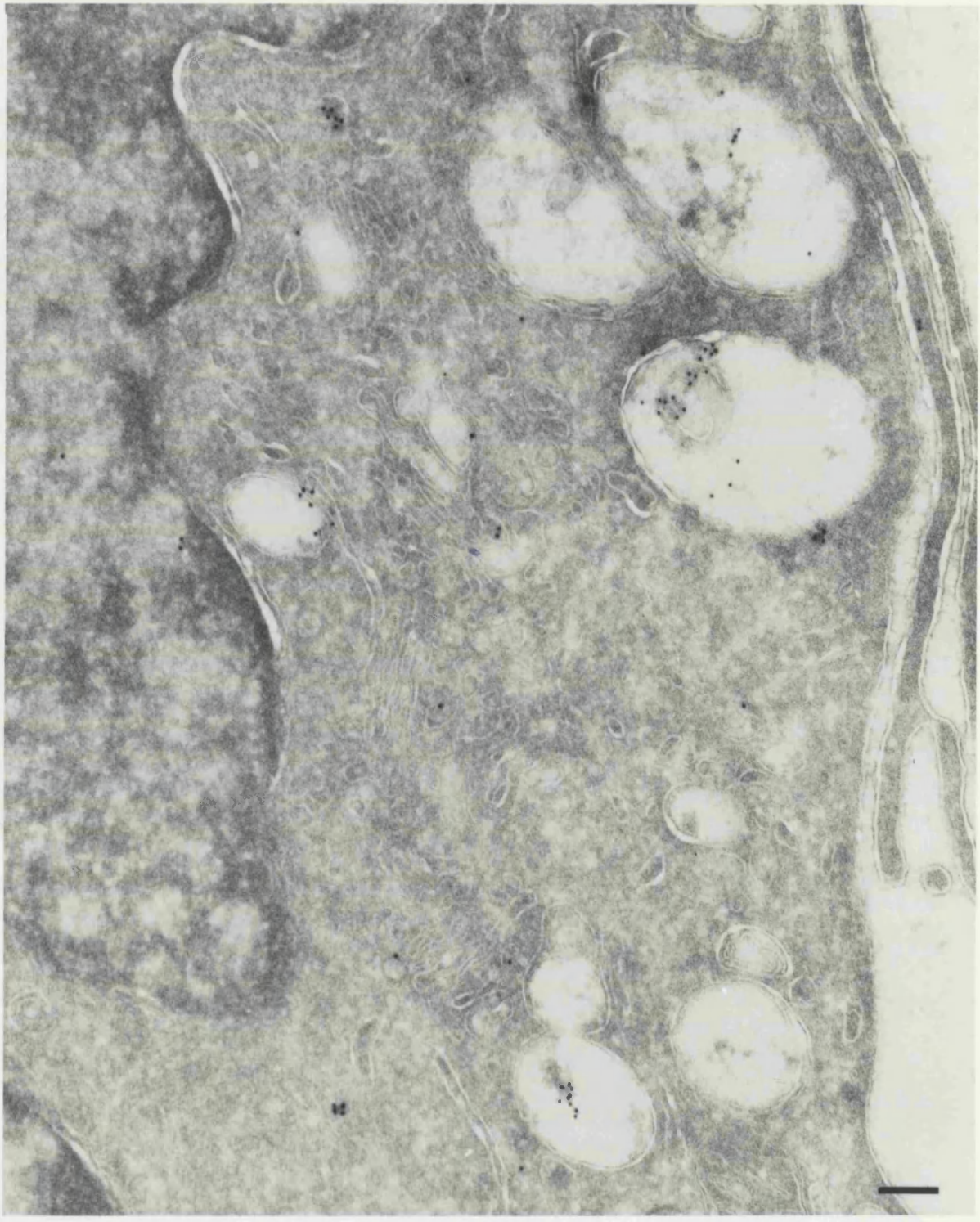

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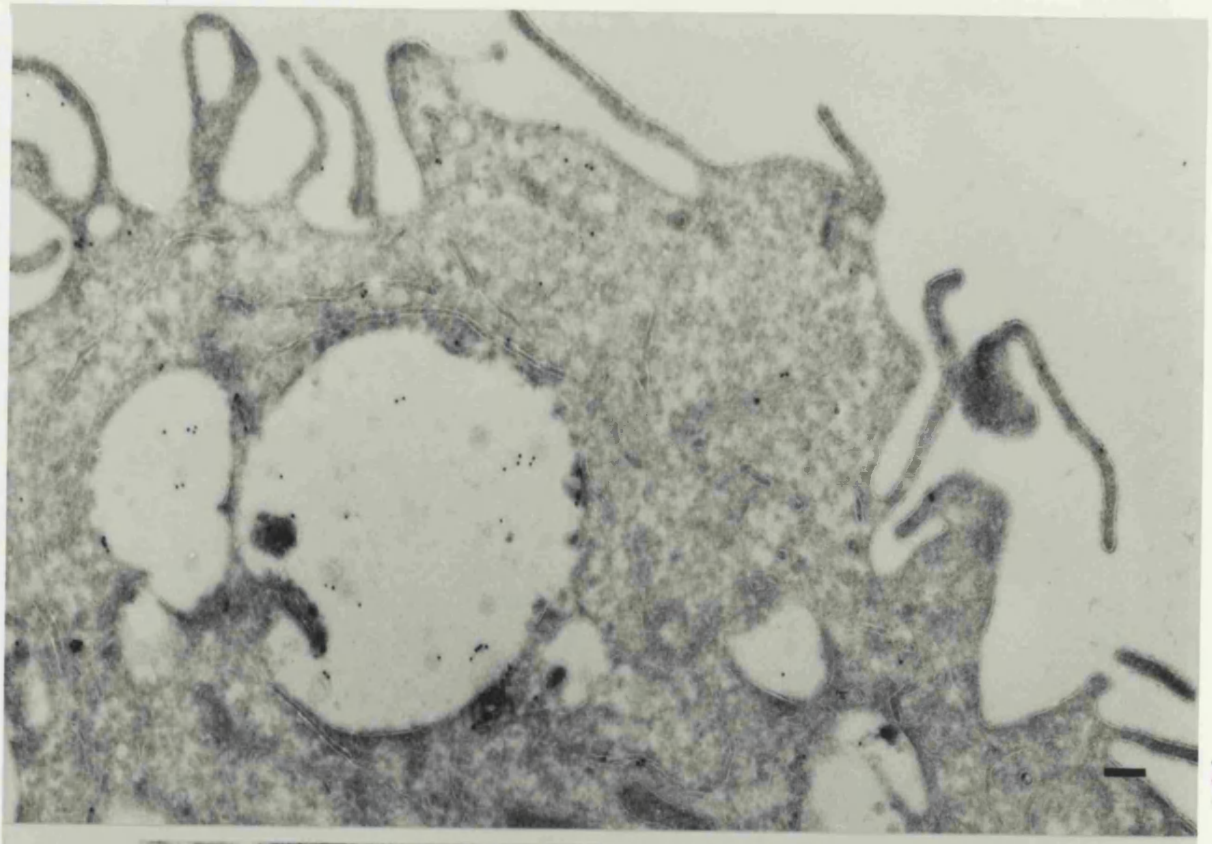

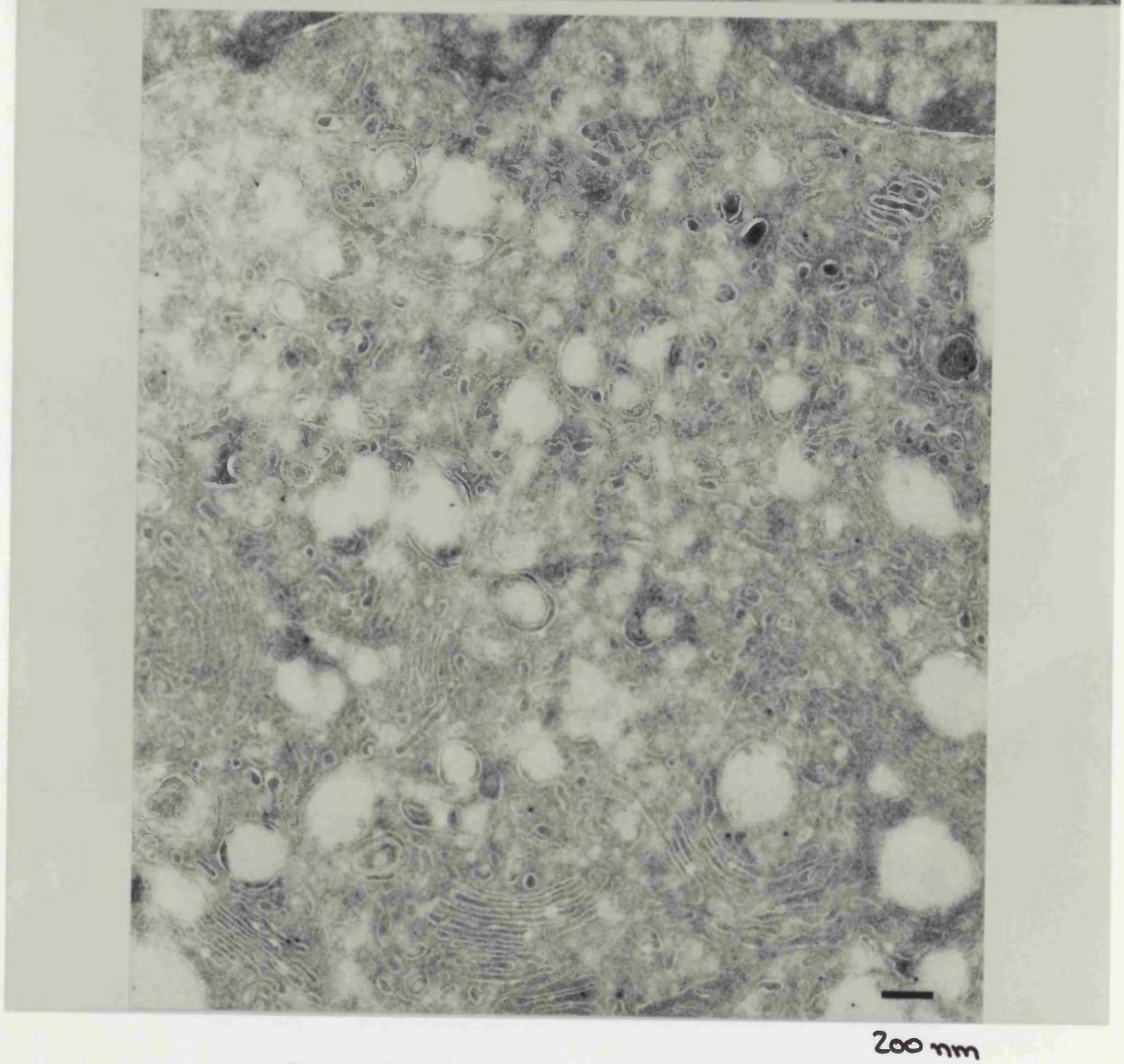

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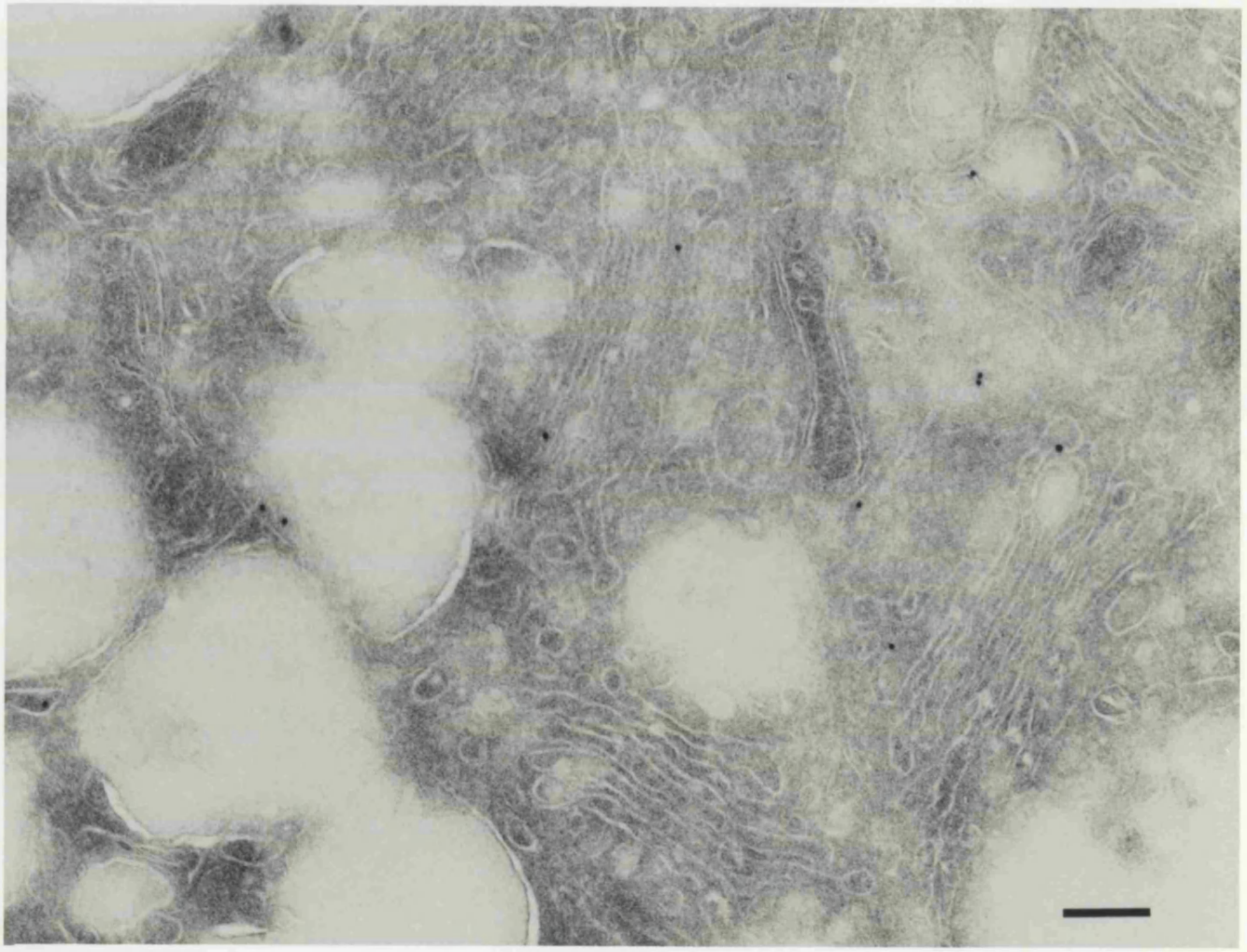

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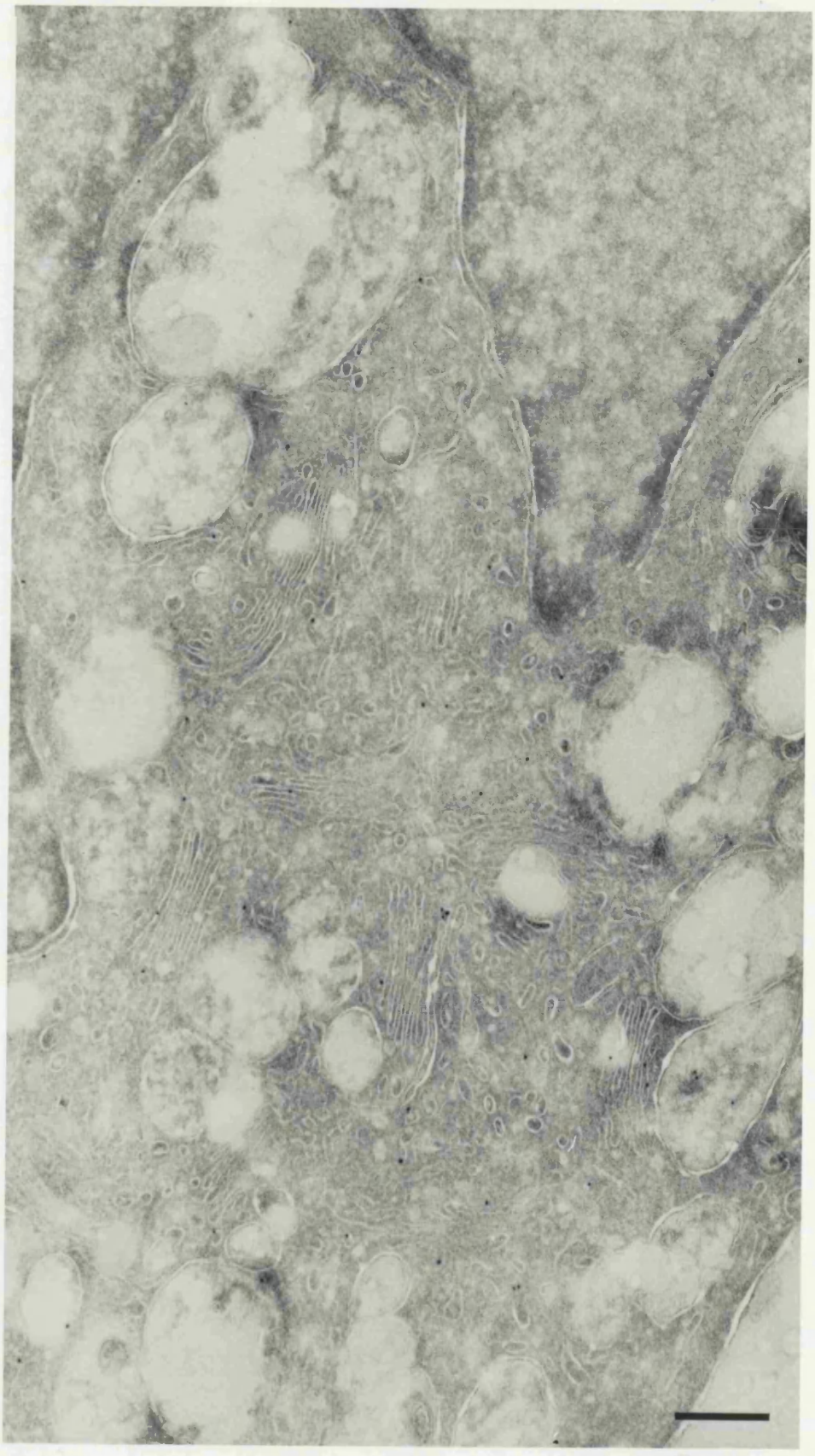

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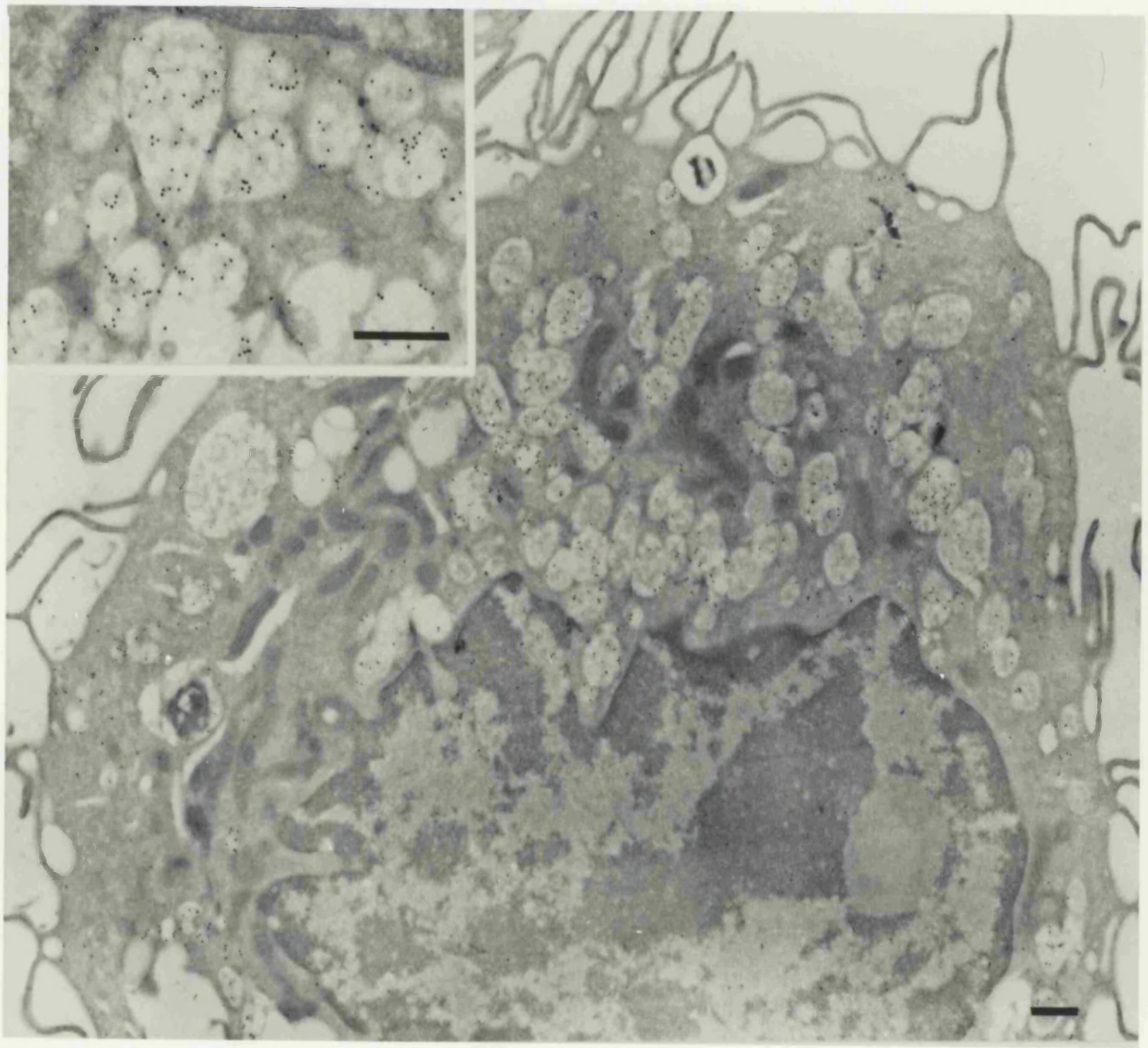

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