*Immunology* 1998 **95** 309–313

# The Ii41 isoform of invariant chain mediates both positive and negative selection events in T-cell receptor transgenic mice

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## SUMMARY

The functional role of invariant chain in T-cell selection events and antigen presentation is well established. The invariant chain gene encodes differentially spliced isoforms, Ii31 and Ii41. The Ii41 isoform has been described to increase the efficiency of antigen presentation. We have analysed the effect of the Ii41 isoform on positive and negative selection of transgenic CD4 T cells with specificity for a natural self antigen (C5) which are crucially dependent on invariant chain for their development and functional antigen recognition. The data show that Ii41 fully substitutes for wild-type invariant chain in both positive and negative selection events during functional maturation of T cells with specificity for a natural, blood-borne self antigen.

## INTRODUCTION

The major histocompatibility complex (MHC) class II-associated invariant chain is essential for the function of MHC class II molecules in T-cell development and immune responsiveness. Invariant chain facilitates folding of class II molecules, is responsible for transport of class II molecules into endosomal compartments and prevents their interaction with peptides prior to arrival in these compartments.[1–5] Invariant chain-deficient mice are severely compromised for MHC class II-associated functions, such as thymic positive selection of the CD4 T-cell lineage and processing/presentation of protein antigens within the class II presentation pathway.[6–8] The mouse invariant chain gene encodes two polypeptides, p31 and p41, which are produced by differential RNA splicing[9] and there is some, albeit not universal, evidence that Ii41 can enhance antigen presentation.[10] Invariant chain-deficient mice expressing only either the Ii31 or the Ii41 isoform have been generated by different means.[11–13] Detailed analysis of these mice indicated that both Ii31 and Ii41 on their own can reconstitute CD4 T-cell development and antigen presentation function in mice with a polyclonal T-cell repertoire. However, while, on the whole, selection of CD4 T cells is dependent on the presence of invariant chain, some CD4 T-cell specificities are invariant chain-independent in their selection.[14] In this paper we are addressing the role of the Ii41 isoform in reconstituting invariant chain function for the T-cell receptor (TCR) transgenic strain A18. This TCR is specific for the fifth component of complement (C5) a natural self antigen present in the blood circulation of normal mice, but absent in C5-deficient mice. This makes it possible to study both positive and negative selection under physiological conditions *in vivo*. The present data illustrate the dependency of C5-specific T-cell responses on the presence of the invariant chain and demonstrate that the Ii41 isoform alone is sufficient for both positive and negative selection.

Received 16 April 1998; accepted 18 June 1998.

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## MATERIALS AND METHODS

*Animals*

The C5 TCR transgenic mouse line A18 was previously described.[15] The invariant chain knockout mice[6] were obtained from Drs Diane Mathis and Christoph Benoist and were crossed onto an MHC H-$2^k$ background with or without C5 by backcrossing with either the C5-deficient ($C5^-$) strain A/J or the $C5^+$ strain CBA. The Ii41 strain described in ref. 16 likewise was crossed onto a $C5^+$ and a $C5^-$ background. A18 TCR transgenic mice were subsequently backcrossed onto the different invariant chain knockout or Ii41 strains to obtain A18 transgenic mice with $C5^+$ or $C5^-$ background without invariant chain or with the Ii41 isoform only. Typing of mice for invariant chain, A18 TCR expression and C5 status was done by polymerase chain reaction (PCR). A/J $C5^-$ mice are bred in the specific pathogen-free unit of the National Institute for Medical Research. All mice are housed under conventional conditions in the Mill Hill animal facilities.

*Fluorescence-activated cell sorter (FACS) analysis of thymus and antigen-presenting cells*

Expression of cell surface antigens on thymus cell suspensions was determined by cytofluorimetric analysis. Cells were triple stained with biotinylated antibody against the transgenic TCR β-chain Vβ8.3[17] fluorescein isothiocyanate (FITC)-conjugated anti-CD8[18] and phycoerythrin (PE)-conjugated anti-CD4 (Boehringer Mannheim, Mannheim, Germany). Aquisition

was performed on a FACScan (Becton Dickinson, San Diego, CA) using forward- and side-scatter characteristics to exclude dead cells.

Spleen antigen-presenting cells (APC) were double stained for cell type-specific markers and MHC class II (anti-H2-$E^k$, 14.4.4. Pharmingen, San Diego, CA). For identification of B cells the anti-B220 antibody RA3-6B2 (Pharmingen) was used, for dendritic cells the anti-CD11c antibody N418[19] and for macrophages the antibody F4/80.[20]

*Culture medium*

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

*Antigen presentation assays*

The function of mature peripheral T cells was tested in two ways. Either spleen cells from transgenic mice were enzyme digested with a cocktail of 1 mg/ml collagenase (Worthington CL4) and 0·2% DNase (Sigma) for 30 min at 37°. Cells were then cultured at a concentration of $2 \times 10^5$/well with different concentrations of C5 protein. Alternatively, the spleens were teased and then cultured at a concentration of $2 \times 10^5$/well with $2 \times 10^4$/well bone marrow-derived dendritic cells from normal A/J (invariant chain wild-type, $C5^-$) mice and different concentrations of protein. After 48 hr of co-culture, 100 μl aliquots of supernatant were transferred to fresh microtitre plates together with 5000 IL-2-dependent ATCC TIB214 CTLL cells per well. Incorporation of [$^3$H]thymidine by CTLL (present for the last 9 hr of culture) was measured 24 hr later.

*Bone marrow-derived dendritic cells*

Bone marrow-derived dendritic cells as APC were generated as previously described[21] with some modifications. Briefly, $5 \times 10^6$ bone marrow cells were cultured in Petri dishes (9-cm diameter, Nunc) in 10 ml culture medium containing 10% supernatant of Ag8653 myeloma cells transfected with murine granulocyte–macrophage colony-stimulating factor (GM-CSF) cDNA (≈25 U/ml). On day 4 of culture non-adherent cells, mostly granulocytes, were removed. Loosely adherent cells were transferred onto a second dish on day 6 of culture. From day 6 to day 10 these transferred cells were used as a source of dendritic cells. Their purity, assessed by MHC class II staining, was about 80%.

## RESULTS

To address the question of how expression of the invariant chain isoform Ii41 influences positive and negative selection of CD4 T cells, we have used transgenic mice expressing an MHC class II (H2-$E^k$)-restricted TCR (A18) specific for a natural, blood-borne self antigen, the serum protein C5. A18 transgenic mice develop $CD4^+$ T cells when crossed onto the $C5^-$ A/J background. In contrast, when crossed with $C5^+$ strains, where circulating C5 protein is present, thymocytes are deleted at the point of transition from double-positive to single CD4-positive cells.[15] As the A18 TCR is dependent on the presence of invariant chain for positive selection and function (unpublished data), this system offers the advantage of testing thymic selection events in the presence or absence of invariant chain and its Ii41 isoform for a naturally expressed self antigen.

### Thymic maturation in TCR transgenic mice dependent on the Ii41 isoform

FACS staining of thymus from six different TCR transgenic mouse strains was performed. These were: wild-type invariant chain A18 mice (wtA18), invariant chain knockout A18 ($Ii^-/^-$A18) or A18 with the Ii41 isoform of invariant chain only (Ii41A18); all of them either on a $C5^-$ (Fig. 1a–c) or a

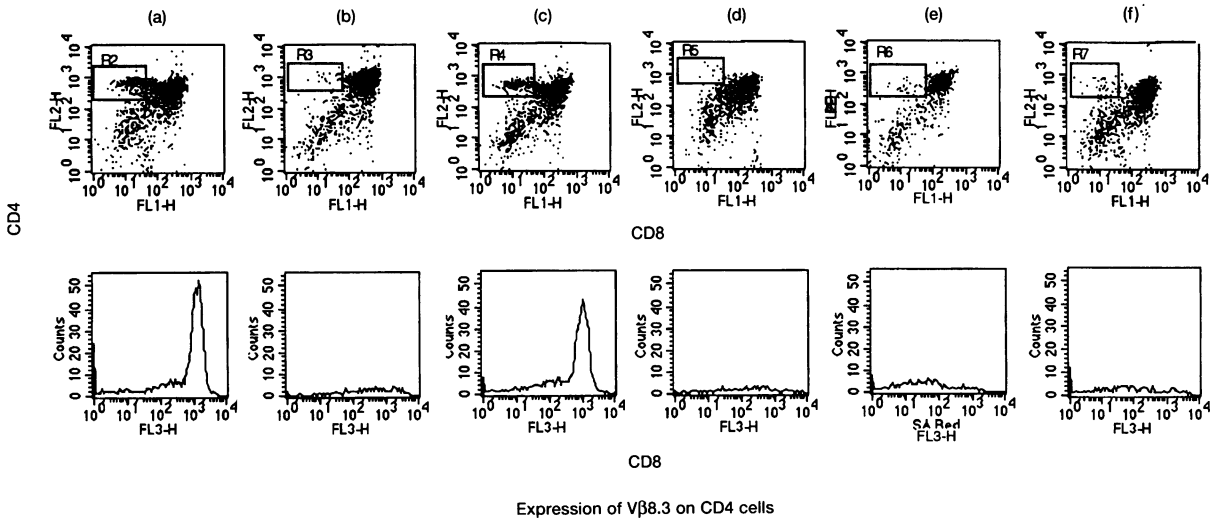


**Figure 1.** FACS analysis of thymus from six strains of mice. (a)–(c) are C5-negative A18 transgenic mice with either wild-type expression of invariant chain (a; wtA18), without invariant chain (b; $Ii^-/^-$A18) or with the Ii41 isoform of invariant chain (c; Ii41A18). (d)–(f) are $C5^+$A18 transgenic mice in the same order. Triple staining for CD4, CD8 and TCR β-chain was performed. Dot blots show the profiles of CD4 against CD8 and the histograms underneath show levels of TCR expression on gated CD4 single-positive cells.

$C5^+$ (Fig. 1 d–f) background. Wild-type A18 transgenic mice on a $C5^-$ background develop preferentially CD4 single-positive T cells due to the presence of the rearranged MHC class II-restricted A18 TCR. In the absence of invariant chain, the development of mature C5-specific T cells is inhibited. As these mice are not on a recombinase gene knockout ($Rag^{-}/^{-}$) background, a few CD4 T cells appear. These do not express the transgenic TCR and are generated by endogenous rearrangements of TCR genes. Thymus from Ii41-expressing A18 mice show normal positive selection indistinguishable from wild-type invariant chain A18 mice.

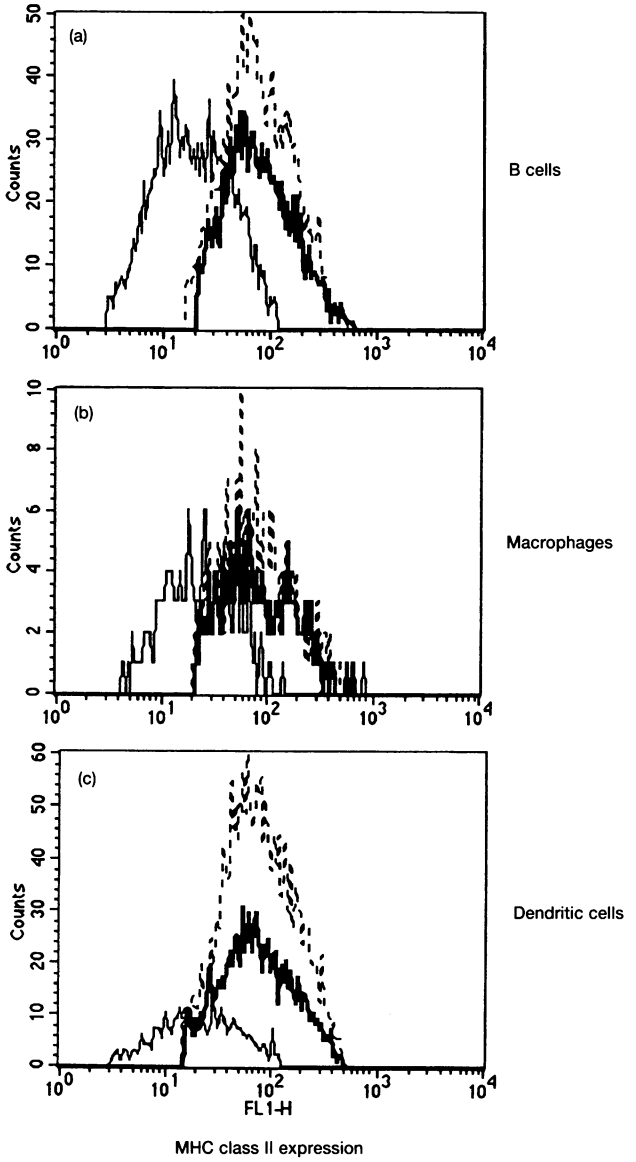


**Figure 2.** FACS analysis for expression of MHC class II on APC populations isolated from enzyme-digested spleen of either invariant chain-negative mice (thin line), invariant chain wild-type mice (stippled line) or Ii41-expressing mice (thick line). Double staining for cell type-specific markers and MHC class II was performed. The histogram overlays show MHC class II expression on cells gated for expression of cell type-specific markers. These are: (a) B220 expression for B cells, (b) N418 expression for dendritic cells and (c) F4/80 expression for macrophages.

Figure 1 (d–f) shows thymus staining for the three strains when crossed onto a $C5^+$ background. In the presence of C5 antigen which reaches the thymus via the blood circulation, mature CD4 T cells do not develop due to negative selection at the transition of immature double-positive to mature single-positive thymocytes.[15] The only CD4 cells visible do not express the transgenic TCR. $C5^+$ A18 mice expressing the Ii41 isoform (Fig. 1f) show negative selection indistinguishable from that seen in wild-type invariant chain A18 mice in Fig. 1(d). Thus, these data indicate that Ii41 can substitute for wild-type invariant chain both for positive selection of the A18 TCR, evident in mice which do not express the self antigen, and for negative selection in the presence of self antigen.

### Functional activity of peripheral T cells

To verify positive and negative selection events evident from the thymus phenotype, we analysed the functional activity of mature peripheral T cells from the six groups of mice. The functional assay was performed in two different ways. First, to assess both the antigen-presenting capacity of endogeneous APC and the responder status of the T cells, spleen cells were enzyme digested to allow optimal recovery of crucial APC such as dendritic cells, and then cultured with C5 protein. Firstly, we show in Fig. 2 FACS staining for expression of $H2\text{-}E^k$ on the three APC populations in the spleen, B cells, macrophages and dendritic cells. While APC from $Ii^{-}/^{-}$ mice show strongly reduced (but not absent) levels of MHC class II, Ii41 reconstitutes MHC class II expression on all types of APC to levels seen with wild-type invariant chain.

Figure 3(a) shows C5 specific T-cell responses following culture of enzyme-digested spleen cells with C5. The only mice which generated a C5-specific T-cell response were wild-type invariant chain A18 mice and mice expressing the Ii41 isoform, both of them on a $C5^-$ background. This emphasizes the role of invariant chain in positive selection and illustrates the capacity of Ii41 to substitute for wild-type invariant chain in this function. Furthermore, the data also show that negative selection in the presence of C5 self antigen causes functional

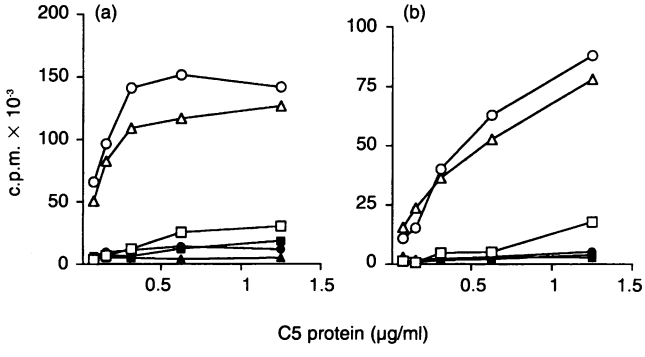


**Figure 3.** (a) Interleukin-2 (IL-2) responses of splenic T cells ($2 \times 10^5$/well) after enzyme digestion of spleen: (○) wild-type invariant chain A18, (□) $Ii^{-}/^{-}$A18 and (△) Ii41 $A18^-$ all on a $C5^-$ background. Closed symbols represent the same order of strains on a $C5^+$ background. (b) IL-2 responses of splenic T cells ($2 \times 10^5$/well) after culture with wild-type invariant chain dendritic cells ($2 \times 10^4$/well). Symbols are identical to those shown in (a). The figure shows incorporation of [$^3$H]thymidine into the IL-2-dependent cell line CTLL, giving the means for triplicate cultures.

tolerance not only in wild-type invariant chain mice, but also in mice carrying only the Ii41 isoform, since neither wild-type A18 nor Ii41 A18 mice on a $C5^+$ background could generate C5-specific T-cell responses.

Figure 3(b) shows splenic T-cell responses of the six strains of mice when cultured in the presence of dendritic cell APC from wild-type invariant chain mice. The presence of dendritic cells with wild-type expression of invariant chain should allow detection of any C5-specific CD4 T cells, which might have escaped detection in the previous assay. This might be the case if the relative deficiency in MHC class II expression of endogenous APC was to compound the defect in positive selection in invariant chain-negative mice. However, the results were identical to the experiments shown in Fig. 3(a), emphasizing that in the absence of invariant chain positive selection of the A18 TCR does not take place. Ii41 fully reconstituted the function of invariant chain, allowing both positive selection and function of CD4 T cells from mice which do not contain the self antigen, as well as negative selection and functional tolerance in cells from mice which express C5.

## DISCUSSION

Despite the fact that the two isoforms of invariant chain, generated by differential splicing, were shown to have equivalent effects on class II biosynthesis, folding, egress from the endoplasmic reticulum and prevention of premature peptide binding, cell biologists and immunologists continue to search for distinctive roles for these two molecules. A number of intriguing features of Ii41 suggested that it might play a distinct role from Ii31. Ii41 contains a cysteine-rich motif, first identified as repeat sequence of thyroglobulin and it was speculated that this motif might play a role as a carrier in the transport of molecules, such as processed antigen.[22] One report suggested that Ii41, but not Ii31, enhances antigen presentation,[10] but this was not universally the case.[11,14,23] Furthermore, Ii41 can influence the degradation of Ii31, suggesting an ability to modify post-translational transport or processing of class II/Ii complexes.[24] A fragment of Ii41 was found to be a potent inhibitor of the lysosomal protease Cathepsin L,[25] but so far no antigen has been found which strictly requires Ii41 instead of Ii31 for presentation. In the meantime, a number of groups have generated transgenic mice expressing the Ii31 or Ii41 isoforms either under the class II promoter[11,13] or under the endogenous Ii promoter.[14,16] Regardless of differences in expression levels found for the various mice, the consensus finding was that in all cases both Ii31 and Ii41 seemed to substitute fully for wild-type invariant chain; that is, they allowed positive selection of CD4 T cells and normal T-cell function as far as this was testable in a normal polyclonal repertoire. Negative selection has not been previously studied in this model.

In this paper we have for the first time investigated the role of the Ii41 invariant chain isoform in positive as well as negative selection of transgenic T cells carrying a TCR-specific for a natural self antigen. We chose to analyse these functions in a transgenic system, since not all CD4 T cells in a polyclonal repertoire are totally dependent on invariant chain, presumably due to the fact that MHC class II can acquire some endogenous peptides in the absence of invariant chain.[26–30] This might obscure the relative role of invariant chain isoforms in rescuing T-cell selection and function. We therefore crossed transgenic mice expressing a TCR which is dependent on invariant chain with either invariant chain knockout or Ii41 transgenic mice. Our data show that the presence of Ii41 was sufficient to allow normal CD4 T-cell selection in the thymus of A18 mice and guaranteed the responsiveness of peripheral transgenic T cells to C5 antigen. MHC class II expression on splenic APC subpopulations from Ii41 or wild-type Ii mice was comparable. Crossing the mice onto a $C5^+$ background in addition gave us the opportunity to assess for the first time the role of Ii41 in thymic negative selection to an endogenously present self antigen. Again we observed no difference in the capacity for thymic negative selection in A18 transgenic mice expressing wild-type Ii compared with Ii41 A18 mice.

Thus we conclude that the Ii41 isoform alone is sufficient to reconstitute all functions of invariant chain in T-cell selection and function of an invariant chain-dependent transgenic TCR.

## ACKNOWLEDGMENTS

This work was supported by the Medical Research Council, UK and NIH grant #AI-19047.

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