Soluble MHC-II proteins promote suppressive activity in $CD4^+$ T cells

Katerina Bakela,[1] Nikos Kountourakis,[2] Michalis Aivaliotis[2] and Irene Athanassakis[1]

[1]*Department of Biology, University of Crete, Heraklion, Crete, and* [2]*Institute of Molecular Biology and Biotechnology, Forth Heraklion, Crete, Greece*

doi:10.1111/imm.12360
Received 18 May 2014; revised 15 July 2014; accepted 18 July 2014.
Correspondence: Prof. Irene Athanassakis, Department of Biology, University of Crete, PO Box 2208, 714-09 Heraklion, Crete, Greece. Email: athan@biology.uoc.gr
Senior author: Irene Athanassakis

Summary

Soluble MHCII (sMHCII) molecules are present in body fluids of healthy individuals and are considered to be involved in the maintenance of self tolerance, and are also related to various diseases. Their concentration increases during *in vivo* antigen-specific tolerogenic stimulation and it was recently shown that exosome-mediated tolerance is MHCII dependent. At the cellular level, sMHCII proteins compete with membrane MHCII for T-cell receptor binding on $CD4^+$ T cells. Immunoaffinity purification techniques isolated sMHCII antigens from the serum of human serum albumin (HSA) -tolerant mice as a single highly glycosylated protein of ~ 60 000 molecular weight, specifically interacting with anti-class II antibodies in Western blotting and ELISA. Mass spectroscopy showed that these sMHCII proteins were loaded with the tolerogenic peptide as well as multiple self peptides. At the cellular level, sMHCII suppressed antigen-specific, and to a lesser degree antigen-non-specific, spleen cell proliferation and induced CD25 in naive T cells. In T cells activated by antigen-seeded macrophages, sMHCII decreased CD28 and increased CTLA-4 protein expression, while decreasing interleukin-2 and increasing interleukin-10 production. In this case, sMHCII proteins were shown to decrease ZAP-70 and LAT phosphorylation. The results presented here for the first time provide evidence for the role of sMHCII proteins in immune response suppression and maintenance of tolerance, revealing novel regulatory mechanisms for immune system manipulation.

Keywords: immunosuppression; soluble MHCII proteins; T cells; tolerance.

Introduction

Class II MHC antigens have been defined as membrane-bound heterodimers composed of glycosylated α and β chains of 30 000–33 000 and 27 000–29 000 molecular weight (MW) respectively, each chain containing two immunoglobulin-like domains, a trans-membrane and a cytoplasmic tail. In early 1967, Calne *et al.*[1] observed that some liver-transplanted pigs could survive for several months after receiving the allograft without any immunosuppressive treatment. This puzzling effect was later found to be due to soluble factors secreted by the liver allograft itself, which were responsible for the development of tolerance to the host and were identified as soluble MHC (sMHC) molecules.[2] Thereafter, many laboratories attempted to define the immunoregulatory effects of the various soluble forms of MHC molecules. It is now widely accepted that body fluids isolated from healthy individuals contain various amounts of soluble MHC class I (sMHCI) and class II (sMHCII) molecules.[2–6]

Although circulating MHCI proteins have been detected in the serum in various isoforms,[7] sMHCII appear to have a molecular weight of ~60 000, which is higher than the molecular weight of membrane MHCII.[8] The origin of these molecules has been hypothesized to

Abbreviations: HSA, human serum albumin; IL-2, interleukin-2; mAb, monoclonal antibody; MBP, myelin basic protein; MS, mass spectroscopy; MW, molecular weight; PE, phycoerythrin; sMHCII, soluble MHCII; TCR, T-cell receptor

be the result of shedding, alternative splicing or active secretion. Although the molecules that circulate in the serum are not engaged into exosomes,[9] there are studies showing that exosomes secreted by antigen-presenting cells contain large amounts of MHC molecules.[9] It has also been demonstrated that trophoblast cells secrete soluble HLA-DR molecules into the culture medium when stimulated by interferon-γ,[10,11] whereas B cells and $CD4^+$ T cells secrete HLA-DR upon interleukin-2 (IL-2) activation.

Some of the physiological roles attributed to sMHC antigens (class I and class II) include maintenance of self tolerance,[12,13] healthy functioning of the central nervous system[14] and mating preference.[15] Mating preference is an important evolutionary task; it has been postulated that choice of partner is based on histocompatibility difference to ensure the propagation of MHC polymorphism and consequently species survival. This property has been attributed to the so-called odortypes which are considered to be sMHC molecules present in the urine.[16]

Changes within the physiological concentrations of sMHC molecules have been recorded in numerous pathological conditions, such as viral encephalitis,[6] rheumatoid arthritis,[17–19] pathological pregnancies,[5,20] asthma,[21] AIDS,[6] chronic hepatitis C and acute chronic uveitis.[22] These results imply that soluble MHC molecules have a special role in the pathology of these diseases, but it is not clear whether they contribute to the pathology or whether they constitute by-products of the pathological condition.

The involvement of sMHCII molecules in the maintenance of tolerance could be related to suppression mechanisms. In the early 1980s, T-suppressors were suggested to exert their effect via suppressor factors bearing class II MHC determinants.[23] Tolerized $CD4^+$ T cells were shown to secrete MHC-dependent tolerosomes,[24] while Almqvist *et al.*[25] suggested that tolerance was mediated by MHCII-dependent exosomes. Other studies have demonstrated that sMHCII could compete with membrane MHCII for T-cell receptor (TCR) binding and modulate cellular and humoral immune responses,[26] induce apoptosis of $CD4^+$ cells,[27] activate $CD8^+$ T cells[28] and down-regulate natural killer cell activity.[29]

Circulating MHCII molecules loaded with self peptides have been hypothesized to maintain tolerance,[12,13] while antigen-specific tolerogenic stimulation has been shown to increase serum MHCII proteins when compared with the corresponding immunogenic stimulus in mice *in vitro* as well as *in vivo*.[30] The present study concentrated on sMHCII molecules isolated from the serum of BALB/c tolerized mice and examined their biochemical features, their immunomodulatory effects as well as their effect on marker expression and signalling of naive and antigen-stimulated CD4-positive cells.

Materials and methods

Animals

BALB/c ($H\text{-}2^d$) inbred mice were purchased from Charles River (Milan, Italy) and bred in the animal facility of the Department of Biology at the University of Crete (Crete, Greece) under standard conditions of temperature (18–25°), humidity (45–50%) and photoperiod of 12 hr light and 12 hr dark. Males 4–8 weeks of age were handled according to the international and national bioethical rules and the study conformed to the bioethics regulations of the University of Crete, approved by the Animal Facility responsible officer of the Department of Biology. Mice were killed by cervical dislocation and spleens were removed under aseptic conditions.

Antibodies

Mouse anti-IA/IE monoclonal antibody (mAb) (HB-225TM hybridoma: *Mus musculus* (myeloma), hamster, Armenian B cell, reacts with a monomorphic determinant on the I-A and I-E region, IgG isotype, generous gift from Dr R Steinman, Rockefeller University, New York, NY) was purified from culture supernatants and used at a concentration 0·1 μg/ml for ELISA experiments, at 0·01 μg/ml for Western blot and was covalently linked to magnetic beads coupled with sheep anti-mouse IgG (see below). For immunofluorescence experiments, phycoerythrin (PE) -labelled mouse anti-CD152 mAb (IgG, produced in Syrian hamster; BioLegend, San Diego, CA), PE-labelled mouse anti-CD28 (IgG, produced in Armenian hamster; BioLegend) and PE-labelled mouse anti-CD25 (IgG1, produced in rat; EuroBioSciences, Friesoythe, Germany) were used at a concentration of 1 μg/ml. Furthermore, FITC-labelled mouse anti-CD4 (IgG2b, produced in rat; EuroBioSciences) was used for cell sorting techniques at 1 μg/ml. Finally, mouse anti-IL-2 (IgG2a, k, produced in rat; ImmunoTools, Friesoythe, Germany) and mouse anti-IL-10 (IgG2b, k, produced in rat, ImmunoTools) were used at a concentration of 0·1 μg/ml for ELISA experiments. Goat anti-mouse IgG (Fab fragment) secondary antibody coupled to peroxidase (Sigma, Munich, Germany) was used at a concentration of 0·02 μg/ml. The antibodies used for TCR signalling evaluation included purified rabbit anti-mouse ZAP-70, purified rabbit anti-mouse phospho-ZAP-70 (Tyr319)/Syk (Tyr352) (65EA), purified rabbit anti-mouse LAT, purified rabbit anti-mouse phosphor-LAT (Tyr191), purified rabbit anti-mouse Lck, purified rabbit anti-mouse phospho Lck (Tyr505) and were purchased from Cell Signaling Technology (Boston, MA). In all cases the above antibodies were used at a concentration of 0·1 μg/ml. Horseradish peroxidase-conjugated anti-rabbit IgG (produced in goat, NIDA,

IMBB-FORTH, Heraklion, Greece) was used at a concentration of 0·02 μg/ml.

Purification of sMHCII proteins

Dynabeads M-280 sheep anti-mouse IgG (Dynabeads M-280, 2·8 μm superparamagnetic beads with affinity-purified polyclonal sheep anti-mouse IgG1, IgG2a, IgG2b; Life Technologies, Carlsbad, CA) were cross-linked with the mouse anti-IA/IE HB-225TM mAb and were used for the isolation of sMHCII proteins following the instructions of the manufacturer. Briefly, 10^8 Dynabeads M-280 sheep anti-mouse IgG were coupled to 15 μg HB-225TM immunoglobulin with rotational mixing for 60 min at 4°. After washing the beads twice using a magnet with 1 ml PBS (pH 7·2), 1 ml 0·2 M triethanolamine (pH 8·2) was added to the magnetic beads with the immobilized HB-225TM immunoglobulin. The beads were thereafter washed twice with 1 ml 0·2 M triethanolamine (pH 8·2), resuspended in 1 ml of 20 mM dimethyl pimelimidate dihydrochloride (DMP; Pierce, Rockford, IL) in 0·2 M triethanolamine, pH 8·2 (5·4 mg DMP/ml buffer) and incubated with rotational mixing for 30 min at 25°. After removing the supernatants, the reaction was stopped by resuspending the beads in 1 ml of 50 mM Tris–HCl, pH 7·5 and incubating for 15 min with rotational mixing. The cross-linked Dynabeads were washed three times with 1 ml PBS, resuspended in 1 ml mouse serum (1 : 1 volume/volume in PBS) and incubated with rotational mixing for 2 hr at 4°. After washing twice with 1 ml PBS, elution was performed using 2 M NaCl, with rotational mixing for 20 min at 25°. The recovered (1 ml) sMHCII protein was dialysed against PBS and concentrated using centrifuge filters (cut off 10 000 MW; centricon 10; Amicon Inc., Beverly, MA).

In some experiments, sMHCII protein was treated with glycoprotein denaturation buffer (10 ×, New England Biolabs, Ipswich, MA) and cleaved with PNGase F protease (New England Biolabs) according to the manufacturer's instructions.

SDS–PAGE/Western blot

Purified (as described in the previous paragraph) sMHCII protein (20 μl) was loaded onto a 12% SDS–polyacrylamide gel and run in an electrophoresis apparatus (GibcoBRL, Gaitherburg, MD). Proteins were visualized by silver staining.[31] For Western immunoblot analysis, the SDS–PAGE-separated proteins were transferred electrophoretically to nitrocellulose membrane and after incubation with the first HB-255 and secondary mouse IgG-POD (20 mU) antibodies, the blot was developed using the Super Signal West Pico chemiluminescence substrate (Thermo Scientific, Waltham, MA). The analysis of bands was performed using the KODAK DIGITAL SCIENCE 1DTM software (Eastman Kodak, Rochester, NY).

Mass spectrospcopic analysis

For mass spectroscopic (MS) analysis, the SDS–PAGE-separated sMHCII proteins were stained with the MS-compatible blue silver staining protocol according to Candiano *et al.*[32] The selected gel bands were excised from the gel and further cut into ~1-mm pieces to perform in-gel tryptic digestion according to Huynh *et al.*[33] without reduction and alkylation due to the absence of cysteines in the protein. The resultant peptide mixture was dried in a SpeedVac Concentrator (Savant ISS110; Thermo Scientific) and reconstituted in 0·5% formic acid aqueous solution before nano liquid chromatography (nLC) MS/MS analysis. As a control, a gel piece from an area not corresponding to the protein band area of the gel was also excised and digested to determine the protein background.

ELISA techniques

Indirect ELISA was performed to verify the purified sMHCII molecules as well as to detect IL-2 and IL-10 cytokines in cell supernatants and the phosphorylated or not isoforms of Lck, ZAP-70 and LAT in $CD4^+$ cell extracts. Briefly, samples were diluted in 0·05 μ $NaHCO_3$, 0·05 M Na_2CO_3 in H_2O_{dist} (pH = 9·6; coating buffer) coated in 96-well flat-bottom plates (Sarstedt, Nümbrecht, Germany), incubated overnight at 4° and washed three times in 5% Tween-20. The remaining protein-free sites in the plate were blocked by 200 μl/well of 2% PBS-BSA (Albumin Fraction V; Applichem, Saxony-Anhalt, Germany) solution upon incubation for 2 hr at room temperature. After washing three times, 100 μl of test antibodies diluted in 0·1% PBS-BSA was added and incubated for 2 hr at room temperature. Extensive washing of the plate was followed by addition of 100 μl of goat anti-mouse IgG coupled to horseradish peroxidase and incubation for 1 hr at room temperature, in the dark. Finally, the reaction was developed by adding 100 μl/well of tetramethylbenzidine-H_2O_2 (TMB Substrate Kit; Thermo Scientific) for 5 min. The enzyme reaction was stopped with 50 ml H_2SO_4 (1 M). Optical density (OD) was measured at 450 nm using a Titertec ELISA photometer (Digiscan, GmbH; ASYSHitech, Engendorf, Austria).

Immunization and tolerization protocols

Human serum albumin (HSA; albumin from human serum; Sigma) was used for *in vitro* culture immunization at the concentration of 1 μg/ml and 100 μg per mouse for the i*n vivo* immunization. SecA protein (SecA, *Staphylococcus aureus*) was used for *in vivo* immunization at

26 μg/mouse and myelin basic protein (MBP; bovine; Sigma) was used for *in vitro* immunization at 1 μg/ml. For the *in vivo* immunization protocols, mice were injected intraperitoneally with HSA or SecA mixed with an equal volume of complete Freund's adjuvant (1·0 mg *Mycobacterium tuberculosis*; Sigma) and 7 days later with HSA or SecA mixed with an equal volume of incomplete Freund' s adjuvant (Sigma). On day 15, spleen cells were isolated. Tolerization was conducted by injecting 100 μg/mouse of HSA in the absence of adjuvant. Blood was collected 6 days later and serum was isolated.

Cell proliferation assays

Spleen cells were cultured in 96-well V-bottomed plates (Sarstedt) at a concentration of 1×10^6 cells/well in RPMI-1640 culture medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum (Gibco) at a final volume of 200 μl with or without HSA, SecA or MBP (all in 1 μg/ml) and with or without sMHCII (30 ng/ml) and processed for [^{3}H]TdR incorporation assays after 4 days of culture. The cultures were pulsed with 1 μCi of [^{3}H]TdR (ICN, Costa Mesa, CA) 18 hr before harvest. After transferring the cells on to cellulose filters, these were put in scintillation fluid (toluene-omnifluor; 1·38 g/l, NEN-PerkinElmer, Waltham, MA) and counted using an LKB beta-counter (Turku, Finland).

CD4$^+$ cell purification and immunofluorescence

Spleen cells were cultured for 24 hr in 10-mm Petri dishes at 1×10^6 cells/ml. Upon elimination of adherent cells, the remaining cells were washed twice in PBS (1 ml) and incubated with PBS-BSA 3% buffer to block the remaining protein-free sites at room temperature for 30 min. After extensive washing with PBS, the cells were incubated with FITC- or PE-conjugated antibodies. After washing, the cells were diluted in the appropriate volume of PBS and processed for FACS Cell-Sorter isolation or flow cytometry analysis. In the case of cell sorting, the cells were set in culture for further experimentation.

CD4$^+$ cell extracts

CD4$^+$ cells (4×10^6) were cultured for 24 hr in presence or not of sMHCII (30 ng/ml) either alone or in co-culture with 4×10^6 macrophages that were previously activated with 10 μg/ml HSA for 2 days. Upon culture termination, CD4$^+$ cells were collected, washed with 1 ml PBS and lysed with 25 μl/10^6 cells 1 × SDS buffer (62.5 mM Tris–HCl, 2% weight/volume SDS, 50 mM dithiothreitol). The cells were sonicated for 10–15 seconds to shear DNA and reduce sample viscosity and immediately stored at −20°. Cell extracts (dilution of 1/40 in coating buffer) were tested for the presence of phosphorylated or not isoforms of Lck, ZAP-70 and LAT by ELISA.

Statistical analysis

Paired Student's *t*-test was used to compare the significance levels (P) between control and test values. Statistical analysis was performed using the ORIGIN PRO 8 program.

Results

Previous studies have defined the *in vitro* and *in vivo* tolerogenic protocols in mice using HSA as the antigenic stimulus. In the present study, sMHCII proteins were isolated from sera of mice that had received the HSA tolerogenic protocol. Upon purification, sMHCII proteins were tested for their specificity and functional activity on *in vitro* as well as *in vivo* developed immune response.

Isolation and identification of sMHCII

Soluble MHCII molecules were isolated from serum of HSA-tolerant mice using anti-IgG magnetic beads cross-linked with an anti-mouse class II mAb. Upon sMHCII isolation, protein concentration was evaluated using the Lowry method. Following the purification procedures described herein, 400–500 μg/ml sMHCII could be isolated from 1 ml of HSA-tolerant mouse serum. The concentration of sMHCII protein isolated from control mouse serum was four times lower than sMHCII isolated from HSA-tolerant mouse serum ($P < 0{\cdot}005$; data not shown). The purity and specificity of sMHCII were evaluated by SDS–PAGE chromatography, Western blot analysis and ELISA (Fig. 1). The SDS–PAGE chromatography revealed a single band at ~60 000 (Fig. 1a) which specifically reacted with an anti-MHCII mAb in Western blot analysis (Fig. 1b). Soluble MHCII molecules underwent further ELISA, where titration of purified sMHCII using a specific anti-MHCII mAb resulted in a linear equation ($y = 272.08x$, $R^2 = 0{\cdot}9773$), verifying at the same time the specific recognition of sMHCII molecules by the HB225 mAb (Fig. 2c).

All efforts to dissociate the heterodimer, including different time-points of boiling at 95°, up to 8 M urea or high salt concentration treatments, failed to reveal the α and β chains of sMHCII molecules (data not shown). However, these proteins were shown to be glycosylated, as *N*-glycosylase digestion revealed two bands in SDS–PAGE chromatography at 60 000 and 40 000 MW, respectively, apparently as the result of incomplete enzymatic reaction (Fig. 2a). The 40 000 band should correspond to the non-glycosylated product, whereas the 60 000 band should represent the initial glycosylated protein. In an effort to sequence sMHCII proteins, MS

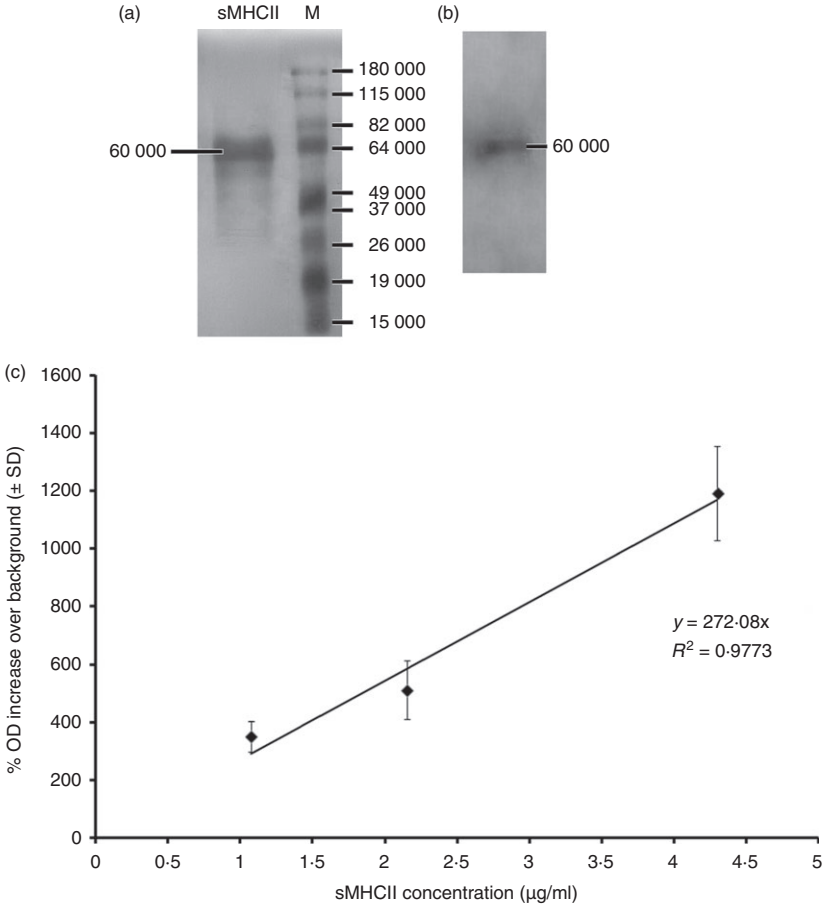

Figure 1. Characterization of soluble MHC class II (sMHCII) proteins isolated from human serum albumin (HSA) -tolerant mouse serum. SDS–PAGE reveals a 60 000 molecular weight band (a) which reacts with the HB225 monoclonal antibody in Western blot analysis (b). The experiments have been repeated at least five times and similar results were obtained. The sMHCII proteins underwent ELISA using the HB225 class II specific monoclonal antibody. The titration curve (c) corresponds to sMHCII proteins isolated from 1 ml of mouse serum. The results represent the mean of five experiments.

analysis was performed. Native or de-glycosylated sMHCII were cut from a specifically prepared SDS–PAGE gel, digested with trypsin and submitted to MS analysis. Except for the identification of the Aβ chain of class II MHC, the analysis of the serum albumin by tandem MS coupled on line with nLC after tryptic digestion resulted in its confident characterization with high sequence coverage of 75·66% (Fig. 2b). The large number of self peptides identified in the sMHCII preparations indicates that sMHCII molecules were indeed loaded with the tolerogenic peptide as well as an abundance of self peptides (Fig. 2b, see Supporting information, Tables S1 and S2).

Effect of sMHCII on *in vitro* and *in vivo* immunized spleen cells

The biological effect of sMHCII on antigen-specific and non-specific cell proliferation was evaluated after *in vitro* as well as *in vivo* immunization protocols, using [^{3}H]TdR incorporation assays. Hence, spleen cells from control mice were treated *in vitro* using HSA or MBP protein at a concentration of 1 μg/ml, which was previously shown to be an immunogenic dose,[30] in the presence or not of sMHCII. In the absence of antigenic stimulus, sMHCII caused a non-statistically significant decrease of cell proliferation. However, sMHCII was able to reduce the antigen-specific response by 65% ($P < 0{\cdot}001$) and antigen-non-specific response by 59% ($P < 0{\cdot}001$) compared with the HSA- and MBP-treated spleen cell cultures, respectively (Fig. 3a). These results indicated that although sMHCII proteins were isolated from HSA-tolerant mice, they could suppress other self-antigen responses. However, such results were expected, because except for increasing the concentration of HSA-loaded sMHCII in the serum of HSA-tolerant mice, natural sMHCII loaded with self peptides should be present in the serum and exert equally well a suppressive activity. Indeed, analysis of MBP by tandem MS coupled on line with nLC after tryptic digestion resulted in its confident characterization by one tryptic peptide corresponding to a 19·53% protein sequence coverage, which was confirmed by its MS/MS spectrum showing confident identification with high fragment coverage (Fig. 3b).

To evaluate whether sMHCII could suppress an immune response generated *in vivo*, spleen cells were isolated from mice immunized *in vivo* with HSA (100 μg/mouse) or a control *Staphylococcus aureus* protein, SecA (100 μg/mouse). Hence, spleen cells from HSA- or SecA-immunized mice were cultured in the presence of HSA or SecA with or without sMHCII (Fig. 3c,d). Using spleen

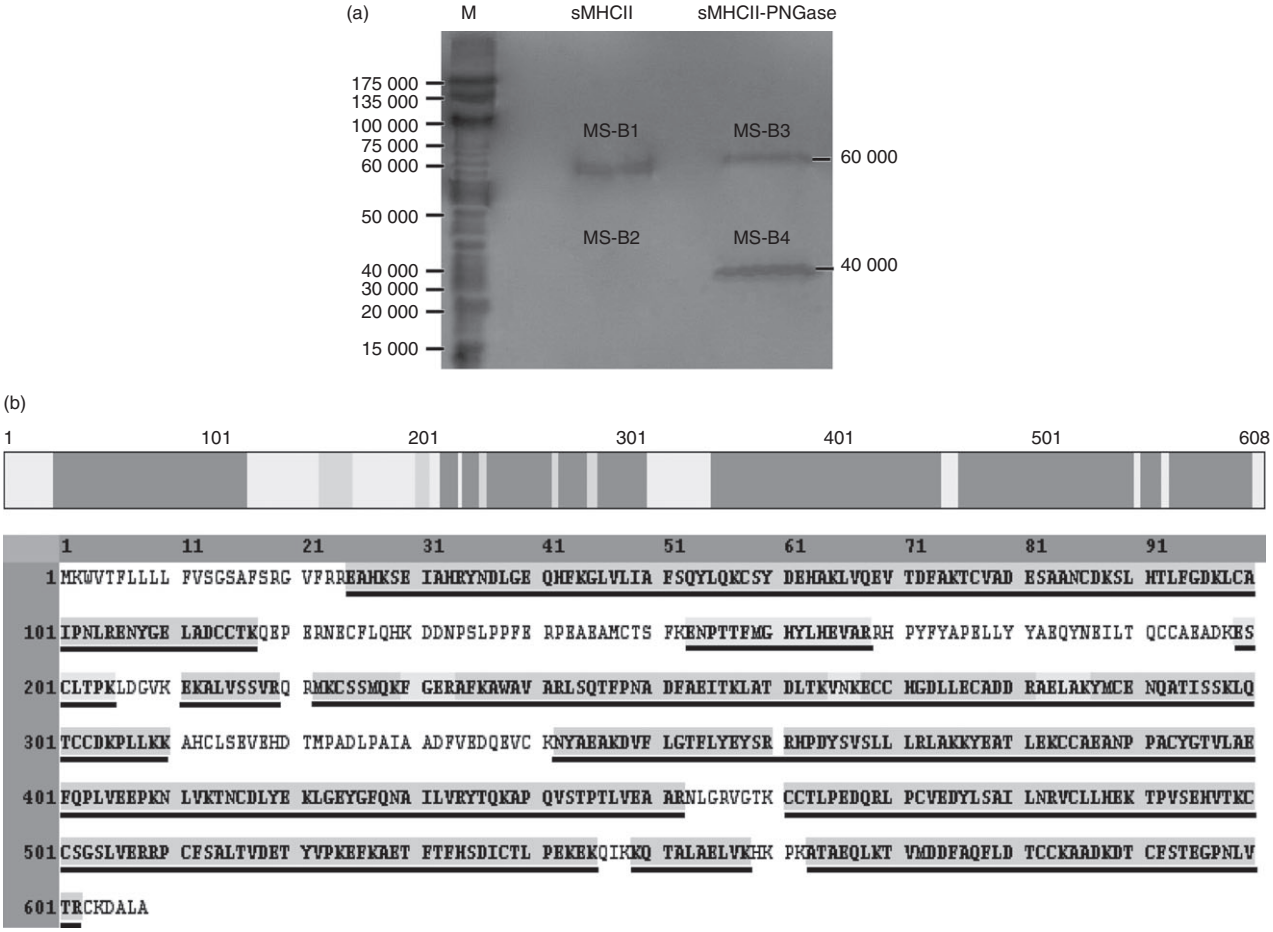

Figure 2. Quality control experiments for soluble MHC class II (sMHCII) proteins isolated from human serum albumin (HSA) -tolerant mouse serum. Treatment of sMHCII proteins with *N*-glycosylase reveals a 40 000 molecular weight band in SDS–PAGE (a). Bands 1 (MS-B1), 2 (MS-B2), 3 (MS-B3) and 4 (MS-B4) were submitted to mass spectrometry (supplemental data). The analysis of serum albumin by tandem mass spectrometry coupled on line with nLC after tryptic digestion resulted in its confident characterization with high sequence coverage of 75·66% (coloured grey and underlined on the protein sequence-lower panel). The light and dark grey (upper panel) indicate the middle and high probabilities of the specific tryptic peptide identification (b). The experiments have been repeated three times and similar results were obtained.

cells from the HSA/*in vivo* immunization protocols, sMHCII could significantly suppress [^{3}H]TdR uptake in the absence of any additional *in vitro* antigenic stimulus (65% decrease, $P < 0{\cdot}001$) and could suppress cell proliferation by 77% ($P < 0{\cdot}001$) in the *in vitro* presence of HSA or SecA (Fig. 3c). Although not expected, these results indicated that sMHCII could exert an antigen-non-specific suppressive effect, because the SecA antigenic stimulus was only provided *in vitro* and represents a foreign, non-self antigen against which the animals could not have previously developed an immune response.

These results were also verified in the case where spleen cells from Sec-A-immunized animals were used. Although in the absence of any additional *in vitro* antigenic stimulus, sMHCII reduced in a statistically non-significant manner [^{3}H]TdR uptake, they suppressed by 30% ($P < 0{\cdot}005$) and 37% ($P < 0{\cdot}005$) cell proliferation in the *in vitro* presence of HSA and SecA respectively (Fig. 3d). These results raised the obvious question about the mode of action of sMHCII molecules, and their effect on the cell targets.

sMHCII molecules promote expression of suppressive markers in CD4$^+$ cells

The results so far showed that sMHCII molecules exert a suppressive activity on total spleen cells. These molecules have been previously shown to antagonize classical membrane MHCII molecules for binding to CD4$^+$ T cells,[26] indicating that CD4$^+$ cells are at least one of their cell targets. To evaluate whether sMHCII molecules could induce CD25 marker expression, CD4$^+$ T cells were isolated from naive mice, purified using flow cytometry cell sorting techniques (purity 97%, Fig. 4a), cultured in presence or not of sMHCII (30 ng/ml) for 48 hr and tested for the expression of CD25 by immunofluorescence. Flow cytometry analysis showed that indeed sMHCII increased

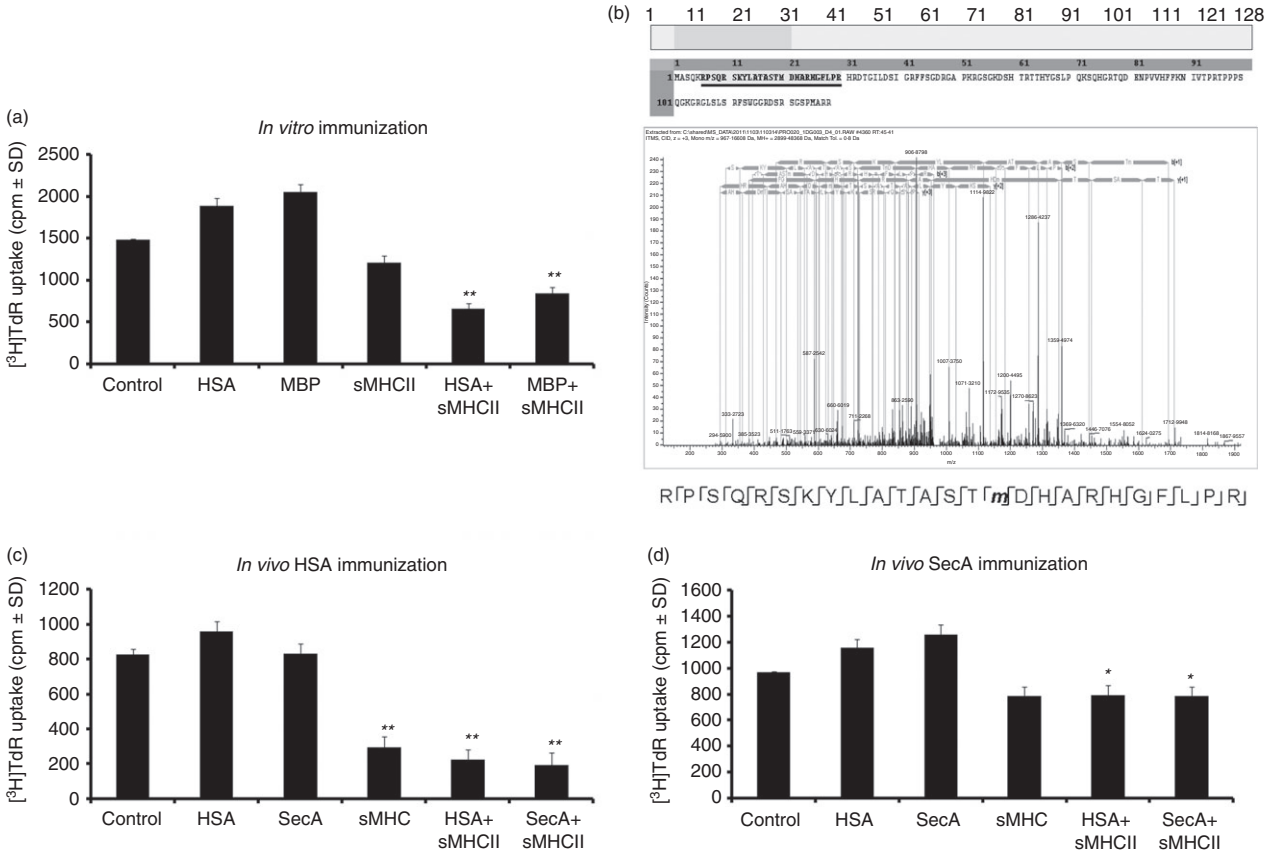

Figure 3. Effect of soluble MHC class II (sMHCII) proteins on *in vitro* and *in vivo* immunization protocols. Control spleen cells were immunized *in vitro* with human serum albumin (HSA) or myelin basic protein (MBP) in presence or not of sMHCII and cell proliferation was assessed by [^{3}H]TdR incorporation assays (a). The results represent the mean of three experiments (**$P < 0{\cdot}01$). Mass spectrometric characterization of MBP by nLC-MS/MS (b). The analysis of MBP by tandem mass spectrometry coupled on line with nLC after tryptic digestion resulted in its confident characterization by one tryptic peptide corresponding to a 19·53% protein sequence coverage (coloured grey and underlined on the protein sequence). The light and dark grey indicate the middle and high probability of the specific tryptic peptide identification. The MS/MS spectrum of the identified tryptic peptide is shown that confirms its confident identification with high fragment coverage. The lower-case 'm' in the sequence of the tryptic peptide indicates oxidation of methionine residue. The proliferative effect of sMHCII was also examined on spleen cells isolated from mice immunized *in vivo* either with HSA (c) or SecA (d) and further stimulated with HSA or SecA *in vitro*. Cell proliferation was assessed by [^{3}H]TdR incorporation assays. The results represent the mean of three experiments (*$P < 0{\cdot}05$).

CD25 expression by 30% compared with the untreated control ($P < 0{\cdot}005$; Fig. 4b,c).

As sMHCII have been isolated from HSA-tolerant animals and were shown to carry HSA determinants, it was then investigated whether these molecules could alter the T-cell activation process during the development of an antigen-specific immune response. Therefore, CD4$^+$ T cells were seeded to macrophage cell cultures activated with HSA for 24 hr, in the presence or not of sMHCII and 48 hr later CD4$^+$ cells were examined for the expression of CD28 and CTLA4 as well as the production of IL-2 and IL-10. Flow cytometry analysis showed that sMHCII increased CTLA-4 expression by 40% ($P < 0{\cdot}005$; Fig. 5a), while reducing CD28 expression by 27% ($P < 0{\cdot}005$; Fig. 5b).

In parallel experiments, culture supernatants isolated from the co-cultures of activated macrophages and CD4$^+$ cells in the presence or not of sMHCII were tested for the production of IL-2 and IL-10 by ELISA. The results showed that sMHCII increased by sixfold IL-10 production ($P < 0{\cdot}001$), while decreasing IL-2 production by 50% ($P < 0{\cdot}005$) (Fig. 5c).

sMHCII molecules inhibit T-cell activation signalling

To further investigate the mechanisms involved in the sMHCII-induced suppression, the early events in T-cell signalling, including Lck, ZAP-70 and LAT phosphorylation, were studied. Hence, CD4$^+$ cells were co-cultured or not with antigen-activated macrophages in the presence or not of sMHCII for 24 hr and upon culture termination CD4$^+$ cell extracts were submitted to ELISA experiments using specific antibodies for the phosphorylated and non-phosphorylated forms of Lck, ZAP-70 and LAT

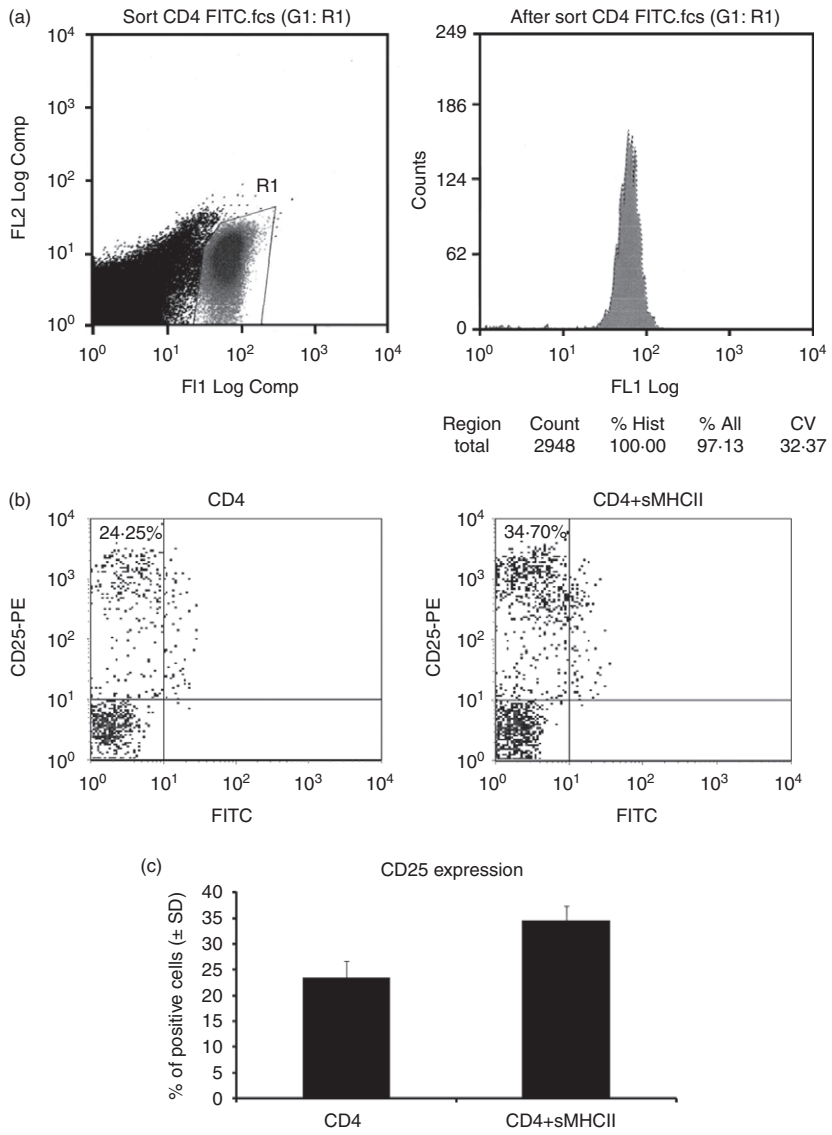

Figure 4. Effect of soluble MHC class II (sMHCII) proteins on specific T-cell populations. Sorting of fluorescent $CD4^+$ T cells in FL1 reached purity by 97% (a). Naive $CD4^+$ cells were incubated with sMHCII (30 ng/ml) for 48 hr and submitted to flow cytometry analysis (b) for the evaluation of CD25 levels. The results give % of positive cells (± SD) and represent the mean of three experiments (c).

proteins (Fig. 6). At the specific time-point tested, naive $CD4^+$ cells did not show any significant difference between the phosphorylated or non-phosphorylated forms of Lck and ZAP-70, whereas there was a decreased expression of phosphorylated LAT, indicating the absence of cell activation (Fig. 6a). In the presence of sMHCII, although the higher amounts of ZAP-70 and LAT were observed compared with naive $CD4^+$ cells, the phosphorylation patterns were not altered (Fig. 6a). When $CD4^+$ cells were co-cultured with HSA-activated macrophages, a significant increase in the phosphorylated ZAP-70 and LAT was observed, indicating an activation state of $CD4^+$ cells. In this case, the presence of sMHCII molecules blocked activation *vis-à-vis* ZAP-70 and LAT because a statistically significant decrease was observed in the phosphorylated form of these proteins (Fig. 6b). In these experimental conditions Lck remained un-altered probably because its activation had preceded the culture termination time-point. These results indicate that sMHCII could block T-cell signalling from the very early stages of ZAP-70 activation.

Discussion

Recent studies on sMHCII have been enlightening complicated regulatory interference with the development, maintenance and suppression of an immune response. As sMHCII molecules seem to be loaded with antigenic peptide, one can easily foresee the different levels of actions and the variety of reactions they could regulate. The present report concentrated on the study of sMHCII molecules isolated from HSA-tolerant mice. The choice of this protocol was based on three reasons: (i) practicality, because the tolerant animals considerably increased the amount of circulating sMHCII protein, which allowed profound experimentation *in vitro* as well as *in vivo*; (ii)

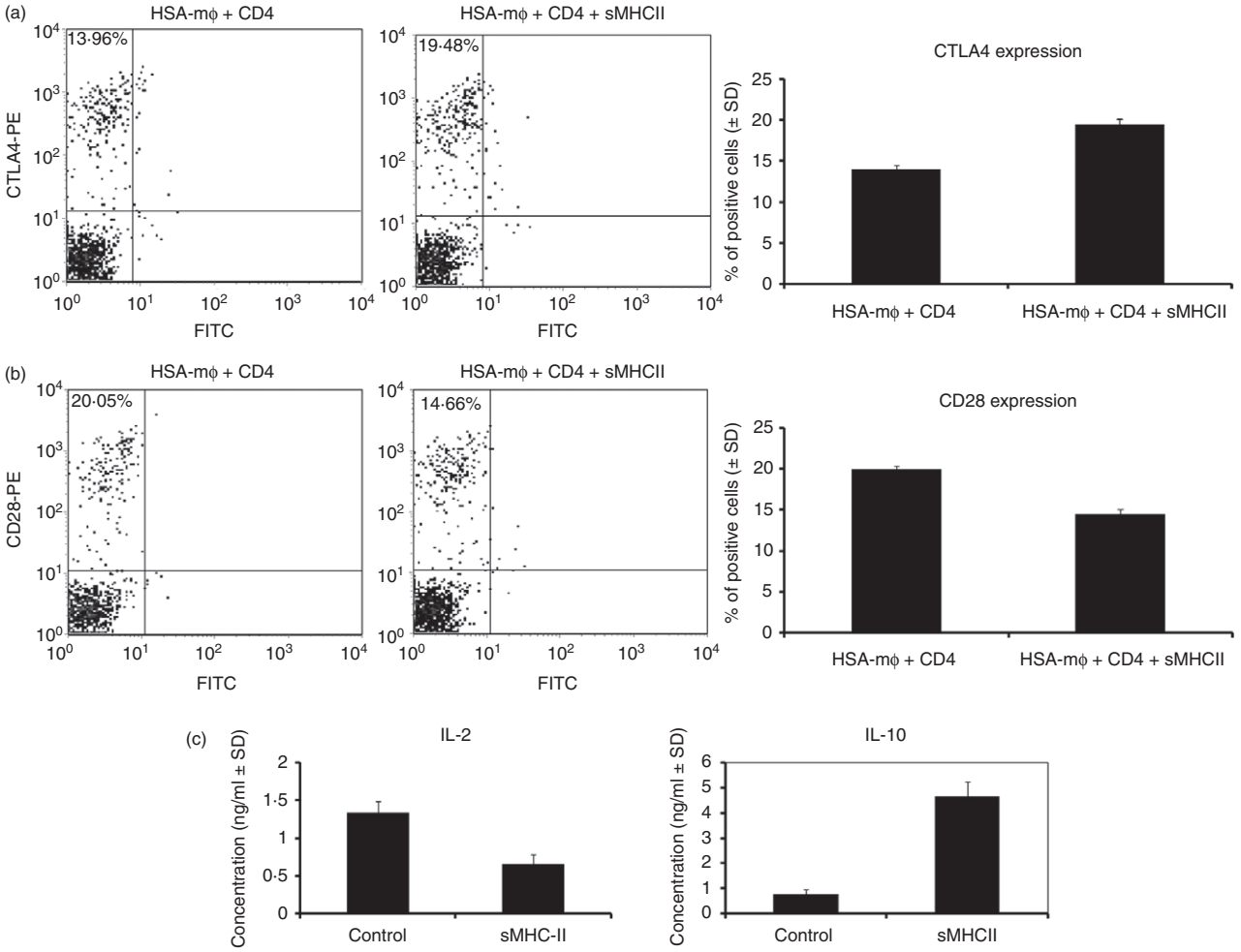

Figure 5. Effect of soluble MHC class II (sMHCII) proteins on surface marker expression of $CD4^+$ cells. Naive $CD4^+$ T cells were seeded to macrophage cell cultures activated with human serum albumin (HSA) for 24 hr, in the presence or not of sMHCII (30 ng/ml) and 48 hr later $CD4^+$ cells were examined for the expression of CTLA4 (a) and CD28 (b) by flow cytometry analysis as well as the production of interleukin-2 (IL-2) and IL-10 by ELISA (c). The bar graphs represent the mean of at least three experiments in each case (± SD).

studying a tolerogenic system would give the opportunity to understand the mode of action of self-peptide-loaded sMHCII, which have been hypothesized to be involved in the maintenance of tolerance; and (iii) working with a specific antigen would be easy to manage immunity versus tolerance and follow up the events leading to the cessation of an immune response.

Indeed, the chosen experimental design allowed sMHCII to be studied at the biochemical as well as the physiological level. It was shown that sMHCII proteins isolated using specific immunoaffinity techniques, although loaded with tolerogenic peptide, resisted well-known denaturing procedures, showing a remarkable stability. At the physiological level, sMHCII proteins were shown to suppress not only an antigen-specific but also antigen-non-specific immune activation. The sMHCII not only promoted suppressive marker expression on $CD4^+$ cells, but also induced production of suppressive cytokines and significantly reduced T-cell activation signalling.

Following the protocols described herein, sMHCII were isolated from mouse serum using a mAb that reacts with a monomorphic determinant on I-A and I-E regions. Specific interaction of the isolated sMHCII with the anti-class II mAb was evaluated by Western blot analysis and ELISA. The isolated product could also react with other class II specific mAbs including, purified anti-mouse $I\text{-}A^d/I\text{-}E^d$, produced in mouse, IgG2a (0.5 mg/ml; BD Pharmingen, San Diego, CA), FITC-labelled anti-mouse $I\text{-}A^d$, produced in mouse, IgG2b (0.5 mg/ml; BD Pharmigen), MK-D6 (ATCC® HB-3TM), anti-mouse $I\text{-}A^d$, IgG2a (data not shown) indicating that the isolated molecules were indeed class II proteins. MS-MS analysis detected the presence of serum albumin peptides as well as the Aβ chain of H-2 class II. Further experiments are needed to define the structural homology to membrane MHCII.

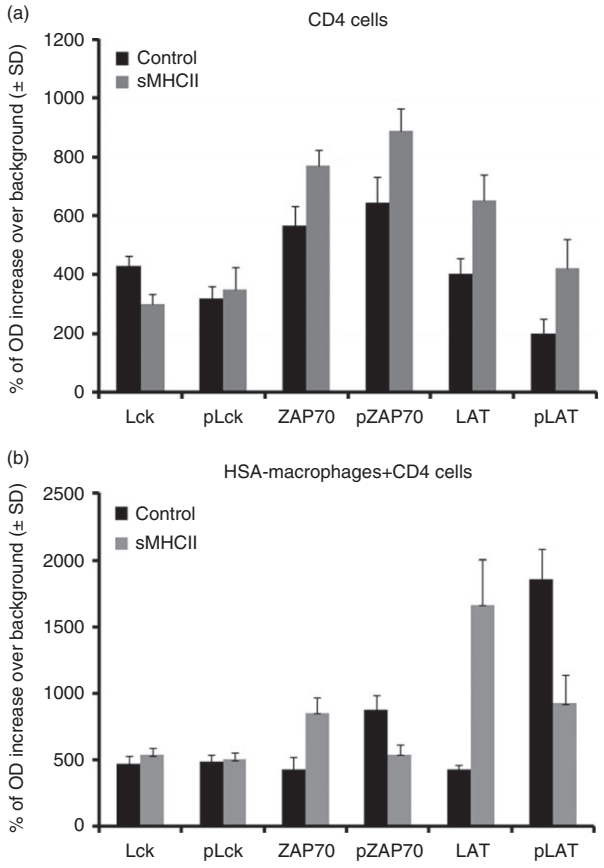

Figure 6. Effect of soluble MHC class II (sMHCII) proteins on early T-cell activation events. Cell extracts from $CD4^+$ cells cultured for 24 hr in presence or not of sMHCII (30 ng/ml) either alone (a) or in co-culture with human serum albumin (HSA) -activated macrophages (b) were submitted to ELISA for the evaluation of phosphorylated or non-phosphorylated lck, ZAP-70 and LAT. The results are expressed as optical density (OD) increase over background (± SD) and represent the mean of three experiments.

Crystallographic analysis, which is currently underway, is expected to provide valuable information.

At the functional level, when sMHCII proteins isolated from HSA-tolerant mice were applied to spleen cells stimulated with either HSA or MBP, which was used as a representative of a natural self antigen, cell proliferation was suppressed. Although suppression reached higher levels when HSA was used as an antigen, the MBP response was also decreased. These results indicated that sMHCII either exerted an antigen-non-specific effect or the sMHCII preparation also contained sMHCII molecules loaded with MBP peptides. Indeed, out of the 35 128 peptides corresponding to 532 self proteins recognized by the MS analysis (see Supporting information, Table S1), 59 peptide sequences corresponded to serum albumin (see Supporting information, Table S2), while MS/MS spectrum identified MBP and confirmed its confident identification with high fragment coverage. On the other hand, in the context of MHCII–TCR interaction, histotope–resitope recognition is independent of the antigenic peptide, which, however, is recognized through the epitope–paratope interaction (Fig. 7a). Therefore, in the context of MHC restriction, the histotope–resitope interaction will occur independently of the antigenic peptide. It can therefore be hypothesized that regardless of the peptidic load of sMHCII, this will be able to interact with TCR with lower affinity.

Such an assumption could be verified in another set of experiments, where sMHCII proteins were added *in vitro* to spleen cells isolated from mice immunized *in vivo* either with HSA or SecA, which represent a real foreign antigen. Hence, sMHCII were able to suppress HSA- or SecA-immunized spleen cell proliferation in the presence of additional *in vitro* administration of HSA or SecA. These results indicated that although the sMHCII proteins used could not contain SecA antigenic epitopes, the histotope–resitope interaction should suffice for suppression.

The obvious cell target should be $CD4^+$ T cells. Indeed, it has been previously shown that sMHCII antagonize classical membrane MHCII molecules for binding to $CD4^+$ T cells. However, depending on the phase of the immune response, a $CD4^+$ cell can initiate, propagate or block an immune reaction. Therefore, the effect of sMHCII on $CD4^+$ T cells depends on co-receptor expression on T cells. The results presented here showed that sMHCII could increase CD25 expression on naive $CD4^+$ cells. However, $CD4^+$ cells isolated from naive mice could display any type of antigenic specificity. To control the experimental conditions, naive $CD4^+$ cells were co-cultured with *in vitro* HSA-activated macrophages in the presence or not of sMHCII. Such manipulation showed that sMHCII decreased CD28 expression, while increasing CTLA. In addition, it was shown that sMHCII decreased IL-2 production, while increasing IL-10 production, which could account for another mechanism of non-specific suppression.

To further exploit the effect of sMHCII molecules, early T-cell signaling in naive $CD4^+$ cells or in $CD4^+$ cells co-cultured with activated macrophages in the presence or not of sMHCII was evaluated. In all cases, Lck could be phosphorylated, indicating that sMHCII binding to TCR could recruit CD4 to stimulate Lck phosphorylation. Indeed, previous results have shown that anti-CD4 antibody could block the effect of soluble class II molecules in T-cell responses.[26] Except, however, from Lck activation, sMHCII inhibited phosphorylation of ZAP-70 and especially LAT proteins in the downstream pathways of T-cell activation signalling.

In conclusion, the present work demonstrated that sMHCII proteins isolated from HSA-tolerant mouse serum exerted antigen-specific as well as antigen-non-specific suppressive effects on $CD4^+$ T cells mediated not only by inhibitory marker expression but also by production of

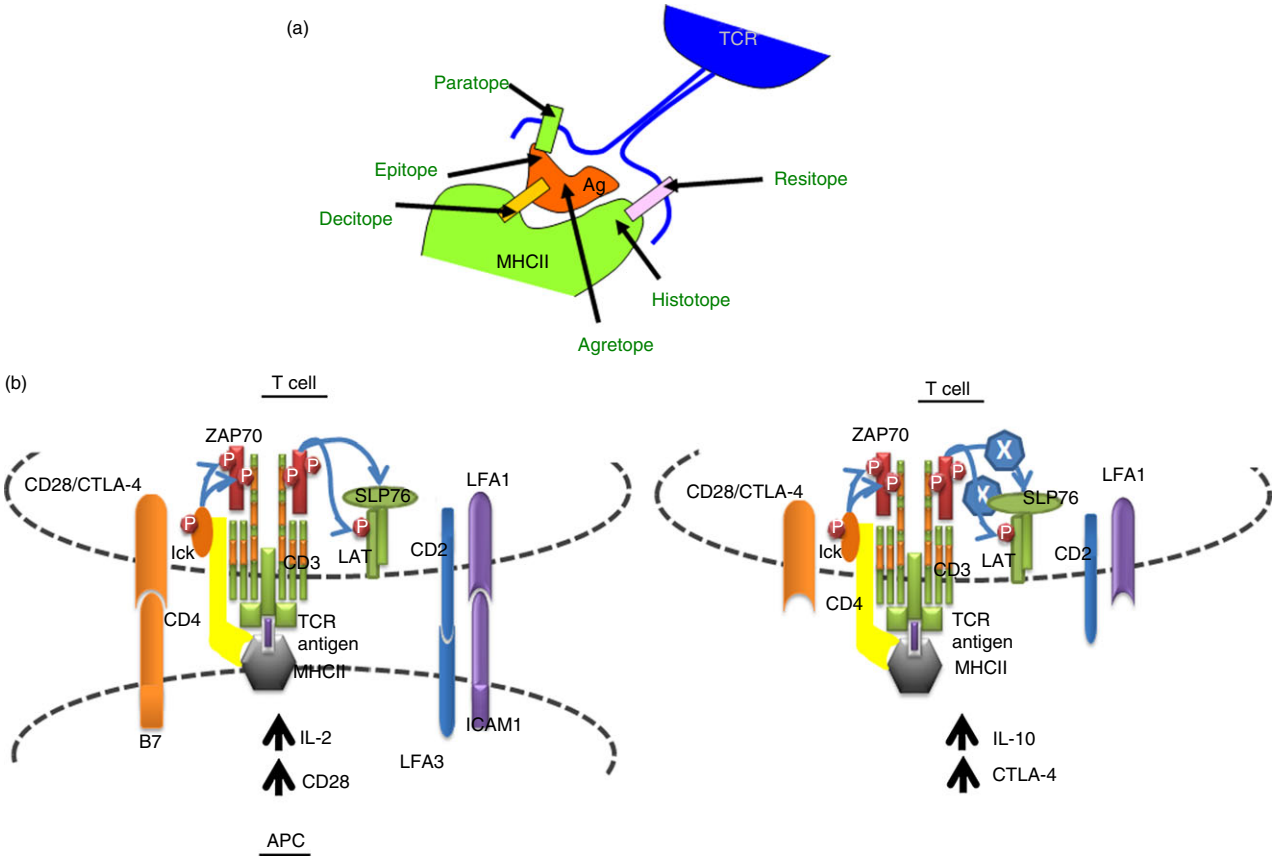

Figure 7. MHCII–T-cell receptor (TCR) interaction (a). The TCR interacts with MHC as well as the antigenic epitope (Ag). Different signal transduction events can be evoked in $CD4^+$ cells by membrane-bound MHCII compared with soluble MHC class II (sMHCII) (b). In the presence of antigen-presenting cells, membrane MHCII interaction with $CD4^+$ cells is fortified by CD4, CD28/CTLA-4/B7, CD2/LFA3 or LFA1/ICAM1 interactions, and promotes an increase in CD28 expression and in production of interleukin-2 (IL-2), while in the absence of antigen-presenting cells and consequently the absence of an immune synapse, sMHCII interact with $CD4^+$ cells to promote CTLA-4 expression and IL-10 production.

suppressive soluble products (i.e. IL-10). Extrapolating these observations, the results provide evidence for the mechanisms of self tolerance maintenance by soluble MHC class II antigens as postulated by Pentcheva-Hoang *et al.*[12] and Fischer *et al.*[13] In the presence of antigen-presenting cells, membrane MHCII interaction with $CD4^+$ cells promote cell activation manifested by an increase of CD28 expression and production of IL-2. In the absence, however, of antigen-presenting cells, sMHCII interact with $CD4^+$ cells to promote CTLA-4 expression and IL-10 production (Fig. 7b). Apparently, soluble MHCII antigens could be considered as potent regulators of the immune response. Preliminary experiments have been showing that administration of sMHCII molecules *in vivo* could decrease antigen-specific antibody production, hence providing novel tools for immune system manipulation and therapy.

Acknowledgements

The authors would like to thank Mrs Vlata Chara FORTH (Crete, Greece) for technical assistance in FACS cell sorting. We thank the Special Account for Research Resources of the University of Crete for covering publication costs. This work was supported by national funding from the Ministry of Education and the Special Account for Research Resources of the University of Crete through services to the private sector.

Author contributions

KB performed all cellular and biochemical experiments and wrote the the first draft of the paper as part of her PhD thesis. NK performed the MS analysis under the supervision of MA, who is responsible for the proteomics facility at IMBB. IA conceived and supervised the project and wrote the paper.

Disclosures

The authors declare that there is no financial or commercial conflict of interest that might be construed as influencing the results or interpretation of this manuscript.

References

1 Calne RY, White HJ, Yoffa DE *et al.* Prolonged survival of liver transplants in the pig. *Br Med J* 1967; **4**:645–8.

2 van Rood JJ, van Leeuwen A, van Santen MCT. Anti-HL-A2 inhibitor in normal human serum. *Nature* 1970; **226**:366–7.

3 Puppo F, Indiveri F, Scudeletti M, Femme S. Soluble HLA antigens: new roles and uses. *Immunol Today* 1997; **18**:154–5.

4 Hagihara M, Shimura T, Yamamoto K, Takebe K, Munkhbat B, Tsuji K. Soluble HLA Class I and Class II in Japanese. *Hum Immunol* 1994; **40**:171–3.

5 Pfeiffer KA, Rebmann V, Passler M, van der Ven K, van der Ven H, Krebs D, Grosse-Wilde H. Soluble HLA levels in early pregnancy after *in vitro* fertilization. *Hum Immunol* 2000; **61**:559–64.

6 Aultman D, Adamashvili I, Yaturu K, Langford M, Gelder F, Gautreaux M, Ghali GE, McDonald J. Soluble HLA in human body fluids. *Hum Immunol* 1999; **60**:239–44.

7 Dobbe LM, Stam NJ, Neefjes JJ, Giphart MJ. Biochemical complexity of serum HLA class I molecules. *Immunogenetics* 1988; **27**:203–10.

8 Jendro M, Goronzy J, Weyand C. Structural and functional characterization of HLA-DR molecules circulating in the serum. *Autoimmunity* 1991; **8**:289–96.

9 MacKay PA, Leibundgut- LS, Koch N, Dunn AC, Reith W, Jack RW, McLellan AD. Circulating, soluble forms of major histocompatibility complex antigens are not exosome-associated. *Eur J Immunol* 2006; **36**:3381.

10 Athanassakis I, Ranella A, Vassiliadis S. IFN-γ facilitates release of class II-loaded intracellular pools in trophoblast cells: a novel property in dependent of protein synthesis. *J Interferon Cytokine Res* 2000; **20**:823–30.

11 Ranella A, Vassiliadis S, Mastora C, Dionyssopoulou E, Athanassakis I. Constitutive intracellular expression of human leukocyte antigen (HLA)-DO and HLA-DR but not HLA-DM in trophoblast cells. *Hum Immunol* 2005; **66**:43–55.

12 Pentcheva-Hoang T, Egen JG, Wojnoonski K, Allison JP. B7-1 and B7-2 selectively recruit CTLA-4 and CD28 to the immunological synapse. *Immunity* 2004; **21**:401–13.

13 Fischer UB, Jacovetty EL, Medeiros RB *et al.* MHC class II deprivation impairs CD4 T cell motility and responsiveness to antigen-bearing dendritic cells *in vivo*. *Proc Natl Acad Sci USA* 2007; **104**:7181–6.

14 Schwartz M, Ziv Y. Immunity to self and self-maintenance: a unified theory of brain pathologies. *Trends Immunol* 2008; **29**:211–9.

15 Restrepo D, Lin W, Salcedo E, Yamazaki K, Beauchamp G. Odortypes and MHC peptides: complementary chemosignals of MHC haplotype? *Trends Neurosci* 2006; **29**:604–9.

16 Vassiliadis MA, Theochari C, Georgiakakis P, Boura V, Athanassakis I, Kouzoupis S. Emotion transfer through transmission of vocal messages in mice. 4th International Conference on Bio-Acoustics 2007; 29 Pt.3.

17 Verbruggen LA, Versaen H, Rebmann V, Duquet W, De CS, Grosse-Wilde H, Demanet C. Soluble HLA-DR levels in serum are associated with therapy and genetic factors in rheumatoid arthritis. *Hum Immunol* 2002; **63**:758–64.

18 Verbruggen LA, Dumarey N, van de Velde H, Rebmann V, Flament J, van Wayenberge C, Grosse-Wilde H, Demanet C. Soluble HLA-DR antigen levels in serum correlate with rheumatoid arthritis disease activity and the presence of disease-associated epitopes. *Tissue Antigens* 2000; **56**:436–40.

19 Wolf RE, Adamashvili IM, Gelder FB, Hall VC, Fraser PA, McDonald JC. Soluble HLA-I in rheumatic diseases. *Hum Immunol* 1998; **59**:644–9.

20 Steinborn A, Rebmann V, Scharf A, Sohn C, Grosse-Wilde H. Soluble HLA-DR levels in the maternal circulation of normal and pathologic pregnancy. *Am J Obstet Gynecol* 2003; **188**:473–9.

21 Rizzo R, Mapp CE, Melchiorri L *et al.* Defective production of soluble HLA-G molecules by peripheral blood monocytes in patients with asthma. *J Allergy Clin Immunol* 2004; **115**:508–13.

22 Heiligenhaus A, Rebmann V, Neubert A, Plewa S, Ferencik S, Vogeler U, Steuhl KP, Grosse-Wilde H. Soluble HLA class I and HLA-DR plasma levels in patients with anterior uveitis. *Tissue Antigens* 2004; **63**:369–75.

23 Athanassakis I, Vassiliadis S. T-regulatory cells: are we re-discovering T suppressors? *Immunol Lett* 2002; **84**:179–83.

24 Ostman S, Taube M, Telemo E. Tolerosome-induced oral tolerance is MHC dependent. *Immunology* 2005; **116**:464–76.

25 Almqvist N, Lonnqvist A, Hultkrantz S, Rask C, Telemo E. Serum-derived exosomes from antigen-fed mice prevent allergic sensitization in a model of allergic asthma. *Immunology* 2008; **125**:21–7.

26 Sardis M, Miltiadou P, Bakela K, Athanassakis I. Serum-derived MHC class II molecules: potent regulators of the cellular and humoral immune response. *Immunobiology* 2010; **215**:194–205.

27 Nag B, Kendrick T, Arimilli S, Yu SCT, Sriram S. Soluble MHC II–peptide complexes induce antigen-specific apoptosis in T cells. *Cell Immunol* 1996; **170**:25–33.

28 Ge Q, Stone JD, Thompson MT, Cochran JR, Rushe M, Eisen HN, Chen J, Stern JL. Soluble peptide–MHC monomers cause activation of $CD8^+$ T cells through transfer of the peptide to T cell MHC molecules. *Proc Natl Acad Sci USA* 2002; **99**:13729–34.

29 Webb BJ, Bochan MR, Montel A, Padilla LM, Brahmi Z. The lack of NK cytotoxicity associated with fresh HUCB may be due to the presence of soluble HLA in the serum. *Cell Immunol* 1994; **159**:246–61.

30 Vevis K, Matheakakis A, Kyvelidou C, Bakela K, Athanassakis I. Characterization of antigen-binding and MHC class II-bearing T cells with suppressive activity in response to tolerogenic stimulus. *Immunobiology* 2012; **217**:100–10.

31 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **277**:680–5.

32 Candiano G, Bruschi M, Musante L *et al.* Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 2004; **25**:1327–33.

33 Huynh ML, Russell P, Walsh B. Tryptic digestion of in-gel proteins for mass spectrometry analysis. *Methods Mol Biol* 2009; **519**:507–13.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of the total proteins identified by nLC-MS/MS in all bands.

Table S2. List of the total serum albumin tryptic peptides identified by nLC-MS/MS.

