

# Activated mouse T-cells synthesize MHC class II, process, and present morbillivirus nucleocapsid protein to primed T-cells

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## Abstract

A pivotal step in the initiation of T-cell immunity is the presentation of antigenic peptides by major histocompatibility complex (MHC) expressed on antigen presenting cells. The expression of MHC class II molecules by mouse T-cells has not been shown unequivocally. In the present work, we demonstrate that activated mouse T-cells synthesize MHC class II molecules de novo and express them on their surface. Further, we have demonstrated that in vitro activated T-cells take up extra-cellular soluble nucleocapsid protein of a morbillivirus. The internalized antigen goes to antigen processing compartment as shown by co-localization of antigen and LAMP-1 using confocal microscopy. We show that activated T-cells express H2M, a chaperone molecule known to have a role in antigen presentation. Further, we demonstrate that activated T-cells process and present internalized extra-cellular antigen to primed T-cells as shown by IL-2 secretion and in vitro proliferation. The presentation of antigen by T-cells may have implications in immuno-regulation, control of infection by lymphotropic viruses and maintenance of immunological memory.

**Keywords:** T-APC; Major histocompatibility complex II; Antigen presentation; CIITA

## 1. Introduction

The most important steps in the initiation of T-cell immune response is the interaction of T-cells with peptide loaded major histocompatibility complex (MHC)<sup>1</sup> molecules present on the antigen presenting cells. In general, MHC class II molecules bind peptides formed in endocytic organelles. Antigen presenting cells (APCs) synthesize MHC class II molecules which accumulate in late endosomal and lysosomal compartments (collectively termed MIICs) together with other components required for antigen processing [1]. However, an increas-

ing number of diverse cell types besides professional APCs have been documented to express class II molecules and act as accessory cells for T-cell stimulation. These include hepatic Kupffer cells [2], epidermal Langerhans cells [3], resting and activated B-cells [4], endothelial cells [5], astrocytes [6], eosinophiles [7], and natural killer cells [8].

The MHC class II expression characterizes the antigen presenting capacity of a cell. Human, guinea pig, and rat T-cells have been shown to synthesize and express class II molecules on their surface after activation by mitogens or alloantigens [9–12]. In contrast, the expression of Ia antigens on murine T-cells is still debated. Although expression of Ia antigen by mouse T-cells has been reported [13–16], subsequent work has shown that Ia antigens might have been acquired passively from contaminating Ia bearing non-T-cells [17,18]. These MHC class II expressing T-cells are able to present antigen to other T-cells as reported for human T-cells [19]. In case of mouse

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<sup>1</sup> Abbreviations used: T-APC, T-cells as antigen presenting cells; LAMP-1, lysosome-associated membrane protein-1; PMA, phorbol 12-myristate 13-acetate; TCR, T-cell receptor; MHC, major histocompatibility complex; CIITA, class II transactivator.

T-cells, most of the studies demonstrating MHC class II expression have been carried out using long term cultured T-cells or specific T-cell clones. Appearance of MHC class II on naïve mouse T-cells due to de novo synthesis after activation has not been reported.

The expression of MHC class II molecules on activated T-cells can bring about various important immunological functions. Direct MHC class II restricted T-cell-T-cell interactions between TCR peptide specific T-cells and MHC class II<sup>+</sup> T-cells expressing the appropriate TCR have been demonstrated in rat [20]. Similar interactions have been postulated to drive immune responses after T-cell vaccination [21,22] and to be of importance in T-cell regulation [23]. TCR peptide immunization, which has been used for protection against multiple sclerosis, appears to function through T-T interactions [24]. Idiotypic and anti-idiotypic T-cells interaction had been proposed for maintenance of immunological memory [25].

In the present work, we report that activated mouse T-cells de novo synthesize and express MHC class II molecules on their surface. Activated T-cells take up extra-cellular soluble antigen (morbillivirus nucleocapsid protein), which is processed in endosome/lysosome for presentation to primed T-cells leading to proliferation and cytokine secretion.

## 2. Materials and methods

### 2.1. Animal

Balb/c and C57BL/6 mice were bred and maintained in the Central Animal Facility, Indian Institute of Science, Bangalore, India. Six- to eight-weeks old male mice were used in all experiments.

### 2.2. Reagents

The following antibodies—anti-mouse CD3-PE (clone 145-2C11), purified anti-mouse I-A/I-E (clone 2G9), anti-mouse I-A/I-E-PE (M5/114), anti-mouse CD69-biotin (clone H1.2F3), anti-mouse CD11c (clone HL3), anti-mouse-IgM-FITC (clone II/41), purified anti-invariant chain (CD74; clone In-1) and isotype control antibodies as well as recombinant mouse IL-2 were from PharMingen, USA. Polyclonal rabbit anti-mouse Ig and anti-rabbit IgG Cy3 conjugated antibody were purchased from Sigma, USA. Donkey anti-rat IgG-FITC was obtained from Jackson ImmunoResearch Laboratories, USA. Anti-mouse TCRβ-PE-Cy5 (clone H57-597), purified anti-mouse CD28 (clone 37.51), anti-mouse I-A/I-E-biotin (clone M5/114), Streptavidin-PE, and Streptavidin-FITC were procured from e-Bioscience (USA). Anti-LAMP-1 (1D4B) antibody was from Santa Cruz Biotechnology, USA. Anti-mouse H2M antibody (clone

2C3a) was a generous gift from Dr. L. Karlsson, Johnson & Johnson Pharmaceutical Research and Development, USA. LAMP-1 and H2M antibodies were conjugated with FITC and TRITC, respectively, using standard protocol [26].

Recombinant Rinderpest virus (RPV) nucleocapsid protein (N) was expressed in *Escherichia coli* and purified as described earlier [27]. RPV nucleocapsid protein has been earlier shown to elicit good CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in vivo and in vitro in the mouse model as well as in cattle [27,28].

### 2.3. Purification of T-cells

Mice were sacrificed by cervical dislocation and lymph nodes (inguinal, posterior axillary, internal axillary, and popliteal) were removed. Single cell suspensions were panned for 1 h in FBS coated plate (Falcon). Unbound cells were harvested and complement mediated lysis was performed using anti-mouse Ig, anti-mouse MHC class II (clone m5/115), anti-mouse CD11c and rabbit complement. Mononuclear cells were purified by density gradient centrifugation on Histopaque-1077 (Sigma, USA). Cells were further panned on anti-mouse Ig antibody coated plate for 1 h in complete medium (RPMI-1640 supplemented with 25 mM Hepes, 100 µg/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin, and 10% FBS). Unbound cells were harvested and purity of T-cells was checked by FACS.

### 2.4. In vitro activation of T-cells

Purified T-cells ( $4 \times 10^6$ ) were treated with PMA (500 ng/ml) and ionomycin (50 ng/ml) for 6 h in complete medium in a final volume of 1.5 ml in 24-well plates. Cells were harvested and washed with Hank's balanced salt solution. Purified T-cells were also activated with IL-2, concanavalin-A, anti-TCRβ antibody or anti-CD28 and plate bound anti-CD3 antibody. Twenty four well plates were coated with 10 µg/ml of either anti-mouse CD3 antibody (145-2C11) or anti-mouse TCRβ antibody (H57.597) in PBS overnight at 4 °C, and then washed with medium. Purified T-cells ( $4 \times 10^6$  cells/well) were cultured in anti-CD3 coated plate with soluble anti-CD28 antibody (1 µg/ml) or into anti-TCRβ antibody coated plates in complete medium for 8 h at 37 °C in 5% CO<sub>2</sub> incubator. Cells were in vitro activated with concanavalin-A (1 µg/ml) or recombinant mouse IL-2 (200 U) in complete medium for 8 h at 37 °C in 5% CO<sub>2</sub> incubator. Cells were harvested, washed and stained for MHC class II, and analyzed by FACS.

### 2.5. In vivo activation of T-cells

Balb/c mice were injected with purified recombinant nucleocapsid protein of Rinderpest virus (50 µg/mouse)

in CFA subcutaneously and sacrificed after 5 days. Single cell suspensions from lymph nodes (inguinal, popliteal, and axillary) and spleen were made. RBCs were removed from spleen using ACK lysis buffer (0.15 mM NH<sub>4</sub>Cl, 1 mM KHCO<sub>3</sub>, and 0.1 mM EDTA). Mononuclear cells were purified by density gradient centrifugation using Histopaque-1077. Cells were surface stained for TCR $\beta$ , CD69, I-A/I-E, and isotype control antibodies. Cells were analyzed by FACS.

## 2.6. FACS analysis

Purified T-cells were surface stained with purified anti-mouse I-A/I-E or anti-mouse CD69-biotin conjugate or isotype control antibodies. Briefly, cells were incubated in 10% FBS containing PBS for 20 min at 4 °C. Cells were washed with PBS containing 0.2% BSA and 0.01% sodium azide, and incubated with antibody conjugates for 30 min on ice. Purified antibody or biotin conjugated antibody were used as primary antibodies followed by anti-rat-IgG FITC conjugate or streptavidin-FITC, respectively. Flow cytometric analysis was performed on FACSScan (BD Bioscience, USA) and the data was analyzed using WinMDI 2.8 software.

## 2.7. Confocal microscopy

In vitro activated T-cells ( $5 \times 10^6$  cells/well) were cultured for 3 h in the presence of N protein (100  $\mu$ g/ml) in complete medium. Cells were washed and fixed with 3% paraformaldehyde for 15 min on ice and permeabilised using permeabilisation solution (e-Bioscience, USA) for 10 min on ice. Cells were incubated with rat anti-LAMP-1-FITC antibody, rat anti-mouse H2M-TRITC antibody, anti-mouse invariant chain antibody, rat anti-I-A/I-E-biotin antibody or rabbit anti-N antibody for 30 min on ice in the presence of permeabilisation solution. Cells were incubated with secondary antibodies, streptavidin-FITC, streptavidin-TRITC, and anti-rabbit IgG-Cy3 conjugates for 30 min on ice and were washed twice with permeabilising solution and finally with PBS. Cells were visualized under confocal microscope using 100 $\times$  objective (Leica Microsystems, Germany). In these experiments, isotype control antibody stainings were performed and visualized under microscope. Cells stained with isotype control antibodies have not shown significant fluorescence (data not shown).

## 2.8. Radiolabelling and immunoprecipitation

Radiolabelling and immunoprecipitations were performed as described earlier [29]. Purified T-cells were activated with PMA/ionomycin for 6 h. Before radio-labeling, cells were incubated for 1 h in methionine-deficient RPMI-1640 (Sigma) supplemented with 5% dialyzed FBS. The cells were then pelleted and re-sus-

pended in the same medium at  $4 \times 10^7$  cells/ml and metabolically labeled by 20 min pulse incubation with 200  $\mu$ Ci/ml [<sup>35</sup>S]methionine protein labeling mix (Perkin-Elmer, USA). After the pulse, the cells were pelleted, unincorporated radiolabel removed by washing, and the cells were resuspended at  $2 \times 10^6$  cells/ml in ice-cold (0 chase) or pre-warmed RPMI medium containing 5% FCS. The cells in the prewarmed media were then incubated for 0.5, 1, 2, or 3 h at 37 °C in a 5% CO<sub>2</sub> atmosphere.

After the pulse-chase, the cells were pelleted and lysed in 0.5% Triton X-100, 300 mM NaCl, 50 mM Tris, pH 7.4, and complete protease inhibitor cocktail (PharMingen, USA). Nuclei and debris were removed by centrifugation. The cell lysates were pre-cleared first, by incubation with rabbit anti-mouse IgG (Sigma, USA) and Protein-A-agarose (Bangalore, Genie, India) overnight at 4 °C. Lysates were incubated with MHC class II antibody (2G9 clone; PharMingen, USA) for 2 h. Immune complexes were precipitated using Protein-G-agarose (Sigma, USA). Aggregate immune-complexes were washed three times in 0.5% NP40, 30 mM NaCl, 50 mM Tris, pH 7.4. The beads were then resuspended in Laemmli sample buffer containing 3% SDS and either incubated for 1 h at room temperature or boiled for 3 min. The immunoprecipitates were analyzed by electrophoresis using 10% SDS-PAGE. The gels were treated with salicilate and dried. The gel was subjected to fluorography and exposed to phosphoimager plate for 16 h and scanned.

To detect synthesis of MHC class II molecule by antigen activated T cells, naïve mouse splenocytes ( $2 \times 10^6$  cells/well) were pulsed with N protein (100  $\mu$ g/ml) in 24-well plates in complete medium. Cells were incubated for 12 h and then fixed with 0.5% paraformaldehyde and used as antigen presenting cells. In vivo antigen primed T-cells were purified from lymph node cells of mice 5 days after immunization with N protein (50  $\mu$ g) in Freund's complete adjuvant. These T-cells were cultured in the presence of fixed antigen presenting cells for 6 h in complete medium and pulse labeled with [<sup>35</sup>S]methionine (200  $\mu$ Ci/ml, specific activity 1175 Ci/mmol) for last 2 h of incubation. Cells were lysed and MHC class II was immuno-precipitated and detected by phosphor-imager as described above.

## 2.9. Purification of CD4 $^+$ and CD8 $^+$ T-cells

CD4 $^+$  T-cells were purified from T-cell populations by complement mediated lysis of CD8 $^+$  T-cells as described earlier [30]. Briefly, purified T-cells were incubated with anti-CD8 antibody (3.155) or anti-CD4 antibody (GK 1.5) on ice for 30 min. Cells were washed and treated with rabbit complement for 20 min on ice, dead cells were removed using Histopaque-1077 density gradient centrifugation. Depletion was checked by FACS using anti-mouse CD4 antibody (clone H129.19) and

anti-mouse CD8 antibody (clone 53-6.7) and purity of CD4<sup>+</sup> T and CD8<sup>+</sup> T cells was >98% (data not shown).

#### 2.10. *In vitro* proliferation assay

Purified, in vitro activated T-cells were incubated with purified N protein (100 µg/ml) in complete medium for 12 h. After incubation, cells were washed with RPMI medium, irradiated (3000 rad) and used as antigen presenting cells (T-APCs) for in vitro proliferation assay. Antigen primed responder T-cells were purified from lymph node cells of N immunized mouse 5 days after immunization. Purified primed responder cells ( $4 \times 10^5$  cells/well) were incubated with different number of irradiated T-APCs in flat bottom 96-well plates in a final volume of 200 µl and incubated for 72 h in a humidified CO<sub>2</sub> incubator. [<sup>3</sup>H]Thymidine (1 µCi/well) (85 Ci/mmol, Perkin-Elmer) was added to cells in the last 16 h of incubation. Cells were harvested on glass fiber filters using semi-automated cell harvester (Nunc) and the incorporated radioactivity was measured in a scintillation spectrophotometer.

#### 2.11. IL-2 secretion assay

Stimulator cells were generated as described above and fixed with 0.5% paraformaldehyde for 5 min on ice. Cells were washed and cultured with responder T-cell ( $5 \times 10^5$  cells/well) in a final volume of 200 µl in flat bottomed 96-well plate in complete medium. Culture supernatant was harvested after 36 h of incubation and IL-2 was detected employing IL-2 ELISA kit (e-Bioscience, USA).

#### 2.12. RT-PCR analysis

Total RNA was prepared from purified PMA/Ionomycin activated T-cells using TRIzol reagent according to manufacturer's instruction (Life Technology, USA). Primer sequences used for amplification of H2M $\alpha$  (NM\_010386.2), Ii (NM\_010545.2), I-A  $\alpha$  chain (AY452201.1), I-A  $\beta$  chain (AY452202.1), I-E  $\alpha$  chain (X54427.1), and I-E  $\beta$  chain (X00958.1) were designed using software from Integrated DNA Technology, USA. The GAPDH, total mCIITA, type I, type III and type IV were as published [31]. The sequence of primers are as follows. GAPDH: sense 5'-AACGACCCCTTCATT GAC-3' and anti-sense 5'-TCCACCGACATACTCAG CAC-3' (predicted size = 191 bp). H2M $\alpha$ : sense 5'-ACC CCAGAACGCCCTGCC-3' and anti-sense 5'-GCTCT TCTGGATGCAAGTCC-3' (predicted size = 312 bp). Invariant chain (Ii): sense 5'-AGGACACGGCAAAT GAAGTC-3' and anti-sense 5'-CCATGATCCTAC AGGCTGCT-3' (predicted size = 319 bp). I-A  $\alpha$  chain: sense 5'-GGACAACATCTCCCCACCTG-3' and anti-sense 5'-GCAGGCCTTGAATGATGAAG-3' (predicted

size = 326 bp). I-A  $\beta$  chain: sense 5'-CAACCACCACA ACACTCTGG-3' and anti-sense 5'-CTGACTCCTGTG ACGGATG-3' (predicted size = 346 bp). I-E  $\alpha$  chain: sense 5'-ACACACCACATCCAGGCAGAGTT-3' and anti-sense 5'-TCTGGAGTGTGTTGGAACGC TCT-3' (predicted size = 234 bp). I-E  $\beta$  chain: sense 5'-A AGGACAGTCTGGACTTCAGCAA-3' and anti-sense 5'-AGGGATAAACAGCCATCAGGAGCA-3' (predicted size = 204 bp). Total mCIITA: sense 5'-AC GCTTCTGGCTGGATTAGT-3' and anti-sense 5'-T CAACGCCAGTCTGACGAAGG-3' (predicted size = 342 bp). Anti-sense primer for type I, III, and IV mCIITA: 5'-GGTCGGCATCACTGTTAAGGA-3'. Sense primer type I mCIITA: 5'-AAGAGCTGCTCTCACGGGAAT-3' (predicted size = 264 bp). Sense primer type III mCIITA: 5'-TCTTACCTGCCGG AGTT-3' (predicted size = 100 bp). Sense primer for type IV mCIITA: 5'-GA GACTGCATGCAGGCAGCA-3' (predicted size = 129 bp). Reverse transcription of RNA (1 µg) into cDNA was performed in the presence of Oligo(dT)<sub>18n</sub> primer and RevertAid M-MuLV Reverse Transcriptase (MBI Fermentas) as per manufacturer's instructions. Reverse transcribed cDNA was amplified in the presence of specific sense and anti-sense primer and DNA Taq polymerase (MBI Fermentas). Thirty cycles of PCR (93 °C for 30 s, 57 °C for 20 s, 72 °C for 20 s) was followed by 5 min extension incubation at 72 °C. Negative controls included all reagents and primers without cDNA. GAPDH was amplified as positive control. PCR products were resolved on 2.0% agarose gels and were confirmed by size comparison with 100 bp ladder.

### 3. Results

#### 3.1. Expression of MHC class II by activated T-cells

To test for cell surface expression of MHC class II molecules by activated mouse T-cells, purified T-cells from naïve mice were stimulated in vitro with phorbol 12-myristate 13-acetate (PMA) and ionomycin. The purity of T-cells was detected by FACS after staining with anti-CD3, anti-CD11c and anti-IgM antibody and they are 99% pure (Fig. 1). The combination of PMA/ionomycin is routinely used as a TCR-independent model to study T-cell activation and proliferation [32,33]. PMA/ionomycin treatment has been shown to positively select CD4<sup>+</sup> T-cells in thymocyte cultures [34] and modulate T helper and T cytotoxic subsets [35]. Activation of T-cells after treatment with PMA/ionomycin was monitored by FACS after staining with CD69, a very early activation marker. Our result shows that all cells are activated after PMA/ionomycin treatment (Fig. 2A). We have monitored the expression of MHC class II molecules on naïve mouse T-cells after in vitro activation with PMA/ionomycin and the results are shown in Fig. 2B, which dem-

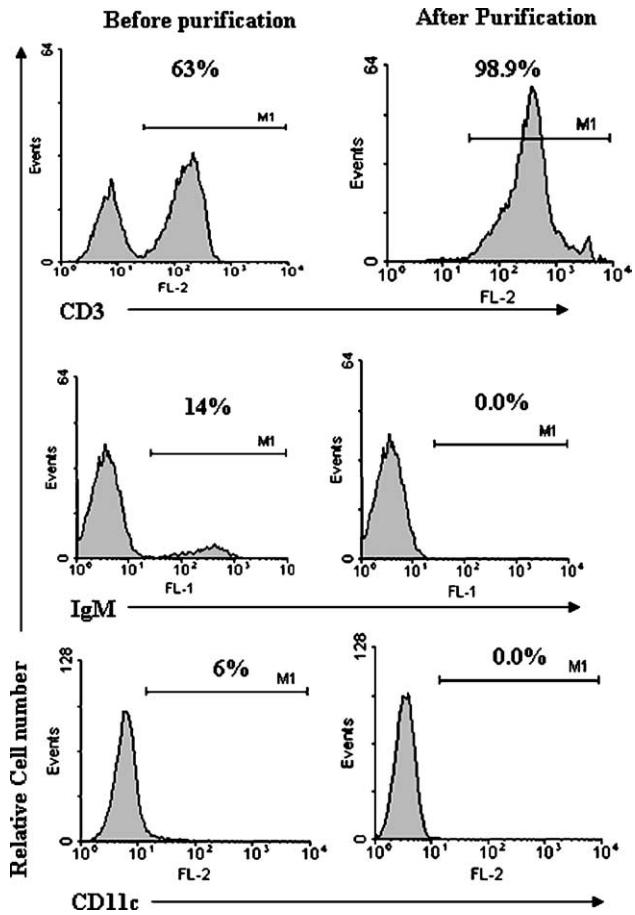


Fig. 1. Purity of T-cells. T-cells were purified from naïve mouse as described in Section 2. Cells were stained with fluorochrome-conjugated antibodies and isotype control antibodies. The purity was detected by FACS. Marker (M1) were set according to isotype control antibody staining.

onstrates the expression of MHC class II molecules. To detect the expression of MHC class II on CD4<sup>+</sup> and CD8<sup>+</sup> T-cells, purified CD4<sup>+</sup> or CD8<sup>+</sup> T-cells were activated in vitro with PMA/ionomycin and stained for MHC class II molecule. The results (Fig. 2C) show that CD4<sup>+</sup> T-cells express MHC class II molecules but not CD8<sup>+</sup> T-cells. We observed that surface expression of MHC class II on purified CD4<sup>+</sup>CD8<sup>+</sup> mixed T-cells (Fig. 2B, iii) is almost similar to that expressed on purified CD4<sup>+</sup> T cell (Fig. 2C). This MHC class II staining in the mixed population is probably due to acquisition of MHC class II by CD8<sup>+</sup> T cells from CD4<sup>+</sup> T-cells as it has been reported earlier that activated CD8<sup>+</sup> T-cells acquire surface molecules from other cells [36–38]. When we analyzed the expression of MHC class II on mixed population of purified T-cells, we see the presence of MHC class II on both CD4<sup>+</sup> and CD8<sup>+</sup> T-cells which could be due to acquisition of MHC class II from CD4<sup>+</sup> T-cells by CD8<sup>+</sup> T cells (Fig. 2B, v and vi). Further, expression of MHC class II in cells activated by concanavalin-A, plate bound anti-CD3 and soluble anti-CD28,

plate bound anti-TCR $\beta$  antibody or purified recombinant IL-2 was tested. The results (Fig. 2D) show that T-cells after activation by the above-mentioned reagents also express MHC class II molecules.

To obtain evidence for the expression of MHC class II by T-cells activated in vivo, mice were immunized with N protein and after 5 days, the expression of MHC class II was tested. The results given in Fig. 3 show that in vivo activated T-cells express MHC class II molecules on their surface. However, this experiment cannot completely rule out the possibility of passive acquisition of MHC class II molecules from other APCs as reported earlier [17,18].

### 3.2. *De novo* synthesis of MHC class II molecules by activated T-cells

Mouse T-cells have been shown to acquire MHC II from professional antigen presenting cells [18,39]. To test for de novo synthesis of MHC class II, purified T-cells from naïve mouse were activated in vitro and metabolically labeled with [<sup>35</sup>S]methionine and MHC class II synthesis in such labeled T-cells was measured by immuno-precipitation and SDS-PAGE. The results show that upon stimulation in vitro, T-cells synthesize MHC class II molecules endogenously (Fig. 4A). Two protein bands (~33 kDa and ~28 kDa) seen in Fig. 4A and B correspond to the MHC class II  $\alpha$  and  $\beta$  chain, respectively. MHC class II expression was further confirmed in antigen driven activated T-cells. The results shown in Fig. 4B demonstrate that antigen driven (N protein) activation also leads to expression of MHC class II molecules.

SDS-stable complex formation was monitored by SDS-PAGE under non-reducing condition. MHC class II antibody specific for  $\beta$  chain was used, which immunoprecipitates both  $\alpha$  and  $\beta$  chain along with Ii molecules [29]. Co-immunoprecipitation of  $\alpha$  chain by MHC class II antibody indicates that a significant fraction of the newly synthesized class II molecules formed dimers shortly after synthesis. The band for  $\alpha$  chain appears denser than the  $\beta$  chain band in the immunoprecipitate because newly synthesized Ii chain co-migrates with  $\alpha$  chain under the condition used for these experiments (Fig. 4A). After 1–2 h, however, an increasing fraction of  $\alpha$  and  $\beta$  chain migrated as a band of 52–55 kDa, (C[ $\alpha$ / $\beta$ ], Fig. 4A). Since these proteins are sensitive to boiling in SDS (Figs. 4A and B), it is indicative of “compact” peptide loaded  $\alpha$ / $\beta$  dimers [29,40–42]. Apart from the C[ $\alpha$ / $\beta$ ], we also see another band F[ $\alpha$ / $\beta$ ] appearing as early as 0.5 h which represents the dimers formed by immature  $\alpha$  and  $\beta$  chain of MHC class II as reported earlier [29,43]. Aggregates present in the not-boiled samples dissociated and migrated at ~47 kDa and above. This is probably due to immature stable dimers of  $\beta$  chains or disulphide bonded  $\beta$  chain with other ER proteins as reported earlier [29,43]. We also see the presence of Ii polypeptide by

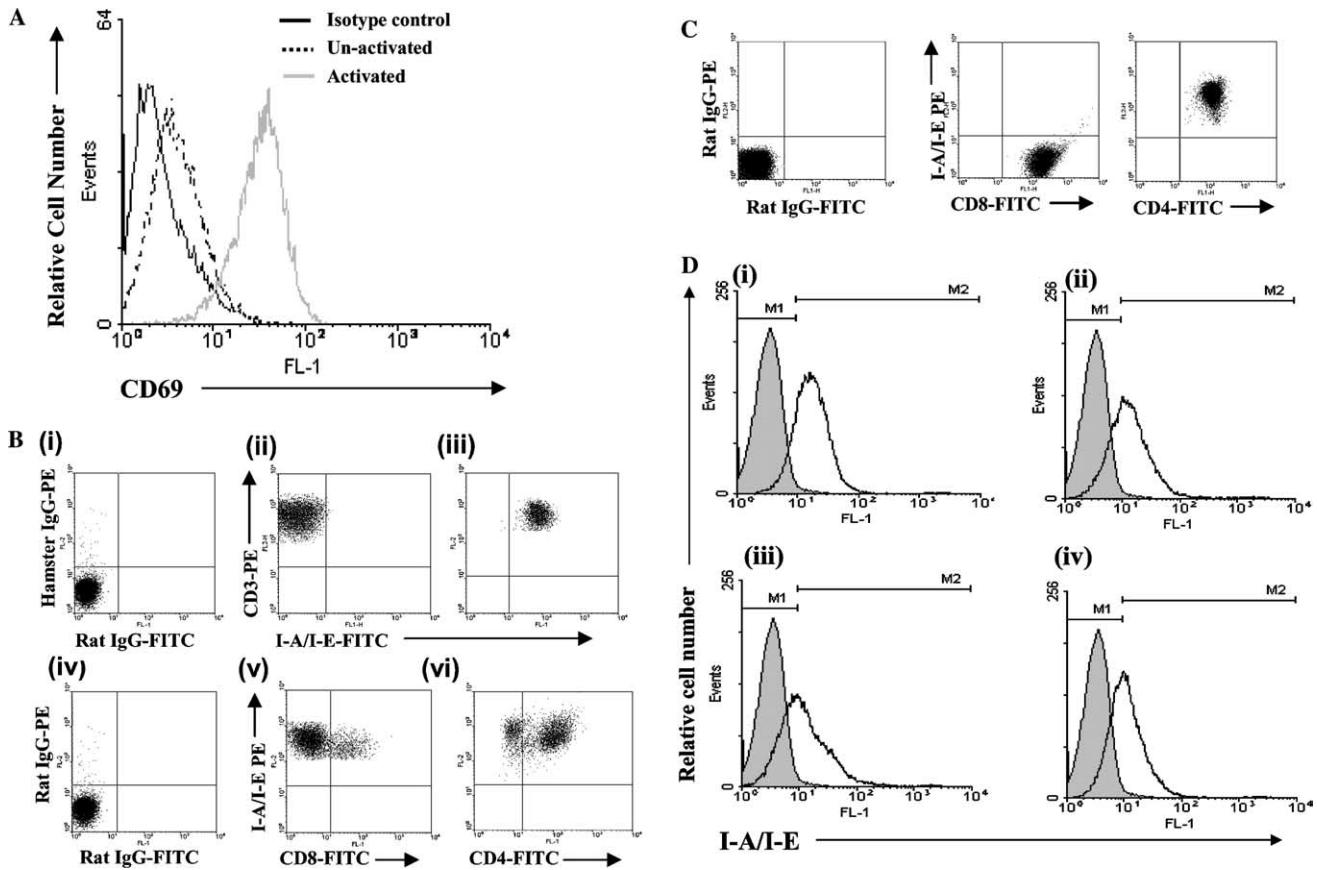


Fig. 2. Expression of MHC II by in vitro activated T-cells. (A) T-cells were purified from naïve mouse lymph nodes and in vitro stimulated with PMA and ionomycin for 6 h. Activation of T cells was detected by surface staining of early activation marker CD69. (B) In vitro activated T-cells showing MHC class II expression. Purified T-cells ( $4 \times 10^6$ ) were stimulated with PMA and ionomycin for 6 h and stained for surface marker and analyzed by FACS. Dot plot (i, iv) represents isotype controls, plot (ii) staining of un-activated T-cells, plot (iii, v, vi) staining of activated T-cells. (C) Purified CD4<sup>+</sup> T-cells or CD8<sup>+</sup> T-cells were activated for 6 h with PMA/ionomycin and surface stained and analyzed by FACS. (D) Purified mouse T-cells were in vitro activated with (i) combination of plate bound anti-CD3 antibody (145-2C11; 10  $\mu$ g/ml) + purified soluble anti-CD28 antibody (1  $\mu$ g/ml), (ii) recombinant mouse IL-2 (200 U/ml), (iii) concanavalin-A (1  $\mu$ g/ml) or (iv) plate bound anti-mouse TCR $\beta$  antibody (H57.597; 10  $\mu$ g/ml). In the presence of these activators, cells were cultured for 8 h and tested for MHC class II expression by FACS. Filled histogram represents staining with isotype control antibody and open histogram represents staining for MHC class II antibody. Data are representative of three independent experiments.

Western blot in the boiled sample using anti-mouse Ii antibody which confirmed the co-migration of Ii with  $\alpha$  chain (data not shown).

To further confirm the finding that MHC class II is endogenously synthesized and transported to the surface of activated mouse T-cells, the cells were treated with brefeldin-A during activation and stained for surface MHC class II. Brefeldin-A is known to block the transport of protein from endoplasmic reticulum to Golgi-complex thereby inhibiting the transport of molecules to the surface of cells [44,45]. The data given in Fig. 4C show that MHC class II expression on the surface of activated T-cells is blocked by brefeldin-A treatment.

### 3.3. Co-localization of LAMP-1, H2M, MHC class II, and antigen

In order to investigate whether external antigens taken up by activated T-cells undergo processing through the

standard pathway for class II presentation, the expression of LAMP-1, a late endosome-lysosome marker was tested in the activated T-cells. To demonstrate that internalized antigen goes through MHC class II processing pathway, cells were pulsed with N antigen and co-localization of LAMP-1 and antigen was visualized by confocal microscopy. The results shown in Fig. 5A demonstrate that LAMP-1 is co-localized with N protein indicating that N protein has gone into the antigen-processing compartment. To detect the number of T-cells which have taken N protein, we have performed FACS analysis and results show that 80% activated T-cells were N positive (data not shown). To provide further support for the MHC class II processing pathway in activated T-cells, the expression of H2M and MHC class II molecules together (Fig. 5B) or individually with Lamp-1 (Fig. 5C and D) in T cells were monitored by confocal microscopy. H2M is a chaperone molecule, which plays a crucial role in loading of peptide on major histocompatibility

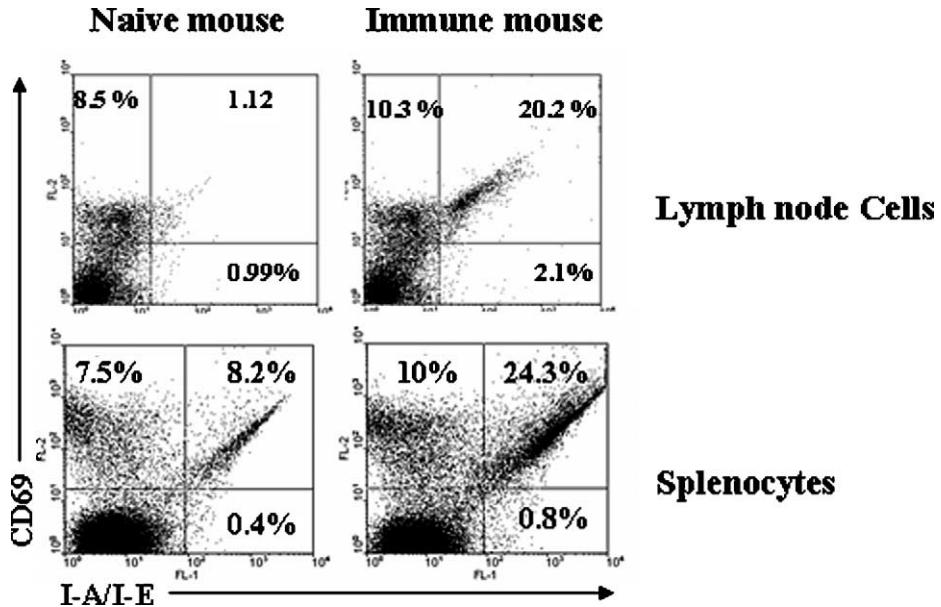


Fig. 3. Expression of MHC class II by in vivo activated T-cells. T-cells were in vivo activated by subcutaneously immunizing mice with 50 µg of N protein in Freund's complete adjuvant. After 5 days, mice were sacrificed, single cell suspension from lymph nodes and spleen were made. Tri-color surface staining of these cells were performed using anti-mouse TCR $\beta$ -Cy5PE, anti-mouse CD69-biotin/streptavidin-PE and anti-I-A/I-E antibody/anti-Rat-FITC. FACS results are gated on TCR $\beta$ -positive cells. Quadrants were set according to isotype control antibody staining. Data are representative of three independent experiments.

class II molecules in lysosomal compartments [46]. It is interesting to note that the expression of H2M and MHC class II are co-localized in the late endosome/lysosome in activated T-cells. Results given in Figs. 5C and D show that H2M and MHC class II molecules are present in this compartment where antigen processing takes place. In all the staining, cells were stained with isotype control antibody, which does not give any significant fluorescence (data not shown).

#### 3.4. Expression of mRNA for Ii, H2M $\alpha$ chain, I-A $\alpha$ chain, I-A $\beta$ chain, I-E $\alpha$ chain, I-E $\beta$ chain and CIITA by activated T-cells

To detect the generation of mRNA for Ii, H2M $\alpha$ , I-A $\alpha$  chain, I-A $\beta$  chain, I-E $\alpha$  chain, I-E $\beta$  chain and CIITA in activated T-cells, RT-PCR of RNA from activated T-cells was carried out. As shown in Fig. 6A, mRNA for these molecules are present in activated T-cells but not in naïve T-cells of Balb/c mice. Since C57Bl/6 inherently lack MHC class II IE because of a naturally occurring defect in the Ea, gene making them class II IA $^+$ IE $^-$  [47]. We have used T-cells from C57BL/6 strain of mice as genetic control for the RT-PCR analysis and shown that activated C57BL/6 T-cells express I-A molecules but not the I-E molecules (Fig. 6B).

The expression of Ii, H2M $\alpha$ , and MHC class II molecules is controlled by class II transactivator protein (CIITA). The CIITA has been referred to as the "master control factor" for these molecules [31,48,49]. Therefore, we have examined the expression of CIITA in activated

mouse T cells. CIITA expression is regulated by three promoters (pI, pIII, and pIV), producing type I, type III and type IV CIITA [31]. Recently it has become clear that the restriction of these promoters to different cell types or with various stimuli is not so simple [31,48]. We observed that activation dependent expression of CIITA in mouse T-cells involves promoter I and IV but to lesser extent promoter III in PMA/ionomycin activated T-cells (Fig. 6C). It is interesting to note that we also observed a very faint band in un-activated T-cells (total CIITA and type IV CIITA). This low level expression of CIITA is probably not sufficient to drive the expression of MHC class II and other molecules as reported earlier [50,51]. In normal activated splenocytes (un-fractionated T cells) the expression of all three type CIITA is seen (data not shown).

#### 3.5. Activated T-cells process and present extra-cellular antigen to other T-cells

To examine whether mouse T-cells can also present external antigen to other T-cells, purified activated T-cells, were pulsed with Rinderpest virus nucleocapsid protein, and irradiated after 12h of culture. These cells were used as antigen presenting cells (T-APCs) in an in vitro proliferation assay. The results shown in Fig. 7A demonstrate that T-APC is able to present antigen to primed, specific T-cells. When activated T-APCs were pulsed with antigen in the presence of chloroquine, they do not stimulate primed T-cells showing that antigen processing has been inhibited in these cells. To test if proliferation signal is coming through MHC class II,

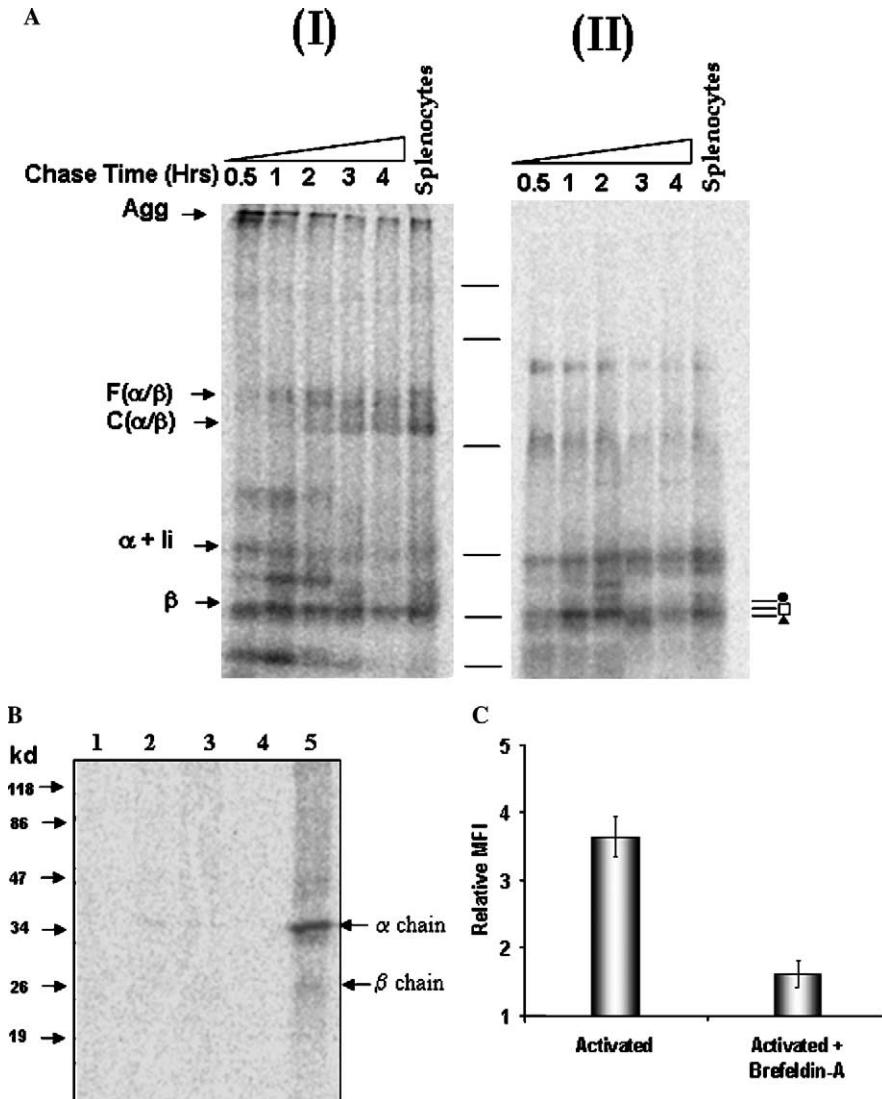


Fig. 4. De novo synthesis of MHC class II by activated T-cells. (A) Purified T-cells ( $5 \times 10^6$ ) from naïve mice were activated with PMA and ionomycin for 6 h. Cells were pulsed with [ $^{35}$ S]methionine for 20 min and chased for different time points. Immune-complexed bead were divided into two fractions. One fraction was incubated for 1 h at room temperature in Laemmli sample buffer (I) and other fraction was boiled for 3 min (II). Samples were analyzed by 10% SDS-PAGE followed by phosphor-image analysis. Splenocytes were activated with PMA/ionomycin for 1 h and pulsed with [ $^{35}$ S]methionine as described above and chased for 3 h and used as positive control for these experiments. Different glycosylation intermediates are also shown. (●) Core glycosylated; (□) trimmed intermediate; (►) terminally glycosylated. Molecular weight markers are indicated by the six horizontal lines shown between boiled and not boiled gels. As shown, the markers indicate the migration of the 118, 86, 47, 34, 26, and 19 kDa, respectively. (B) Splenocytes from naïve mouse were pulsed with N protein (100  $\mu$ g/ml) and cultured for 12 h in complete medium. As a control, splenocytes pulsed with antigen were treated with chloroquine (40 mM) during culture. Fixed splenocytes ( $4 \times 10^6$  cells/well) were co-cultured with purified antigen specific T-cells ( $2 \times 10^6$  cells/well) in complete medium in 24-well plates. Cells were cultured for 6 h and in the last 2 h, pulsed with [ $^{35}$ S]methionine. Lysate from these cells were immuno-precipitated with anti-MHC class II antibody and analyzed on 12% SDS-PAGE followed by fluorography and phosphorimaging. Lane 1, immunoprecipitation with isotype control antibody; lane 2, with anti-MHC class II antibody from fixed splenocytes only; lane 3, responder immune cells only; lane 4, N protein pulsed splenocytes in the presence of chloroquine along with responder immune T cells; lane 5, fixed splenocytes with responder T-cells. Data are representative of three independent experiments. (C) Purified T cells were in vitro activated with PMA and ionomycin in the presence or absence of brefeldin-A (10  $\mu$ g/ml). After 6 h of incubation, cells were harvested and stained for surface MHC class II. Cells were analyzed by FACS and relative MFI (mean fluorescence intensity) were calculated by dividing the MFI of activated cells value with MFI of un-activated cells.

antigen pulsed T-APCs were co-cultured with primed T-cells in the presence of purified anti-mouse MHC class II antibody. In vitro stimulation of primed T-cells with antigen pulsed T-APC leads to secretion of IL-2 as shown in Fig. 7B. Un-stimulated antigen pulsed T-APC

and stimulated antigen pulsed T-APC treated with anti-MHC class II antibody are not able to induce IL-2 secretion by antigen specific T cells. These results show that activated mouse T-cells process and present antigen to other T-cells in a MHC class II dependent manner.

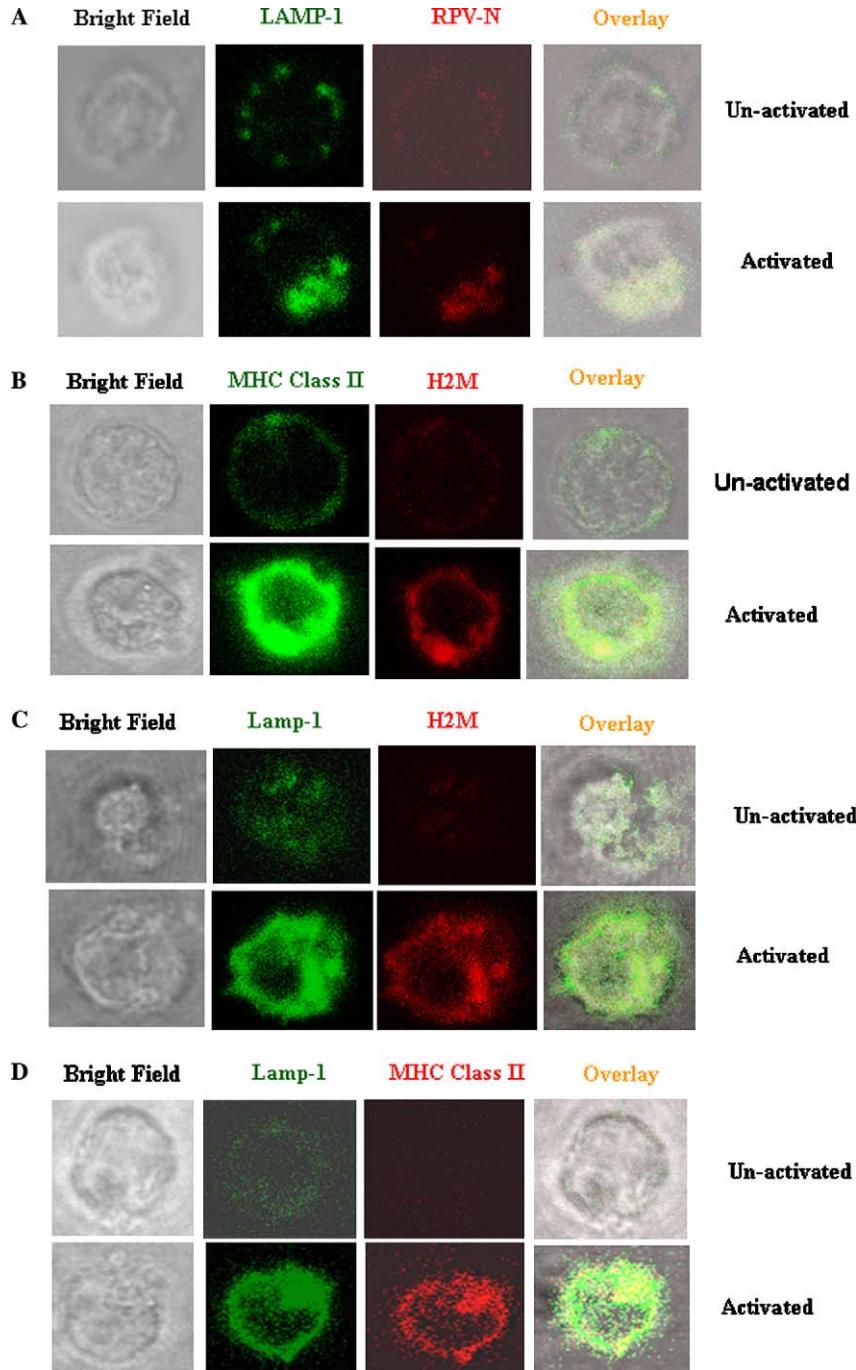


Fig. 5. Co-localization of H2M, MHC class II, LAMP-1, and antigen. Purified mouse T-cells were in vitro activated with PMA/ionomycin for 6 h. Intra-cellular staining was performed. Cells were visualized under Confocal microscopy (100 $\times$  objective). Double staining is indicated by a yellow colour in the overlay image. (A) Activated T-cells were cultured with N protein (100  $\mu$ g/ml) for 3 h. Intracellular staining of cells with anti-mouse LAMP-1-FITC and rabbit anti-N antibody/anti-rabbit IgG-Cy3 was performed. (B) Cells were stained with anti-mouse H2M-TRITC and anti-mouse MHC II-biotin/streptavidin-FITC. (C) Anti-mouse LAMP-1-FITC and anti-mouse H2M-TRITC. (D) Anti-mouse LAMP-1-FITC and anti-mouse MHC class II-biotin/streptavidin-TRITC.

#### 4. Discussion

In the present work, we have examined the expression of MHC class II molecules and explored the antigen presenting capacity of activated mouse T-cells. The expression of MHC class II molecules in mouse T-cells has

been reported using T-cell clones or T-cell lymphoma but not in naïve T-cells [13–16,52,53]. In most of these studies, T-cells were cultured in the presence of mitogens or IL-2. In the present work, we show that culturing of T-cells in the presence of various mitogens leads to expression of MHC class II molecules. Earlier reports on

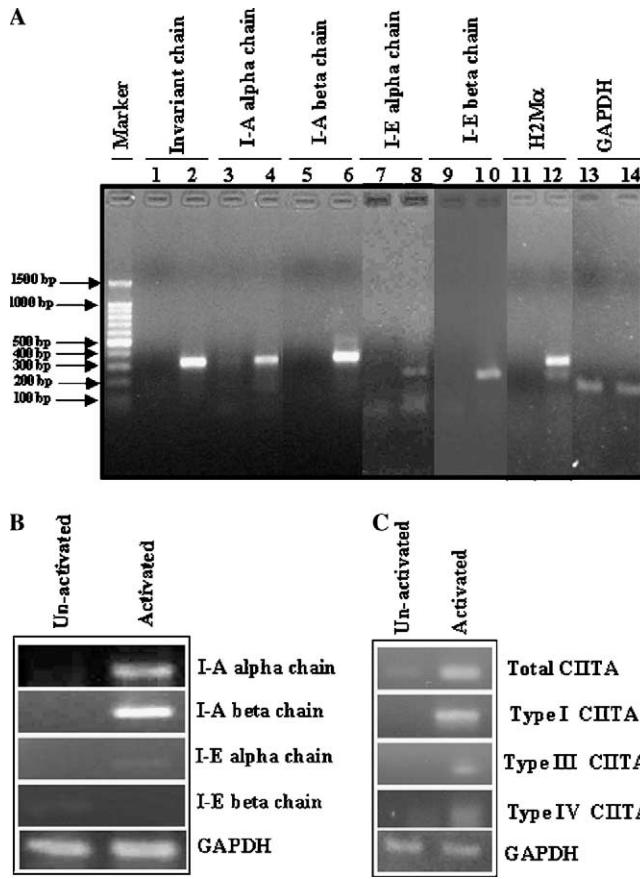


Fig. 6. Activated T-cells synthesize mRNA encoding Ii, I-Ad  $\alpha$  chain, I-Ad  $\beta$  chain, H2M $\alpha$ , and CIITA. Purified T-cells were activated with PMA/ionomycin for 3 h. cDNA were synthesized using oligo(dT)<sub>18</sub> primer and expression of transcripts were detected by RT-PCR using gene specific sense and anti-sense primers. GAPDH was used as an internal control. Products were electrophoresed through 2% agarose gel. (A) Lanes 1, 3, 5, 7, 9, 11, and 13 represent the profile of transcript from the naïve T-cells. Lanes 2, 4, 6, 8, 10, 12, and 14 represent profile of activated T-cells. (B) T-cells from C57BL/6 mice were purified and activated in vitro. Expression of gene was detected by RT-PCR as described above. (C) Expression of CIITA was tested as described earlier [31].

absence of MHC class II expression in long-term cultured specific T-cell clone or -cell lines, needs to be re-examined in the context of expression or defect in the expression of CIITA, which is a master controlling factor. There are reports that activated T-cell acquires MHC class II molecules from other antigen presenting cells [17,18,39]. We also observe the presence of MHC class II molecules on ex vivo activated T-cells by FACS. However, FACS analysis does not differentiate between de novo synthesized and passively acquired MHC class II molecules.

In our experimental system, since the cells were activated in vitro in the absence of professional antigen presenting cells, increased expression of MHC class II on the surface is possible via intracellular synthesis, and not by acquisition of MHC class II molecules from other

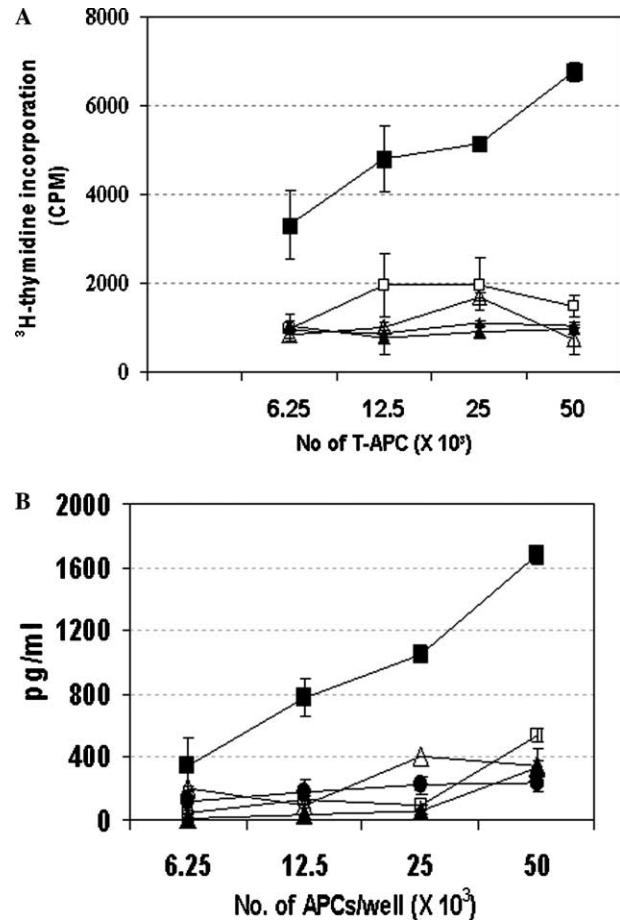


Fig. 7. Processing and presentation of soluble antigen by activated T cells to primed T-cells. (A) In vitro proliferation assay. Purified T-cells ( $5 \times 10^6$  cells/well) from naïve mouse were in vitro activated with PMA and ionomycin for 6 h, pulsed with purified soluble nucleocapsid protein (100  $\mu\text{g}/\text{ml}$ ) in 24-well plates. After 12 h of incubation, T-APC were washed and irradiated. Responder T-cells ( $4 \times 10^5$  cells/well) were co-cultured with different number of T-APCs for 72 h in flat bottom 96-well plate. Result shown is the mean  $\pm$  SEM of triplicates. (B) IL-2 secretion. T-APC and responder cells were generated as mentioned above. T-APC was fixed with 0.5% paraformaldehyde for 10 min on ice. Responder T cells ( $5 \times 10^5$  cells/well) were incubated with different number of T-APCs in flat bottom 96-well plates for 36 h in final volume of 200  $\mu\text{l}$  of complete medium. Supernatant was collected and IL-2 secretion was detected by ELISA kit (e-Bioscience, USA). Data shown is the mean  $\pm$  SEM of triplicates. ( $\triangle$ ) represents activated T-cells without N protein, ( $\blacksquare$ ) activated T-cells pulsed with RPV-N protein, ( $\square$ ) normal T-cells pulsed with RPV-N protein, ( $\blacktriangle$ ) activated T-cells pulsed with N protein in presence of chloroquine (40 mM), ( $\bullet$ ) Activated T-cells pulsed with RPV-N protein but co-cultured in the presence of MHC class II antibody (clone M5/115; 1  $\mu\text{g}/\text{ml}$ ).

APCs as has been reported earlier [17,18]. Metabolic labeling of newly synthesized MHC class II with [ ${}^{35}\text{S}$ ]methionine followed by immunoprecipitation confirms de novo synthesis. The results demonstrate that class II molecules are synthesized endogenously after activation with either PMA/ionomycin or with antigen pulsed APCs. Our result also correlates with earlier published report for other species [11,12,19]. This is further

confirmed by inhibition of surface expression of MHC class II by brefeldin-A. Thus, naïve mouse T-cells are capable of synthesizing MHC class II de novo after activation with mitogens or even by antigen driven activation. Although, MHC class II expression in long term cultured cloned T-cells or T-cell lymphoma or thymus derived cells has been shown [13,14,16], the present work demonstrates that activated mouse T-cells can synthesize MHC class II molecules de novo. Using pulse labeling, immunoprecipitation and analysis of immune complexes under denaturing and non-denaturing condition, we show that newly synthesized MHC class II molecules form SDS-stable complexes similar to that reported for other antigen presenting cells [29]. In addition, the synthesis of transcripts for I-A  $\alpha$  chain, I-A  $\beta$  chain, I-E  $\alpha$  chain, I-E  $\beta$  chain, Invariant chain, H2M $\alpha$  in activated mouse T-cells was confirmed by RT-PCR analysis. The expressions of these molecules are controlled by class II transactivator protein (CIITA) [48,54]. It has also been reported that CIITA knockout mice express MHC class II albeit at a five-fold reduced level [55]. We have examined the expression of CIITA by RT-PCR and interestingly, we find that mouse T-cells use predominantly type I and type IV exon of CIITA but very little of type III exon in comparison to rat T-cells where the usage of type III and type IV exon of CIITA has been reported [12]. Though mouse T-cells express class II transactivator (CIITA), a ‘master regulator factor’, yet it is not sufficient to drive the expression of MHC class II molecules in naïve cells [48]. The functional activation of CIITA involves phosphorylation of CIITA which can be achieved from signaling through mitogenic triggering [56–58]. Recently, it has been shown that over-expression of CIITA in mouse T-cells leads to induction of tolerance and altered Th1/Th2 balance [59].

Professional antigen presenting cells are known to take up, process and present extra-cellular soluble antigens to T-cells. T-cells are known to express CD40, CD40L and B7 co-stimulatory molecules in activated state, which are needed for activation of responder T-cells, enhanced cell cycle progression and survival [19,33,60]. Antigen presentation by resting or naïve T-cells is known to induce anergy to responder T cells [61] and apoptosis in other T-cells [62] perhaps because of lack of co-stimulatory molecules on T-APCs. We explored the possibility of intake of soluble antigen by activated mouse T-cells by pulsing with Rinderpest virus nucleocapsid protein in complete medium for 3 h. The results show that activated T-cells take up antigen. Subsequently by confocal microscopy, we have shown that LAMP-1, a late endosomal and lysosomal marker [63], is seen as part of a cluster in vesicles, indicating that vesicles have been formed during the antigen processing pathway. Co-localization of LAMP-1 with antigen in activated T-cells demonstrates that antigen has entered into the endosomal compartment for processing. We

show that H2M is also expressed in activated T-cells, which is an essential molecule for presentation of peptide on MHC class II. These results show that activated mouse T-cells express H2M molecules required for antigen presentation. We have also examined the expression of Invariant chain (Ii) in activated mouse T-cells by confocal microscopy. The results demonstrate that Ii molecules are present in activated T-cells (data not shown). The invariant chain functions as a chaperone protein for MHC class II molecules during folding, assembly and transport from endoplasmic reticulum to Golgi complex [29,43]. It has been reported earlier that Ii chain is not only required for dimerization or at least partial folding of  $\alpha$  and  $\beta$  chains but also required to allow newly synthesized class II molecules to escape the quality control mechanism in the ER that prevents the transport of misfolded proteins [29]. The presence of lysosomal enzymes associated with antigen processing remains to be demonstrated.

The functional importance of processing and presentation was examined by in vitro proliferation of primed T-cells and IL-2 secretion. The results show that activated T-cells are capable of presenting antigen to primed T-cells as has been reported in case of human and rat [11,19,64]. Further inhibition of processing of extracellular antigen was demonstrated using chloroquine, a known inhibitor of lysosomal enzymes by increasing the pH [65]. Proliferation and IL-2 secretion are shown to be MHC class II dependent since anti-MHC class II antibody inhibits this function. This shows that the antigen was processed by lysosomal proteolytic enzymes and presented on surface, which is able to functionally trigger the antigen specific T-cells.

We have demonstrated that activated murine CD4 $^{+}$  T cells can process and present antigen through MHC class II molecules. Antigen presentations by T-cells have far-reaching biological consequences. Antigen presentation by T-cells may play a key role in controlling infection by lymphotropic viruses [66,67]. Perhaps, lymphotropic viruses on infecting T-cells regenerate a quick response bringing in both CD4 $^{+}$  help and CTL attack on infected T-cells. Therefore, antigen presentation by T-cells provides an ideal mechanism to protect immune machinery involving T-cells, which needs experimental verifications. The presentation of antigen by T-cells leads to T-cell–T-cell interactions which may lead to proliferation and/or regulation of cognate T-cells as found in auto-immune diseases [20]. T-cell–T-cell interaction has also been proposed to be the basis of T cell vaccination [21].

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