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Auto-presentation of Staphylococcal enterotoxin A by mouse CD4$^+$ T cells

——Auto-presentation by CD4$^+$ T cells

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ABSTRACT

The currently accepted model for superantigen (SAg) induced T cell activation suggests that SAg, without being processed, cross link both MHC class II, from Antigen Presenting Cells (APC), and V-*β*, from T-cell receptor (TCR), initiating nonspecific T-cell activation. This T-cell proliferation induces a massive cytokine release associated with several human diseases. It is thought that murine CD4$^+$ T cells do not express MHC class-II molecules. However, we discovered that a subtype of mouse naïve CD4$^+$ T cells expresses MHC class II on their cell surface and that these CD4$^+$ T cells can perform the role of both APC and T cells, able to present Staphylococcal enterotoxin A (SEA) to itself or neighboring CD4$^+$ T cells via MHC class II, thus inducing massive CD4$^+$ T cell proliferation. Treatment with neutralizing anti MHC class II antibody inhibits this CD4$^+$ T cell proliferation response. The fact that murine CD4$^+$ T cells express MHC class II offers new insight about SAg activity. Based on our findings, we propose revising and extending previous models for SAg induced T cell activation, altering previous models of MHC class II restriction of T cell responses to SEA as well as the requirement for SAg processing.

Keywords: MHC Class II; T-Cell; Enterotoxin

1. INTRODUCTION

Staphylococcus aureus is a major bacterial pathogen causing several diseases including food-borne illnesses [1]. *S. aureus* produces a group of 21 (known) staphylococcal enterotoxins (SEs) that have two separate biological activities: they cause gastroenteritis in the gastrointestinal tract and act as a superantigen (SAg) causing massive activation and proliferation of T-cells and cytokine release [1]. Several studies have shown that there is a relation between emetic and superantigenic activities [2,3]. SAg plays an important role in pathogenesis by undermining the specificity of the adaptive immune response. They are reported to be associated with etiology of several human diseases including toxic shock syndrome, Kawasaki disease, guttate psoriasis, eczema, rheumatoid arthritis and scarlet fever [4].

The current model for superantigen activity suggests that native, unprocessed SAg bind directly to the α-helical chain of the MHC class II, outside the peptide binding groove of the antigen presenting cell (APC) [5]. This binding takes place without proteolytic degradation and fragmentation, internalization, and re-expression of the SAg degraded short fragments on the cell surface. This native SAg is recognized and interacts with a large number of T-cells, which all share particular sequences within the variable region of the β chain (V-*β*) of the T cell receptor (TCR), thereby stimulating ~20% of the naïve T-cell population [6]. This trimolecular interaction triggers massive proliferation of the T-cells subsets and production of large quantity of cytokines that can have pathological effects. The currently accepted model, therefore, suggests that both types of cells; APC and T cells, are needed for SAg induced T-cell proliferation [7,8].

The currently acceptable view is that murine T-cells do not express MHC class-II molecules [9-13], and that professional APCs expressing MHC class II are required for SAg induced T cell proliferation [7]. However, the present study demonstrates for the first time that mouse naïve CD4$^+$ T cells *αβ* TCR express MHC class II on their cell surface. Our data suggest that these T cells act as APCs, capture staphylococcal enterotoxin A (SEA), and present the SEA-MHC class II complex to itself or

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neighboring $CD4^+$ T cells targeting their own surface molecules. These events then cause proliferation. When anti MHC class II antibodies are added to purified double positive $CD4^+$ T cells, they block T-cell auto presentation and inhibit T cell proliferation.

2. MATERIAL AND METHODS

2.1. Chemicals and Reagents

SEA was obtained from Toxin Technology (Sarasota, FL). Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) was obtained from Calbiochem (San Diego, CA). Phytohemagglutinin (PHA) was obtained from Sigma Aldrich (St Louis, MO). Anti-CD3 epsilon chain labeled (PE), anti-$\alpha\beta$ TCR labeled pacific blue, anti-CD4 antibody labeled with APC anti-MHC class II antibody I-Ek, anti MHC class II antibody against the subregion encoded glycoproteins I-A^b, I-A^d, I-A^q, I-E^d, I-E^k. And anti-MHC class II antibody labeled with FITC or APC were obtained from eBioscience (San Diego, CA). Anti-Vβ3 T-cell receptor was obtained from BD Pharmingen (Franklin Lakes, NJ). Imunomagnetic beads conjugated with antibodies directed against CD3 and CD28 were obtained from Invitrogen (Carlsbad, CA), as well as $CD4^+$ T-cell positive and negative isolation kits.

2.2. Splenocyte Isolation

Spleens from C57BL/6 female mice were aseptically removed and disrupted using a syringe and needle in Russ-10 cell culture medium (made by combining 450 ml of RPMI 1640 medium without glutamine (Gibco, Carlsbad, CA), 50 ml Fetal bovine serum (Hyclone, Logan, UT), 5 ml 200 mM glutamine (Gibco), 5 ml antibiotic-antimycotic (Gibco; containing penicillin, streptomycin, and fungizone), 5 ml nonessential amino acid mix (Gibco), 5 ml sodium pyruvate (Gibco), and 0.25 ml of 100 mM beta mercaptoethanol (Sigma)). Cells were centrifuged at 200 × g at 4˚C for 10 min. Red blood cells were then lysed by adding 5 mL of red cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA). Cells were again centrifuged and resuspended in Russ-10 medium, and viable cells were counted using trypan blue and a hemocytometer.

2.3. Positive or Negative Isolation of Murine $CD4^+$ T Cells

Murine $CD4^+$ T cells were isolated using either a positive (Dynabeads Mouse CD4 L3T4) or negative selection kit (Dynal Mouse CD4 Negative Isolation Kit), according to the manufacturer's instructions. Briefly, for positive isolation, splenocytes were resuspended in isolation buffer (PBS supplemented with 0.1% BSA and 2 mM EDTA) at a concentration of 1 × 10^7/mL, and incubated with washed Dynabeads (25 µl of Dynabeads per 10^7 cells) for 20 mins on ice with gentle rotation. After incubation the cells and Dynabeads were placed on a magnet for 2 mins. The supernatant was removed and the bead-bound cells were washed with isolation buffer 3 times. The bead-bound cells were resuspended in Russ-10 media (10^7 cells per 100 µl of media) and DETACHaBEAD mouse CD4 was added (10 µl per 10^7 cells) and incubated for 45 mins with gentle rotation at room temperature. The detached cells were washed 3 times and resuspended in media. For negative isolation of CD4 cells heat inactivated FBS and antibody were added to splenocytes and incubated for 20 mins on ice. The cells were washed with isolation buffer, and pre-washed mouse depletion dynabeads were added and incubated for 15 mins with gentle rotation at room temperature. The cells and dynabeads were placed on a magnet and the supernatant was obtained and washed. The supernatant contained the negatively isolated mouse CD4 T cells.

2.4. Superantigen Induced Naive T-Cell Proliferation Assay

Cells were placed in 96-well plates (1 × 10^6/mL, 0.2 mL) in Russ-10 medium and treated with various concentrations of SEA ranging from 0.5 to 200 ng/ml following incubation at 37˚C in a 5% CO_2 incubator. After incubation for 48 h, cell proliferation was measured by adding Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU), which was incorporated into the DNA of dividing cells, 4 h before fixation as described by manufacturer instructions (Calbiochem, San Diego, CA). Spectroscopic measurements were made at an optical density of 620 nm and 450 nm.

2.5. Flow Cytometry

One and a half million cells were placed through a strainer and labeled with prospective antibodies for 40 mins. Cells were washed twice in 200 µL of PBS and resuspended in 0.5 mL of PBS. Flow cytometry and cell sorting were performed using a FACS Vantage SE (Becton Dickinson) fitted with a Cobolt Calypso™ 100 mW 491 nm laser (Cobolt AB, Sweden) and a cube 40 mW 640 nm laser (Coherent, Auburn, CA). The fluorescence of FITC and PE labels were quantifed by excitation at 491 nm using 530/30 and 585/42 bandpass filters, respectively. Fluorescence of APC was quantified by excitation at 640 nm using a 676/29 bandpass filter (Semrock, Rochester, NY).

2.6. Expansion of Cell Sorted Cells

After sorting cells were stimulated with immunomagnetic beads coated with anti-CD3/28 in Russ-10 con-

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taining IL-2 (30 U/mL).

2.7. Statistical Analysis

Statistical analysis was performed using SigmaStat 3.5 for Windows (Systat Software, San Jose, CA). Multiple comparisons were made of PHA or with increasing concentrations of SEA that induce splenocytes or purified T cell proliferation. The experiments were repeated at least three times, and results with $p < 0.05$ were considered statistically significant.

3. RESULTS

3.1. The Effect of APC-CD4$^+$ T Cells Ratio on Superantigen Induced Naive T-Cell Proliferation

In order to activate CD4$^+$ T-cells, a 10 to 1 ratio of APCs to T cells is needed [14]. Our hypothesis is that the ratio between APC and T cell is important for efficient T-cell proliferation, and that there would be a reduction in CD4$^+$ T-Cell proliferation response after altering this ratio, because reduction in APCs limits the number of CD4$^+$ T cells that can bind to the SEA-APC complex. To test this hypothesis, we increased CD4$^+$ T cell concentration by utilizing two types of CD4$^+$ T cell isolation methods; negative selection which increases the concentration of CD4$^+$ T cell from 22% in the average spleen to 90%, or positive selection with an average purity of 95%. The isolated CD4$^+$ T cells from a single spleen preparation were cultured with three concentrations of the superantigen SEA 0.5, 1 and 200 ng/ml. Proliferation was measured on day 1 by BrdU incorporation. As shown in **Figure 1**, SEA induced proliferation in a dose dependent manner in all three cell cultures. Surprisingly, the highly enriched positive isolated CD4$^+$ T, which presumably has the lowest concentration of APC cell, provided the highest signal with highest signal-to-noise ratio. These experiments raise the questions whether depleting APC paradoxically increases proliferation, and how complete elimination of APCs will effect proliferation.

3.2. The Superantigen Staphylococcal Enterotoxin A Induces Proliferation of CD4$^+$ T Cells in the Absence of APC

To determine how elimination of APCs affects SEA induced T cell proliferation, CD4$^+$ T cells were purified from splenocytes by positive and negative selection. In these experiments, SEA was used at a concentration of 200 ng/ml. To determine the absence of APC, APC-dependent mitogen PHA was used at concentration of 10 μg/ml. Antibody against MHC class II was used to block the interaction between MHC class II and the variable region of the TCR. As shown in **Figure 2**, the APC-de-

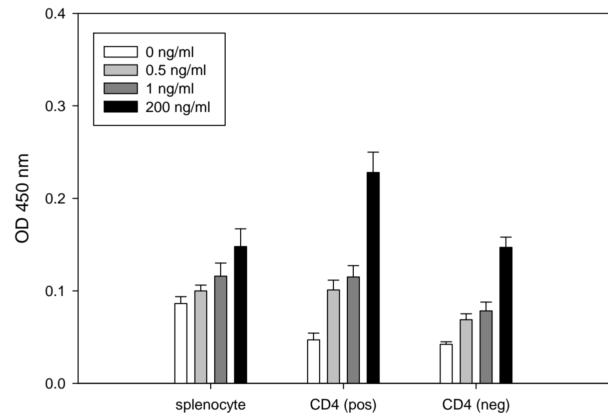

Figure 1. SEA activation of purified CD4$^+$ T-cells. Splenocytes, positively selected, and negatively selected CD4$^+$ T-cells were incubated with increasing concentrations of SEA. After incubation for 1 day newly synthesized DNA was measured. Error bars represent standard errors and $n = 3$.

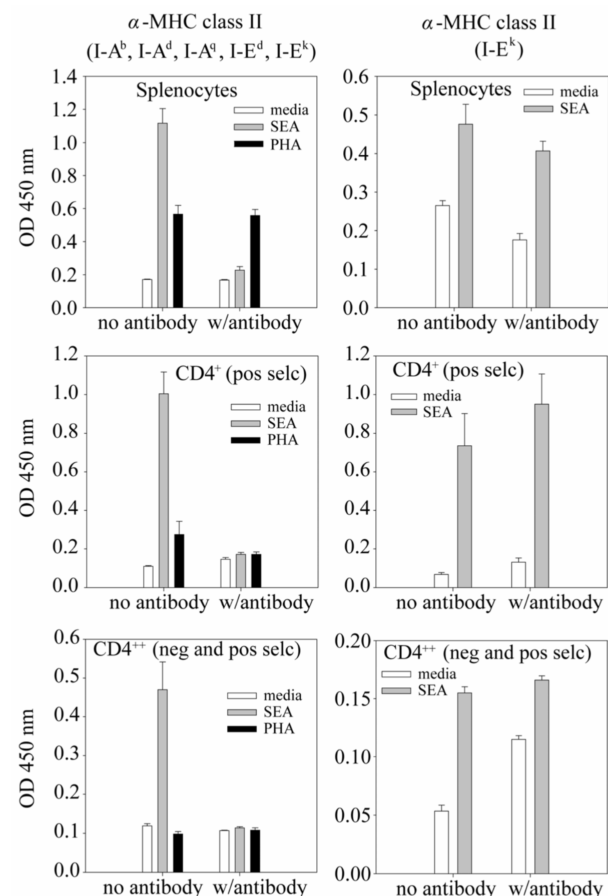

Figure 2. SEA induces proliferation of double purified CD4$^+$ T cells Splenocytes, positive selected and double positive selected CD4$^+$ T cells were incubated with SEA in the presence or absence of antibody against MHC class II subregion glycoproteins I-A^b, I-A^d, I-A^q, I-E^d, I-E^k or I-E^k alone. After incubation for 2 days, newly synthesized DNA was measured. Error bars represent standard errors and $n = 3$.

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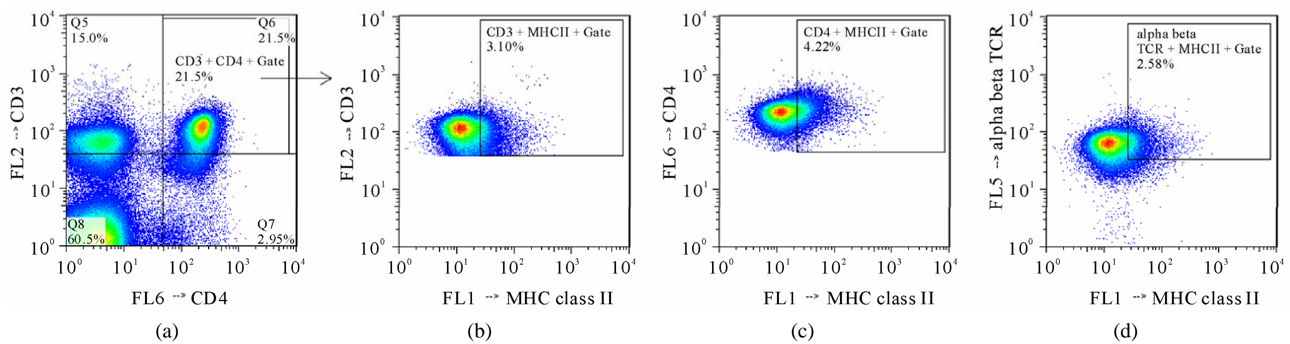
pendent mitogen, PHA has high proliferative effect on splenocytes, which contain large number of APCs. The data also show a low effect on $CD4^+$ T cells with positive isolation, and no proliferative effect on positive and negative selection of $CD4^+$ T cells. These observations suggest that the double positive purified $CD4^+$ T cells lack APCs. This result is novel and interesting because SEA was able to induce proliferation of purified $CD4^+$ T cells in the absence of MHC-class II expressed on APC. However, anti MHC class II antibody against the subregion encoded glycoproteins I-A^b, I-A^d, I-A^q, I-E^d and I-E^k, but not I-E^k alone, blocked proliferation of the doubled purified murine $CD4^+$ T cells. This shows that the SEA induced proliferation response is MHC class II dependent. These observations suggest the presence of MHC class II despite the lack of APCs.

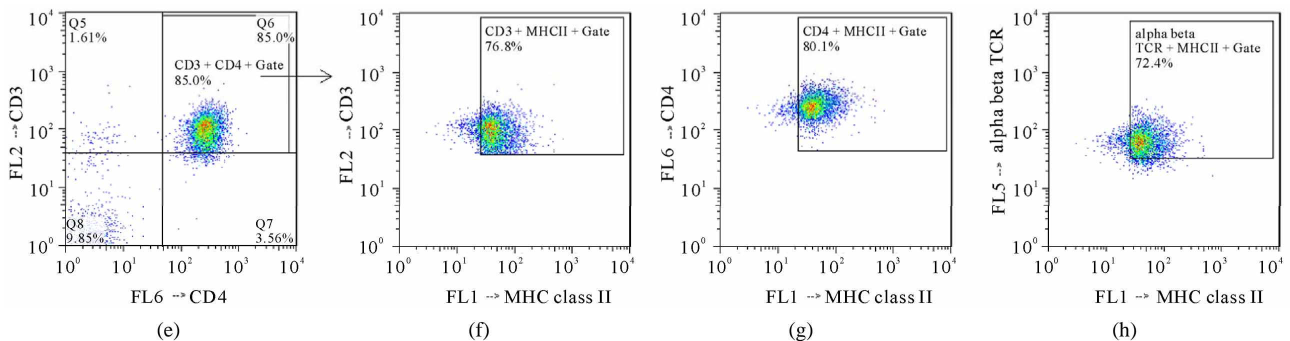
3.3. Naïve CD4⁺ T Cells Can Express MHC Class II

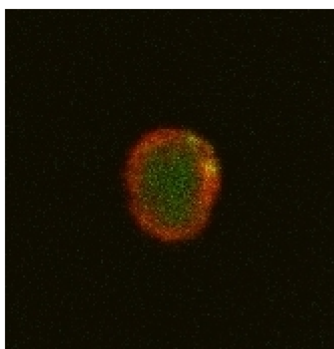
The presence of MHC class II in purified naïve $CD4^+$ T cells without APC raises the question of the source of the MHC class II molecules. Our hypothesis was that

Presort analysis of naïve splenocyte cells



Post sort analysis of naïve splenocyte cells





(i)

Figure 3. Naïve $CD4^+$ T cells can express MHC class II Mouse splenocyte were immunostained with anti CD4, anti CD3, anti αβ TCR and anti MHC class II antibody were simultaneously analyzed by flow cytometry in a single analysis. The data prior to sorting show that 2.6% of the gated splenocytes are positive for all 4 antibodies (A-D). Cell sorting enrichment increased concentration of T cells expressing MHC class II (E-H). (I) fluorescent microscopy of $CD4^+$ T-cells, anti-CD4 antibody (is labeled with APC (red) and anti-MHC class II is labeled with FITC (green). The data suggest that these are indeed $CD4^+$ T cells expressing MHC class II.

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$CD4^+$ T-cells become activated when they are presented with SAg by MHC class II molecules that are expressed on their surface. To test this hypothesis we labeled naïve mouse splenocyte cells with anti CD4 fluorescein labeled (APC) antibody, anti MHC class II fluorescein labeled (FITC), anti CD3 labeled (PE) and anti $\alpha\beta$ TCR labeled (pacific blue). These cells were analyzed by flow cytometer FACS Calibur. Representative data from three independent experiments show 22% of splenocytes are CD3 and CD4 positive (**Figure 3(a)**). From this population 3.1% are positive for CD3 and MHC class II, (**Figure 3(b)**). 4.2% are positive for CD4 and MHC class II (**Figure 3(c)**). And 2.6% are positive for $\alpha\beta$ TCR cells and MHC class II (**Figure 3(d)**). Cell sorting increased the concentration of this fluorescin labeled T cell subtype (**Figure 3(e)-(h)**). We also used confocal fluorescent microscopy (**Figure 3(i)**) and demonstrated that these highly purified $CD4^+$ T cells are double labeled with anti-CD4 antibody (labeled with APC (red) and anti-MHC class II is labeled with FITC (green), suggesting these murine $CD4^+$ T cells express MHC class II.

3.4. Expansion of $CD4^+$ T Cells Expressing MHC Class II

We performed an ex *vivo* assay that studied long term expansion of this cell and demonstrated that isolated $CD4^+$ T cells expressing MHC class II can proliferate without using autologous APCs, and can be expanded with immunomagnetic beads conjugated with costimulatory signals anti-CD28 mAb and T cell activation anti-CD3 mAb. Without this antibody the cells did not proliferate. Our result also shows that the exponential proliferation of this $CD4^+$ T cell was maintained only for 13 days with an expansion rate of 40-fold (**Figure 4(a)**). Flow cytometry indicated that during this expansion time $CD4^+$ T cells expressed high levels of MHC class II molecule on their surface (**Figure 4(b)**).

4. DISCUSSION

Our studies demonstrate for the first time a subtype of mouse naïve $CD4^+$ T cells express MHC class II molecules on the cell surface. We showed that SEA was able to induce high T cell proliferation in dose dependent manner on both negatively and positively selected mouse naïve $CD4^+$ T cells. The proliferative response of these selected $CD4^+$ T cell was similar to the response from splenocytes. SEA was able to induce $CD4^+$ T cell proliferation even in the absence of APCs. This may suggest that the safety mechanism requiring cellular interaction between two different types of cells, accessory cell and T cell does not occur in the response to SAg, consequently inducing T cell over-activation with massive cytokine release.

Treatment with neutralizing anti MHC class II antibody totally inhibits the proliferation of these $CD4^+$ T cells in the presence of SEA. These data suggest our $CD4^+$ T cells were able to present SEA via MHC class II and provide sufficient accessory signals to themselves or neighboring $CD4^+$ T cells, triggering proliferation. Consequently, they performed the roles of both professional APCs and T cells. This may suggest that this T cell may

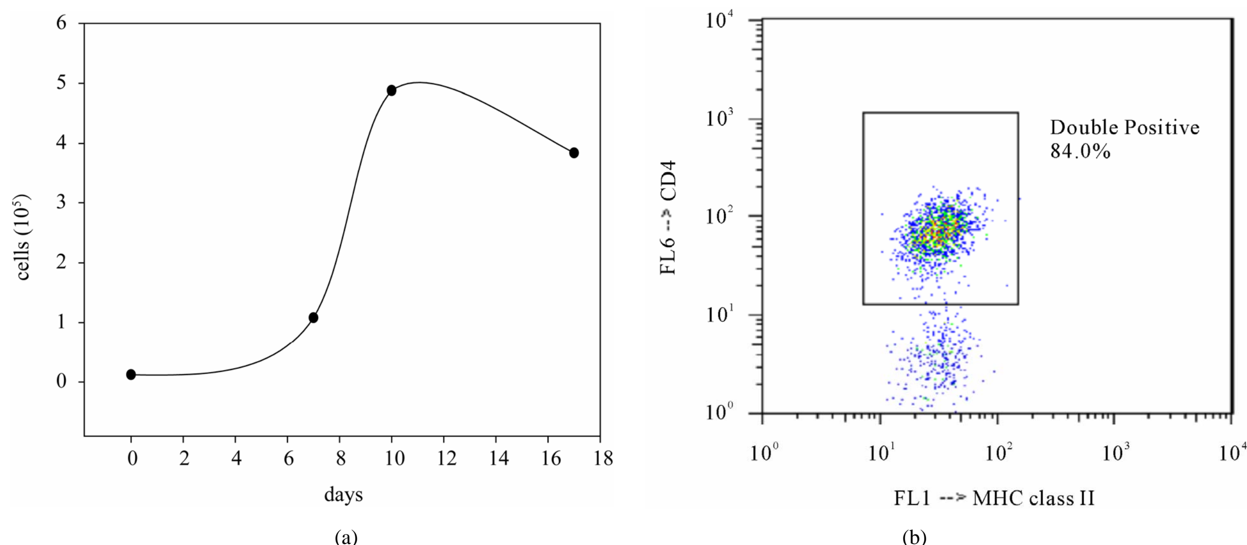

Figure 4. Expansion of $CD4^+$ T cell expressing **MHC** class II. Naïve CD4+ T cells expressing MHC class II molecule were isolated from C57BL/6 mouse splenocytes by FACS Calibur. The purified Naïve $CD4^+$ T cells were cultured in 96 well microplates. At day 0, 7, 10, 13 and 17, cells were stimulated with anti-CD3/anti-CD28 mAb-coated beads and counted using the trypan blue dye exclusion test to evaluate the fold increase in their numbers. Error bars represent standard errors and $n = 3$.

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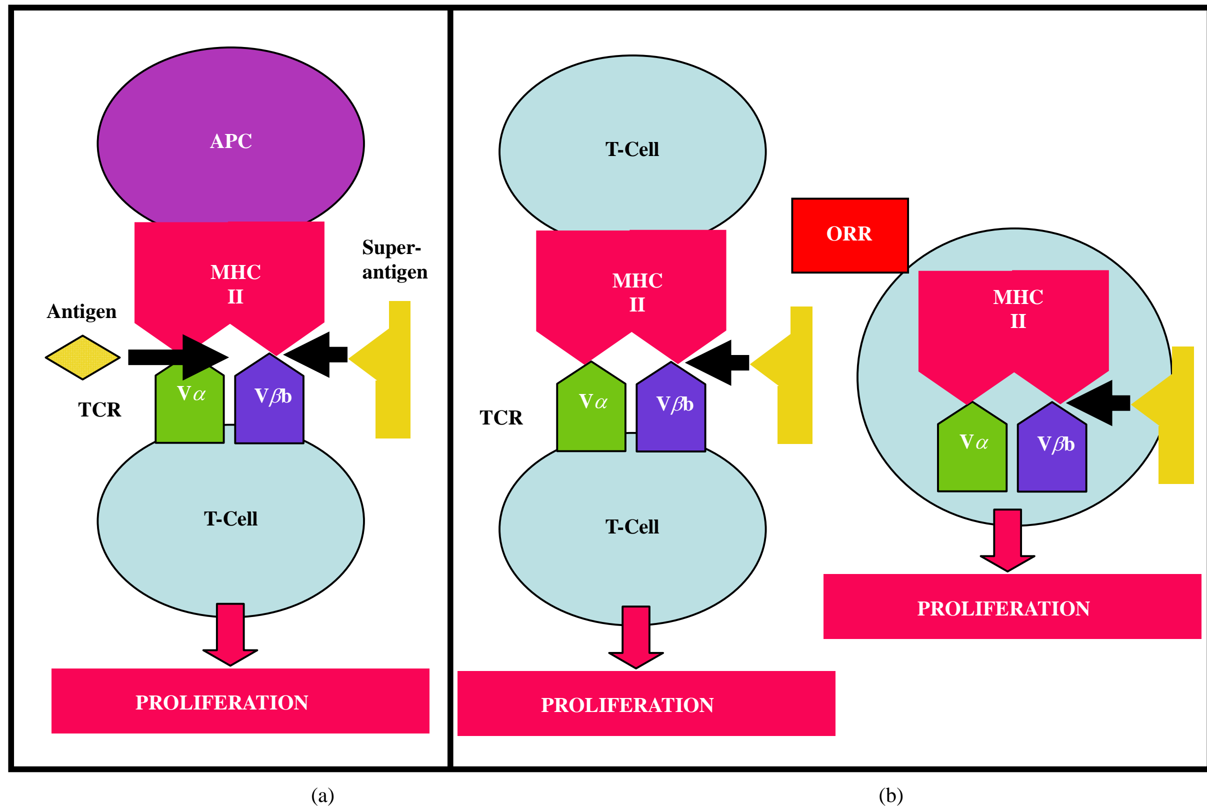

Figure 5. The current model (a) and the proposed model (b in addition to a) explain how SEA induces T cell proliferation. The current model suggests that SAg binds to two molecules on two separate cells; the V-β portion of the TCR and the MHC class II on APC forms a bridge between the T cell and APC induces proliferation to large number of T cells. The proposed mechanism suggests that T cells express both molecules; MHC class II and TCR. Therefore, activation by SAg may require only one type of cell performing the role of both APC and T cell. SAg binds to both molecules in neighboring T cells or to both the TCR and MHC class II on the same cell causing massive T cell proliferation.S

play a role as a sensor cell and first line of host defense against *S. aureus* infection.

Our findings demonstrate that subtype of CD4$^+$ T cells express MHC class II and may act as professional APC, process SAg and present them to neighboring T cells and induce CD4$^+$ T cell proliferation even in the absence of macrophages, dendritic cells or B lymphocytes. This observation may change the interpretation of earlier experiments that used fixed APCs that are metabolically incapable to uptake and process the superantigens, but were able to present SEs and activate T cells. Therefore, the question as to whether processing of SEs is not required was not addressed by the previous studies.

White *et al.* [15] and Fleischer *et al.* [16] demonstrated that T cell response to SEA are not MHC class II restricted and that mouse TCR can recognize non host APC expressing MHC class II alleles from different species. However, these authors did not take into consideration that CD4$^+$ T cells that express MHC class II molecules on their cell surface can act as APCs. Therefore, their experimental data cannot evaluate whether MHC restriction is violated in response to SEs.

In conclusion the paradigm (**Figure 5(a)**) that suggests the only possible mechanism that SAg induces T cell proliferation is by binding to two separate cells, APC and T-cell, may be need to be revised. Our revised model (**Figure 5(b)**) suggests that alongside the above mechanism SAg may be able to induce T cell proliferation by binding to only one type of CD4$^+$ T-cell expressing MHC class II. Performing the role of both accessory cell and T cell, these cells present SAg via MHC class II to themselves or neighboring CD4$^+$ T-cells, triggering nonspecific massive T-cell proliferation. The revised model offers further insight into the *in vivo* mechanism of SAg activity.

5. ACKNOWLEDGEMENTS

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