Mol Cell Biochem (2010) 338:1–10
DOI 10.1007/s11010-009-0314-z

Adaptive immune responses during *Shigella dysenteriae* type 1 infection: an in vitro stimulation with 57 kDa major antigenic OMP in the presence of anti-CD3 antibody

Ashim Kumar Bagchi · Ajoy Kumar Sinha · Rushita Adhikari · Joydeep Mukherjee

Received: 4 June 2009 / Accepted: 29 October 2009 / Published online: 14 November 2009
© Springer Science+Business Media, LLC. 2009

Abstract An effort was made to understand the role of the 57 kDa major antigenic fraction of *Shigella* outer membrane protein (OMP) in the presence of T-cell antigen receptor in activation of adaptive immune responses of the cell mediated immune (CMI) restored patients. The expression of HLA-DR/CD4 out of $CD3^+$ T-cells was significantly dominant over the HLA-DR/CD8 and comparable to unstimulated cells of infected or healthy controls. $CD4^+$ T-cell activation together with HLA-DR is associated with the expression of $CD25^+$ (IL2Rα) for IL-2 growth factors with decreased IL-4 levels, required for maintaining the homeostasis of $CD4^+$ T cell. Furthermore, the positive expression of the CD45 antigen is possibly required for acquiring the memory for $CD4^+$ cells signals and facilitates the interaction with CD54 antigen. As a result, antigen-specific secondary signal is generated for B-cell activation to produce IgG2a and IgG2b. This suggests that antibody mediated-adaptive immune responses are generated due to anti-CD3 induced helper T-cell activity. The above mentioned findings reflect that the antigen alone might not exacerbate the selective T-cell responses. But these antigens in the presence of anti-CD3 antibody might help to elicit adaptive immune response via T-cell receptor (TCR) activation.

Keywords 57 kDa OMP · Anti-CD3 antibody · Adaptive immune response · Shigellosis

A. K. Bagchi
Microbial-Immunology Division, Research and Development, Nutratech Inc, Winnipeg, MB R3Y1M5, Canada

A. K. Bagchi · A. K. Sinha
Division of Immunology and Vaccine Development, National Institute of Cholera and Enteric Diseases, Beliaghata, Kolkata 700010, India

R. Adhikari
Department of Biological Sciences, University of Lethbridge, Lethbridge, AB T1K 3M4, Canada

J. Mukherjee
School of Environmental Studies, Jadavpur University, Kolkata 700032, India

A. K. Bagchi (✉) · R. Adhikari
Institute of Cardiovascular Sciences, St. Boniface General Hospital and Research Centre, 351 Taché Avenue, Winnipeg, MB R2H 2A6, Canada
e-mail: ashim_k_bagchi333@yahoo.co.in

A. K. Sinha
Department of Experimental Haematology, NSCB Cancer Research Institute, Kolkata, India

R. Adhikari
Department of Physiology, Faculty of Medicine, University of Manitoba, Winnipeg, MB R3E 0J9, Canada

Introduction

In shigellosis, the existing knowledge about host immune mechanism is limited to mucosal antibody responses during natural or experimental infection. An increase in the number of intraepithelial T-cell populations in gut mucosal lymphocytes during natural [1] or in experimental infection to guinea-pigs [2] was reported. Whereas many *Shigella* surface components are used as mucosal vaccine candidate but their immunostimulatory effects are less defined. Earlier, in vitro experiments have shown that the bacterial antigens or super antigens might induce T-lymphocyte proliferation and activation [3] but they fail to induce antigen-specific responses. Later, in vitro cellular changes in TCR Vβ repertoire of T-cell during the course of *Shigella* infection have also been reported [4] but they did not show any response generating for Th1,

which is important in elicitation of cell-mediated immunity as host defense against shigellosis. We have also established recently that exogenous IFN-γ could efficiently promote the lymphoid-cell activity to induce the cytokine secretions like IFN-γ and IL-2 in CMI restored acute *Shigella* patients [5]. We have reported that the 57 kDa major antigenic component of *S. dysenteriae* OMPs was found to be important in producing a typical delayed type hypersensitive (DTH) reaction, which may also activate the cellular immune responses during shigellosis [6]. Recently, we have also evaluated its significance in generating MHC-II restricted T-cell responses [7]. However, the immunoregulatory role of this specific antigen in the pathomechanism is elusive.

The lack of immunostimulatory features of the target candidates often does not elicit strong mucosal immunity, which causes T-cell suppression [8]. Therefore, immunosuppressive mode of S*higella* infection is due to lack of antigen reactive T-cells. Despite many efforts, there is a profound sum of T-cell suppression exclusively undermining its protective memory Th1 cells. Hence, the immunoregulatory role of memory T-cells needs to be considered. Memory T-cells play an important role in mediating antigen recall responses for the helper cells to acquire memory and specificity towards bacteria. Anti-CD3 has been shown to exhibit receptor-like properties in proliferating memory T-cells through directed signals [9]. Recently, we have demonstrated that phosphotidylinositol-3 kinase-mediated signals upregulated TCR ligand-induced phosphorylation of PKC during memory T-cell activation in immunized BALB/c mice [10]. Although anti-CD3 ligand have been shown to be immunosuppressants [11], but in the presence of co-stimulatory signals they can activate memory T-cells via TCR activation [12]. Anti-CD3 ligand stimulation to the murine T-cells has been shown to activate and proliferate the memory T-cells and require co-stimulatory signals to induce IL-2 secretion [13]. The ligand-induced TCR activation might suppress the naïve or memory $CD8^+$ T-cells as a result of selecting only type 1 $CD4^+$ T-cells [14]. On the other hand, anti-CD3 induced T helper cell activity showed production of IgG and IgM by B-cells [15]. Data suggest that antibody can provide B-lymphocyte mediated adaptive immunity. Adaptive immunity to intracellular pathogens is also mediated by sensitized T-cells in which both $CD4^+$ and $CD8^+$ T cells contribute to protection against the subsequent challenge [16]. The T-cell independent adaptive immunity to *S. flexneri* 2a in vaccinated TCR $\beta^{-/-}$ and TCR $\delta^{-/-}$ mice was exclusively antibody-mediated [17]. On the contrary, T-cell dependent antibody responses were also demonstrated in anti-CD3 stimulated T-cells of Balb/c immunized with the 57 kDa major antigen, though the secretory antibody levels due to the effect of such stimulation were not measured [7]. Thus, anti-CD3 antibody can act as an immunostimulatory molecule to induce adaptive immune responses through TCR activation. Further, we assumed that ligand-induced T-cell modulation could efficiently promote the ability of the used antigen in achieving immunity to shigellosis. Hence, in this study, anti-CD3 was used as an immunoadjuvant and supplemented with 57 kDa antigen to acquire memory of the CMI restored patients and gain access to the antigen-specific signals for Th1 of the Th0 subsets.

The aim of our study was to evaluate the in vitro cellular changes due to the effect of the 57 kDa antigenic fraction in presence of anti-CD3 on macrophages and thymic subsets in patients with acute *S. dysenteriae* 1 infection. The activated cell expressions signify the degree of immune response, which provide a rationale for the development of a new approach to activate T- and B-cell adaptive immunity against shigellosis.

Materials and methods

Patients blood samples

Heparinized blood samples were collected as per approval from the institutional animal ethical committee from six *S. dysenteriae*-1 infected male patients with ages between 12 and 18 years after they were bacteriologically and clinically confirmed and admitted to the Infectious Diseases Hospital, Kolkata. Three healthy individuals (serologically negative to *Shigella* antibody) of the same age group were also included as control.

Bacterial culture and preparation of 57 kDa major antigenic fraction

Serovar-specific strain (IPC-31) of *S. dysenteriae* type 1 were isolated and identified from the faeces of patients with bacillary dysentery using API-20E biochemical test (BioMérienx, Durham, NC, USA) and different antisera (Difco, USA). After guinea-pig passage, virulent smooth colonies were grown at 37°C in Tryptic soy broth (TSB, Difco, USA). OMPs were isolated as described earlier [18] from *S. dysenteriae* type 1. In brief, after harvesting the cells from culture, they were sonicated and treated with sarkosyl (sodium lauryl sarcosinate, 1% w/v, Sigma Chemical Co., St. Louis, MO, USA) for 30 min at 24°C to dissolve the inner membrane, followed by centrifugation at $100{,}000 \times g$ for 2 h. The pellet containing OMP was washed with distilled water and stored at −20°C. The major antigenic fraction (57 kDa) was eluted from the gel slices electrophoretically using an electro-eluter (Bio-Rad, Richmond, CA, USA), as described previously [6]. The protein was concentrated using Speed-Vac (Savant No. SC-210A, Savant Instruments, Holbrook, NY, USA) and the concentration was measured using 1% BSA as standard [19]. To

ascertain whether the eluted protein contained trace amounts of bound lipopolysaccharide (LPS), the *Limulus* amoebocyte lysate (Sigma, USA) assay was performed using *Escherichia coli* O55:B5 LPS (Sigma, USA) as control [20]. The negligible amount of LPS (<10 pg per 10 μg protein) did not show any interference in biological responses.

Isolation of mononuclear cells and in vitro stimulation

Mononuclear cells (MNC) were isolated from heparinized blood samples by layering on Histopaque-1077 density gradient solution (Sigma, USA) and centrifuged in cold at $400\times g$ for 10 min. Monolayer at the interface was recovered and washed twice in cold supplemented RPMI-1640 (Sigma, USA) at $400\times g$ for 10 min at 4°C. The viability of the cells was checked with 0.2% trypan blue and counted using haemocytometer. More than 90% of cells were obtained viable. Finally, 1×10^6/ml of cells were suspended in complete RPMI-1640 containing 10% Fetal Bovine Serum (FBS, Sigma, USA) and 50 U gentamicin/ml. Later, the mononuclear cells (MNCs) were stimulated in duplicate with standard dosages of phytohaemagglutinin (PHA) as positive control (5 μg/ml), the 57 kDa alone (10 μg/ml), anti-CD3 antibody (10 μg/ml; Pharmingen, San Diego, CA, USA) alone and the 57 kDa antigen with equal amount of anti-CD3 and PBS as negative control. The stimulated and unstimulated cells were incubated for 24 h at 37°C in 5% CO_2. Lymphocyte proliferation was measured at different time points (6–36 h) with concentration (5–25 μg) of stimulants. Briefly, the cells were pulsed for 6 h with 0.5 μCi of [^{3}H] TdR (Amersham, Buckinghamshire, UK). The cells were harvested onto glass fiber mats and per minute counts were measured in liquid scintillation counter (Beckman, Fullerton, CA, USA). Results were expressed as the mean proliferative index (proliferation of stimulated cells divided by proliferation of unstimulated cells per minute) or as percentage proliferation. Further, secretory antibody levels for IgG2a and IgG2b (Sigma, USA) were determined by ELISA at a 1:3200 dilution in triplicate using 10 ng/ml/well isotype specific antibody in microtiter plates (Nunc, Denmark).

Fluorescence activated cell sorting (FACS) analysis

Quantitative analysis of MNCs and in vitro stimulated cells was determined by FACSorter (Becton–Dickinson, Mountain View, CA, USA) using FITC conjugated-specific monoclonal antibody for the target population. Briefly, 1×10^6 cells were washed with cold PBS with Tween 20 (PBS-T) containing 1% BSA and stained with FITC labeled-specific antibody for 1 h at 4°C after incubating with 2% BSA for 30 min at 37°C. Then, the cells were washed twice with PBS-T and suspended in PBS containing 1% paraformaldehyde [21]. The cells were counted and fluorescence intensity was detected, optimized and read through database-Lysys II analysis software (Becton–Dickinson, USA) against specific negative as well as experimental controls. For each set of experiments, single parameter histogram (specific stained cell number versus fluorescence intensity) was plotted for 20,000 events per sample and wherever applicable the data was represented as a single color dot-plot to measure cell proliferation as described [14].

Immunofluorescent staining

Stimulated or non-stimulated cells were allowed to adhere to 22 mm glass coverslips in 6-well tissue culture plates (Corning Co, Corning, NY, USA) in complete RPMI-1640 medium (Sigma, USA) supplemented with 10% FBS in 12 mM Hepes and 50 U gentamicin/ml at 37°C in 5% CO_2. Coverslips were washed with RPMI medium and once with PBS-T containing 1% BSA to remove the non-adherent cells, then fixed with 0.25% glutaraldehyde. The cells were then incubated with 5% BSA for 30 min at 37°C water bath to block the non-specific sites and washed twice. The cells were again incubated with 50 μl of FITC labeled HLA-DR antibody (Sigma, USA) in duplicate for 30 min at 37°C. Finally, the cells were washed twice with PBS-T containing 1% BSA. Expression of HLA-DR antigen on the macrophage surface was quantified by using automated video microscopic analysis (Olympus Optical Co., Ltd., Tokyo, Japan) in 100 μm^2 area as described [22].

Immunoassay for IL-2 and IL-4

The assay was carried out as described [23]. In brief, 96-well microplates (Nunc, Denmark) were coated with 100 μl/well of captured antibody (5 μg/ml in PBS; pH 7.2) and incubated overnight at 4°C. On the next day, the plate was taken out at room temperature and washed twice with washing buffer (PBS-T containing 1% BSA). Unbound sites were blocked with 5% BSA (IgG free, Sigma, USA) in PBS for 30 min at 37°C. After blocking, the plates were washed with washing buffer thrice and incubated for 2 h at 37°C with different serial dilutions (twofolds) of standard and differently formulated culture (described earlier) in duplicate with PBS and 1% BSA. Later on, the wells were washed thrice with washing buffer and incubated with 1 μg/ml of biotinylated antibody (Sigma, USA) in PBS containing 1% BSA. Excess antibody was removed by washing buffer. In total, 100 μl of a 1:2000 dilution of Streptavidin-Horse Radish Peroxidase conjugate (Sigma, USA) was added to each well. After washing three times, the color developed after adding 50 μl per well of 0.1% substrate [2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), Sigma, USA] in 0.1 M sodium citrate buffer (pH 4.5) and 0.1% H_2O_2 for 15 min at

37°C in dark. Finally, the reaction was stopped by adding 20 µl of 10% SDS. The absorbance was recorded at 492 nm in ELISA reader (Bio-Rad, USA). The graphs were plotted with standard curve against concentration of IL-2 and IL-4 levels in the culture supernatant.

Statistical analysis

A two-way analysis of variance and Student's *t*-test was performed to compare the effect of 57 kDa antigenic fraction in presence or absence of anti-CD3 for each variable. The data for immunoassays were processed using a software package (Epistat, Microsoft Co., Redmond, WA, USA) to generate a curve using linear regression analysis and expressed as a mean ± SE for three consecutive experiments.

Results

Dose- and time-dependent lymphocyte proliferation

Figure 1 reveals that different concentrations of the 57 kDa antigen in the presence or absence of anti-CD3 antibody could generate increasing number of CD4 or CD8 cells. Initially at 12 h, the lymphocyte proliferation to either stimulation was noted to increase (Fig. 1b). Later, the proliferative responses to the 57 kDa antigen in the presence of anti-CD3 were found to be highest (proliferative index = 7.4) after 24 h of stimulation. However, in the absence of anti-CD3 (57 kDa alone), the proliferative index was about 6.3, which was comparatively higher than that of PHA or anti-CD3 alone (proliferative index ≤ 5.0) presented in Fig. 1a. It seems that the 57 kDa antigen (10 µg/ml) in the presence of an equal concentration of anti-CD3 antibody (10 µg/ml) significantly stimulates the cell proliferation at 24 h (Fig. 1b). An intermediate dose of such stimulant was considered for each experiment at which the cell proliferation was noted to be high.

Secretory IgG subclass responses to in vitro stimulation

Levels of IgG2a and IgG2b were measured after 24 h of differently stimulated culture supernatant. The relative level of secretory IgG subclasses to the 57 kDa antigen in the presence of anti-CD3 significantly ($P < 0.01$) increased in the *Shigella*-infected patients than those of unstimulated S*higella* patients and the stimulated healthy controls. The mean level of secretory IgG2a/IgG2b ratio in such stimulated healthy control was a little higher than that of other stimulated groups. The unstimulated absorbance level in both the patients and the healthy controls was negligible (not shown) as compared to that in PHA, 57 kDa antigen and anti-CD3 (no significant difference in all six conditions) ($P < 0.01$, patients vs. controls). However, the mean level of secretory IgG2a/IgG2b ratio after 24 h of such stimulation was comparable to PHA and anti-CD3 stimulation and increased by 2- to 3-folds (Fig. 2). The Ig2a/IgG2b ratio in such stimulated *Shigella*-infected patient was corresponding to the 57 kDa

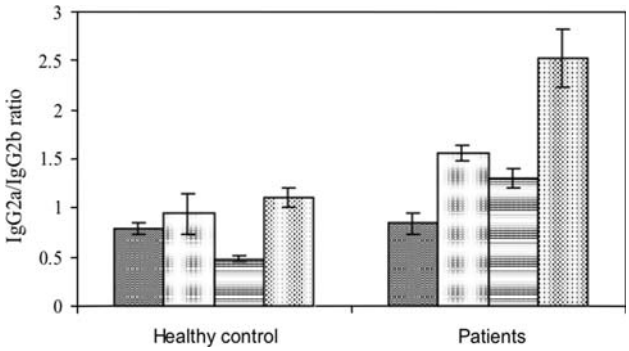

Fig. 2 Determination of secretory IgG1a/IgG2b ratio after 24 h of stimulation with different stimulants: PHA (▨), 57 kDa (⊡), Anti-CD3 (⊟) and 57 kDa plus anti-CD3 (◧) using 500 ng of anti-human subclass specific antibody in ELISA. IgG1a/IgG2b ratio for unstimulated healthy and patient cells were <0.5. IgG1a/IgG2b ratio was represented as mean ± SE (mean of variance) for the three consecutive experiments

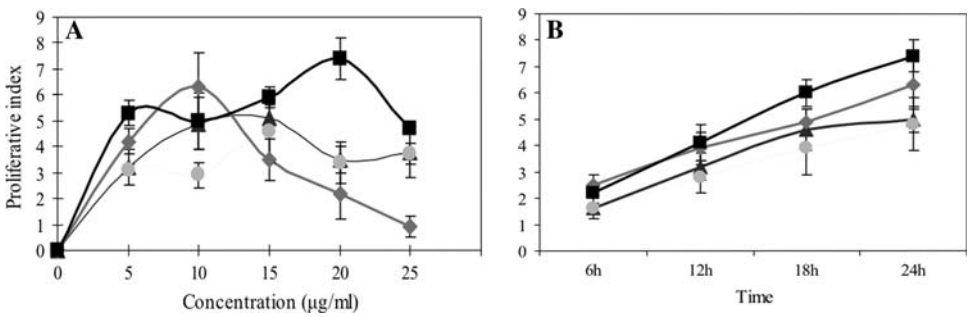

Fig. 1 [^{3}H]-thymidine incorporated lymphocyte proliferations: **a** measured after 24 h of stimulation to healthy MNCs using different concentration of stimulants PHA (—▲—), 57 kDa (—◆—), anti-CD3 (—●—) and 57 kDa plus anti-CD3 (—■—); **b** measured at different time points (6–24 h) of stimulation with an intermediate dosages of stimulants (PHA; 5 µg/ml), (57 kDa; 10 µg/ml), (anti-CD3; 10 µg/ml) and (57 kDa plus anti-CD3; 20 µg/ml). Lymphocyte proliferation represented as mean ± SE for the three consecutive experiments

antigen stimulation and insignificant. Antibody secreting cell response to such antigen helps in elicitation of IgG antibody response until after 24 h, suggesting that the 57 kDa antigen in the presence of anti-CD3 has significance in achieving humoral immunity against shigellosis.

Antigen driven thymocyte differentiation and functional maturation of macrophages

Expression of CD4, CD8 and CD25 (interleukin-2R) [IL-2R]

Phenotypic expression of CD4, CD8, and CD25 in differently stimulated MNCs of *Shigella*-infected patients and healthy controls were evaluated by flow cytometry. Significant shift in the expression of $CD4^+$ cells was noted in the presence of anti-CD3 stimulated cells than that of unstimulated cells of patients. While no shift was seen in the expression of CD4 in healthy control cells exposed to the 57 kDa antigen plus anti-CD3 (Fig. 3, panel a-i), a significant increase (10-folds) was seen in the cells isolated from patients (Fig. 3, panel a-ii). Data for anti-CD3 and the 57 kDa alone were not different from unstimulated controls (not shown).

On the other hand, the expression of $CD4^+$ T-cells was found to be significantly greater than $CD8^+$ T-cells in such stimulated patients despite a little right shift in CD8 cell expression (Fig. 3, panels a-ii and b-ii). As compared to $CD4^+$ cell expression, the corresponding $CD8^+$ T-cells number in such stimulated patients was observed to have an opposite relation, i.e. the level of such expression significantly decreases by 2-fold than that of unstimulated cells (Fig. 3, panel b-i). The pattern of the $CD8^+$ T-cell expression in healthy controls was almost similar to that in infected patients (Fig. 3, panel b-i). Other stimulated cells from both the groups were shown to be insignificant and fluctuating when compared together.

The changes in expression of $CD25^+$ T-cells were found to be insignificant to the 57 kDa antigen plus anti-CD3 stimulated cells as compared to the unstimulated cells of patients (Fig. 3, panel c-ii). In such stimulated cells of patients, the level of CD25 expression was noticeably higher (3- to 4-fold) than that of PHA and unstimulated healthy controls (Fig. 3, panel c-i and ii).

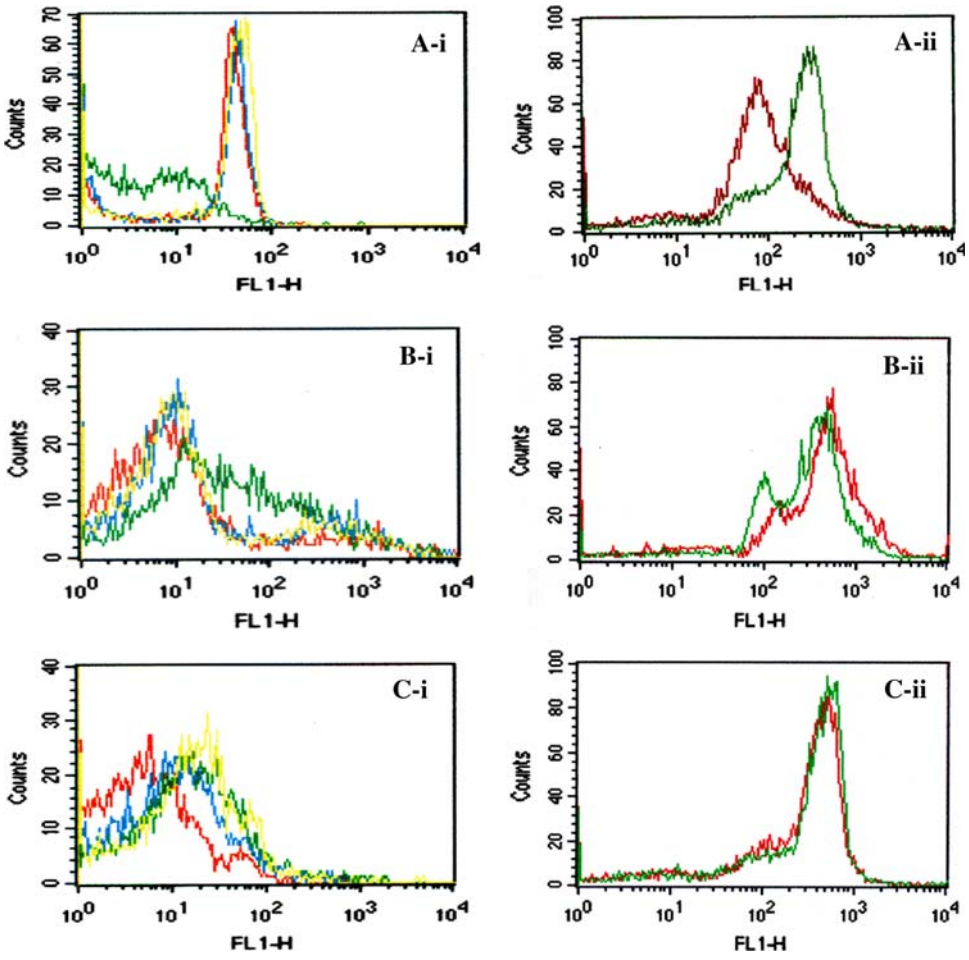

Fig. 3 Single-parameter histogram were generated after three consecutive experiments for the expression of CD4 (**a**), CD8 (**b**) and CD25 (**c**) after 24 h of stimulated cells of healthy controls (i) with PBS (*red*), PHA (*green*) and 57 kDa antigen with (*yellow*) or without (*blue*) anti-CD3 antibody and *Shigella*-infected patients (ii) with PBS (*red*) and 57 kDa antigen in the presence of anti-CD3 (*green*). Data for anti-CD3 were not shown as found insignificant expressions

Expression of HLA-DR antigens

The macrophages of *Shigella*-infected patients were strongly positive for HLA-DR in the presence of anti-CD3 than in the absence of anti-CD3 (Fig. 4, panels b and d). In 66.7% cases, HLA-DR antigens were expressed on macrophage surface stimulated with 57 kDa antigen in the presence of anti-CD3 and showed significant changes than the unstimulated or PHA stimulated cells of both the groups (Fig. 4, panel d compared subsequently with panels a, e, b, and f). However, the expression of HLA-DR antigen due to PHA stimulation (50%) was found to be insignificant to the 57 kDa antigen stimulated cells of both the groups but significant ($P < 0.05$) to unstimulated healthy controls (Fig. 4, panels b and f compared with panels c and g and respectively with panels a and e). No significant changes were observed in the 57 kDa antigen alone stimulated cells of both the groups due to its immunomodulatory effect on the macrophages. On the other hand, significant changes in HLA-DR antigen expression in anti-CD3 stimulated cells of *Shigella*-infected patients were correspondent to the increasing number of $CD4^+$ cells than that of $CD8^+$ cells.

Expression of CD45 and CD54

The $CD45^+$ expression was found to be 2- to 3-fold more in anti-CD3 stimulated patients and was significant ($P < 0.01$) than the expressions found in healthy controls and unstimulated cells (Fig. 5, panel l compared individually with panels d, a and i). As compared to the healthy control, we

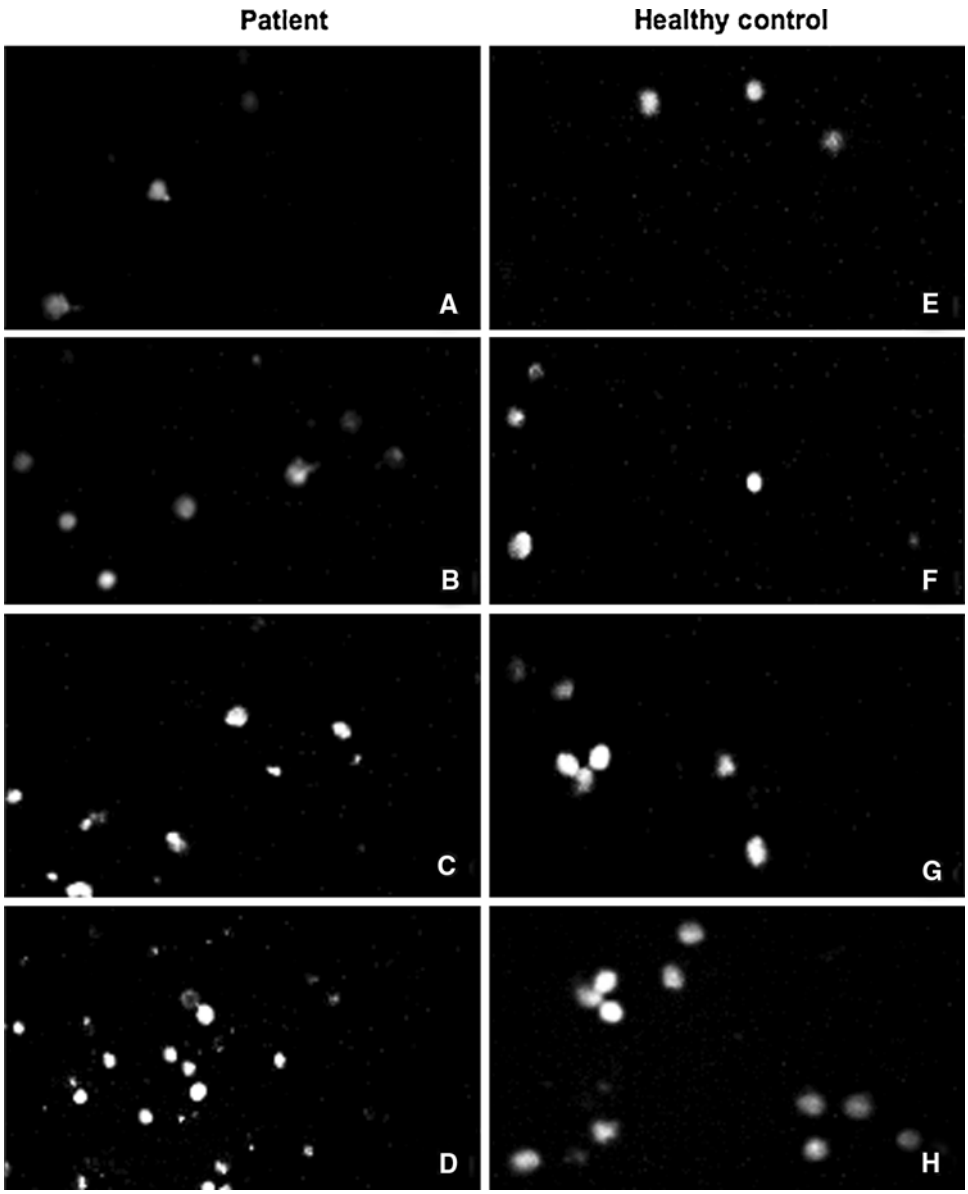

Fig. 4 Immunofluorescent staining with anti-FITC labeled HLA-DR monoclonal antibodies. Expression of HLA-DR antigen on the surface of macrophages from the *Shigella*-infected patients (**a**–**d**) and healthy controls (**e**–**h**) after 24 h of stimulation with: PBS as negative control (**a** and **e**), PHA as positive control (**b** and **f**), 57 kDa antigen (**c** and **g**) and 57 kDa antigen coupled anti-CD3 antibody (**d** and **h**). Original magnification 1712 × 1368 pixels

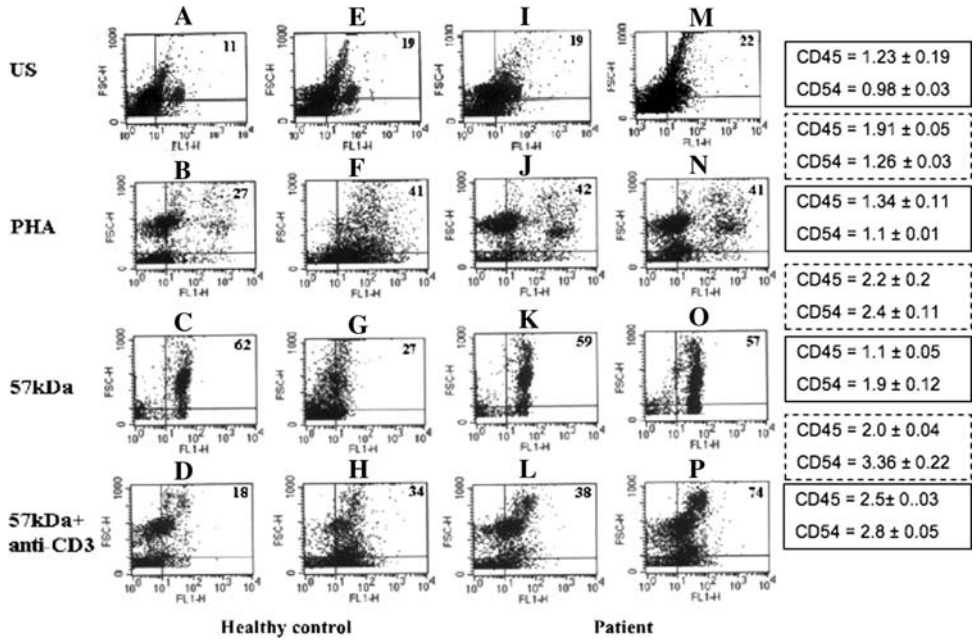

Fig. 5 Representative dot plots of CD45 (**a–d** and **i–l**) and CD54 (**e–h** and **m–p**) after 24 h of stimulated cells from a healthy control (**a–h**) or a *Shigella*-infected patients (**i–p**) stimulated with PHA (**b**, **f**, **j** and **n**), 57 kDa antigen (**c**, **g**, **k** and **o**), 57 kDa antigen plus anti-CD3 antibody (**d**, **h**, **l** and **p**) as compared to unstimulated; US (**a**, **e**, **i** and **m**). Numbers at the upper right hand represent percentage (%) of CD45 or CD54 expression out of the 20,000 cells gated in the panel. Expression levels are shown in mean ± SD folds increase or decrease. *Solid line* healthy control group compared individually with patient and *dotted line* each stimulated group was again individually compared with unstimulated group

did not find any significant changes in 57 kDa stimulated patients despite a little change in PHA stimulated group (Fig. 5, panels a–c individually compared with panels i–k). Furthermore, expression pattern of CD54 was found to be similar to that of CD45 in the presence of anti-CD3 stimulation as in 57 kDa antigen alone-stimulated patients (Fig. 5, panels p and o compared with panels g and h). The results were found to be significant ($P < 0.01$) when anti-CD3 stimulated cells of patients were compared to stimulated and unstimulated cells of the healthy group (Fig. 5, panel p compared with panels e–h) but insignificant when compared to other stimulated cells of shigellosis patients (Fig. 5, panel p compared with panels o and n).

Cytokine responses during T-cell stimulation

In vitro T-cell responses to various stimulated MNCs of *Shigella*-infected patients were compared with healthy controls. After 24 h of stimulation, it was observed that the IL-2 levels significantly increased ($P < 0.05$) in the 57 kDa antigen plus anti-CD3 stimulated patients than that of the stimulated and unstimulated healthy controls (Fig. 6a). On the other hand, the level of IL-4 from anti-CD3 stimulated cells of patients significantly ($P < 0.05$) decreased as compared to unstimulated and other stimulated cells of patients (Fig. 6b). An elevated response to IL-4 levels was noted in other stimulated cells of patients with decreasing IL-2 levels. Although as for IL-2 generation the same responses for IL-4 were noted in the absence of anti-CD3 stimulation but comparatively lower than in the presence of anti-CD3 stimulation, which was statistically deviated from the mean of variance (Fig. 6a, b). As compared to the healthy controls, the trend of IL-2 was found to be inversely proportional to IL-4 in such stimulated cells of patients.

Discussion

An in vitro study showed that *Shigella* 57 kDa major antigen could preferentially promote the cell proliferation for CD4 subsets [24]. We observed that different doses of the 57 kDa OMPs in the presence or absence of anti-CD3 could stimulate the functional $CD4^+$ and $CD8^+$ T-cells from the memory precursor cells. An intermediate dose (10 μg/ml/10^6cells each) of the 57 kDa antigen plus anti-CD3 can activate mature T-cell proliferation followed by cytokine production as at this concentration [^{3}H]-thymidine uptake for MNCs was observed to be highest after 24 h (Fig. 1). TCR ligand stimulation of MNC of immunized Balb/c mice with 57 kDa antigen has been shown to increase $CD4^+$ cells, which was predominant over $CD8^+$ cells [7]. During T-cell development in the thymus, CD4

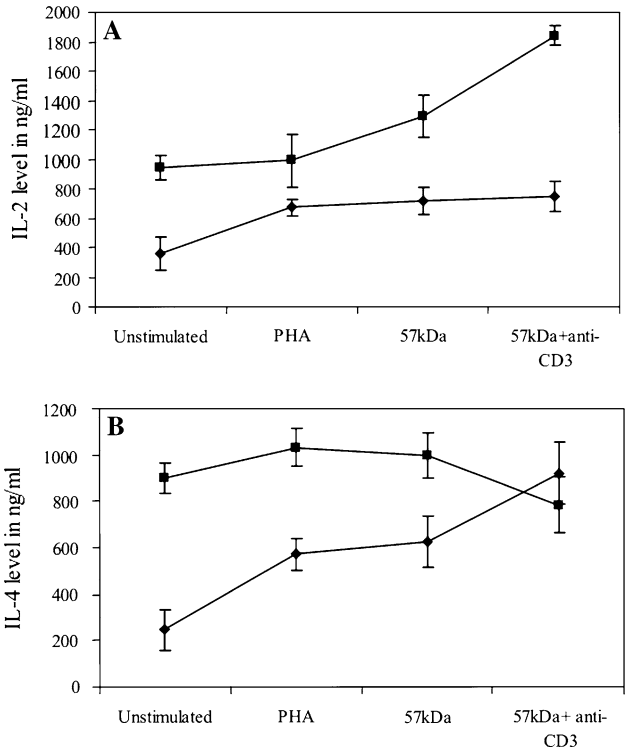

Fig. 6 Patterns of IL-2 (**a**) and IL-4 (**b**) response after 24 h of unstimulated and stimulation with different stimulants to the MNCs of *Shigella*-infected patients (*filled square*) and of healthy controls (*filled circle*). The data for anti-CD3$^+$ alone were not shown as found insignificant expressions. Data were expressed as mean ± SE for three consecutive experiments

and CD8 T-cells are selected from a population of lymphocyte precursors that require TCR engagement by suitable ligands [25]. The relative expression of CD4$^+$ cells together with HLA-DR antigens was markedly dominant over HLA-DR/CD8$^+$ in the presence of anti-CD3 stimulation in *Shigella*-infected patients (Figs. 2 and 4). This reveals that there is a generalized memory CD4$^+$ T-cell activation in the presence of APC as a result of which IL-2 is produced (Fig. 6). While, in unstimulated MNCs of *Shigella* infected patients, the proportion of CD8$^+$ T-cells was significantly ($P < 0.05$) higher by 1- to 2-fold than that of stimulated healthy controls (Figs. 2b and 3b). This signifies that such stimulation might also trigger CD4 antigen in immunocompromised or immunodeficient *Shigella*-infected patients via CD8$^+$ T-cell suppression [26] or via induction of the signals for CD45 memory cells [27].

T-cell activation is associated with the expression of IL-2R for IL-2 growth factors, which is required for T-cell regulation [28]. Down-regulation in CD25$^+$ T-cells resulting in increased IL-2 level in such stimulated cells from infected patients may be due to anti-CD3 stimulation [15]. However, the increased level of IL-2 with its lowered receptor expression was noted in both the unstimulated groups. But this induced decrease in IL-4 level in the presence of anti-CD3 stimulation and generated signals for the re-expression of its receptors which may be due to ligand-induced down-regulation [29]. In the presence of anti-CD3, the *Shigella*-infected patients were observed to have upregulated T-cell responsiveness towards the release of specific cytokine for Th1 of Th0 subsets.

The presence of HLA-DR antigens together with CD4$^+$ T-cells in gut mucosa of acute shigellosis [30] signifies that HLA-DR antigens are involved in the modulation of cellular immune responses through antigen presentation [31]. Similar to the expression of CD4$^+$ cells, the HLA-DR expression was noted to be much higher and prominent in the presence of anti-CD3 stimulation compared to the 57 kDa antigen alone of *Shigella*-infected patients and healthy controls (Fig. 4c and d compared with 5g and h respectively). The induction in HLA-DR antigen might help to express the peptide on the macrophages surface and in phosphorylation of the memory T-cells through cytokine production [32]. This hypothesis supports previous reports on CD4$^+$ T-cells dependent host defense against cholera-toxin induced disease in mice [33] and in HIV co-infected *Shigella* patients [34]. The result also reveals that 57 kDa antigen alone can activate macrophage function to express antigen through class-II molecules [24] as the expression of HLA-DR antigens was observed in patients and in healthy controls. The cellular activation persistent in such stimulated MNCs of control was indeed found to be higher in ligand-induced cells of acute shigellosis patients. The persistence of HLA-DR antigens together with CD4$^+$ T-cells may probably help in activation of CD45 during acute infection as we found significant changes in the expression of CD45$^+$ in such stimulated patients compared to the healthy control (Fig. 5l and d). The positive expression of the CD45 antigen is possibly required for acquiring memory for generating signals for CD4$^+$ cells [35] just 24 h after the ligand stimulation with homologous antigen. Further, the expression of CD45 facilitates interaction with CD54, a cell adhesion marker. The increased CD54$^+$ expression in the presence of anti-CD3 stimulated patients (Fig. 5p) indicated that there is an involvement of CD11a lymphocyte with activated CD18 and, consequently, enhancing the homing property of the cells through released pro-inflammatory cytokines [36]. Thus, antigen-specific secondary signal is generated for B-cell activation to produce more of IgG2a than IgG2b. The antibody production by B-cells may be due to anti-CD3 induced helper activity in T-cells [15] as the ratio of secretory IgG2a/IgG2b was noted to be highest after 24 h of stimulation in patients and was significant ($P < 0.05$) in healthy controls (Fig. 2). These antibody-producing cells are actively engaged in antibody-mediated adaptive immunity, which confers serotype-specific protection against *Shigella flexneri* 2a

[17]. Increased IgG2a/IgG2b ratio indicated delayed B-cell activation with low affinity of IgG or IgA receptors expression to acquire adaptive immunity. Although we have not estimated IgA in our experiments but we may assume that IgA antibodies would have been secreted in the culture supernatants as far as mucosal immunity is concerned. It suggests that IgG2a subtype antibodies are induced just after 24 h of such stimulation in the CMI restored *Shigella* patients to recall antigen-specific IL-2 producing memory cells. Data support the report that IgG2a and IgG2b are produced during Th1 and Th2 dominated responses, respectively [37]. TCRs of high affinity prefer Th1 type immune response by the relative level of IL-2/or IL-2 receptor expression [38]. In other words, we can say that CD25 (IL-2 receptor), a marker of B-cell activation, was observed to have significance in T-cell activation due to the effect of TCR ligands.

Anti-CD3 ligand stimulation of healthy control cells activated mature T-cell proliferation and its co-receptors such as CD4 or CD8 resulting in lymphokine production. Hence, it might have some mitogenic activity, like PHA and Concanavalin A(Con A), to induce immune response. Along with 57 kDa antigen, anti-CD3 will act better upon the CMI restored shigellosis patients towards the activated response that would be antigen specific. Overall cellular responses before and after the 57 kDa antigen with or without anti-CD3 stimulation have been demonstrated here and the findings suggested that two-way signals are generated for T-cell activation after 24 h of ligand stimulation. On the one hand, CD54 receives a continuous signal from the antigen and concomitantly stimulates IL-2R. This might help in the selection of CD45 memory T-cells and later evoke IL-2 secretion rather than IL-4 and activate the $CD4^+$ T-cells. Another way is to activate the HLA-DR with peptide to present memory cells via CD54 in turn suppressing $CD8^+$ T-cells by inducing IL-2 or by other cytokines. Our data support the earlier work on cellular activation of B- and T-cells and their related changes during the course of *Shigella* infection [1]. Later on, they defined the role of bacterial antigens in skewing the TCR Vβ repertoire towards the Th2 response with increased humoral level [4]. Besides, we were able to evaluate the ligand induced T-cell activation by triggering the affinity rate of TCR molecules as a result selecting only the antigen-specific responding $CD4^+$ T-cells and possibly trigger CD16 or CD23 antigen on B-lymphocytes to produce IgG2a and IgG2b. The above findings suggest that the 57 kDa antigen together with anti-CD3 would help in achieving B- and T-cell adaptive immune response towards the cellular target against shigellosis. Further, in vivo experiments will be carried out to prove the efficacy of the used antigen in the presence of anti-CD3 antibody.

Acknowledgments The authors are thankful to Dr. S.K. Bhattacharya, former Director and Dr. G. B. Nair, Director, National Institute of Cholera and Enteric Diseases for providing the facilities.

References

1. Islam D, Bardhan PK, Lindberg AA, Christensson B (1995) *Shigella* infection induces cellular activation of T and B cells and distinct species-related changes in peripheral blood lymphocyte subsets during the course of the disease. Infect Immun 63:2941–2949
2. Sinha AK, Chakrabarti MK, Chakrabarti S (1992) Gut mucosal lymphocyte sub population in the host-defense of *Shigella* infected guinea-pigs. Immunol Lett 32:65–68
3. Herman A, Kappler JW, Marrack P, Pullen AM (1991) Superantigens: mechanism of T-cell stimulation and role in immune responses. Ann Rev Immunol 9:745–772
4. Islam D, Wretlind B, Lindberg AA, Christensson B (1996) Changes in the peripheral blood T-cell receptor Vβ repertoire in vivo and in vitro during Shigellosis. Infect Immun 64:1391–1399
5. Sinha AK, Bagchi AK (2005) Cytokine release induced by killed bacteriae associated with anti-IFN-γ antibody in shigella infection. Cytokine 31:87–93. doi:10.1016/j.cyto.2005.03.004
6. Sinha AK, Chakraborti S, Chakraborti MK (1994) Delayed hypersensitivity in relation to host cellular immune responses in *Shigella* infected mice. Immunol Infect Dis 4:149–154
7. Sinha AK, Bagchi AK (2004) Role of anti-CD3 in modulation of Th1 type immune response in *Shigella dysenteriae* infection. J Med Microbiol 53:1075–1081. doi:10.1099/jmm0.05420-0
8. Borisova EV (1999) The role of the structural components of bacterial lipopolysaccharide in its inductive immunosuppressive activity. Mikrobiol Z 61:36–41
9. Tamura T, Yanagid T, Nariuchi H (1993) Difference in signal transduction pathway for IL-2 and IL-4 production in T helper 1 and T helper 2 cell clones in response to anti-CD3. J Immunol 151:6051–6061
10. Bagchi AK, Sinha AK (2005) Phosphotidylinositol-3 kinase-mediated signals in mice immunized with the 57 kDa major antigenic outer-membrane protein of *Shigella dysenteriae* type 1. J Med Microbiol 54:631–637. doi:10.1099/jmm.0.46014-0
11. Alegre ML, Peterson LJ, Xu D, Sattar HA, Jeyarajah DR, Kowalkowski K, Thistlethwaite JR, Zivin RA, Jolliffe L, Bluestone JA (1994) A non-activating "humanized" anti-CD3 monoclonal antibody retains immunosuppressive properties in vivo. Transplantation 15:1537–1543
12. Lohoff M, Schmitt E, Reske-Kunz AB, Rollinghoff M (1990) Different response of TH1 cells for stimulation with anti-CD3 antibodies. Eur J Immunol 20:653–658
13. Nguyen DD, Beck L, Spiegelberg HL (1995) Anti-CD3-induced anergy in cloned human Th0, Th1, and Th2 cells. Cell Immunol 165:153–157. doi:10.1006/cimm.1995.1199
14. Bommhardt U, Cole MS, Tso JY, Zamoyska R (1997) Signals through CD4 or CD8 can induce commitment to the CD4 lineage in the thymus. Eur J Immunol 27:1152–1163
15. van Lier RA, Brouwer M, Rebel VI, van Noesel CJ, Aarden LA (1989) Immobilized anti-CD3 monoclonal antibodies induce accessory cell-independent lymphokine production, proliferation and helper activity in human T lymphocytes. Immunology 68:45–50
16. Scott P, Casper P, Sher A (1990) Protection against *Leishmania major* in BALB/c mice by adoptive transfer of a T cell clone recognizing a low molecular weight antigen released by promastigotes. J Immunol 144:1075–1079

17. Way SS, Borczuk AC, Goldberg MB (1999) Thymic independence of adaptive immunity to the intracellular pathogen *Shigella flexneri* serotype 2a. Infect Immun 67:3970–3979
18. Johnston KH, Gotschlich EC (1974) Isolation and characterization of the outer membrane of *Neisseria gonorrhea*. J Bacteriol 119:250–257
19. Markwell MA, Hess SM, Beiber LL, Tolbert ME (1978) A modification of the lowery procedure to simplify protein determination in membrane and lipoprotein samples. Annal Biochem 87:206–210
20. Yin ET, Galanos C, Kinsky S, Bradshaw RA, Wessler S, Luderritz O, Sarmiento ME (1972) Picogram sensitivity assay for endotoxin: gelation of *Limulus polyphemus* blood cell lysate induced by purified lipopolysaccharides and lipid A from Gram-negative bacteria. Biochim Biophys Acta 261:284–289
21. Lanier LL, Warner NL (1981) Paraformaldehyde fixation of haematopoietic cell for quantitative flow cytometry (FACS) analysis. J Immunol Methods 47:25–30
22. Raqib R, Ekberg C, Sharkar P, Bardhan PK, Zychlinsky A, Sansonetti PJ, Andersson J (2002) Apoptosis in acute shigellosis is associated with increased production of Fas/Fas ligand, perforin, caspase-1 and caspase-3 but reduced production of Bcl-2 and interleukin-2. Infect Immun 70:3199–3207
23. WHO (1999) Vaccinology and Biotechnology applied to Infectious Diseases. Module 5 (Practicals) 4–7 October. Advanced Course in Immunology. WHO–IRTC, Lausanne, Switzerland
24. Bagchi AK, Sinha AK (2004) Role of 57 kDa major antigenic component of *Shigella dysenteriae* OMP in induction of major histocompatibility complex II -restricted T-cell response. Arch Med Res 35:428–434. doi:10.1016/j.arcmed.2004.05.007
25. Lovatt M, Tian-Hui Y, Stauss HJ, Fisher AG, Merkenschlager M (2000) Different doses of agnostic ligand drive the maturation of functional CD4 and CD8 T-cells from immature precursors. Eur J Immunol 30:371–381. doi:10.1002/1521-4141(200002)30:2<371:AID-IMMU371>3.0.CO;2-T
26. Mayer L, Eisenhardt D (1990) Lack of induction of suppressor T-cells by intestinal epithelial cells from patients with inflammatory bowel disease. J Clin Invest 86:1255–1260. doi:10.1172/JCI114832
27. Clement LT (1992) Isoform of the common CD45 leukocyte antigen family markers for human T-cell differentiation. J Clin Immunol 12:1–5
28. Rose JS, Heinrich PC (1994) Soluble receptors for cytokines and growth factors: generation and biological function. Biochem J 300:281–290
29. Taniguchi T, Minami Y (1993) The IL-2/IL-2R receptor system: a current overview. Cell 73:5–8
30. Raqib R, Reinholt FP, Bardhan PK, Kamell A, Lindberg AA (1994) Immunopathological patterns in the rectal mucosa of patients with shigellosis: expression of HLA-DR antigens and T-lymphocyte subsets. APMIS 102:371–380
31. Germain RN (1994) MHC-dependent antigen processing and peptide presentation: providing ligands for T-lymphocyte activation. Cell 76:287–299
32. Brandtzaeg P, Sollid LM, Thrane PS, Kvale D, Bjerke K, Scott H, Kett K, Rognum TO (1988) Progress report: lymphoepithelial interactions in the mucosal immune system. Gut 29:1116–1130
33. Hornqvist E, Goldschmidt TJ, Holmdahl R, Lycke N (1991) Host defense against cholera toxin is strongly $CD4^+$ T-cells dependent. Infect Immun 59:3630–3638
34. Luc K, Guido V, Gigase P, Young G, Hannet I, Vanlangendonck F, Hulstaert F, Bach BA (1992) Expression of activation antigens, HLA-DR and CD38, on CD8 lymphocytes during HIV-1 infection. AIDS 6:793–797
35. Dianzani U, Funaro A, DiFranco D, Garbarino G, Bragardo M, Redoglia V, Buonfiglio D, De Monte LB, Pileri A, Malavasi F (1994) Interaction between endothelium and $CD4^+$ $CD45RA^+$ lymphocytes. J Immunol 153:952–959
36. Diamond MS, Staunton DE, de Fougerolles AR, Stacker SA, Garcia-Aguilar J, Hibbs ML, Springer TA (1990) ICAM-1 (CD54) a counter receptor for Mac-1 CD11b/CD18). J Cell Biol 111:3129–3139
37. McGhee JR, Lamm ME, Strober W (1999) Mucosal immune responses: an overview. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR (eds) Mucosal immunology. Academic Press, San Diego, pp 485–506
38. Smith KA, Cantrell DA (1985) Interleukin 2 regulates its own receptors. Proc Natl Acad Sci 82:864–868