View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1762 (2006) 46 – 53

http://www.elsevier.com/locate/bba

Antigen presenting cells (APCs) from thermally injured and/or septic rats modulate CD4+ T cell responses of naive rat

Nadeem Fazal [a,b,*], Syed Raziuddin [a], Mehdi Khan [a], Walid M. Al-Ghoul [b]

[a] *Burn and Shock Trauma Institute, Department of Surgery, Stritch School of Medicine, Loyola University Chicago, Maywood, IL, USA*
[b] *Department of Biological Sciences, Chicago State University, 9501 South King Drive, Chicago, IL 60628, USA*

Received 3 November 2004; received in revised form 25 July 2005; accepted 26 July 2005
Available online 11 October 2005

Abstract

Regulation of immune response is marked by complex interactions among the cells that recognize and present antigens. Antigen presenting cells (APCs), the antigen presenting cell component of the innate immune response plays an important role in effector CD4+ T cell response. Thermal injury and/or superimposed sepsis in rats' leads to suppressed CD4+ T cell functions. We investigated modulations of CD4+ T cell function by APCs (purified non-T cells) from thermally injured and/or septic rats. Rats were subjected to 30% total body surface area scald burn or exposed to 37 °C water (Sham burn) and sepsis was induced by cecal-ligation and puncture (CLP) method. At day 3 post-injury animals were sacrificed and CD4+ T cells and APCs from mesenteric lymph nodes (MLN) were obtained using magnetic microbead isolation procedure. APCs from injured rats were co-cultured with sham rat MLN CD4+ T cells and proliferative responses (thymidine incorporation), phenotypic changes (Flow cytometry), IL-2 production (ELISA) and CTLA-4 mRNA (RT-PCR) were determined in naive rat CD4+ T cells. The data indicate that APCs from thermally injured and/or septic rats when co-cultured with CD4+ T cells suppressed CD4+ T cell effector functions. This lack of CD4+ T cell activation was accompanied with altered co-stimulatory molecules, i.e., CD28 and/or CTLA-4 (CD152). In conclusion, our studies indicated that defective APCs from thermally injured and/or septic rats modulate CD4+ T cell functions via changes in co-stimulatory molecules expressed on naive CD4+ T cells. This altered APC: CD4+ T cell interaction leads to suppressed CD4+ T cell activation of healthy animals.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Antigen presentation; Dendritic cell; Burn injury; Sepsis; IL-2 production; Mesenteric lymph node; Costimulatory molecule; CD28; CTLA-4

1. Introduction

Burn injury-induced T cell effector responses modulate both CD4+ T cells and antigen presenting cells [1]. Immunosuppression after burns has been associated with imbalanced T-helper immune response, altered cytokine response and decreased resistance to infection [2]. Severe injury through their interactions with T lymphocytes induces adaptive immune response to pathogens, tolerance to self-antigens and the induction of autoimmunity [3–5]. At lymph nodes draining mucosal surfaces such as mesenteric lymph nodes, APCs constantly survey and process commensal bacteria and pathogens and by their morphology attract and interact with naive T cells [6]. The ligands presented by APCs include a proper MHC–antigen peptide complex, as well as a requisite set of co-stimulatory molecules [7,8]. Three cell types expressing MHC class II complexes (macrophages, dendritic cells and B cells) have the potential to activate CD4 T cell. Although much circumstantial evidence shows that dendritic cells are the professional antigen presenting cells, and various subsets of dendritic cells (DCs) have been identified in the intestinal lymphoid organs, it is still not certain which cells first present MHC complexes to naive CD4+ T cells [8,9]. APCs in the lymph nodes undergo phenotypic and functional changes, including up-regulation of cell surface expression of co-stimulatory and adhesion molecules and production of cytokines [9–11].

T-cell responses predict the adaptive immune response after injury. Naive T cells generally reside in the lymph node whereas effector and memory T cells migrate preferentially to peripheral tissues [12–14]. The lack of ability of an APC to precisely regulate naive T cell activation may provide an explanation for

* Corresponding author. Department of Biological Sciences, Chicago State University, 9501 South King Drive, Chicago, IL 60628, USA. Tel.: +1 773 995 2148; fax: +1 773 995 3759.
E-mail address: nfazal@csu.edu (N. Fazal).

0925-4439/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2005.07.005

the burn-induced derangements found in T cell activation observed in our previous studies [15]. The attenuated proliferative responses by T lymphocytes in burn/sepsis injured hosts may also arise as a result of their inappropriate interaction with dysfunctional APCs. In this study, we have evaluated the capacity of APCs isolated from burn and/or septic rats to activate naive CD4+ T cells. Activation of resting CD4+ T cells is organized through interaction of co-stimulatory molecules, both stimulatory (CD28) and inhibitory (CTLA-4) expressed on surface of CD4+ T cells and their corresponding ligands presented on antigen presenting cells. These APCs likely represent the population of cells draining to the node from peripheral intestinal mucosal sites where they would have encountered injury related non-self antigen including intestinal bacteria/bacterial products. The use of T cells also from uninjured animals' MLN that represent a significant population of naive T cells has allowed us to evaluate relatively specifically how naive T cells may be affected by interactions with injured animals APCs in the MLN habitat.

In the present study, we utilized clinically relevant rat model of burn plus sepsis to examine the impact of such individual conditions as well as that of a combined burn injury plus sepsis on function of APCs during their interaction with naive CD4+ T cells. We hypothesized that injury causes modulation of APCs and alter their ability to prime T cell for an effective immune response. Thus, burn plus sepsis causes a more profound defect in APCs in their ability to stimulate naive CD4+ T cells than individual burn or sepsis injuries. Such an APC defect may be attributed to altered co-stimulatory molecules expressed on CD4+ T cells leading to a more profound immunosuppression found in burn plus septic condition.

2. Methods

2.1. Animal models

Male Sprague–Dawley rats (250–300 g) were housed and used in compliance with the regulation of the Animal Care Facility of Chicago State University, Chicago. Burn injury: This rat model has been previously described as a suitable model demonstrating the response associated with burn injury [15]. Rats were anesthetized with sodium pentobarbital [40–50 mg/kg intraperitoneal (i.p)]; their dorsal body surface hair shaved off, and placed in an appropriately sized template device such that the shaved area of the skin on the animals' back was exposed. Adequacy of anesthesia was tested by the absence of withdrawal response to toe pinching. The template device was then lowered into a hot water bath (95–97 °C) to immerse the exposed skin area in hot water for 10 s. With this technique, full thickness 3rd degree burns comprising 30% of the total body surface were obtained. Sham rats were subjected to identical anesthesia and other treatments, except that they were immersed in 37 °C water. The animals were dried immediately, and given fluid resuscitation with 0.9% saline (3 cc/kg/total body surface area) to maintain urine output. 10 animals were included in each group. The animals were sacrificed at day 3 post-injury. Untreated and un-operated rats were controls.

2.2. Cecal-Ligation-Puncture (CLP)

A mid-line laparotomy was performed on anesthetized rats in the lower abdomen, and abdominal cavity was opened along striae alba. The cecum was identified and ligated with 2-0 sutures silk at 8–10 mm from the tip of the cecum. The tip of the ligated cecum pouch was punctured with 22G needle once. When the needle was pulled back, small amounts of cecum content were expressed, and cecum pouch returned to the abdominal cavity. Muscle layers were sutured and skin closed. Animals were given fluid resuscitation with 0.9% saline (3-cc/kg/total body surface area). The animals were sacrificed at day 3 post-injury. CLP only rats were given Cecal-Ligation-Puncture without burn.

2.3. Magnetic microbead T cell isolation method

MLN were aseptically isolated from rats and crushed under sterile stainless steel sieve. The crushed tissues were passed through cell strainer (35 μm pore size) to remove clumps. The filtered cell suspension was spun at 300×g for 10 min and pellet suspended in 1 ml RPMI-1640 media. Cell population counted with a trypan blue exclusion method to determine cell viability. Cells were adjusted to 10^7 cells in 80 μl and incubated with 20 μl of CD+ T cell Magnetic Micro-beads (Miltenyi Inc.) at 4 °C for 15 min. The cells were washed with elution buffer (Miltenyi, Inc.) by centrifugation at 300×g for 10 min. The micro-beads and cell mixture was passed through pre-wet columns. The columns were flushed with 0.5 ml of elution buffer 3 times. The cells passed through the columns (negative selection) were discarded. Columns were removed from magnetic field; the cells (positive selection) were eluted by running 0.5 ml of elution buffer 2 times with gentle pressure from plunger. The eluted cells were washed with elution buffer by centrifugation at 300×g for 10 min. Positive selection cells (10^6) were taken and incubated with FITC-labeled anti-CD4+ antibody Biosource Inc, CA. (1 μl/10^6 Cells) at 4 °C for 10 min to determine positive yield by FACS analysis. This method repeatedly yielded 99% purity of CD4+ T cells.

2.4. Isolation of antigen presenting cells (APC)

Rats mesenteric lymph nodes (MLN) were crushed under stainless sieve and single-cell suspensions were prepared. Total T cells were depleted using Pan T cell magnetic micro-beads as described by the manufacturers (Miltenyi Inc.). The details are given above in Methods. Briefly, total T cells were positively selected and discarded while the negative selection of cells was used as potential antigen presenting cells. The cell were suspended in complete RPMI-1640 medium with 10% FCS and incubated at 37 °C in tissue culture Petri dishes (Fibrinogen-treated) for 2 h to allow adherence to dishes. The supernatants containing non-adherent cells were discarded and adherent cell population was obtained by repeated pipetting with warm RPMI-1640 medium. The adherent cell population (total T-cell free) was used as (APC) in the subsequent experiments.

2.5. Proliferation assays

APC and CD4+ T cells at 1:10 effector target ratios were cultured in 96 well flat-bottomed microtiter plates (Falcon, Lincoln Park, NJ) for 72 h/37 °C in 5% CO_2. Anti-CD3 antibody (1 μg/ml), rIL-2 (1 μg/ml), anti-CD28 (5 μg/ml) purchased from Biosource Inc. were used in the subsequent experiments as activators. T cell proliferation was assessed by adding Tritiated Thymidine 1 μCi (37Bq) to the growing cultures in the last 16–18 h. Cells were then harvested at the end of culture period onto filtermats (Skatron, Sterling, VA) with a semi-automatic PHD cell harvester (Cambridge Technology Inc). The counts per minute (cpm) of the filter membrane were measured in scintillation liquid on a Beckman LS 6500 liquid scintillation counter (Fullerton, CA).

2.6. IL-2 Measurement (ELISA)

The supernatants were harvested from APC: CD4+ T cells co-cultures after 24 h and IL-2 content determined by using Enzyme-Linked Immunosorbent Assay (ELISA) kits (Biosource Inc, CA).

2.7. Reverse-transcription polymerase chain reaction (RT-PCR)

Determination of CTLA-4 mRNA was performed by isolation of total RNA aseptically from CD4+ T cell co-cultures using RNA Easy kit (QIAGEN, Inc, Valencia, CA) as per manufacturers protocol. High quality RNA was eluted in

RNAse-free water. The concentration and purity of RNA was determined by measuring the absorbance at 260 nm (A260) and 280 nm (A280) in a spectrophotometer. PCR amplification was performed using the Gene-Amp RNA PCR kit obtained from Perkin-Elmer (Roche, NJ). In brief, 1 μg of total RNA was reverse transcribed in a total volume of 20 μl containing 25 mM $MgCl_2$, 50 nM KCl, 10 mM Tris–HCl (pH 8.3), 2.5 μM oligo dT primers, 1 μM deoxynucleotide triphosphate, 1 μ/μl RNase inhibitor and 2.5 U/ml MLV reverse transcriptase. Samples were incubated for 15 min at 42 °C, 5 min at 99 °C and 5 min at 5 °C. The cDNA products obtained were incubated with a specific pairs of primers designated to amplify cDNA coding for HPRT (sense 5′-GCG AAG TGG AAA AGC CAA GT-3′, antisense 5′-GCC ACA TCA ACA GGA CTC TTG TAG-3′), CTLA-4 (sense 5′-GGC AGA CAA ATG ACC AAG TGA C-3′ antisense, 5′-TCT GAA TCT GGG CAT GGT TCT-3′). All primers were obtained from Integrated DNA Technology Inc. (IDT, Coralville, IA). Amplification was carried out in 80 μl reaction mixture containing 25 mM $MgCl_2$, 2.5 U/100 μl ampli-Taq DNA polymerase and 1 μM of each sense and antisense primers. cDNA were amplified in a Perkin-Elmer thermocycler (GeneAmp, PCR System 2400). Cycle conditions: one cycle at 94 °C for 5 min followed by 32/36 cycles at 94 °C for 30 s, 55 °C for 60 s and 72 °C for 60 s. After amplification, the PCR products were electrophoresed in 1.0% agarose gel (GIBCO-BRL), stained with ethidium bromide and photographed on Polaroid 667 film. The individual specific mRNA bands were identified using a standard DNA marker of 100–1500 base pair (bp). After complete electrophoresis, the PCR gels image were captured and analyzed using NIH image software with a multi scan Sony computer. Experiments were repeated following treatment of mRNA with DNAse to exclude DNA contamination.

2.8. Statistical analysis

All statistical analyses were carried out using the Statistical Package, Social Sciences Software Program (SPSS, Sigma Stat version 2.0, Chicago, IL). The statistical analysis of the different experimental groups included the comparison of sham-burned, day 3-postburn, CLP and Burn plus CLP rats in n = number of animals. Statistical significance was assigned at P values < 0.05.

3. Results

3.1. The effects of co-cultures of sham animal APCs on the proliferation of sham animal (naive) CD4+ T cells

These control studies were carried out using APCs and CD4+ T cells from uninjured sham rats to determine naive T cell activation through TCR and costimulatory signals as affected by APCs from naive rats. Purified CD4+ T cells were obtained from sham rats by magnetic microbead isolation procedure and stimulated with αCD3 alone or in the presence of αCD3 and APCs, ex vivo. Fig. 1 shows proliferative responses by naive rat MLN T cells stimulated with the combination of αCD3 and APCs also harvested from uninjured rat MLN. Activation of CD4+ T cells by αCD3 alone failed to demonstrate an effective proliferative response in the absence of co-stimulation. The additional signal for co-stimulation was efficiently provided by APCs. Hence, CD4+ T cells proliferated following stimulation with αCD3 and interaction with APC. In these experiments APC to CD4+ T cell ratio (1:10) was found to have an optimum proliferative response such that addition of exogenous stimulatory anti-CD28 antibody did not further increase proliferation of CD4+ T cells. The IL-2 signaling pathway was checked by addition of exogenous IL-2 that provided maximal proliferation response in these APC: CD4+ T cell co-culture assays (P < 0.05).

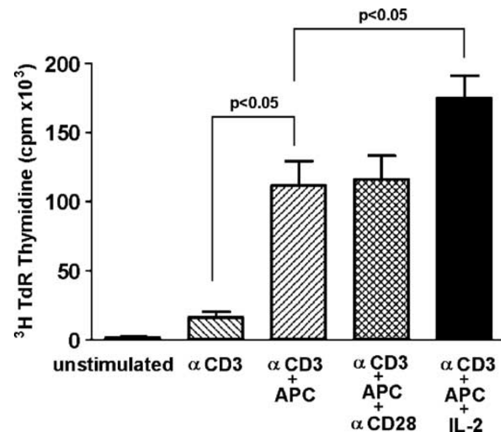

Fig. 1. Proliferation of sham rat CD4+ T cells following co-culture with sham rat APCs over 72 h as assessed by ^{3}H Thymidine incorporation expressed in counts per minute (cpm). Control un-stimulated (U), stimulated with αCD3, and/or incubation with APCs from sham rats with and without exogenous IL-2 or anti-CD28. Data represent Mean ± S.E. (n = 6) from 3 experiments. Effector to target ratio was 1:10 (1 APC for 10 CD4+ T cell).

3.2. The effects of co-cultures of sham animal APCs on IL-2 production of sham animal (naive) CD4+ T cells

Fig. 2 shows IL-2 release in the supernatants of APC: CD4+ T cell co-culture assays over 24 h in the presence of αCD3. Detectable levels of IL-2 were obtained following αCD3 stimulation of purified CD4+ T cells (Fig. 2). Interaction of APCs in the presence of αCD3 provided the requisite co-stimulatory signal to activate CD4+ T cells for an enhanced IL-2 production (P < 0.05). APCs caused IL-2 production to 3–4× higher level than with αCD3 stimulus alone. Supplement of additional co-stimulatory signal like anti-CD28 antibody to APC: CD4+ T cells could not significantly elevate IL-2 levels (P > 0.05) to any further than combination of αCD3 and APCs. These data show effectiveness of allogeneic interaction between naive rat CD4+ T cell and APCs for the optimum production of IL-2.

3.3. Proliferative responses of naive CD4+ T cells following co-culture with injured-APC obtained from Burn, CLP or Burn + CLP

To explore the effect of injured rat APCs (day-3 post-injury) on naive CD4+ T cells, experiments were performed where APCs were obtained from burn, CLP or burn + CLP animals and then cocultured with naive CD4+ T cells for 72 h in the presence of αCD3. Results in Fig. 3 show when the naive CD4+ T cell were co-cultured with APCs from burn, CLP or burn + CLP, proliferation of CD4+ T cells was significantly reduced (P < 0.05) as compared to sham APC: CD4+ T cell co-cultures. The magnitude of suppression was ∼50% for burn APCs, ∼40% for CLP APCs and ∼75% for burn plus CLP APCs, as compared to sham APC co-cultured with sham CD4+ T cells. These findings clearly show that injured APCs are less effective at inducing CD4+ T cell proliferation. Interestingly, when burn injury was super-imposed with septic injury (naive rat CD4+ T cells co-cultured with APCs from burn + CLP animals) the suppressive effect was found to be most significant (P < 0.05). In

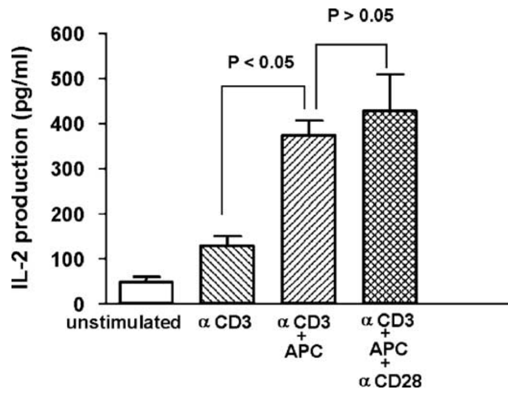

Fig. 2. IL-2 production of sham rat CD4+ T cells following co-culture with sham rat APCs over 24 h as assessed by rat-specific IL-2 ELISA. Concentration of IL-2 in the culture supernatants expressed as pg/ml in the experimental groups of un-stimulated, stimulated with αCD3, αCD3 + anti-CD28 and/or APC from sham rats. Data represent Mean ± S.E. (n = 6) from 3 experiments.

order to test if this suppression induced by injured APCs was due to alteration in CD28 co-stimulatory signal, we supplemented these co-cultures with a stimulatory anti-CD28 antibody. Our results showed that addition of stimulatory anti-CD28 antibody failed to reverse the inhibition demonstrated by APCs from burn, and CLP-injured animals (Fig. 3). The partial restoration observed in burn plus CLP APC co-cultured with sham CD4+ T cell was found to be statistically insignificant ($P < 0.05$), whereas the effect of addition of anti-CD28 in case of sham APC plus CD4+ T cells co-cultures was statistically non-significant ($P > 0.05$). We then tested if addition of exogenous IL-2 could restore this CD4+ T cell proliferative response. Our data confirmed that suppressed T cell activation was reversed by addition of IL-2 to injured APCs co-cultures obtained from burn and CLP such that comparable levels of T cell activation to the sham APC co-cultures were achieved. However, in combined injury model (burn + CLP) addition of IL-2 to CD4+ T cells plus burn + CLP co-cultures failed to restore proliferative responses ($P > 0.05$). The effect of addition of IL-2 to sham APCs plus naive CD4+ T cells was significant ($P < 0.05$) in these experiments similar to results obtained in the initial experiments documented in Fig. 1.

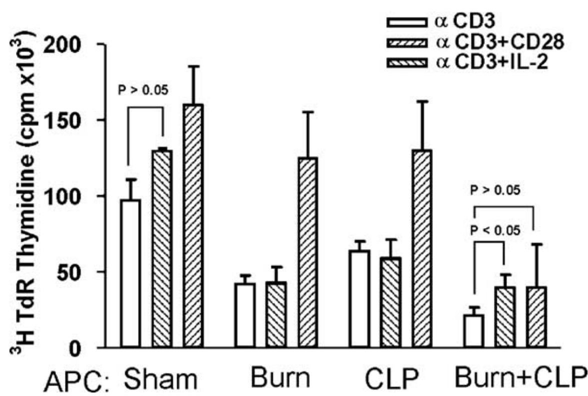

Fig. 3. Proliferation of sham rat CD4+ T cells following co-culture with injured rat APCs over 72 h as assessed by ^{3}H Thymidine incorporation assay. Results are expressed in counts per minute (cpm). CD4+ T cells and APCs were immunomagnetically isolated from rat MLN of sham and experimental groups. APCs were obtained from MLN of the following experimental groups; Sham (S), Burn (B), CLP (sepsis) or Burn + CLP rats. Data represent Mean ± S.E. (n = 6) from 3 separate experiments. APC: CD4+ T cell (1:10 effector target ratio) co-cultures were stimulated with αCD3, or anti-CD28, and/or incubation with IL-2. Sham APCs and sham CD4+ T cell were used as controls. Data represent Mean ± S.E. (n = 6) from 3 experiments. $P < 0.05$ was considered significant while $P > 0.05$ as non-significant.

3.4. IL-2 production by naive CD4+ T cells following co-culture with injured APC obtained from Burn, CLP or Burn + CLP

To explore the effect of injured rat APC (day-3 post-injury) on IL-2 production capability by naive CD4+ T cells, experiments were performed where APCs were isolated from sham, burn, CLP or burn + CLP animals and then co-cultured with sham CD4+ T cells for 24 h in the presence of αCD3. When the naive CD4+ T cells were co-cultured with APCs from burn, CLP or burn + CLP, IL-2 production by sham CD4+ T cells was significantly reduced ($P < 0.05$). The present results demonstrated a ∼3–4× decrease in IL-2 production in burn and CLP groups of APC co-cultures, and ∼7× inhibition in APC co-cultures of burn plus CLP animals (Fig. 4). Our findings showed that the combined burn and burn/sepsis injury lead to maximal suppression of IL-2 production ($P < 0.05$) as compared to IL-2 production in any other experimental groups. Addition of anti-CD28 to the APC: CD4+ T cell co-cultures from injured animals' groups were unable to reverse the down-regulation of IL-2 production in burn and CLP experimental groups. However, addition of anti-CD28 to APC co-culture obtained from burn plus CLP animals, documented a 3-fold increase in IL-2 production, which was found to be statistically significant ($P < 0.05$), yet it did not bring it to the sham IL-2 levels. When sham rat APCs were co-cultured with naive CD4+ T cells, maximum IL-2 production was observed (Fig. 4). Addition of stimulatory anti-CD28 failed to significantly enhance this maximal IL-2 production ($P > 0.05$).

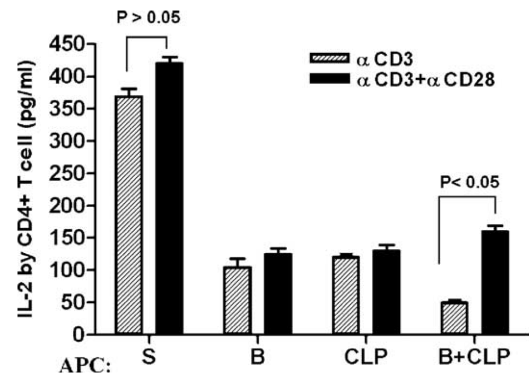

Fig. 4. IL-2 production of sham rat CD4+ T cells following co-culture with injured rat APCs over 24 h as assessed by rat-specific IL-2 ELISA and expressed as pg/ml in the culture supernatants. Co-cultures were stimulated with αCD3, and/or αCD3 + αCD28 and/or incubated with APC from Sham (S), Burn (B), CLP (sepsis), or Burn + CLP rats. Data represent Mean ± S.E. (n = 6) from 3 separate experiments. $P < 0.05$ was considered significant while $P > 0.05$ as non-significant.

3.5. Surface expression of co-stimulatory molecule (CD28) on naive CD4+ T cells following co-culture with APCs from injured rats

Flow cytometry analysis was performed to explore alterations in the expression of co-stimulatory molecule (CD28) on naive CD4+ T cells following incubation with APCs from sham, burn, CLP and burn + CLP. In the following experiments APC: CD4+ T cells co-cultures were stimulated with αCD3 for 72 h. Results represented as a histogram in Fig. 5 showed that surface expressions of CD28 receptor on naive CD4+ T cells following co-incubation with sham (Fig. 5a) and burn (Fig. 5c) injured APCs remained unchanged, i.e. same as observed with αCD3 alone. APCs from burn + CLP (Fig. 5d) significantly ($P < 0.05$) and CLP (Fig. 5b) to a non-significant degree ($P > 0.05$) down-regulated the surface expression of CD28 receptor as evidenced by a remarkable shift to left in the flow plots. This pattern of down-regulation of CD28 receptor expression on CD4+ cells caused by interaction with injured APCs was noticed to be consistent in repeat experiments. Statistical analyses in these experiments were performed after gating out dead cells (10–12%).

3.6. Surface expression of co-stimulatory molecule (CTLA-4) on naive CD4+ T cells following co-culture with APCs from injured rats

Flow cytometry data as depicted by representative quadrant analyses (Fig. 6) was performed to explore alterations in the expression of CD152/CTLA-4 on naive CD4+ T cells following incubation with APCs obtained from sham, burn, CLP and burn + CLP experimental animals. APC: CD4+ T cells co-cultures were stimulated with αCD3 for 72 h and dually labeled with PE-labeled CTLA-4 and FITC-labeled CD4+ T cell. When naive CD4+ T cells were co-cultured with burn APCs, 11% of CD4+ T cells were CTLA-4 positive (Fig. 6b), 15% in case of sepsis APCs (Fig. 6c), and 25% following co-culture with APCs obtained from burn plus CLP animals (Fig. 6d). CTLA-4 (CD152) surface expressions on naive CD4+ T cells when co-cultured with sham APCs expressed 5% CTLA-4 positive T cells (Fig. 6a), the significance of which was not found. Owing to a long co-culture assay conditions (72 h) we did encounter an increased percentage of dead cells (10–12%). However, by labeling with ethidium bromide, we gated dead cells out and were not included in statistical analyses. Furthermore, the number of dead cells excluded remained quite similar among different experimental groups which again emphasize the effect of lengthy co-culture conditions.

3.7. Determination of mRNA CTLA-4 on naive CD4+ T cells by RT-PCR

Up-regulation of receptor expression of CTLA-4 (CD152) on naive CD4+ T cells following co-culture with APCs obtained from injured animals, lead to investigation of CTLA-4 mRNA status of naive CD4+ T cells. Since these cell-mixing studies contained populations of both APC and CD4+ T cells in a 1:10 effector target ratio, no attempt was made to separate CD4+ T cells from APCs in experimental co-cultures. However, our data suggested that no CTLA-4 (CD152) mRNA was detected in lysates of either CD4+ T cells or APC alone. RT-PCR data indicated an up-regulation of CTLA-4 mRNA in CD4+ T cells co-cultured (24 h in the presence of αCD3) with injured APCs obtained from burn, CLP and burn + CLP groups (Fig. 7). In the control experiments, there was no detection of mRNA CTLA-4 when naive CD4+ T cells were co-cultured with APCs from sham rats in the presence of αCD3 or sham CD4+ T cells alone. Experiments were repeated twice with similar results.

4. Discussion

The present series of experiments was performed to test the effects of burn injury or burn injury combined with experimental sepsis on changes in mesenteric lymph node antigen presenting cells functional capacity. Following TCR/CD3-mediated signaling, CD4+ T cell responses were compared in the presence of APCs from burn, CLP and Burn + CLP experimental animals. Allogenic T-cell effector responses measured T-cell activation by APCs. Our findings clearly showed that injured APCs are less effective at inducing CD4+ T cell proliferation and IL-2 production. Combined burn and sepsis lead to higher suppression in T cell responses than burn or sepsis alone. Addition of exogenous IL-2 reversed the suppression induced by injured APCs from burn or CLP but not burn plus CLP experimental animals. Our studies linked CD4+ T cell dysfunctions to alterations in the co-stimulatory molecules (CD28 and CTLA-4) induced by injured animals' APC.

The rat injury model had limitations due to paucity of rat-specific reagents. This co-culture system allowed us a model whereby we could assess the role of APCs based on their functional capacity to activate T cells. Mesenteric lymph node APCs located at the portals of antigen entry in intestine sample, process, display MHC complex and migrate to the T cells areas of secondary lymphoid organs [8,9]. Although much circumstantial evidence shows that dendritic cells are the professional antigen presenting cells, it is still not certain which cells first present MHC complexes to naive CD4+ T cells [8]. Mucosal APCs sample antigen in the intestinal mucosa and migrate to MLN to prime naive T cells [16]. Furthermore, commensal bacteria colonize the gastrointestinal tract, therefore, MLN (APC/CD4+ T cell system) regularly encounter gut micro flora as well as food antigen load and hence provide a microenvironment for host immune responses [17]. In this study, CD4+ T cells and APC were isolated from mesenteric lymph nodes, which mimic a microenvironment of APC: CD4 T cell cross-talk in a lymph node.

Our studies suggested that APCs present in the mesenteric lymph nodes control proliferation and differentiation of naive or resting CD4+ T cells of MLN. CD3/TCR system was maximally stimulated in our proposed APC: CD4+ T cell co-culture system since our data showed that sham APCs interact with naive CD4+ T cells in the presence of anti-CD3 in such a

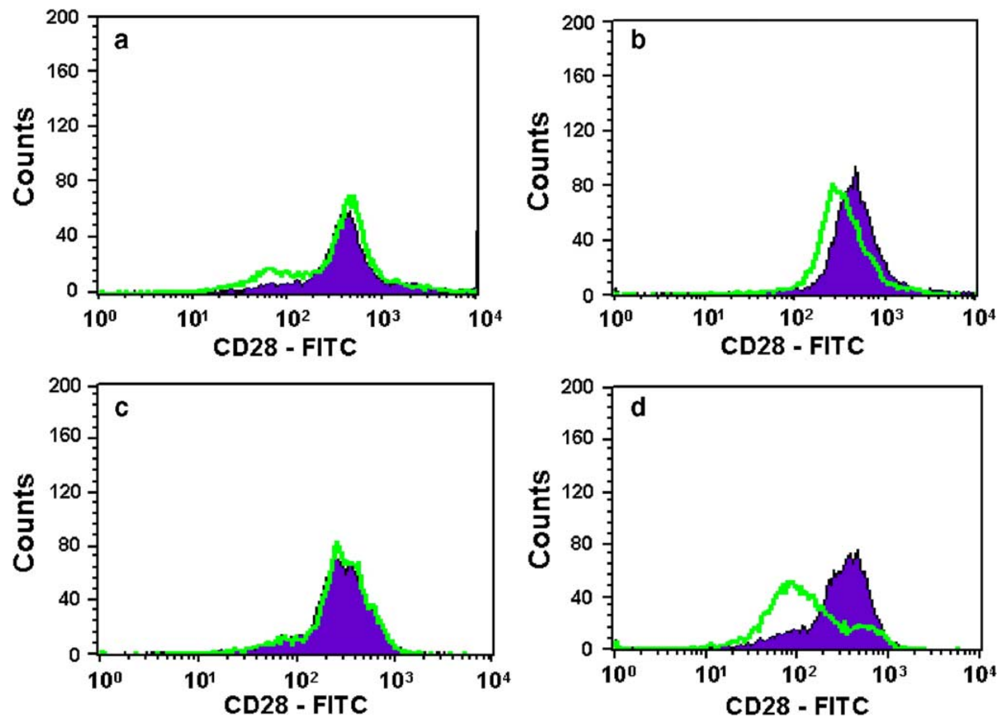

Fig. 5. Representative flow cytometry plots of CD28 surface expression on sham rat CD4+ T cells following 72 h co-culture with rat APCs from Sham (a), CLP (b), Burn (c) or Burn+CLP (d). x axis (log scale) represents FITC-CD28 labeled CD4+ T cells while y axis show fluorescence counts. Closed lines (filled/purple) indicate (αCD3-stimulated), open lines (empty/green) indicate αCD3-stimulated plus APCs from different experimental groups. Shift towards left was considered significant. Similar results were obtained in 3 separate experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

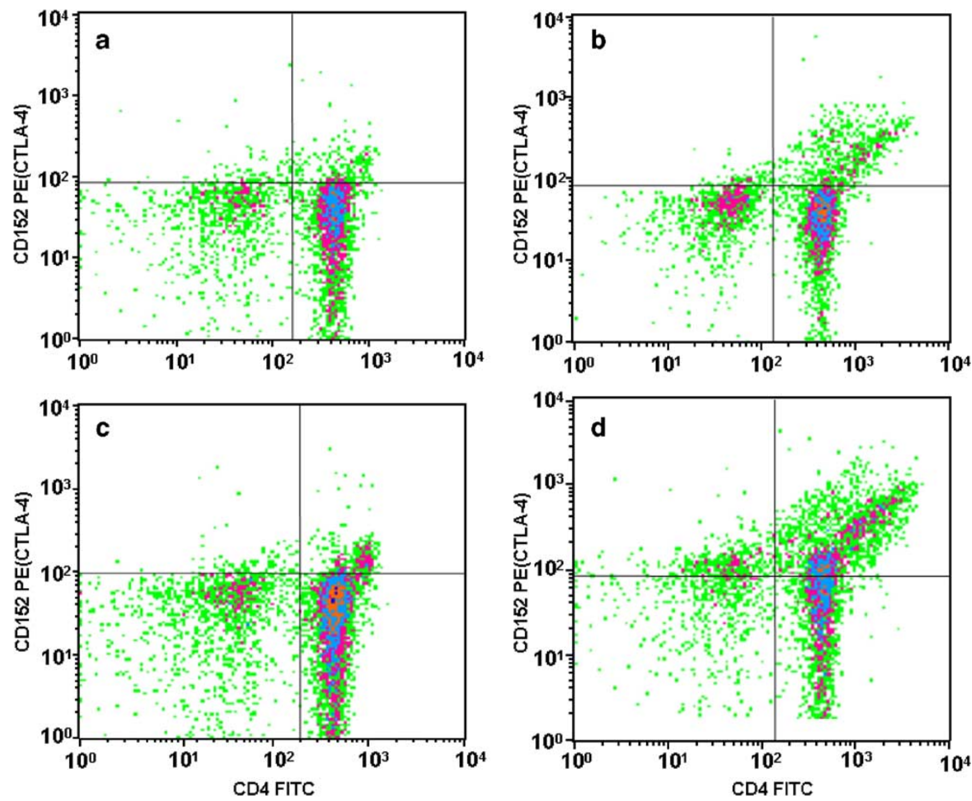

Fig. 6. Representative FACS quadrant plots of MLN CD4+ T cell illustrating CTLA-4 expression on sham rat CD4+ T cells following 72 h co-culture with rat APCs from Sham (a), Burn (b), CLP (c), or Burn+CLP (d). All the co-cultures were αCD3-stimulated. x axis (log scale) represents FITC-labeled CD4+ T cells while y axis shows PE-labeled CTLA-4 (CD152) positive cells. Cells in the right upper quadrant of all the quadrant plots represent dually labeled CD4+ (FITC) and CTLA-4 (PE) positive cells. Similar results were obtained in 3 separate experiments.

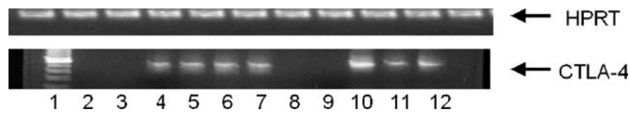

Fig. 7. CTLA-4 mRNA of sham rat CD4+ T cell lysates as determined by RT-PCR. CD4+ T cells were co-cultured for 24 h with injured APCs obtained from MLN of the following experimental groups; Sham (S), Burn (B), CLP (sepsis) or Burn+CLP rats. Co-cultures were maintained in the presence of αCD3. Lane 1 (molecular marker), lanes 2–3 (CD4+ T cell only), lanes 4–5 (CD4+ T cell plus burn APCs), lanes 6–7 (CD4+ T cell plus sepsis APCs), lanes 8–9 (CD4+ T cell plus sham APCs), lanes 10–12 (CD4+ T cell plus burn/sepsis APCs). HPRT was used as a housekeeping gene. Results were verified in 2 separate experiments.

way that no further activation could be demonstrated by the addition of anti-CD28. Interestingly, APC: CD4+ T cell co-cultures remained significantly responsive to supplemental IL-2 as evidenced by addition of IL-2 which further enhanced proliferation of naive CD4+ T cells. Thus, APC: T cell co-culture system provided a model to study functional responses of CD4+ T cells following interaction with APCs obtained from injured animals. Injured APCs variably effected activation of naive CD4+ T cells as judged by proliferation and IL-2 production. Burn and sepsis APCs suppressed CD4+ T cells proliferation without a significant restorative effect by the addition of anti-CD28. Maximal inhibition of proliferation was produced by burn plus sepsis APCs; however anti-CD28 antibody reversed this suppressive effect. On the contrary, addition of supplemental IL-2 though reversed CD4+ T cell suppression following interaction with sham, burn and sepsis-injured APCs yet failed to reverse suppression induced by burn plus sepsis APCs. Our studies of septic animals support previous observations where modulation of antigen presenting cells and CD4+ T cells have been documented in immunosuppressive septic patients [18,19].

Further experiments were performed to judge the status of co-stimulatory molecules, stimulatory (CD28) and inhibitory (CTLA-4), on CD4+ T cells. The results verified that the interaction of CD4+ T cells with injured APCs altered co-stimulatory molecules. Flow cytometry analysis confirmed that burn plus sepsis APCs significantly down-regulated surface expression of CD28, while it remained unchanged in other injuries. The alteration of CD28 receptor expression following burn plus septic injury may explain suppression seen in this combined injury module. We then determined CTLA-4 status of CD4+ T cells as this negative co-stimulatory molecule is known to compete for the same receptor on corresponding APCs. Interestingly, an increased CTLA-4 mRNA activity with an up-regulation of CTLA-4 surface receptor expression with burn plus sepsis injured APCs was observed. The signaling pathways leading to CTLA-4 up-regulation in CD4+ T cells are not completely understood, however, it is known that following TCR/CD3-mediated signaling, the transcription of CTLA-4 gene increases and is further enhanced by either CD28-mediated co-stimulation or IL-2 [11,19]. Our data showed although naive CD4+ T cells expressed resting CTLA-4 levels, the surface levels were increased upon activation by anti-CD3 and APC co-culture, probably due to both redistribution of an intracellular pool and increased synthesis of CTLA-4 as noticed by others [20]. We found CTLA-4 mRNA levels increased within 24 h of co-culture with injured APCs; however no signal was detected in CD4+ T cells following interaction with sham APCs. We confirmed that injured APC could up-regulate CTLA-4 in naive CD4+ T cells in an ex vivo environment. Thus CTLA-4 could then compete with CD28 for interaction with CD80/86 molecules present on APCs as CTLA-4 is known to bind to these ligands with greater affinity (~20×) than CD28 [20], which may account for the abrogation of the co-stimulatory effects of CD28 in the face of expression of CTLA-4 on activated T cells. CTLA-4 could also interfere with IL-2 production of T cells and cause arrest of T cell cycle in the G1 phase. Unlike CD28, which is constitutively expressed and is stable, CTLA-4 is induced in activated T cells and is relatively unstable with a much shorter half-life [19]. We have not looked into other potential effects of injured APCs causing alteration of CD4+ T cell function, i.e., anergy, apoptosis, generation of T regulatory activity, and cytokine response, etc. Our results are in line with studies showing the role of co-stimulatory-ligand binding, i.e., CD28/B7 in the regulation of immunosuppression observed in these studies [21].

Our simplistic approach to study the functional ability of injured APCs to modulate naive CD4+ T cells also tested their ability to regulate IL-2 as a potential growth factor. Naive T cells produced IL-2 in the presence of APCs from sham rats and αCD3/anti-CD28, while presence of injured APCs hampered this capacity of CD4+ T cells. These findings suggested that IL-2 signaling pathway (IL-2 transcription/translation) was down-regulated by injured APCs while IL-2 receptor activation remained intact as presence of injured APCs allowed the effect of exogenous IL-2 on restoring proliferation of CD4+ T cells. Disturbance in signaling pathway downstream to CD3-plus co-stimulatory molecules, or IL-2 transcription/translation could thus be considered. We recently demonstrated immunosuppression of T cell in burn injury due to down-regulation of signaling pathways (MAPKs, Erk 1/2 and p38). The role of calcium signaling was proposed as MAPK-signaling and effector functions of T cells were restored by activation of T cells with PMA plus ionomycin [15]. Similar observations were made in resting CD4+ T cells where cyclic AMP- and calcium-mediated induction led to up-regulation of CTLA-4 [19].

There is overwhelming evidence that injury causes suppressed T-cell activation, especially thermal injury that leaves burn patients susceptible to infection with opportunistic bacteria. Data from burned patients and animal models of burn injury show immunological alterations linked to decreased T-cell proliferation [22–25]. We conclude that APCs from injured animals failed to support T cell activation from healthy rats following an increase of CTLA-4 on naive CD4+ T cells and a relative decrease of CD28 co-stimulatory molecule in burn plus sepsis injury. Although we have linked T-cell suppression in injury states with altered APC function, vice versa, T cell causing modulation of healthy APCs could occur. One study provided evidence for sepsis induced deletion of CD4+ T cell to account for immunosuppression [19]. Thus,

increased susceptibility of burn and sepsis hosts could be due to APC dysfunction and T cell-dependent cell mediated immunity, as there could be a compromise of not only the T cell-dependent cell-mediated immunity (against viral/intracellular infections) but also a linked weakening of APC mediated defense against extracellular bacterial invasions. Recently, burn injury-induced changes in T cell responses have documented changes in both CD4+ T cells as well as antigen presenting cells [1]. Although APCs could be the major contributor to prime naive CD4+ T cells in lymph nodes, there remain more subtle ways for the host to mount an effective immune response to burn injury [10,21]. This study has addressed the role of antigen presenting cells ex-vivo, however, in vivo conditions; soluble factors like cytokines, other professional antigen presenting cells like dendritic cells, NK cells and yet unknown factors direct an effective CD4+ T cell response. More studies in rat are needed to further characterize functional responses that these injured APCs may provoke in the naive CD4+ T cells and vice versa [26].

Acknowledgment

This work was supported by NIH MBRS SCORE Grant No. 3 S06 GM008043-34S1.

References

[1] Y. Zang, S.M. Dolan, N.N. Choileain, S.J. Kriynovich, T.J. Murphy, P. Sayles, J.A. Mannick, J.A. Lederer, Burn injury initiates a shift in superantigen-induced T cell responses and host survival, J. Immunol. 172 (8) (2004 Apr. 15) 4883–4892.

[2] B.G. Sparkes, Immunological responses to thermal injury, Burns 23 (1997) 106–113.

[3] B.L. Kelsall, C.A. Biron, O. Sharma, P.M. Kaye, Dendritic cells at the host–pathogen interface, Nat. Immunol. 3 (2002) 699–702.

[4] M.R. Neutra, A. Frey, J.P. Kraehenbuhl, Epithelial M cells: gateways for mucosal infection and immunization, Cell 86 (1996) 345–348.

[5] E.G. Kavanagh, J.L. Kelly, A. Lyons, C.C. Soberg, J.A. Mannick, J.A. Lederer, Burn injury primes naive $CD4^+$ T cells for an augmented T-helper 1 response, Surgery 124 (1998) 269.

[6] J.H. Niess, S. Brand, X. Gu, L. Landsman, S. Jung, B.A. McCormick, J.M. Vyas, M. Boes, H.L. Ploegh, J.G. Fox, D.R. Littman, H.C. Reinecker, CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance, Science 307 (5707) (2005 Jan 14) 254–258.

[7] M.K. Jenkins, A. Khoruts, E. Ingulli, et al., In vivo activation of antigen-specific CD4 T cells, Annu. Rev. Immunol. 19 (2001) 23–45.

[8] A.A. Itano, M.K. Jenkins, Antigen presentation to naive CD4 T cells in the lymph node, Nat. Immunol. 4 (2003) 733–739.

[9] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, Nature 392 (6673) (1998 Mar 19) 245–252.

[10] P. Efron, L.L. Moldawer, Sepsis and the dendritic cell, Shock 20 (2003) 386–401.

[11] M.L. Alegre, K.A. Frauwirth, C.B. Thompson, T-cell regulation by CD28 and CTLA-4, Nat. Rev., Immunol. 1 (2001) 220.

[12] J.E. Gretz, A.O. Anderson, S. Shaw, Cords, channels, corridors and conduits: critical architectural elements facilitating cell interactions in the lymph node cortex, Immunol. Rev. 156 (1997) 11–24.

[13] P. Revy, M. Sospedra, B. Barbour, A. Trautmann, Functional antigen-independent synapses formed between T cells and dendritic cells, Nat. Immunol. 2 (2001) 925–931.

[14] P. Kourilsky, P. Truffa-Bachi, Cytokine fields and the polarization of the immune response, Trends Immunol. 22 (2001) 502–509.

[15] N. Fazal, M.A. Choudhry, M.M. Sayeed, Inhibition of T cell MAPKs (Erk 1/2, p38) with thermal injury is related to down-regulation of Ca (2+) signaling, Biochim Biophys Acta 30 1741 (1–2) (2005 Jun) 113–119.

[16] R.L. Reinhardt, A. Khoruts, R. Merica, T. Zell, M.K. Jenkins, Visualizing the generation of memory CD4 T cells in the whole body, Nature 410 (6824) (2001 Mar 1) 101–105.

[17] A.J. Stagg, A.L. Hart, S.C. Knight, M.A. Kamm, The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria, Gut 52 (10) (2003 Oct) 1522–1529.

[18] R.S. Hotchkiss, K.W. Tinsley, P.E. Swanson, M.H. Grayson, D.F. Osborne, T.H. Wagner, J.P. Cobb, C. Coopersmith, I.E. Karl, Depletion of dendritic cells, but not macrophages, in patients with sepsis, J. Immunol. 168 (2002) 2493.

[19] R.S. Hotchkiss, K.W. Tinsley, P.E. Swanson, R.E. Schmieg Jr., J.J. Hui, K.C. Chang, D.F. Osborne, B.D. Freeman, J.P. Cobb, T.G. Buchman, I.E. Karl, Sepsis-induced apoptosis causes progressive profound depletion of B and $CD4^+$ T lymphocytes in humans, J. Immunol. 166 (2001) 69.

[20] S. Vendetti, A. Riccomi, A. Sacchi, L. Gatta, C. Pioli, M.T. De Magistris, Cyclic adenosine 5′-monophosphate and calcium induce CD152 (CTLA-4) up-regulation in resting CD4+ T lymphocytes, J. Immunol. 169 (2002) 6231.

[21] Q. Tang, J.A. Smith, G.L. Szot, P. Zhou, M.L. Alegre, K.J. Henriksen, C.B. Thompson, J.A. Bluestone, CD28/B7 regulation of anti-CD3-mediated immunosuppression in vivo, J. Immunol. 170 (2003) 1510.

[22] S.G. Santucci, S. Gobara, C.R. Santos, C. Fontana, A.S. Levin, Infections in a burn intensive care unit: experience of seven years, J. Hosp. Infect. 53 (2003) 6.

[23] R.L. Bang, P.N. Sharma, S.C. Sanyal, I. Al Najjadah, Septicaemia after burn injury: a comparative study, Burns 28 (2002) 746.

[24] J.A. Teodorczyk-Injeyan, B.G. Sparkes, G.B. Mills, W.J. Peters, R.E. Falk, Impairment of T cell activation in burns patients: a possible mechanism of thermal injury-induced immunosuppression, Clin. Exp. Immunol. 65 (1986) 570–581.

[25] S. Zedler, R.C. Bone, A.E. Baue, G.H. von Donnersmarck, E. Faist, T-cell reactivity and its predictive role in immunosuppression after burns, Crit. Care Med. 27 (1999) 66.

[26] E. Turnbull, G. MacPherson, Immunobiology of dendritic cells in the rat, Immunol. Rev. 184 (2001) 58–68.