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Growth Factors, Cytokines, Cell Cycle Molecules

Suppression of Activation and Costimulatory Signaling in Splenic CD4$^+$ T Cells after Trauma-Hemorrhage Reduces T-Cell Function

A Mechanism of Post-Traumatic Immune Suppression

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Reduced immune function is frequently a consequence of serious injury such as trauma-hemorrhage (T-H). Injury may lead to reduced T-cell activation, resulting in decreased engagement of costimulatory molecules after antigen recognition and in subsequent immunological compromise and anergy. We hypothesized that inhibition of CD28 expression is one possible mechanism by which immune functions are suppressed after T-H. Male C3H/HeN mice (with or without ovalbumin immunization) were subjected to sham operation or T-H and sacrificed after 24 hours. Splenic T cells were then stimulated with concanavalin A or ovalbumin *in vivo* or *in vitro*, and CD28, cytotoxic T-lymphocyte antigen 4 (CTLA-4), CD69, and phospho-Akt expression was determined. T-cell proliferation/cytokine production was measured *in vitro*. Stimulation-induced CD69, CD28, and phospho-Akt up-regulation were significantly impaired after T-H compared with sham-operated animals; however, CTLA-4 expression was significantly higher in the T-H group. Over a 3-day span, stimulated T cells from sham-operated animals showed significantly higher proliferation compared with the T-H group. IL-2 and IFN-γ were elevated in sham-operated animals, whereas IL-4 and IL-5 rose in the T-H group, revealing a shift from T_H1 to T_H2 type cytokine production after T-H. Dysregulation of the T-cell costimulatory pathway is therefore likely to be a significant contributor to post-traumatic immune suppression. *(Am J Pathol 2009, 175:1504–1514; DOI: 10.2353/ajpath.2009.081174)*

It is now generally accepted that serious injury in humans or experimental animal models may be followed by systemic inflammatory response syndrome and may frequently lead to subsequent multiple organ dysfunction syndrome, which remains a major cause of death despite numerous advances in trauma research.[1–5] Perturbations of both the innate and adaptive immune systems after trauma-hemorrhage such as hyperactivity of macrophages and Kupffer cells, depressed dendritic cell functions, and impaired T-cell activities have been widely recognized as the cause of multiple organ dysfunction syndrome.[6–9] However, interactions between innate and adaptive immune systems and their relative contribution to multiple organ dysfunction syndrome after trauma-hemorrhage are not completely understood.

The immune system has the remarkable ability to defend against various microbial pathogens and yet not to respond to self. This is due to the tightly regulated activation of T cells. T-cell activation requires two signals that are delivered by antigen-presenting cells (APCs).[10] The first signal is the recognition by T-cell receptors of antigen presentation in the context of major histocompatability complex. The subsequent secondary or costimulatory signal is provided by molecules on APCs that engage cognate receptors on T cells. It is known that T cells will

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fail to recognize an antigen or enter a state of anergy in the absence of costimulation. The best-characterized T-cell costimulatory pathway involves the CD28 receptor, which binds to CD80 and CD86.[11] The interaction of CD80 and CD86 with CD28 promotes the expansion of activated T cells and up-regulates cell survival protein expression.

Our previous studies revealed that the antigen-presenting capacity of dendritic cells is suppressed after trauma-hemorrhage because of the down-regulation of major histocompatability complex II expression and the decreased ability to stimulate T-cell proliferation.[8] However, in that study, we also found that the expression of CD80 and CD86 on dendritic cells was not influenced by trauma-hemorrhage. However, in keeping with an overall pattern of immune unresponsiveness, we showed that T-cell activities were suppressed after trauma-hemorrhage.[9,12] Hence, it is not clear whether posttraumatic T-cell suppression is secondary to suppression of APC functions or alternatively whether costimulatory molecules on T cells also play an important role in producing T-cell dysfunction. We hypothesized that the costimulatory factor CD28 is down-regulated after trauma-hemorrhage even though the expression of CD80 and CD86 on APCs is not affected under those conditions, and the decreased expression of CD28 on T-cell surface is another key mechanism that explains the impaired T-cell function after trauma-hemorrhage.

Materials and Methods

Animals, Experimental Groups, and Study Design

Male C3H/HeN mice 8 weeks old and weighing 22 to 25 g (Charles River Laboratories, Wilmington, MA) were fasted overnight before the experiment but were allowed water *ad libitum*. All experiments were performed in adherence with National Institutes of Health (Bethesda, MD) *Guidelines for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (Birmingham, AL). Animals were subjected to either the trauma-hemorrhage procedure or a sham operation. To study the impact of trauma-hemorrhage on T-cell activation and CD28 expression, T cells were activated either *in vivo* or *in vitro* and were activated either by concanavalin A (ConA) or by ovalbumin (OVA) immunization followed by OVA challenge.

Trauma-Hemorrhagic Shock Model

Animals were anesthetized with isoflurane (Minrad, Bethlehem, PA) and restrained in a supine position.[13] A midline laparotomy (2 cm) was performed, which was then closed in two layers with sutures (Ethilon 6/0, Ethicon, Somerville, NJ). Both femoral arteries and a femoral vein were cannulated with polyethylene-10 tubing (Becton Dickinson, Sparks, MD). Blood pressure was measured via one of the arterial catheters using a blood pressure analyzer (Micro-Med, Louisville, KY). The animals were then restrained in a supine position in a small plastic box covered with a dark cloth to prevent the mice from seeing the surrounding environment and thus keeping them calm. On awakening, the mice were bled rapidly through the other arterial catheter to a mean arterial blood pressure of 35 $\pm$ 5 mmHg within 10 minutes. The rapid bleeding puts the animals in a state of depressed sensibility. Blood was further withdrawn slowly to reach a blood loss of 60% of the total circulating blood volume while still maintaining mean arterial pressure at 35 $\pm$ 5 mmHg. The entire hemorrhagic shock time lasted for 90 minutes. At the end of that interval, the animals were resuscitated with four times the shed blood volume with Ringer's lactate over 30 minutes via the venous line. After resuscitation, the restraint was removed, and the animals were lightly anesthetized with isoflurane. After the blood vessels were ligated, catheters were removed, and the incision sites were bathed with lidocaine and closed with sutures. The animals were then allowed to awaken and were returned to their cages. Sham-operated animals underwent the same surgical procedures but were neither subjected to hemorrhage nor resuscitated. The animals were sacrificed at 24 hours after resuscitation or sham operation.

In Vivo *Stimulation of Lymphocytes*

For *in vivo* ConA stimulation, a nonhepatotoxic dosage of Con A (3 mg/kg b.wt.[14]) dissolved in 100 μl of Ringer's lactate was injected via the venous line at the end of resuscitation. Sham-operated animals were also injected at the same time point. For OVA immunization, each mouse was injected s.c. with 0.1 ml of complete Freund's adjuvant mixed with 0.1 mg of OVA 14 days before trauma-hemorrhage. After trauma-hemorrhage, mice were challenged with 0.1 mg of OVA again at the end of resuscitation or at the respective time point for sham-operated animals. ConA, OVA, and complete Freund's adjuvant were all purchased from Sigma-Aldrich (St. Louis, MO).

Harvesting of Spleen and Isolation of Splenocytes

The animals were anesthetized with isoflurane 24 hours after resuscitation or sham operation. Spleens were removed aseptically and were isolated as described previously.[15] In brief, spleens were gently ground to produce a single cell suspension, which was centrifuged at 400 $\times$ *g* for 10 minutes at 4°C. The erythrocytes were lysed with lysis buffer (BD Biosciences, San Jose, CA), and the remaining cells were washed with PBS by centrifugation (400 $\times$ *g*, 10 minutes, 4°C). After centrifugation, cells were resuspended in PBS to get a final concentration of 1×10^7 cells/ml. Splenocyte suspensions were divided into two parts for either flow cytometric analysis or further purification of $CD4^+$ T cells and macrophages by magnetic separation.

Magnetic Separation of CD4$^+$ T Cells and CD11b$^+$ Macrophages

Isolated splenocytes were resuspended in MACS buffer (Miltenyi Biotec, Auburn, CA) and incubated with CD4 or CD11b microbeads (Miltenyi Biotec) at 4°C for 15 minutes according to the manufacturer's instructions. After washing steps, CD4$^+$ T cells and CD11b$^+$ macrophages were positively selected by applying splenocytes onto MS columns that were placed on a MACS separator (all from Miltenyi Biotec). The magnetically labeled CD4$^+$ T cells and CD11b$^+$ macrophages were eluted and collected. The CD11b$^+$ macrophages were used as antigen-presenting cells for experiments involving *in vitro* stimulation of T cells (see below). Only macrophages of sham animals were isolated.

Isolation of CD4$^+$ T Cells and Regulatory T Cells

A regulatory T-cell staining kit was used to stain CD4$^+$ T cells and regulatory T cells (Tregs) (eBioscience, San Diego, CA). The fraction of Tregs in CD4$^+$ T cells was analyzed for splenocytes in the sham and trauma-hemorrhage groups. Furthermore, Tregs and CD4$^+$ T cells were purified from splenocytes by using a CD4$^+$CD25$^+$ regulatory T-cell isolation kit and anti-CD4 microbeads according to the manufacturer's instruction (all from Miltenyi Biotec, Auburn, CA).

Cell Surface Marker Staining and Flow Cytometric Analysis

Three anti-mouse antibodies against surface markers were used in this analysis: APC anti-CD4 antibody (Ab), phycoerythrin (PE) anti-CD28 Ab, and PE Cy7-anti-CD69 Ab (all from eBioscience). Splenocytes were washed and resuspended in staining buffer (PBS containing 0.5% bovine serum albumin and 0.09% sodium azide, Sigma-Aldrich) at a concentration of 1×10^7 cells/ml and were stained as described previously.[8] In brief, after blocking with anti-CD16/32 Ab (eBioscience) for 15 minutes on ice, cells were stained with the above indicated antibodies for 45 minutes at 4°C in the dark. Cells were then washed, resuspended in staining buffer, and analyzed using a BD LSRII flow cytometer (BD Biosciences, Mountain View, CA). A total of 50,000 events were collected for analysis.

Staining of Cytotoxic T-Lymphocyte Antigen-4 and Phospho-Akt for Flow Cytometric Analysis

A PE-conjugated American hamster anti-mouse cytotoxic T-lymphocyte antigen-4 (CTLA-4) monoclonal antibody (eBioscience) and an Alexa Fluor 488-conjugated anti-phospho-Akt (p-Akt) rabbit monoclonal antibody (Cell Signaling Technology, Danvers, MA) were used in this study. Cells were surface-stained with APC anti-CD4 Ab as described in the previous section and were then fixed in 2% formaldehyde for 20 minutes at room temperature. After washing, cells were resuspended in permeabilization buffer (eBioscience) and incubated with either anti-p-Akt Ab or anti-CTLA4 Ab for 30 minutes. After 2 additional washes, cells were resuspended in staining buffer and analyzed on a flow cytometer. A total of 50,000 events were collected for analysis.

In Vitro *Stimulation and Determination of CD4$^+$ T-Cell Proliferation*

To determine any changes in proliferative capacity of T cells after trauma-hemorrhage or sham operation, T cells were cultured and challenged with either ConA or OVA. For ConA stimulation, purified CD4$^+$ T cells from sham or trauma-hemorrhage animals were resuspended in complete RPMI 1640 medium (10% fetal bovine serum, 2 mmol/L glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 20 μg/ml gentamicin, all from Invitrogen, Carlsbad, CA) with or without 5 μg/ml of ConA and plated in a 96-well plate at a cell density of 1×10^5 cells/well. For OVA challenge, isolated splenic macrophages from a sham mouse were first incubated for 30 minutes (37°C, 5% CO_2, in the dark) with 50 μg/ml mitomycin C (Sigma-Aldrich) to serve as APCs. After washing, these APCs were cultured with T cells (1×10^5 cells/well at a 1:1 ratio) purified from OVA-immunized sham or trauma-hemorrhage animals in culture medium with or without 0.1 mg/ml of OVA. The proliferation capacities of T cells were measured after 24, 48, and 72 hours of culture, respectively. Cell proliferation was determined using a Cell Proliferation Biotrak ELISA System (Amersham Biosciences Inc., Piscataway, NJ) as described previously.[8] In brief, 5-bromo-2′-deoxyuridine was added to the cell suspension, which was then given 3-hour exposure to the substrate. The cells were fixed, and their DNA was denatured and then incubated with peroxidase-labeled anti-5-bromo-2′-deoxyuridine Ab according to the manufacturer's instructions. The optical density of each well was determined by a spectrophotometer at 450 nm after the peroxidase substrate 3,3′,5,5′-tetramethylbenzide was added to the cells.

Determination of CD28 Expression and Akt Activation on CD4$^+$ T Cells after in Vitro *Stimulation*

CD28 and Akt activation were measured in cultured T cells by flow cytometry. Cells were cultured in the same way as those for measuring cellular proliferation capacity. After 3 days of culture, cells were harvested, stained for CD4, CD28, and p-Akt, and analyzed using flow cytometry as described in the previous section. The mean fluorescence intensity (MFI) of CD28 and p-Akt of those events gated as CD4$^+$ were measured.

Flow Cytometric Analysis of Cytokine Concentration

The cytokine concentrations in culture supernatant were determined with a cytometric bead array using flow cy-

tometry according to the manufacturer's instructions (BD Pharmingen, San Diego, CA) as described previously.[16] In brief, 50 μl of mixed capture beads were incubated with 50 μl of sample for 1 hour at 25°C, and then 50 μl of mixed PE-conjugated detection antibody was added. After incubation for 1 hour at 25°C in the dark, the complexes were washed twice and analyzed using the LSRII flow cytometer. Data analysis was performed using the FACSDiva and FCAP array software (BD Biosciences).

Statistics

Statistical analysis was performed using Sigma-Stat computer software (SPSS, Chicago, IL). Statistical significance was assumed when probability values of less than 0.05 were obtained. Comparisons between groups were performed using one-way analysis of variance followed by Tukey's test. Results are expressed as mean ± SE.

Results

Cellular Activation and Expression of Costimulatory Molecules on in Vivo*-Stimulated T Cells after Trauma-Hemorrhage*

The expression of CD69, CD28, and CTLA-4 on splenic $CD4^+$ T cells was analyzed 24 hours after *in vivo* stimulation. As shown in Figure 1, A and B, CD69 expression was significantly increased after ConA stimulation compared with that for matched unstimulated controls. In addition, when one compares both stimulated groups, CD69 expression by sham-treated animals was significantly higher than that in the trauma-hemorrhage group. There were no differences between unstimulated sham and trauma-hemorrhage groups. The expression of CD28 with or without ConA stimulation showed a similar pattern as observed for CD69. As shown in Figure 1, C and D, although ConA stimulation induced a significant increase in CD28 expression, such an increase was suppressed by the trauma-hemorrhage insult. No differences in CD28 expression were noted between the unstimulated groups. In contrast, as shown in Figure 1, E and F, the expression of CTLA-4 was significantly higher in the ConA-stimulated trauma-hemorrhage group than in all other groups. The OVA challenge induced increased CD69 and CD28 expression on T cells similar to those stimulated by ConA; however, such increases were suppressed by the trauma-hemorrhage insult (Figure 2, A–D). On the other hand, although CTLA-4 expression was elevated after OVA challenge in sham and trauma-hemorrhage groups, trauma-hemorrhage was found to induce a further increase in CTLA-4 compared with that in the sham group (Figure 2, E and F).

Activation of Akt after in Vivo *Stimulation*

As shown in Figure 3, A and B, the expression of p-Akt was significantly increased in ConA-stimulated sham animals compared with that in other groups. However, in the ConA-stimulated trauma-hemorrhage animals, it was significantly lower than in sham-operated animals and was similar to that in those without stimulation. In the experiments in which animals were challenged with OVA *in vivo*, the p-Akt levels increased significantly after OVA challenge compared with those without OVA challenge. Moreover, sham animals challenged with OVA had significantly higher p-Akt levels compared with trauma-hemorrhage animals challenged with OVA (Figure 3, C and D).

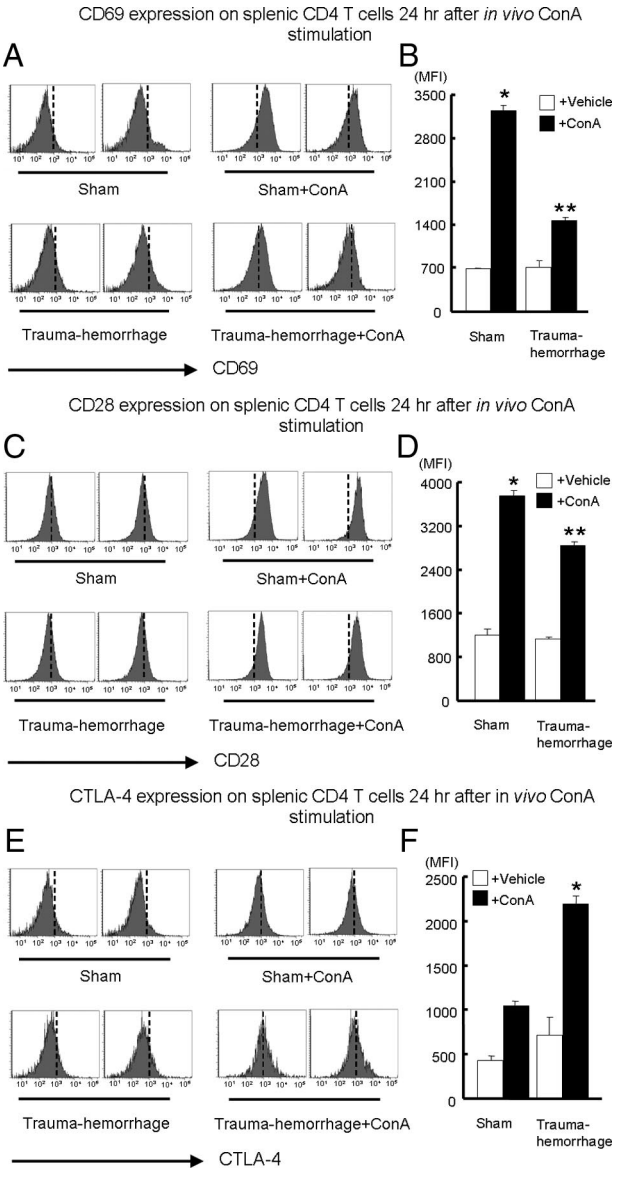

Figure 1. Expression of CD69, CD28, and CTLA-4 on splenic $CD4^+$ T cells after *in vivo* ConA stimulation. For *in vivo* stimulation, 3 mg/kg of ConA were injected i.v. at the end of resuscitation in trauma-hemorrhage groups or at corresponding time points in sham groups. Twenty-four hours after the end of resuscitation, splenocytes with or without stimulation were isolated and stained with APC anti-CD4 Ab plus either PE Cy7-anti-CD69 Ab, PE anti-CD28 Ab, or PE anti-CTLA-4 Ab as described in *Materials and Methods*. Representative histograms were gated on $CD4^+$ T cells and showed MFIs of CD69 (**A**), CD28 (**C**), and CTLA-4 (**E**). MFI data for CD69 (**B**), CD28 (**D**), and CTLA-4 (**F**) are shown as mean ± SEM. $^*P < 0.05$ versus all of the other groups. $^{**}P < 0.05$ versus vehicle groups. $n = 5$/group. The vertical dotted line in each histogram is intended for better recognition of the shift of MFI among different treatment groups.

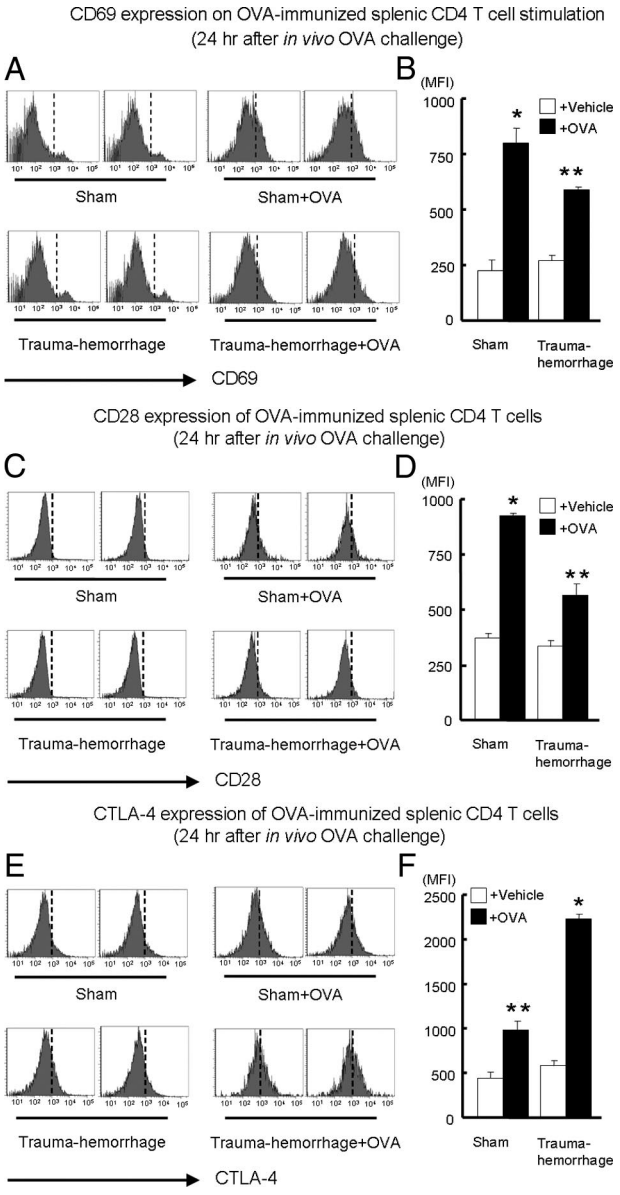

Figure 2. Expression of CD69, CD28, and CTLA-4 on splenic $CD4^+$ T cells after OVA immunization and *in vivo* OVA challenge. In this experiment, all of the animals were immunized with OVA 14 days before trauma-hemorrhage by injection of 0.1 ml of complete Freund's adjuvant mixed with 0.1 mg of OVA. Another 0.1 mg of OVA or vehicle (PBS) was injected at the end of resuscitation in trauma-hemorrhage groups or at corresponding time points in sham groups. Twenty-four hours after the end of resuscitation, splenocytes with or without OVA challenge were isolated and stained with APC anti-CD4 Ab plus either PE Cy7-anti-CD69 Ab, PE anti-CD28 Ab, or PE anti-CTLA-4 Ab as described in *Materials and Methods*. Representative histograms were gated on $CD4^+$ T cells and showed MFI of CD69 (**A**), CD28 (**C**), and CTLA-4 (**E**). MFI data for CD69 (**B**), CD28 (**D**), and CTLA-4 (**F**) are shown as mean ± SEM. $^*P < 0.05$ versus all of the other groups. $^{**}P < 0.05$ versus vehicle groups. $n = 5$/group. The vertical dotted line in each histogram is intended for better recognition of the shift of MFI among different treatment groups.

Expression of CD28 on in Vitro *Stimulated T Cells after Trauma-Hemorrhage*

Although we demonstrated that trauma-hemorrhage suppressed the responses of T cells to *in vivo* activation, we further examined whether this suppression also occurred *in vitro*. As shown in Figure 4, A and B, ConA significantly

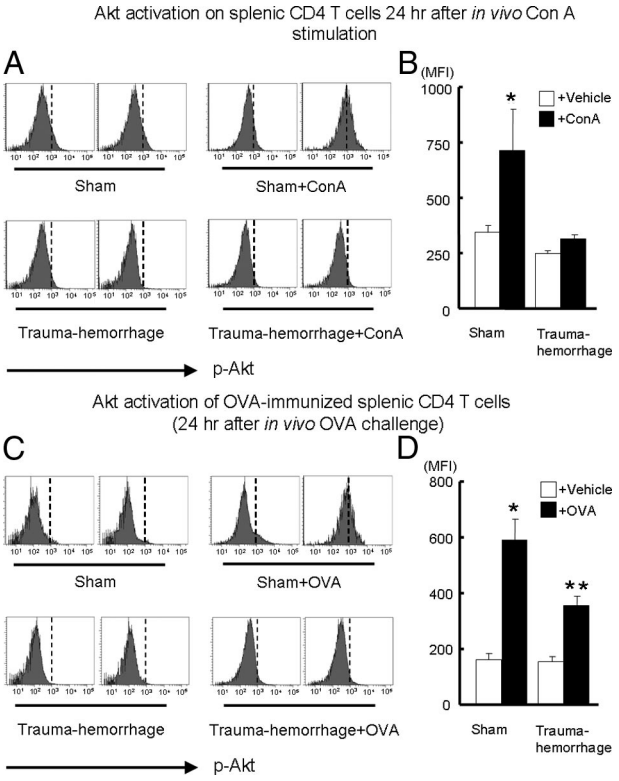

Figure 3. Expression of phospho-Akt on splenic $CD4^+$ T cells after stimulation with ConA or OVA *in vivo*. Splenic T cells were activated either by *in vivo* ConA stimulation or by OVA challenge after prior OVA immunization as described in *Materials and Methods*. Twenty-four hours after the end of resuscitation, splenocytes with or without stimulation were isolated and surface stained with APC anti-CD4 Ab. Cells were than fixed, permeabilized and stained with Alexa Fluor 488-conjugated anti-p-Akt Ab as described in Materials and Methods. Representative histograms were gated on $CD4^+$ T cells and showed MFI of p-Akt after ConA stimulation (**A**) or OVA challenge (**C**). MFI data for p-Akt after ConA stimulation (**B**) or OVA challenge (**D**) are shown as mean ± SEM. $^*P < 0.05$ versus. all of the other groups. $^{**}P < 0.05$ versus vehicle groups. $n = 5$/group. The vertical dotted line in each histogram is intended for better recognition of the shift of MFI among different treatment groups.

induced an increase in CD28 expression in both sham and trauma-hemorrhage groups compared with unstimulated groups. We also found that cells of the ConA-stimulated sham group had higher CD28 expression compared with that of the ConA-stimulated trauma-hemorrhage group. Moreover, OVA stimulation induced a marked increase in CD28 expression in cells of the sham group, an effect not seen in the OVA-stimulated trauma-hemorrhage group (Figure 4, C and D).

Activation of Akt after in Vitro *Activation*

As shown in Figure 5, A and B, comparison between the stimulated and unstimulated subsets, ConA treatment induced a marked increase in p-Akt in both sham and trauma-hemorrhage groups. Moreover, the increase was more pronounced in the sham group compared with that in the trauma-hemorrhage group. There was no difference in p-Akt expression in unstimulated groups. In the experiments in which the cells were stimulated by OVA, a result similar to that in the ConA-stimulated group was observed. Whereas p-Akt levels of T cells were increased

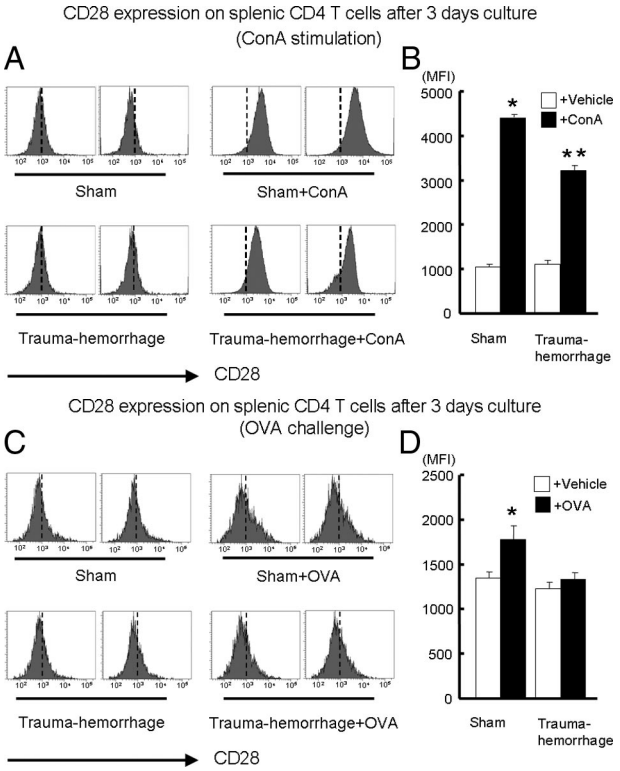

Figure 4. Expression of CD28 on cultured splenic $CD4^+$ T cells after *in vitro* ConA or OVA stimulation. For ConA stimulation, purified $CD4^+$ T cells from sham or trauma-hemorrhage animals were resuspended in complete RPMI 1640 medium with or without 5 μg/ml of ConA and plated in a 96-well plate at a cell density of 1×10^5 cells/well. For OVA stimulation, isolated splenic macrophages from a sham mouse were first incubated for 30 minutes with 50 μg/ml mitomycin C to serve as APCs. After washing, these APCs were cultured with $CD4^+$ T cells (1×10^5 cells/well at a 1:1 ratio) purified from OVA-immunized sham or trauma-hemorrhage animals in RPMI 1640 medium with or without 0.1 mg/ml of OVA. After 3 days of culture, cells were harvested and stained with APC anti-CD4 Ab and PE anti-CD28 Ab as described in *Materials and Methods*. Representative histograms were gated on $CD4^+$ T cells and showed MFI of CD28 after ConA stimulation (**A**) or OVA challenge (**C**). MFI data for CD28 after ConA stimulation (**B**) or OVA challenge (**D**) are shown as mean ± SEM. $^*P < 0.05$ versus all other groups. $^{**}P < 0.05$ versus vehicle groups. $n = 5$/group. The vertical dotted line in each histogram is intended for better recognition of the shift of MFI among different treatment groups.

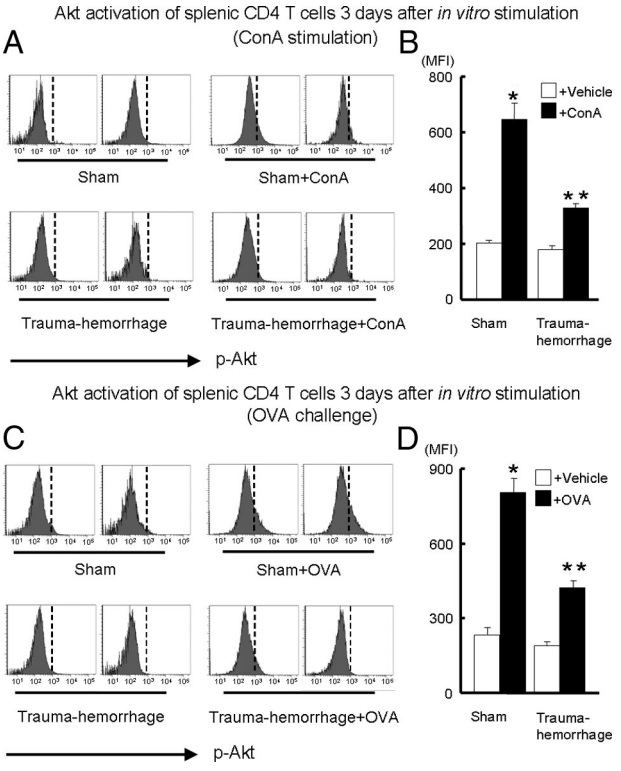

Figure 5. Expression of p-Akt on cultured splenic $CD4^+$ T cells after *in vitro* ConA or OVA stimulation. For ConA stimulation, purified $CD4^+$ T cells from sham or trauma-hemorrhage animals were resuspended in complete RPMI 1640 medium with or without 5 μg/ml of ConA and plated in a 96-well plate at a cell density of 1×10^5 cells/well. For OVA stimulation, isolated splenic macrophages from a sham mouse were first incubated for 30 minutes with 50 μg/ml mitomycin C to serve as APCs. After washing, APCs were cultured with $CD4^+$ T cells (1×10^5 cells/well at a 1:1 ratio) purified from OVA-immunized sham or trauma-hemorrhage animals in RPMI 1640 medium with or without 0.1 mg/ml of OVA. After 3 days of culture, cells were harvested and stained with APC anti-CD4 Ab. Cells were then fixed, permeabilized, and stained with Alexa Fluor 488-conjugated anti-p-Akt Ab as described in *Materials and Methods*. Representative histograms were gated on $CD4^+$ T cells and showed MFI of p-Akt after ConA stimulation (**A**) or OVA challenge (**C**). MFI data for p-Akt after ConA stimulation (**B**) or OVA challenge (**D**) are shown as mean ± SEM. $^*P < 0.05$ versus all of the other groups. $^{**}P < 0.05$ versus vehicle groups. $n = 5$/group. The vertical dotted line in each histogram is intended for better recognition of the shift of MFI among different treatment groups.

after OVA stimulation, they were significantly elevated in the sham versus the trauma-hemorrhage group (Figure 5, C and D).

T-Cell Proliferation Capacities With or Without Stimulation

As shown in Figure 6A, T cells proliferated minimally throughout 3 days of culture in nonstimulated groups. Beginning 24 hours after culture, the proliferation of ConA-stimulated T cells was markedly increased compared with that in those without stimulation. Moreover, although the extent of T-cell proliferation in the stimulated sham and trauma-hemorrhage groups was similar after 24 hours of culture, the ConA-stimulated sham T cells showed increased proliferative capacity after 48 and 72 hours of culture compared with ConA-stimulated trauma-hemorrhage T cells. For those T cells stimulated by APC-processed OVA (Figure 6B) after 48 hours in culture, T cells of the OVA-stimulated sham group proliferated more compared with all of the other groups. However, by 72 hours after culture the proliferation of T cells in OVA-stimulated trauma-hemorrhage groups was significantly increased compared with that in the nonstimulated groups.

Cytokine Production Capacities of T Cells With or Without Ova Stimulation

As shown in Figure 7A, unstimulated T cells produced minimal amounts of interleukin (IL)-2, interferon (IFN)-γ, IL-4, and IL-5. The cytokine response of T cells to OVA stimulation was different between sham and trauma-hemorrhage groups. T cells in the sham group produced significantly more IL-2 compared with those in the trauma-hemorrhage group. In contrast, cells in the trauma-hemorrhage group produced more IL-4 and IL-5 compared with those in the sham group. Furthermore, T_H1

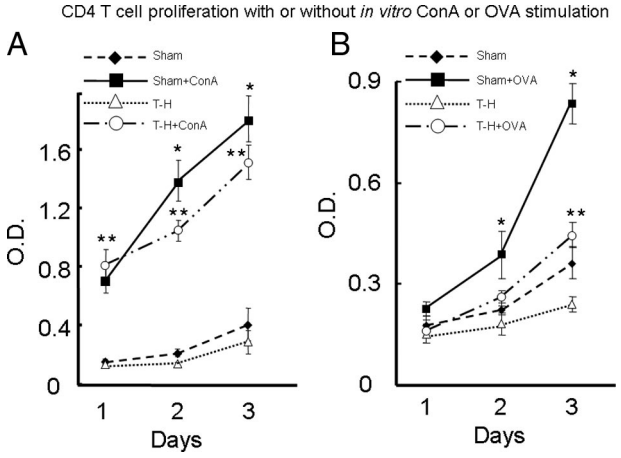

Figure 6. Proliferation of splenic $CD4^+$ T cells after *in vitro* ConA or OVA stimulation. The isolation, stimulation, and culture of T cells were the same as in Figures 4 and 5 and are described in *Materials and Methods*. The proliferation capacities of T cells were measured after 24, 48, and 72 hours of culture, respectively, and were determined using a 5-bromo-2′-deoxyuridine-based, commercially available ELISA kit as described in *Materials and Methods*. **A:** T-cell proliferation with or without ConA stimulation. **B:** T cell proliferation with or without OVA stimulation. Data are shown as mean ± SEM. $^*P < 0.05$ compared with all of the other groups on the same day. $^{**}P < 0.05$ compared with nonstimulated groups on the same day. $n = 5$/group.

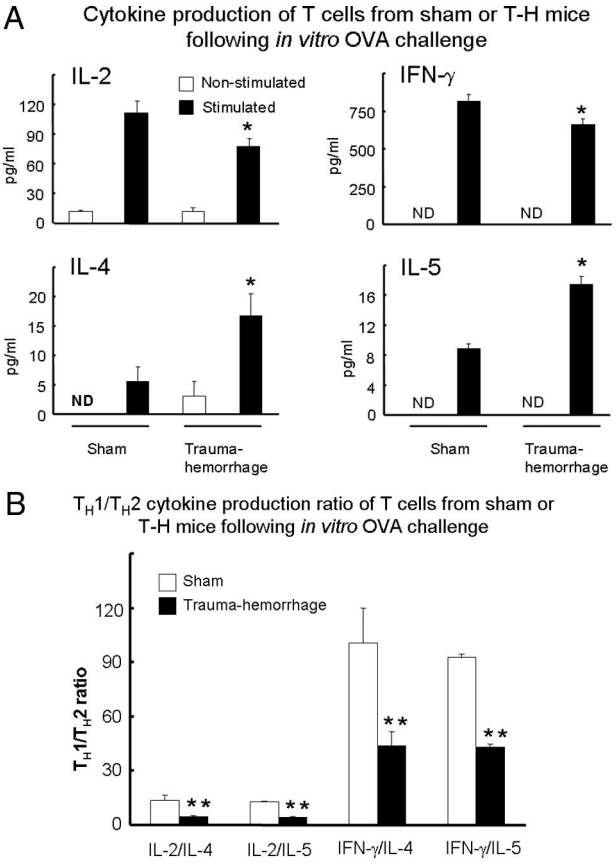

Figure 7. Cytokine production capacities of splenic $CD4^+$ T cells with or without OVA stimulation. Purified OVA-immunized splenic $CD4^+$ T cells were cultured with mitomycin-treated macrophages with or without OVA challenge as described in *Materials and Methods*. After 3 days of culture, cytokine concentrations of culture supernatant were determined with cytometric bead arrays. **A:** Concentrations of IL-2, IFN-γ, IL-4, and IL-5. **B:** ratios of IL-2 or IFN-γ to IL-4 or IL-5. Data are shown as mean ± SEM. $^*P < 0.05$ compared with other groups in **A**. $^{**}P < 0.05$ compared with sham animal of the same group in **B**. $n = 5$/group; ND, nondetectable.

(IL-2 and IFN-γ)/T_H2 (IL-4 and IL-5) cytokine production ratios in the trauma-hemorrhage group were significantly lower than those in the sham group, indicating that T-cell cytokine production shifted from T_H1 type to T_H2 type after trauma-hemorrhage (Figure 7B). In addition, to exclude the possibility that the increased IL-2 and IFN-γ production in the sham group was due to increased numbers of T cells being generated during culture, intracellular cytokine staining was performed to determine cytokine production on a per cell basis. These measurements revealed that intracellular cytokine production of IL-2 and IFN-γ was significantly suppressed after trauma-hemorrhage compared with those of the sham group (supplementary Figure 1, A and B, see *http://ajp.amjpathol.org*).

Discussion

The immune system is well designed to defend against diverse pathogens, while avoiding a response to self. In their role as the key mediators of the immune system, T-cell activation is tightly regulated to maintain immune homeostasis.[17] As an example of a fail-safe mechanism, T-cell activation requires two signals that are delivered by APCs. Whereas the first signal mediates the recognition of a specific antigen by T-cell receptors, the second signal (costimulatory signal) stimulates the expansion and differentiation of T cells.[17] CD28 interaction with either of its two ligands (CD80 and CD86) borne by APCs is widely recognized as the major T-cell costimulatory pathway and thus is important for initiating T-cell responses, such as enhancing the production of IL-2 as well as T-cell proliferation.[11] Therefore, there is great interest in manipulating these costimulatory signals to either enhance antimicrobial immunity or treat autoimmune diseases.[18–21]

In contrast, trauma-hemorrhagic shock induces serious and widespread dysregulation of immune functions,[22] including suppression of T-cell activation in response to a variety of stimuli.[9] In this study, the up-regulation of CD69 expression (a marker of early-stage activation) in stimulated T cells was suppressed after trauma-hemorrhage. This observation indicates that trauma-hemorrhage inhibited T-cell activation shortly after the insult at an upstream level of signal transduction. Whereas CD28 costimulatory factor plays an important role in enhancing the efficiency of T-cell activation, our results suggest that one of the mechanisms by which trauma-hemorrhage suppresses T-cell activation is by altering the balance between costimulatory and regulatory signals. It is well known that to maintain immune homeostasis and prevent harmful inflammatory responses, the expression of CD28 also leads to the up-regulation and expression of CTLA-4,[10] a competitive negative T-cell regulator. CTLA-4 antagonizes the major events mediated by CD28 signal cascades, including T-cell proliferation and IL-2 production, therefore preventing or dampening uncontrolled T-cell activities.[10] In keeping with this

role, when we compared the stimulated T cells of the sham group with those from the trauma-hemorrhage group, we observed a decreased level of CD28 expression and a corresponding increased level of CTLA-4 expression. This finding suggests that the balance of CD28/CTLA-4 signaling had shifted in favor of inducing T-cell anergy after trauma-hemorrhage.

It should be reiterated that, in contrast to the surface-stained CD28, the level of CTLA-4 expression was determined by intracellular staining. CTLA-4 is primarily an intracellular antigen whose surface expression is tightly regulated by restricted trafficking to the cell surface and rapid internalization.[23] As minor changes in surface expression levels may have major effects on the outcome of T-cell activation, CTLA-4 levels are known to rapidly decrease in the absence of new protein translation, and the molecule is quickly degraded on internalization.[24,25] Although our results revealed increased intracellular CTLA-4 expression after trauma-hemorrhage, we were not able to demonstrate increased surface expression of CTLA-4 under those conditions (supplemental Figure 2, see *http://ajp.amjpathol.org*). One possible explanation for this observation is that CTLA-4 was up-regulated and expressed on the T-cell surface, accounting for T-cell suppression soon after trauma-hemorrhage but was subsequently rapidly internalized within 24 hours after the insult.

Our results were consistent with the works of Laudanski et al,[26] who conducted a genome-wide expression analysis to reveal the alteration of T-cell signaling pathways of human subjects after severe blunt trauma. In that study, decreased CD28 gene expression of T cells was noted in trauma patients compared with those from healthy individuals.[26] Moreover, our previous studies showed that the expression of the CD28 ligands CD80 and CD86 on dendritic cells was not decreased after trauma-hemorrhage.[8] Taken together, these results further emphasize the significance of CD28 down-regulation as the likely mechanism that, through negatively affecting T-cell activity (rather than APCs), accounts at least in part for the reduced immunological responsiveness after trauma-hemorrhage. In addition, other than CD28 and CTLA-4, numerous other pathways and molecules have been proposed to be involved in the suppression of T-cell responsiveness after trauma-hemorrhage.[27] To further clarify how these pathways interact with each other, an extension of the current study may be to determine the relative contribution of each molecule important to T-cell suppression. More specifically, it will be illuminating to study the effects of hypoxia per se on T cells *in vitro* with the specific objective of observing the effects of hypoxia/hypoxia-inducible factor-1α on CD28 expression.

It could be argued that because the animals were sacrificed 24 hours after trauma-hemorrhage, it was unclear whether this posttraumatic period had a different impact on cellular responses compared with those immediately after trauma-hemorrhage. Because trauma-hemorrhage induces physiological derangements such as systemic inflammatory response syndrome and multiple organ dysfunction syndrome within hours and days after the insult, our current results suggest that the dysregulation of CD28 expression and associated altered T-cell activities were also part of such immune derangements and were a direct result of the trauma-hemorrhage insult. Furthermore, our previous findings demonstrated a continuity of alteration in cellular responses, ie, if a depression or enhancement of cellular function was evident at 2 hours, it was also evident at 24 hours after injury.[12,13] Based on these observations, although we only investigated one time point (24 hours after injury) in the current study, it would be reasonable to assume that the differences in T-cell activation and regulation after sham and trauma-hemorrhage would be sustained between 2 and 24 hours.

Recently, the phosphatidylinositol 3-kinase/Akt pathway has been recognized as an important regulatory mechanism involving cell survival, activation, and glucose metabolism.[28–32] Previous studies from our laboratory have shown that Akt activation in various tissues and cell types such as intestine, cardiomyocytes, hepatocytes, and Kupffer cells was decreased after trauma-hemorrhage.[33–36] This investigation showed consistently that the ConA- or OVA-stimulated T cells expressed less p-Akt after trauma-hemorrhage than with sham animals. Although studies have shown that Akt is one of the key molecules that mediate CD28 costimulatory signaling,[37,38] several regulatory mechanisms may account for the down-regulation of p-Akt after trauma-hemorrhage. First, phosphatidylinositol 3-kinase is known to be the upstream molecule that regulates Akt activation. Our previous publications revealed that inhibition of phosphatidylinositol 3-kinase activity could suppress the activation of Akt.[34,35] Furthermore, Parry et al[39] demonstrated that the Akt-mediated CD28 signaling was phosphatidylinositol 3-kinase-dependent. In addition, up-regulated CTLA-4 is able to block Akt activity by inhibiting Akt phosphorylation,[40–42] which could be mediated by engaging inhibitory signal transduction machinery such as protein phosphatase type 2A and SHP-2.[43–45] Our data therefore suggest that the suppressed T-cell activities after trauma-hemorrhage could be accounted for by a shift from a pattern dominated by CD28 expression into one favoring CTLA-4, which subsequently down-regulates Akt activity.

Helper T cells (T_H) are divided into two opposing phenotypes: T_H1 cells are characterized by the production of IL-2 and IFN-γ, and, conversely, T_H2 cells produce IL-4 and IL-5. Our results revealed that the cytokine production profiles were different between stimulated T cells in the sham and trauma-hemorrhage groups. Those in the sham group produced significantly higher amounts of T_H1-type cytokines (IL-2 and INF-γ), whereas those in the trauma-hemorrhage group produced significantly higher amounts of T_H2-type cytokines (IL-4 and IL-5). The observed dysregulation of T_H1/T_H2 phenotype and cytokine production balance after trauma-hemorrhage has also been seen in human/animal subjects after major injuries or surgery.[46,47] Studies of Kane et al[41] showed that Akt mediated the CD28 costimulatory signal for up-regulation of IL-2 and IFN-γ but not T_H2 cytokines,[41] indicating that the suppressed p-Akt expression after trauma-hemorrhage might be a contributor to the decreased T_H1 cytokine production of T cells under those conditions. Fur-

thermore, because IL-2 is a well recognized promoter of T-cell activation and proliferation,[48,49] it was not surprising that the increased concentration of IL-2 resulted in the progressive increase in proliferation capacities of sham group T cells over a 3-day span compared with those of the trauma-hemorrhage group.

Although splenic $CD4^+$ T cells exhibited a diminished proliferative capacity and altered cytokine production profile after trauma-hemorrhage, one significant question is whether there were changes in splenic $CD4^+$ T-cell numbers under those conditions. Although splenic $CD4^+$ T-cell numbers could be affected by different events such as acute, severe blood loss with attendant lymphopoietic compensation, as well as inflammatory responses after trauma-hemorrhage, we noticed that there were no significant differences in the number of total splenocytes and T cells from each spleen harvested, either from sham or trauma-hemorrhage animals (data not shown). Consistent with our observation, Abraham and Freitas[50] reported that there were no changes in the total number of splenocytes or of splenic B cells after hemorrhage; however, the number of B cells secreting immunoglobulins was significantly decreased during the period from 2 to 96 hours after hemorrhage. Taken together, these results indicate that the most plausible explanation for the response patterns of splenic $CD4^+$ T cells to trauma-hemorrhage is adversely altered proliferation competence combined with reduced cytokine production capacity, whereas splenic $CD4^+$ T-cell numbers would remain unchanged within the 24 hour interval after trauma-hemorrhage.

One may also argue as to whether there are differential responses to trauma-hemorrhage among each subset of $CD4^+$ T cells, such as the differences between Tregs and T helper cells, as well as differences among naive, memory, and effector T cells. Enhanced Treg activity has been reported to be an element of immune suppression after injury.[51,52] Our ongoing study of splenic Tregs has revealed that the activities of the Tregs were enhanced after trauma-hemorrhage. We found that although the IL-2 and IFN-γ production capacities of T-helper cells alone were decreased after trauma-hemorrhage, they were further suppressed by approximately 50% if T-helper cells were co-cultured with Tregs (supplemental Figure 3, A and B, see *http://ajp.amjpathol.org*). Furthermore, because it is known that naive $CD4^+$ T cells are less sensitive to antigenic stimulation than memory T cells and that the cytokine production capacities are different among naive, central memory T cells and effector memory T cells,[53,54] it is possible that suppression of effector T-cell function(s) correlates with the decreased cytokine production after trauma-hemorrhage. Thus, it would be of great interest to determine the differential effects of trauma-hemorrhage on the various $CD4^+$ T-cell subsets to gain insights into their roles in immune suppression after injury.

ConA is a polyvalent tetrameric lectin that binds to oligosaccharides on the cell surface and triggers a direct but nonspecific activation of T cells.[55] In contrast, T cells in the animals immunized with OVA would be activated via a classic antigen-specific pathway, involving antigen presentation by APC and T-cell receptor recognition, followed by costimulatory factor engagement and signal transduction. Our results showed that regardless of whether T cells were specifically or nonspecifically activated, the expression of CD28 on T cells after trauma-hemorrhage was suppressed compared with that of sham T cells. Thus, in a hierarchical sense, these findings suggest that the insult of trauma-hemorrhage could suppress T-cell activities globally and lead to widespread immunological dysfunction. Furthermore, the relative difference in T-cell proliferative capacity between OVA-stimulated sham and trauma-hemorrhage groups was greater than that seen in the respective ConA-stimulated groups, even though the magnitude of response was greater with ConA. This result probably occurs because ConA is regarded as a strong mitogen[56] and thus operates outside of the normal physiology of T-cell activation in adaptive immunity (ie, antigen presentation via the T-cell receptor and second signals). Thus, we speculate that mitogen stimulation may circumvent some of the vulnerability to trauma-hemorrhage compared with that of the sham group.

Our results demonstrate that the suppression of T-cell activation after trauma-hemorrhage was apparent in cells immediately after harvesting or 3 days after culture. The inhibition of CD28 expression and Akt activation followed a similar temporal pattern. It is thus interesting to note that, although the trauma-hemorrhage insult occurred only *in vivo*, we showed that its effects on the explanted cells persisted for at least 3 days after culture. This matches the clinical experience in that patients are at high risk of immune suppression and subsequent infection even though their major injury has been successfully treated.[57] In addition to the well known fact that post-traumatic suppression of adaptive immunity is due to reduced major histocompatability complex II expression and impaired antigen presentation,[8,58,59] our current study indicated that dysregulation of T-cell costimulatory signaling may also play a crucial role in the induction of T-cell anergy after trauma-hemorrhage.

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