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Lymphocytes in the Development of Lung Inflammation: A Role for Regulatory CD4+ T Cells in Indirect Pulmonary Lung Injury[1]

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Abstract

Although roles for myelocytes have been suggested in the pathophysiology of indirect acute lung injury (ALI not due to a direct insult to the lung), the contribution of various regulatory lymphoid subsets is unknown. We hypothesized a role for lymphocytes in this process. Using a sequential model of indirect ALI induced in mice by hemorrhagic shock followed 24 h later by polymicrobial sepsis; we observed a specific and nonredundant role for each lymphocyte subpopulation in indirect ALI pathophysiology. In particular, we showed that $CD4^+$ T cells are specifically recruited to the lung in a dendritic cell-independent but IL-16-dependent process and diminish neutrophil recruitment through increased IL-10 production. Most importantly, this appears to be mediated by the specific subpopulation of $CD4^+CD25^+Foxp3^+$ regulatory T cells. Although indirect ALI has constantly been described as a proinflammatory pathology mediated by cells of the innate immune system, we now demonstrate that cells of the adaptive immune response play a major role in its pathophysiology as well. Most importantly, we also describe for the first time the nature of the regulatory mechanisms activated in the lung during indirect ALI, with $CD4^+$ regulatory T cells being central to the control of neutrophil recruitment via increased IL-10 production.

Acute lung injury (ALI)[3] and acute respiratory distress syndrome (ARDS) are the two parts of a serious and progressive syndrome associated with the development of lung damage and organ failure (1). This syndrome is characterized by the rapid onset of severe respiratory failure with acute dyspnea, tachypnea, tachycardia, and profound arterial desaturation.

Every year, 74,500 persons die of ALI/ARDS in the United States (2). Among all causes, sepsis is associated with the highest risk of progression to ALI (~40%) and sepsis-associated ARDS carries the highest mortality rates from ARDS (1–3).

ALI can be differentiated into direct (pulmonary) and indirect (nonpulmonary) ALI. Epidemiologically, direct ALI accounts for 57% of all cases and is caused mainly by pneumonia, aspiration, and lung trauma. Indirect ALI accounts for the residual 43% with nonpulmonary sepsis and trauma being the most frequent underlying diseases (4). Importantly,

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[3]Abbreviations used in this paper: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; Hem, hemorrhagic shock; CLP, cecal ligation and puncture; BALF, bronchoalveolar lavage fluid; siRNA, small interfering RNA; IT, intratracheal(ly); DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate; DC, dendritic cell; MHC II, MHC class II; KC, keratinocyte-derived chemokine; Treg, regulatory T cell; EGFP, enhanced GFP; MPO, myeloperoxidase; DTR, diphtheria toxin receptor.

patients developing ALI after nonpulmonary sepsis (indirect ALI) present with a higher mortality rate than patients with pulmonary sepsis (direct ALI) (5).

From a pathophysiological point of view, pulmonary vs nonpulmonary sepsis may differ in mechanisms leading to ALI, with pulmonary infections causing lung injury directly via the pathogen and host response vs nonpulmonary infections causing lung injury indirectly via systemic inflammation (1). In fact, the early development of indirect ALI is characterized by the recruitment to the lung of activated neutrophils and macrophages, experiencing a delay in apoptosis and an increase in respiratory burst (6,7). Concomitantly, lung epithelial cells undergo apoptosis (1,8), thus contributing to destruction of the pulmonary epithelium and compromised barrier function.

Based on this observation, anti-inflammatory therapies such as glucocorticoids, ketoconazole, Abs to endotoxin, cytokines, and adhesion molecules have been tested in the clinical management of ALI/ARDS without showing any benefits (1,3). This failure may reflect the complexity of the pathophysiological process occurring in indirect ALI and the amount of work remaining before acquiring a complete picture of this process.

In particular, beside neutrophils and macrophages, the involvement of other immune cells that may be present or recruited to the lung has been largely ignored. Lymphocytes are present in the lung and have been shown to play a role in several lung diseases (asthma, allergy, chronic obstructive pulmonary disease (COPD), and tuberculosis) in both humans and mice (9,10). More specifically, lymphocytes are recruited to the lung in response to pulmonary infections (*Aspergillus fumigatus*, *Klebsiella pneumoniae*) (11). However their role in the pathophysiology of indirect ALI has not been emphasized.

We hypothesized a role for the different lymphocyte subpopulations present and/or recruited to the lung in the development of indirect ALI. In this study, we investigated this using a sequential model of indirect ALI induced in mice by hemorrhagic shock (Hem) followed 24 h later by a polymicrobial septic challenge (cecal ligation and puncture (CLP)).

Materials and Methods

Animals

Male mutant mice, lacking the RAG (B6.129S7-Rag1tm1Mom/J) or deficient in $\gamma\delta$ T (B6.129P2-Tcrd^{tm1Mom}/J), CD8^{+} T (B6.129S2-Cd8a^{tm1Mak}/J), CD4^{+} T (B6.129S6-Cd4^{tm1Knw}/J), or B lymphocytes (B6.129S2-Igh-6^{tm1Cgn}/J), transgenic enhanced GFP (EGFP)-Foxp3 (B6.Cg-Foxp3^{tm2Tch}/J), and CD11c-diphtheria toxin receptor (DTR) mice (B6.FVB-Tg(Itgax-DTR/EGFP)57Lan/J), 8–10 wk old, were used in comparison to age-matched control mice (C57BL/6J; The Jackson Laboratory). Experiments were done in accordance with National Institutes of Health (Bethesda, MD) guidelines and were approved by the local animal use committee.

Indirect ALI

Indirect ALI was induced as described previously (6,12) by Hem followed by CLP 24 h later.

Hemorrhage—In brief, mice were anesthetized with isoflurane, restrained in a supine position, and catheters were inserted into both femoral arteries (all incisions bathed in lidocaine during this protocol). Anesthesia was discontinued and blood pressure was continuously monitored through one catheter attached to a blood pressure analyzer (BPA; MicroMed). When fully awake, as determined by a mean blood pressure of ~95 mm Hg, the mice were bled over a 5- to 10-min period to a mean blood pressure of 30 mm Hg (±5 mm Hg) and were kept stable for 90 min. Immediately after hemorrhage, mice were resuscitated with Ringer's lactate at four

times the drawn blood volume. After resuscitation, arteries were ligated, catheters were removed, catheter sites were sutured, and mice were returned to their cages. Sham hemorrhage was performed as control and these mice were anesthetized, restrained, and their femoral arteries were ligated but no blood was drawn.

Polymicrobial sepsis—CLP was performed as previously described in this laboratory (13). To summarize, mice were anesthetized with isoflurane and were restrained in a supine position. A 1-cm midline incision was made and the cecum was ligated with 5-0 silk threads and punctured twice with a 22-gauge needle. The cecum was then replaced, the incision was sutured, and lidocaine was applied. Mice were resuscitated with 1 ml of Ringer's lactate (s.c.) and were returned to their cages. For sham controls, the cecum was extracted but neither ligated nor punctured.

Importantly, although we show only the sham Hem plus CLP (which we have not found to differ from Hem plus sham CLP) or sham Hem plus sham CLP groups as our controls for the studies here, we have previously documented that neither Hem nor CLP alone is capable of inducing substantial/consistent increases in the indices of lung inflammation, apoptosis, or injury (6,12,14).

Sample acquisition

Twenty-four hours after CLP, mice were euthanized with an overdose of CO_2. Blood was collected via cardiac puncture into heparinized syringes. The left lobe of the lung was harvested and fixed in 4% formalin, stained with H&E, and examined by light microscopy. Bronchoalveolar lavage fluid (BALF) was then collected to assess protein concentration as an index of lung permeability (injury). The trachea was exposed via a midline incision and cannulated with a sterile polypropylene 18-gauge catheter. The lungs were lavaged with 0.6 ml of saline two times for an average of 1 ml of lavage fluid per lung. Lavage fluid was centrifuged at 1500 × *g* for 10 min at 4°C. Lung tissue was subsequently harvested for digestion, assessment of cytokine levels, neutrophil influx, and apoptosis.

Intratracheal instillation of Abs or small interfering RNA (siRNA)

Intratracheal (IT) instillations of functional grade purified rat anti-mouse CD4 Abs (clone GK1.5, 50 μg; eBioscience), anti-IL-16 Abs (clone 14.1, 75 μg; BD Biosciences, the cross-reactivity of this Ab between human and mouse IL-16 has been demonstrated previously (15)) or isotype control (rat IgG2b) were performed as previously described (12). Foxp3 targeting (4 nmol; sense, 5′-P-GAAUUUGAGUUUCGCAAGAUUdTdT-3′; anti-sense, 5′-P-PUCUUGCGAAACUCAAAUUCUUdTdT-3′) or nontargeting control siRNA (Dharmacon) were also administered IT. Efficient transfection was obtained using cationic liposomal transfection (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP); Roche). Efficient and nontoxic transfection of lung cells using DOTAP instillation has been already described without inducing any lung inflammation (16).

Intraperitoneal injection of Abs or diphtheria toxin

Intraperitoneal injections of diphtheria toxin (100 ng/mouse, 4 μg/kg) in CD11c-DTR and in control mice and of anti-IL-10R-neutralizing Ab (250 μg, clone 1B1.3a; BD Pharmingen) or corresponding IgG (Sigma-Aldrich) in C57BL/6J mice were performed 12 h before the induction of indirect ALI. The ablation of dendritic cells (DCs) was monitored 24 h after CLP by the measurement of the percentage of $CD11c^+MHC\ II^+$ cells in lung by flow cytometry (17).

Lung histology

The left lobe of the lung was harvested and fixed in 4% formalin, stained with H&E, and examined by light microscopy (6,12).

Quantification of cytokines/chemokines

Mouse keratinocyte-derived chemokine (KC; R&D Systems), MCP-1, TNF-α, IL-6, IL-10 (BD Biosciences) and IL-16 (BioSource International) were measured on lung-tissue homogenate using commercially available sandwich ELISA kits (6,12).

Lung myeloperoxidase (MPO) activity-caspase 3 activity assay

An assessment of lung myeloperoxidase activity, a marker for neutrophil influx, was measured according to established protocols (18). Protein concentration was assessed using the Bradford dye-binding procedure (Bio-Rad). Caspase 3 activity in lung tissue was quantified as described elsewhere (19).

Enzymatic lung digest and flow cytometry

Lung tissue cells were isolated from PBS-perfused whole lungs using methods described by Summer at al (20) and De Paepe et al. (21). Each lymphocyte subpopulations was then monitored by flow cytometry (BD Biosciences FACSArray or BD Biosciences FACSAria). mAbs and their respective isotype controls were used according to the manufacturer's recommendation: PE- or allophycocyanin-labeled anti-CD45, PECy7- or allophycocyanin-labeled anti-CD4, PECy7-labeled anti-CD8, PE-labeled anti-B220, PE- or allophycocyanin-labeled anti-CD3, PE-labeled anti-CD49d (DX5-Pan NK), PE-labeled anti-CD25, PE-labeled anti-CD11c, PE-labeled anti-GR1, and allophycocyanin-labeled anti-MHC class II (MHC II) Abs (eBioscience).

Cell purification and RT-PCR

Following incubation with PE-labeled anti-CD31 Ab (BD Biosciences), lung endothelial cells (ECs) were purified thanks to a positive purification using an anti-PE immunomagnetic purification kit (Stem Cell Technology). Alternatively, lung $CD4^+CD25^+$ T cells were purified using a CD4-negative immunomagnetic selection followed by a CD25- positive immunomagnetic selection (Miltenyi Biotec). RNA was immediately extracted from either isolated lung ECs or lung $CD4^+CD25^+$ T cells by using TRIzol reagent (Invitrogen) and cDNA was synthesized with SuperScript Transcriptase III (Invitrogen). Then mRNA expression of IL-16, IL-10, or TGF-β was evaluated by RT-PCR. Primers (IDT) and amplification parameters were: IL-16, 5′-AAACAAGGT GATCACGGTT (sense) and 5′-CACCCTGTTCTGTCCCTT (antisense); 94°C for 5 s, 55°C for 30 s, 72°C for 25 s, 25 cycles; IL-10, 5′-AGTGATTTTAATAAGCTCCA AG (sense) and 5′-TGGAAACAGCTT AAACACAG (antisense); 94°C for 5 s, 56°C for 30 s, 72°C for 25 s, 30 cycles; TGF-β, 5′-CCAAGGAGACGGAATA CAG (sense) and 5′-AGAGGGCAAGGAC CTTG (antisense); 94°C for 5 s, 56°C for 30 s, 72°C for 25 s, 25 cycles; and β -actin, 5′-GAAATCGT GCGTGACATC (sense) and 5′-GCTGATCCACATCTGCTG (antisense); 94°C for 5 s, 55° C for 30 s, 72°C for 30 s, 25 cycles. GoTaq polymerase was used according to the manufacturer's instructions (Promega).

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by Mann-Whitney *U* test. A value of $p < 0.05$ was considered significant.

Results

T lymphocytes are recruited to the lung after Hem plus CLP

Since essentially no data has been published regarding the possible role of lymphocytes in the pathophysiology of indirect ALI, we initially performed a descriptive study of the different lymphocyte subpopulations present/recruited to the lung in our model.

We first observed that the number of lymphocytes in the lung was significantly increased after Hem plus CLP in comparison to naive animals (Fig. 1*A*). Among these lymphocytes, no clear change was observed regarding the number of NK, NKT, and B cells (Fig. 1*B*), whereas a significant increase in the number of lung T lymphocytes was observed after Hem plus CLP in comparison to mice after CLP alone or with naive mice (Fig. 1*B*). Among T cells, despite a trend toward an increase after CLP alone and after Hem plus CLP, no significant change was observed regarding the number of lung $\gamma\delta$ T cells (Fig. 1*C*). In contrast, the ratio of $CD4^+$ vs $CD8^+$ T lymphocytes was significantly increased after Hem plus CLP (Fig. 1*D*), suggesting that $CD4^+$ T cells are specifically recruited to the lung after Hem plus CLP in mice.

The recruitment of T lymphocytes to the lung after Hem plus CLP is independent of the presence of DCs but depends on lung IL-16

The generation and recruitment of effector T cells to the lung in inflammatory contexts such as asthma or COPD have been described to be dependent on an interaction with DCs (22). We therefore monitored, in our model, the recruitment of T cells to the lung after Hem plus CLP in CD11c-DTR mice. In these mice, diphtheria toxin injection led to the selective depletion of lung DCs after Hem plus CLP (Fig. 2*A*) (23). We observed that the recruitment of T cells to the lung after Hem plus CLP was similar in DC-depleted and in control mice (Fig. 2*B*), suggesting that DCs do not play a role in this process. To note, the ratio of $CD4^+$ vs $CD8^+$ T cells in DC-depleted mice (1.43 ± 0.39, $n = 5$ mice) was not different than that in C57BL/6 mice after Hem plus CLP (Fig. 1*D*). Meanwhile, the lung IL-16 concentration, a potent chemoattractant for $CD4^+$ cells (24), was increased in mice after Hem plus CLP (Fig. 2*C*), and pretreatment of mice with anti-IL-16-blocking Abs administered IT 12 h before the induction of indirect ALI was associated with a significantly decreased number of lung $CD4^+$ T cells in comparison to mice treated with IgG (Fig. 2*D*). ECs have been shown to be able to produce IL-16 (24). In our model, after lung EC purification based on CD31 expression, we observed that IL-16 mRNA expression in these cells was increased after Hem plus CLP in comparison to cells purified from mice after CLP alone (Fig. 2*E*). This suggests that IL-16, produced locally in the lung in part by ECs, might be responsible for the specific recruitment of $CD4^+$ T cells during indirect ALI.

Lymphocyte subpopulations have specialized functions in the lung after Hem plus CLP

To investigate the role of T and B lymphocytes in lung dysfunctions induced after indirect ALI in mice, RAG-deficient mice (lacking mature B and T cells) were submitted to Hem plus CLP. BALF protein concentration and lung histology (as markers for lung injury/protein leakage), caspase 3 activity (as a marker for apoptosis), MPO activity (as an index of neutrophil accumulation), and proinflammatory cytokine expression (TNF-α, IL-6, and MCP-1) were measured in the lung 24 h after CLP.

We observed that RAG-deficient mice presented with an increased BALF protein concentration, consistent increase in alveolar septal thickening, associated with marked cellular infiltrates, and alveolar congestion/collapse in comparison to control animals after Hem plus CLP (Fig. 3, *A* and *B*). This suggests that the severity of indirect ALI was increased in RAG-deficient mice in comparison to controls. Concomitantly, an increased lung apoptosis was observed in these mice (Table I and Fig. 3*C*). By contrast, lung MPO activity was not different

in RAG-deficient mice vs background animals (Table I and Fig. 3*D*). Finally, regarding proinflammatory cytokine expression, only lung MCP-1 showed a significant increase in RAG-deficient mice after Hem plus CLP (Table I and Fig. 3*E*).

To investigate the cell-specific involvement of the different lymphocyte subpopulations in the pathophysiology of indirect ALI, mice deficient for $CD8^+$, $\gamma\delta$, $CD4^+$ T, or B cells were then subjected to Hem plus CLP.

We first observed that $CD8^+$ T cell deficiency was associated with a significant increase in lung apoptosis, whereas no difference was observed for the other deficient animals (Table I and Fig. 4*A*). In addition, CD8 deficiency and B cell deficiency were associated with a significant decline in lung IL-6 levels (and TNF levels in the latter) after Hem plus CLP in comparison to control mice (data not shown). $CD4^+$ T cell deficiency was associated with a significantly increased neutrophil recruitment to the lung after Hem plus CLP, whereas MPO activity was reduced in B cell-deficient mice (Table I and Fig. 4*B*). In CD4-deficient mice, this was associated with a decreased IL-10 concentration after Hem plus CLP (Fig. 4*C*).

Since lineage-deficient mice often present with compensatory mechanisms and immune abnormalities, this observation was confirmed in CD4-depleted mice. In these mice, histological examination of lung sections after IT instillation of anti-CD4 Abs (leading to specific $CD4^+$ T cell depletion, as measured by a significant decrease in the percentage of lung $CD4^+$ cells by flow cytometry; data not shown) after Hem plus CLP showed a consistent increase in alveolar septal thickening, marked cellular infiltrates, and alveolar congestion/collapse (Fig. 4*D*). This was associated with an increased lung MPO activity and with a decreased IL-10 concentration as observed in CD4-deficient mice (data not shown).

Blocking IL-10's effect recapitulates the observations made in CD4-deficient/depleted mice after Hem plus CLP

The regulatory role of endogenous IL-10 on the recruitment of neutrophils to the lung has been previously observed in a different pathophysiological context (25). We, therefore, hypothesized that the increased recruitment of neutrophils observed in CD4-deficient and CD4-depleted mice after Hem plus CLP could be due to the decreased lung IL-10 concentration measured in these mice. To investigate this, neutralizing Abs against the IL-10R were administered to the mice before the induction of indirect ALI.

As observed in CD4-deficient and CD4-depleted mice, anti-IL-10R Ab-treated mice presented with an increased BALF protein concentration, consistent increase in alveolar septal thickening associated with marked cellular infiltrates and alveolar congestion/collapse in comparison to IgG-treated mice after Hem plus CLP (Fig. 5, *A* and *B*). This was associated with an increased recruitment of neutrophils to the lung as illustrated both by the increased percentage and number of lung $GR1^+$ cells (Fig. 5*C*) and by increased lung MPO activity (Fig. 5*D*) in comparison to control mice. Finally, we observed that the lung KC concentration was increased in these mice as well (Fig. 5*E*).

Decreasing Foxp3 expression in the lung recapitulates the observations made in CD4-deficient/depleted and anti-IL-10R Ab-treated mice after Hem plus CLP

Among $CD4^+$ lymphocytes, the subpopulation of $CD4^+CD25^+$ $Foxp3^+$ T cells (Treg) has been described to be central to the control of the peripheral T cell response in part due to their large production of IL-10 (26). We thus hypothesized that this small subpopulation of $CD4^+$ T lymphocytes might be responsible for the effects observed in CD4-depleted/deficient and anti-IL-10R Ab-treated mice. Mice coexpressing EGFP and the regulatory T cell-specific

transcription factor Foxp3 (27) were, therefore, submitted to Hem plus CLP after IT instillation of Foxp3 targeting siRNA.

We first observed that the percentage and the number of lung $CD3^+CD4^+CD25^+Foxp3^+$ cells were significantly increased after Hem plus CLP (Fig. 6, *A* and *B*). Interestingly, this was associated with a significant increase in the mean of fluorescence intensity for Foxp3 in these cells (data not shown). IT instillation of Foxp3 targeting siRNA but not of control nontargeting siRNA was associated with a significant decrease in the percentage of Treg (fold change ≈30%), as well as with a decrease in their number after Hem plus CLP (Fig. 6, *A* and *B*). Interestingly, as observed in CD4-deficient/depleted mice, as well as in anti-IL-10R Ab-treated mice, histological examination of lung sections after Hem plus CLP illustrated a consistent increase in alveolar septal thickening associated with marked cellular infiltrates and alveolar congestion/collapse that was not observed in sham and to a lesser extend in mice after control siRNA administration (Fig. 6*C*). Most importantly, in these mice also, the lung IL-10 concentration was decreased (Fig. 6*D*).

Finally, purified lung $CD4^+CD25^+$ cells showed a markedly increased IL-10 mRNA expression after Hem plus CLP in comparison to cells purified from mice after CLP alone (Fig. 6*E*). This was also associated with an increased TGF-β expression (Fig. 6*E*).

Discussion

Our data suggest that $CD4^+$ T lymphocytes are specifically recruited to the lung during indirect ALI in mice in part due to the increased production of IL-16 by ECs and play an inhibitory role on the recruitment of neutrophils through increased IL-10 production. Most importantly, the specific subpopulation of $CD4^+CD25^+$ $Foxp3^+$ regulatory T cells appears to be responsible for this effect.

Beside the involvement of cells of the innate immune system leading to acute intense inflammation (10), the involvement of cells of the adaptive immune response and of regulatory/anti-inflammatory mechanisms in the pathophysiology of indirect ALI has been essentially ignored. The only sparse data available regarding the involvement/production of anti-inflammatory cytokines and cytokine antagonists in ALI however suggest an important role for anti-inflammatory mediators in counterbalancing the proinflammatory response (28). Significantly increased concentrations of sTNF-RI and sTNF-RII (TNF antagonists) have been measured in BALF from patients with ARDS (29). Importantly, low concentrations of IL-10 and IL-1Ra in BALF from patients with ARDS were found to be associated with increased mortality. Similarly, Park et al. (30) observed a major anti-inflammatory response (increased anti-inflammatory cytokines and cytokine antagonists/soluble receptors release) in the BALF of patients with ARDS. Importantly, this response peaked early after the onset of ARDS and exceeded the proinflammatory response. However, the involvement of cellular/regulatory mechanisms and of cells of the adaptive immune response in the pathophysiology of ALI has never been investigated.

Using a sequential model of indirect ALI induced in mice by Hem followed 24 h later by CLP, we observed a clear recruitment of lymphocytes to the lung after Hem plus CLP (as illustrated by their increased number measured in lung digest). This increase was not present after CLP alone or Hem alone (data not shown), therefore illustrating the major priming effect of the Hem on the lung immune response in our double-hit model (31). Similarly, in a model of direct LPS-induced lung inflammation, Morris et al. (32) observed an influx of activated lymphocytes to the lung during the resolution of neutrophilic pneumonitis. Among the different lymphocyte subpopulations, we observed that only the number of T cells was markedly increased after Hem plus CLP, with T cells actually becoming the most frequent lymphocyte subpopulation in the

lung. Among T cells, although we observed a small increase after CLP alone as reported by Hirsh et al. (33), after Hem plus CLP, no significant difference was observed in the number of lung $\gamma\delta$ T cells. Meanwhile, the ratio of $CD4^+$ vs $CD8^+$ T cells was significantly increased after Hem plus CLP, therefore, demonstrating for the first time that, in addition to neutrophils and macrophages, $CD4^+$ T cells are specifically recruited to the lung during indirect ALI.

In an attempt to clarify the mechanisms involved in this specific recruitment, we investigated the role of DCs in this process. In pathologies such as asthma or COPD, the generation and recruitment of effector T cells to the lung has been described to be dependent on an interaction with DCs (22). However, in our model, the recruitment of T cells to the lung was still observed in DC-depleted mice, therefore suggesting that the specific recruitment of $CD4^+$ T cells to the lung during indirect ALI is independent of Ag-specific presentation involving an interaction with DCs. This illustrates the difference in the pathophysiological mechanism occurring in our model and other models of direct inflammatory lung diseases (22).

This recruitment might then be due to the induced expression in the lung of specific chemotactic factors. In accordance, we observed an increased IL-16 level in the lung after Hem plus CLP. IL-16 is a potent and specific chemoattractant for $CD4^+$ cells that can be produced by ECs (24). Interestingly, IL-16 concentration in the lung was not increased after CLP alone or after Hem alone (data not shown) but was increased after Hem plus CLP. Moreover, IL-16 gene expression was increased in ECs purified from the lungs of mice after Hem plus CLP in comparison to cells purified from mice after CLP alone; therefore, not only suggesting that ECs play a major role in the specific recruitment of $CD4^+$ T cells to the lung in our model, but also once again illustrating the priming effect of Hem on the immune response and ECs in the lung during indirect ALI (31). Most importantly, the role of this chemokine in the recruitment of $CD4^+$ T cells to the lung in our model was confirmed by the observation that the administration of anti-IL-16-blocking Abs before the induction of indirect ALI was associated with a decreased number of lung $CD4^+$ T cells after Hem plus CLP in comparison with IgG-treated mice. Moreover, in accordance with a causal link from recruitment of $CD4^+$ T cells to the lung to depression of neutrophil recruitment by IL-10, we observed that anti-IL-16-treated mice presented with higher neutrophil recruitment (as measured by MPO assay and flow cytometry staining) and lower lung IL-10 concentration than IgG-treated mice after Hem plus CLP (data not shown).

Based on the above, we hypothesized a role for T cells in the pathophysiology of indirect ALI. RAG-deficient mice (lacking B and T cells) were, therefore, submitted to Hem plus CLP. In these mice, markers of lung injury and lung apoptosis were increased when compared with control mice (Table I), suggesting that B and/or T cells are playing a protective role in the pathophysiology of indirect ALI. Although an increased apoptosis was observed in RAG-deficient mice after Hem plus CLP, as observed by Morris et al. (32) in a model of direct LPS-induced lung inflammation, neutrophil recruitment or lung IL-10 concentration (data not shown) was not modified in comparison to controls.

Therefore, to clarify the role of each lymphocyte subpopulation, CD8 T-, $\gamma\delta$ T-, CD4 T -, and B cell-deficient mice were subsequently submitted to Hem plus CLP. We observed that the absence $CD8^+$ T cells was associated with an increased lung apoptosis and might therefore have been responsible for the increased apoptosis observed in RAG-deficient mice after Hem plus CLP (Table I). This result was very surprising considering the cytotoxic properties usually described for $CD8^+$ T cells. It has, for example, been shown in a model of direct ALI induced by inhalation of *Staphylococcus aureus* enterotoxin that the massive $CD8^+$ T cell accumulation in the lung was responsible for the pulmonary alveolitis and inflammation (34). Also, lung memory $CD8^+$ T cells have been shown to play a central role in the recall response to respiratory viral infections (35). However, in accordance with our data, it has also been described that

$CD8^+$ T cells that are resident in the lung airways actually lack cytolytic activity, whereas they remain capable of producing cytokines (35).

In parallel, we observed that B cells and $CD4^+$ T cells differentially regulated neutrophil recruitment to the lung: whereas B cells played an activating role in the recruitment of neutrophils, $CD4^+$ T cells appeared to suppress this process (Table I). This result was also confirmed in CD4-depleted mice. This net effect may therefore explain why no difference in MPO activity (and IL-10 concentration; data not shown) was initially observed after Hem plus CLP in RAG-deficient mice.

The regulation of neutrophil recruitment to the lung plays a central role in the pathophysiology of ALI. Clinical and experimental studies have provided circumstantial evidence of the occurrence of neutrophil-mediated injury in ALI (1,36). Histological studies of lung specimens obtained early in the course of the disorder showed a marked accumulation of neutrophils (1). Neutrophils predominate in the pulmonary edema fluid and BALF obtained from affected patients and many animal models of ALI are neutrophil dependent (1). Neutrophils also appear to be important contributors to lung inflammation, as shown by the deterioration of lung pulmonary function in patients with lung injury as neutropenia resolves (36). Our observation of an opposite role for B cells and $CD4^+$ T cells in this process is, thus, a very interesting result. We can then postulate that B cells, which are originally present in high number in the lung, might play an active role in the initiation of the neutrophilic response during indirect ALI, whereas $CD4^+$ T cells, which are recruited to the lung later during this process, may play a regulatory role on neutrophil recruitment and initiate resolution of lung inflammation.

Interestingly, we observed that in CD4-deficient and CD4-depleted mice, the increased neutrophil recruitment was associated with a decreased IL-10 concentration in the lung. IL-10 is a pleiotropic cytokine that inhibits proinflammatory cytokine release (IL-1, TNF-α, IL-8), blocks cytokine-induced chemotaxis and oxidative burst, and therefore interferes with neutrophil-mediated tissue injury (16,25,37–39). Increased IL-10 concentrations have been measured in the BALF of patients with ARDS (30), whereas low concentrations were found to be associated with increased mortality, suggesting an important role for this mediator in counterbalancing the proinflammatory response (28). We hypothesized that, in our model, IL-10 may be responsible for the effect of $CD4^+$ T cells on neutrophil recruitment. Anti-IL-10R-blocking Abs were, therefore, administered to the mice before the induction of indirect ALI. In accordance with the work of Kobbe et al. (40) in IL-10-deficient animals, we observed that IL-10 blockage was associated with increased lung dysfunctions and neutrophil recruitment after Hem plus CLP. Moreover, as observed by Shanley et al. (25), in the absence of an IL-10 effect, KC levels were increased in the lung, suggesting that IL-10's inhibitory effect on neutrophil recruitment to the lung might be mediated via its regulation of chemokine expression. Moreover, this suggests that, in our model, $CD4^+$ T cells regulate the recruitment of neutrophils to the lung via IL-10 production that in turn down-regulates chemokine production.

Among $CD4^+$ T lymphocytes, the minor population of $CD4^+$ $CD25^+Foxp3^+$ T cells possess potent regulatory properties and are thought to play a major role in lung pathologies such as tuberculosis, sarcoidosis, and hypersensitivity pneumonitis (Th1-type diseases) and also in Th2-type lung diseases such as asthma (10). They are recruited to the lung and their Foxp3 expression is increased (10). Most importantly, Treg have been shown to be major producers of IL-10 (26). We observed that $CD4^+CD25^+$ $Foxp3^+$ T cells were recruited to the lung in indirect ALI and that Foxp3 expression as well as IL-10 and TGF-β gene expressions in these cells were increased during this process, therefore suggesting an increased regulatory activity (10). Interestingly and in accordance with our data, it has been shown that IL-16 possesses a preferential chemotactic activity for Treg (41). Most importantly, we were able to show that

the specific down-regulation of the Treg's function using Foxp3 targeting siRNA reproduced the results obtained in CD4-deficient and depleted mice and in particular was associated with a reduction in lung IL-10 concentration. This suggests that, among $CD4^+$ T cells, the small population of $CD4^+CD25^+Foxp3^+$ T cells may be responsible for the effect observed in CD4-deficient and depleted mice. A regulatory/anti-inflammatory role for Treg has been observed in several models of lung diseases: allergic diseases/asthma (42–44), *Pneumocystis* infection (45), and tuberculosis (10,46); however, this study is the first to demonstrate a role for these cells in indirect ALI.

In conclusion, although indirect ALI has been constantly described as a proinflammatory pathology mediated by cells of the innate immune response, with the migration of activated neutrophils to the lung being central to this process, we now describe that cells of the adaptive immune response are playing a role in its pathophysiology as well. It appears from our work that T lymphocytes are specifically recruited to the lung during indirect ALI and that there is a specific and nonredundant role for each lymphocyte subpopulation in the pathophysiology of indirect ALI (Table I). Most importantly, we also describe for the first time that regulatory mechanisms are activated in the lung during indirect ALI, with $CD4^+$ T cells/Treg being central to the control of neutrophil recruitment via increased IL-10 production.

Because under septic conditions lung dysfunction often is the first step in the development of multiple organ failure and because the migration of neutrophils to the lung plays a key role in the cascade of events leading to ARDS (1,18), it is, therefore, extremely important to better understand the basic cellular mechanisms that are not only involved, but also regulate this process. We believe our study represents a significant step forward toward a better understanding of the pathogenesis of this devastating clinical syndrome that may help in the design of effective strategies to minimize the severity and mortality of ARDS and ALI resultant from traumatic injury/shock.

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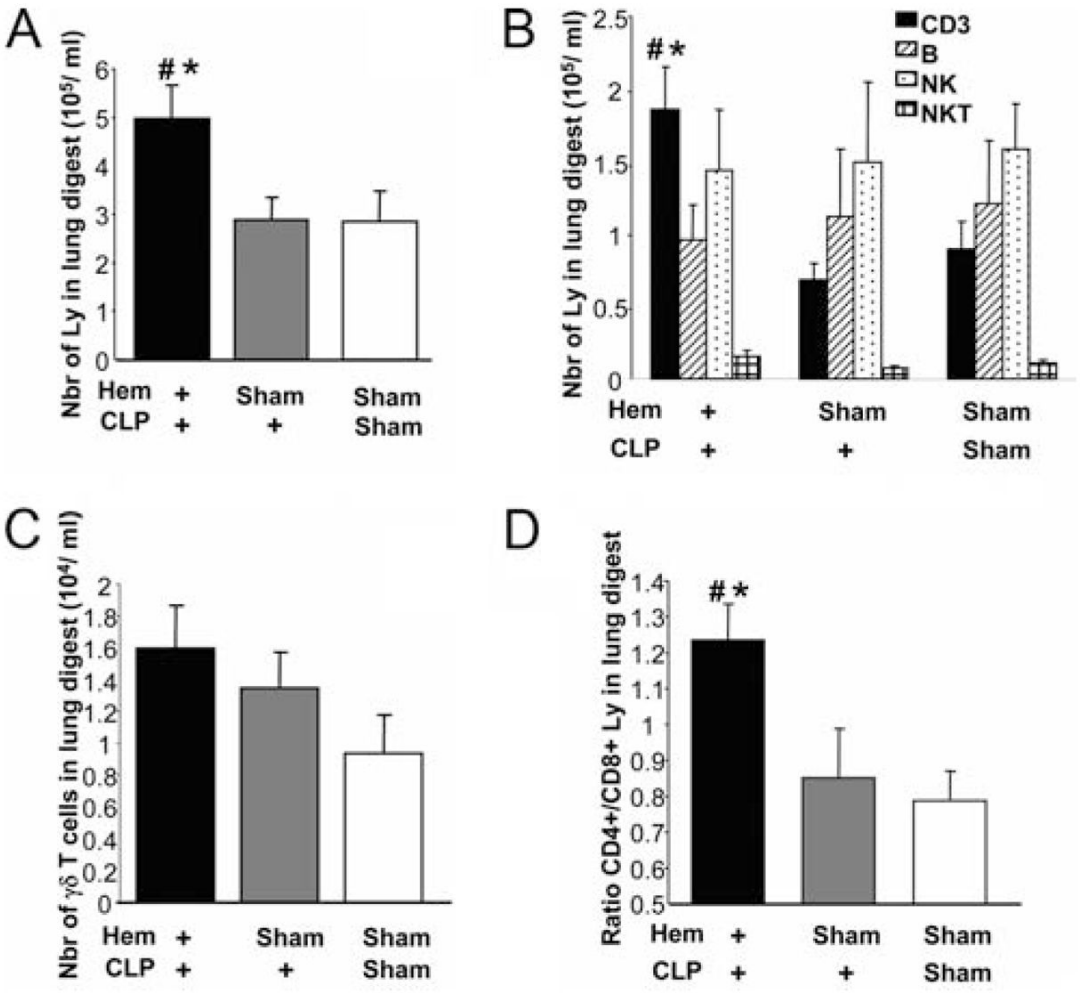

FIGURE 1.

T lymphocytes are recruited to the lung during indirect ALI in mice. Indirect ALI was induced in mice by Hem followed 24 h later by a polymicrobial septic challenge (CLP). As controls, mice were submitted to sham Hem + CLP or sham Hem + sham CLP. Lungs were harvested 24 h after CLP, enzymatically digested, and flow cytometry was performed. *A*, Total number of lymphocytes (Ly) per ml was calculated based on cell count and percentage of lymphocytes determined on a CD45/side scatter flow cytometry dot plot. Results are expressed as number of 10^5 lymphocytes per ml of lung digest. *B*, The respective percentages of T, B, NK, and NK T cells were then measured by flow cytometry and total numbers were calculated. Results are expressed as number of 10^5 lymphocytes per ml of lung digest. *C* and *D*, The percentages of $\gamma\delta$ T cells and of $CD4^+$ and $CD8^+$ lymphocytes were then measured and absolute numbers were calculated. *C*, Results are expressed as number of 10^4 $\gamma\delta$ T lymphocytes per ml of lung digest and *D*, as ratio between numbers of $CD4^+$ and $CD8^+$ T cells. Values are expressed as mean ± SEM (n = 7–9 mice/group). #, $p < 0.05$ vs sham Hem-sham CLP.*, $p < 0.05$ vs sham Hem + CLP (Mann-Whitney U test).

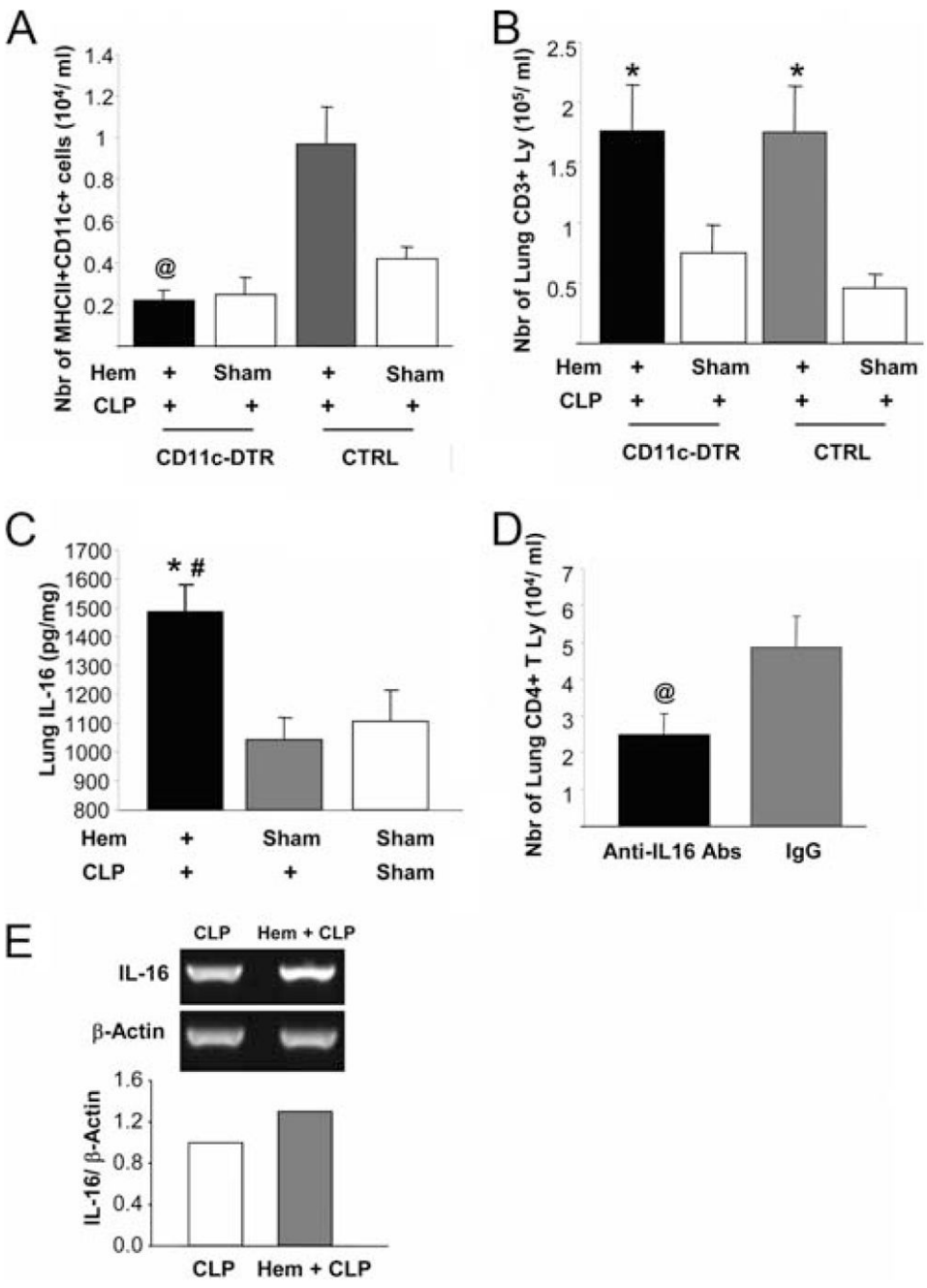

FIGURE 2.

T cell recruitment to the lung during indirect ALI is independent of the presence of DCs but depends on lung IL-16. *A* and *B*, CD11c-DTR and C57BL/6J (CTRL) mice were injected with diphtheria toxin (4 μg/kg) 12 h before the induction of indirect ALI by Hem followed 24 h later by a polymicrobial septic challenge (CLP). As controls, mice were submitted to sham Hem + CLP. Lungs were harvested 24 h after CLP, enzymatically digested, and flow cytometry was performed (n = 7–9 mice/group). *A*, The percentage of lung $CD11c^+MHC\ II^+$ cells was measured and absolute numbers were calculated. Results are presented as number of 10^4 $CD11c^+MHC\ II^+$ cells per ml of lung digest. *B*, The percentage of $CD3^+$ T cells was monitored by flow cytometry and absolute numbers were calculated. Results are expressed as number of

10^5 T lymphocytes (Ly) per ml of lung digest. *C*, C57BL/6J mice were submitted to Hem followed 24 h later by CLP. As controls, mice were submitted to sham Hem + CLP or sham Hem + sham CLP. Lungs were harvested and IL-16 levels were measured by ELISA in lung homogenates. Results are expressed as pg/mg of IL-16 in lung homogenate ($n = 4$ mice/group). *D*, C57BL/6J mice were injected IT with anti-IL-16-blocking Abs (anti-IL16 Abs) or IgG (75 μg/mouse) 12 h before the induction of indirect ALI by Hem followed 24 h later by CLP. Lungs were harvested 24 h after CLP, enzymatically digested, and flow cytometry was performed. The percentage of lung $CD3^+CD4^+$ cells was measured and absolute numbers were calculated. Results are presented as number of 10^4 $CD4^+$ T cells per ml of lung digest ($n = 4$–7 mice/group). *E*, Lung ECs were purified from C57BL/6J mice after Hem + CLP or CLP alone. mRNA expression for IL-16 was measured by densitometric evaluation of PCR results. β-Actin served as internal control ($n = 3$ mice/group). Values are expressed as mean ± SEM. #, $p < 0.05$ vs respective sham Hem-sham CLP. *, $p < 0.05$ vs respective sham Hem-CLP. @, $p < 0.05$ vs control or IgG Hem + CLP group (Mann-Whitney *U* test). Ly, Lymphocyte.

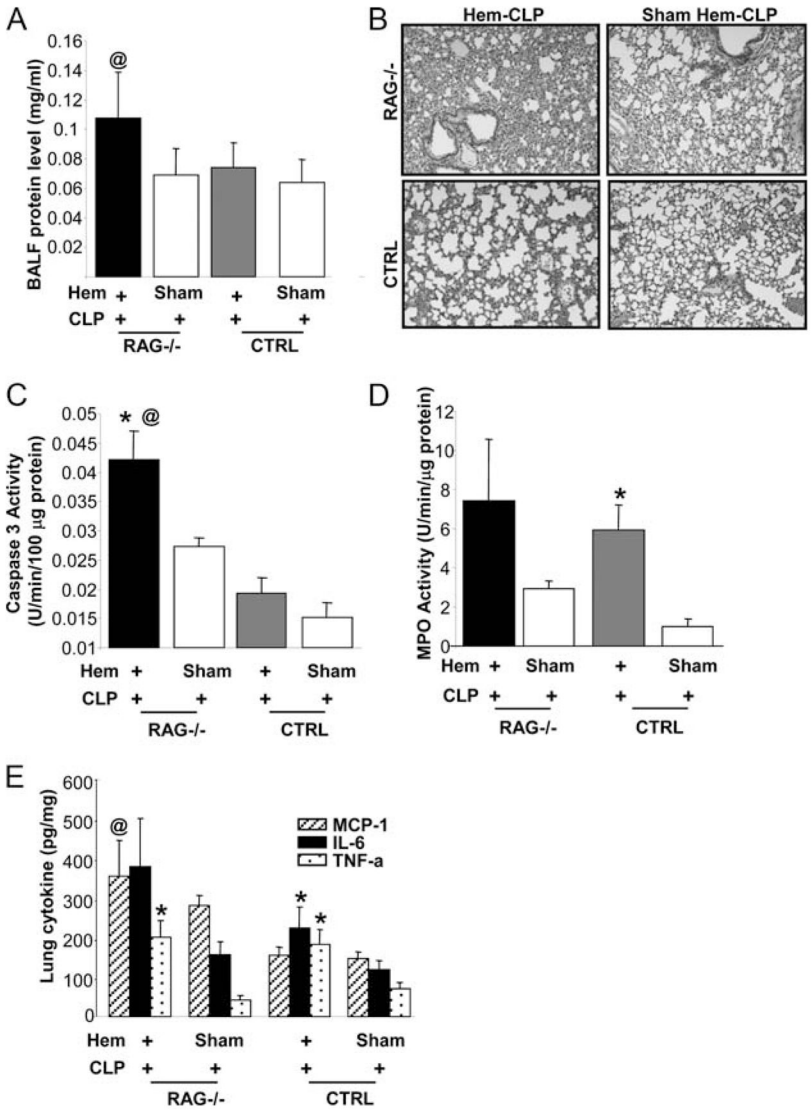

FIGURE 3.

RAG-deficient mice present with increased lung dysfunctions during indirect ALI. Indirect ALI was induced in C57BL/6J mice (CTRL) and RAG-deficient mice by Hem followed 24 h later by a polymicrobial septic challenge (CLP). As controls, mice were submitted to sham Hem + CLP. *A*, Protein concentration, as a maker for lung leakage, was measured in the BALFs. *B*, Lungs were fixed, sectioned, and stained with H&E. Representative sections are shown for each mouse group (original magnification, ×200). *C*, Lung apoptosis was monitored in whole lung homogenate by the measurement of caspase 3 activity. *D*, Neutrophil recruitment was monitored by the measurement of MPO activity in lung homogenate. *E*, Cytokine expressions were measured in lung homogenate by ELISA. Values are expressed as mean ± SEM (n = 7–9 mice/group). @, $p < 0.05$ vs control Hem + CLP group.*, $p < 0.05$ vs respective sham Hem-CLP group (Mann-Whitney U test).

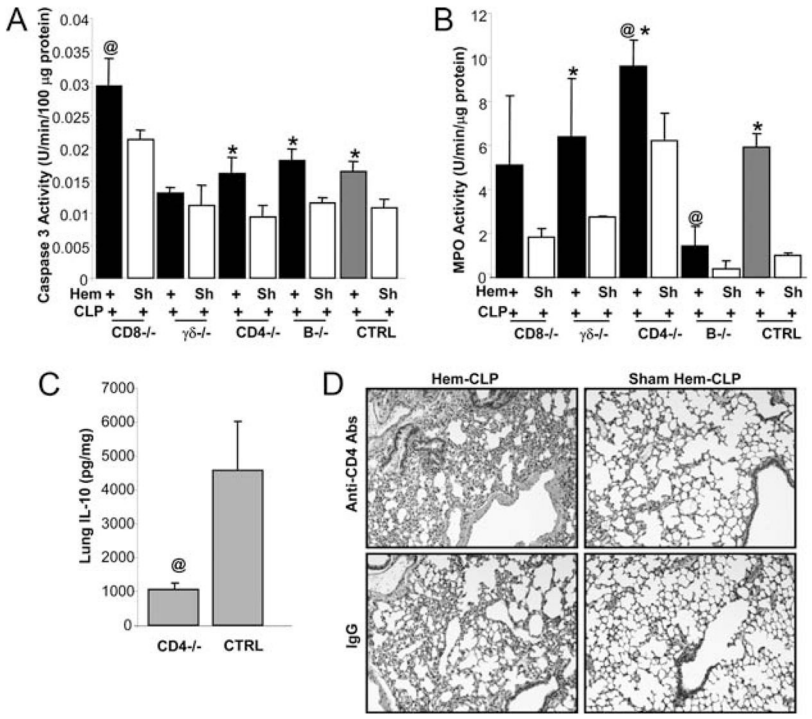

FIGURE 4.

Specific and nonredundant roles of lymphocyte subpopulations in the pathophysiology of indirect ALI. $CD8^+$ T cell ($CD8^{-/-}$)-, $\gamma\delta$ T cell ($\gamma\delta^{-/-}$)-, $CD4^+$ T cell ($CD4^{-/-}$)-, B cell ($B^{-/-}$)-deficient and C57BL/6J (CTRL) mice were submitted to Hem followed 24 h later by a polymicrobial septic challenge (CLP) to induce indirect ALI. As controls, mice were submitted to sham Hem (Sh) + CLP. *A*, Lung apoptosis was monitored in whole lung homogenate by the measurement of caspase 3 activity. *B*, Neutrophil recruitment was monitored by the measurement of MPO activity in lung homogenate. *C*, IL-10 level was measured in lung homogenate by ELISA. *D*, Control mice were submitted to IT instillation of anti-CD4-depleting Abs or IgG (50 μg/mice) 12 h before Hem. Anti-CD4 Abs led to the significant down-regulation of lung $CD4^+$ lymphocyte percentage as monitored by flow cytometry (data not shown). Lungs were fixed, sectioned, and stained with H&E. Representative sections are shown for each mouse group (original magnification, ×200). Values are expressed as mean ± SEM (n = 5–9 mice/group). @, $p < 0.05$ vs control Hem + CLP group.*, $p < 0.05$ vs respective sham Hem-CLP group (Mann-Whitney U test).

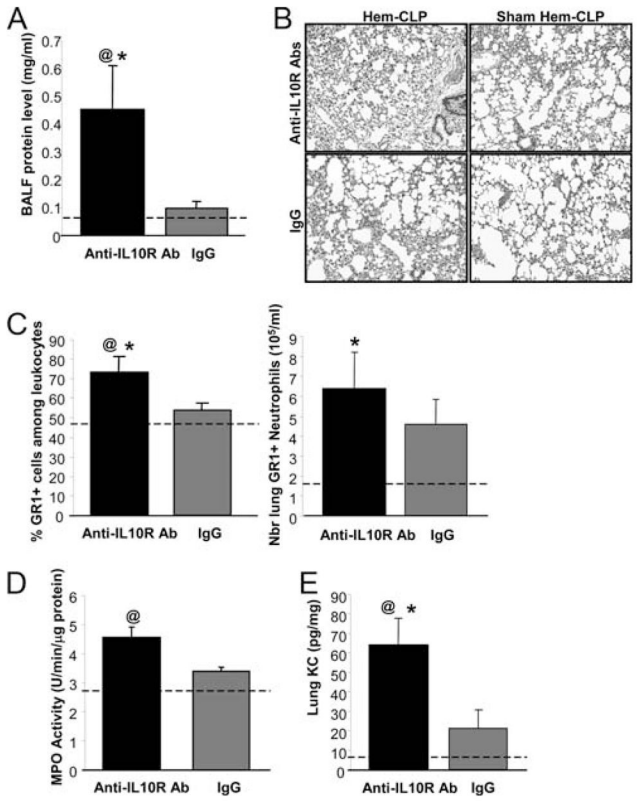

FIGURE 5.

IL-10 blockade recapitulates the observations made in CD4-deficient/depleted mice after Hem + CLP. C57BL/6J mice received an i.p. injection of anti-mouse IL-10R-neutralizing Ab (anti-IL10R Abs) or IgG (250 μg/mice) 12 h before the induction of indirect ALI by Hem followed 24 h later by polymicrobial septic challenge (CLP). As controls, mice were submitted to sham Hem + CLP. *A*, Protein concentration, as a maker for lung leakage, was measured in the BALFs. *B*, Lungs were fixed, sectioned, and stained with H&E. Representative sections are shown for each group (original magnification, ×200). *C*, Lungs were digested and cells were phenotyped for GR1 expression as a marker for neutrophils. Absolute counts were then calculated. Percentage of $GR1^+$ cells among total leukocytes ($CD45^+$ cells) and number of $GR1^+$ cells (10^5) per ml of lung digest are shown. *D*, Neutrophil recruitment was monitored in lung homogenate by the measurement of MPO activity. *E*, KC levels were measured in lung homogenate by ELISA. Values are expressed as mean ± SEM (n = 4 mice/group). Dashed line represents sham Hem + CLP mice. @, $p < 0.05$ vs IgG-treated Hem + CLP group. *, $p < 0.05$ vs sham Hem-CLP group (Mann-Whitney U test).

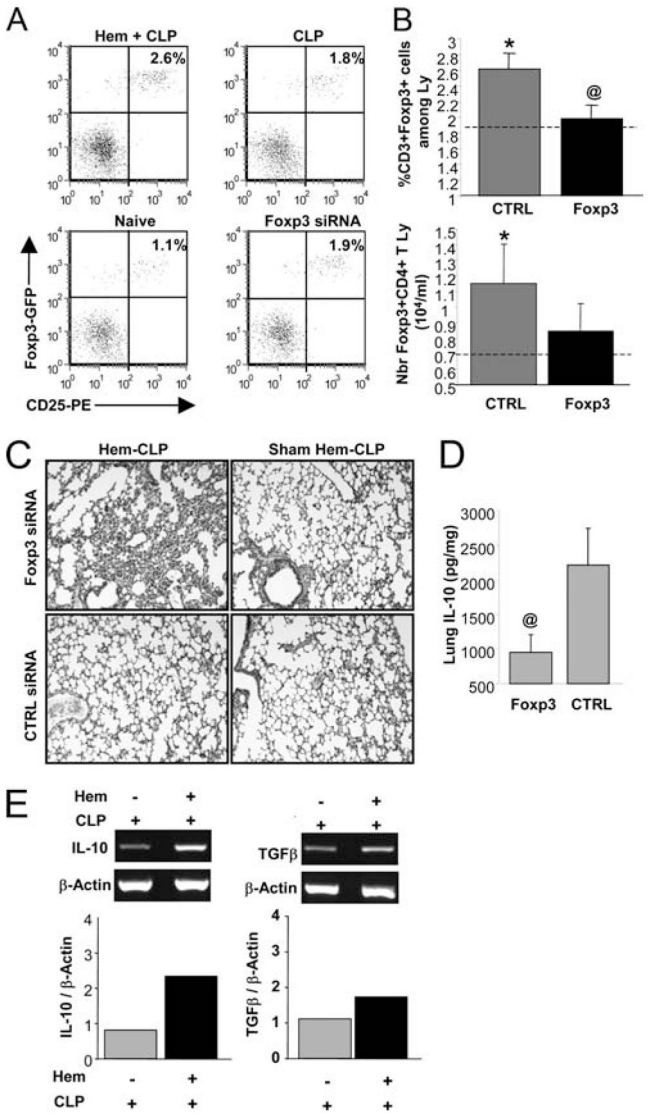

FIGURE 6.

Decreasing Foxp3 expression in the lung recapitulates the effects observed in CD4-deficient/depleted mice and in anti-IL10R Ab-treated mice after Hem + CLP. *A–D*, EGFP-Foxp3 mice were submitted to IT instillation of cationic liposome (DOTAP)- encapsulated Foxp3-targeting (Foxp3) or nontargeting control siRNA (CTRL) 12 h before the induction of indirect ALI by Hem followed 24 h later by polymicrobial septic challenge (CLP). As controls, mice were submitted to sham Hem + CLP. *A*, Lungs were digested and cells were phenotyped for Foxp3, CD3, CD4, and CD25 expressions. Representative dot plots are shown for coexpression of CD25 and Foxp3. Percentages in the *top right quadrants* refer to percentages of cells positive for the four markers measured among total lymphocytes selected based on a CD45/side scatter dot plot. *B*, The percentage of $Foxp3^+CD4^+CD25^+$ T cells among total lymphocytes was measured by flow cytometry and the absolute count was calculated. Results are expressed as percentages of $Foxp3^+CD4^+CD25^+CD3^+$ cells among total lymphocytes and as number of $Foxp3^+CD4^+CD25^+$ T cells (10^4) per ml of lung digest.. *C*, Lungs were fixed, sectioned, and stained with H&E. Representative sections are shown for each group (original magnification, ×200). *D*, The IL-10 level was measured in lung homogenate by ELISA. *E*, Lung

$CD4^+CD25^+$ cells were purified from C57BL/6J mice after Hem + CLP or CLP alone. mRNA expression for IL-10 and TGF-β was measured by densitometric evaluation of PCR results. β -Actin served as internal control. Values are expressed as mean ± SEM (n = 4 mice/group). Dashed line represents sham Hem + CLP mice. @, $p < 0.05$ vs control Hem + CLP group.*, $p < 0.05$ vs sham Hem-CLP group (Mann-Whitney U test).

Table I

Summary of the observations made in lymphocyte-deficient mice after Hem plus CLP[a]

In Comparison to C57BL/6J Mice after Hem + CLP	Lung Apoptosis	Lung Neutrophil Recruitment	Lung Inflammation
$RAG^{-/-}$ mice	↑	No change	↑ MCP-1
$CD8^{-/-}$ mice	↑	No change	↓ IL-6
$\gamma\delta^{-/-}$ mice	No change	No change	No change
$CD4^{-/-}$ mice	No change	↑	No change
$B^{-/-}$ mice	No change	↓	↓ IL-6 and TNF-α

[a]Indirect ALI was induced in C57BL/6J mice (control), RAG, $CD8^+$ T cell ($CD8^{-/-}$)-, $\gamma\delta$ T cel- ($\gamma\delta^{-/-}$)-, $CD4^+$ T cell ($CD4^{-/-}$)-, and B cell ($B^{-/-}$)-deficient mice by Hem followed 24 h later by a polymicrobial septic challenge (CLP). Apoptosis was monitored in whole lung homogenates by the measurement of caspase 3 activity, neutrophil recruitment by the measurement of MPO activity, and cytokine expressions by ELISA.