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MAJOR ARTICLE

Regulatory T Cells Expressing Tumor Necrosis Factor Receptor Type 2 Play a Major Role in CD4$^+$ T-Cell Impairment During Sepsis

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Sepsis causes inflammation-induced immunosuppression with lymphopenia and alterations of CD4$^+$ T-cell functions that renders the host prone to secondary infections. Whether and how regulatory T cells (Treg) are involved in this postseptic immunosuppression is unknown. We observed in vivo that early activation of Treg during *Staphylococcus aureus* sepsis induces CD4$^+$ T-cell impairment and increases susceptibility to secondary pneumonia. The tumor necrosis factor receptor 2 positive (TNFR2^{pos}) Treg subset endorsed the majority of effector immunosuppressive functions, and TNRF2 was particularly associated with activation of genes involved in cell cycle and replication in Treg, probably explaining their maintenance. Blocking or deleting TNFR2 during sepsis decreased the susceptibility to secondary infection. In humans, our data paralleled those in mice; the expression of CTLA-4 was dramatically increased in TNFR2^{pos} Treg after culture in vitro with *S. aureus*. Our findings describe in vivo mechanisms underlying sepsis-induced immunosuppression and identify TNFR2^{pos} Treg as targets for therapeutic intervention.

Keywords. Treg; sepsis; immunosuppression; TNFR2; pneumonia.

Sepsis occurs when an infection leads to a systemic inflammatory response with organ failure, which can lead to septic shock (organ failure and hypotension) and death [1]. The majority of patients who survive the early systemic inflammatory response of sepsis develop a sustained compensatory anti-inflammatory response that is actually a protracted immunosuppressive state that may last for years [2, 3]. During the immunosuppressive phase of sepsis, current data indicate that bacterial pneumonia occurs in 10%–30% of sepsis patients. Interestingly, it has been assumed that the immune function did not recover in those patients who died during the late phase of sepsis [4]. The question to be addressed is why sepsis-induced immunosuppression renders the septic patient prone to secondary pneumonia.

Severe alteration of the lymphocyte compartment is a hallmark of sepsis-induced immune suppression [5, 6]. One of the prevailing theories to explain that lymphopenia induces poor outcomes in septic patients relies on the occurrence of secondary nosocomial infections caused by this sepsis-induced immune impairment [7]. FoxP3$^+$ regulatory T cells (Treg) are good candidates to explain the impairment of the CD4$^+$ T-cell compartment observed during sepsis owing to their highly suppressive function [8, 9]. We found that Treg induce tolerogenic dendritic cells and increase susceptibility to secondary pneumonia following a severe primary infection [10]. However, the mechanisms of early Treg activation during sepsis and whether these cells are responsible for CD4$^+$ T-cell depletion have not been thoroughly investigated to date. Our findings, obtained in a mouse model of *Staphylococcus aureus* septicemia and in human Treg, have shown an early maintenance and activation of Treg with a highly suppressive profile. Our results also suggest that among Treg, tumor necrosis factor receptor 2 positive (TNFR2^{pos}) Treg endorse the majority of effector immunosuppressive functions. The specific blockade or deletion of TNFR2 decreased the susceptibility to secondary infection and could be a therapeutic target to overcome sepsis-induced immunosuppression.

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MATERIALS AND METHODS

Mice

Mice RjOrl:SWISS and C57BL/6NRj were purchased from Janvier Laboratories. C57BL/6-Tg (FoxP3-DTR/EGFP)23.2Spar/Mmjax (later named DEREG) were purchased from Charles River Laboratories and C57BL/6 Tnfrsf1b^{tm1Mwm} (later named TNFR2KO) by B. S. OT-II.Ly5.1.FoxP3EGFP mice (provided by C. L.) were obtained by crossing ovalbumin-specific TCR-transgenic OT-II mice (B6.Cg-Tg(TcraTcrb)425Cbn/J) [11] with Ly5.1 mice (B6.SJL-Ptprca Pepcb/BoyCrl) (Charles River Laboratories) and FoxP3EGFP reporter mice (from Bernard Malissen, Centre d'Immunologie de Marseille-Luminy, Aix Marseille Université, INSERM, CNRS; Centre d'Immunophénomique, Aix Marseille Université, INSERM, CNRS, [12]). Mice were maintained in specific pathogen-free conditions at the IRS2 Institute Animal Care Facility, Nantes, France, following institutional guidelines and were used for experiments between 6 and 14 weeks of age. Experimental procedures were approved by the Animal Ethics Committee of the University of Nantes (protocol number 2126–2130). Mice were used with a male to female ratio of 1:1 for each experiment.

Sepsis Model

S. aureus ATCC29213 and *Pseudomonas aeruginosa* PAO1 strains were grown in brain heart infusion broth overnight at 37°C with agitation. Immediately before use, the bacterial pellet was washed twice with phosphate-buffered saline (PBS) and the inoculum was calibrated by nephelometry.

After general anesthesia by continuous inhalation of isoflurane (3% fresh gas flow 0.8 L/min), the mouse was placed in prone position and 200 µL *S. aureus* suspension (at 10^9 colony-forming units [CFU]/mL) was intravenously injected through the suborbital venous plexus. When required, pneumonia was induced as previously described [13]. Briefly, mice were anesthetized with isoflurane (3% fresh gas flow 0.8 L/min) and placed in dorsal recumbency. Intratracheal insertion of a 24-gauge feeding needle was used to inject 75 µL *P. aeruginosa* suspension adjusted to 10^8 CFU/mL (7.5×10^6 CFU/mouse). Importantly, the overall mortality remained below 5% at the time of analysis: day 3 for T-cell phenotype and day 8 for OT-II cell proliferation in sepsis-positive mice, and day 4 for lung injury measurement in the double-hit model (Supplementary Figure 1G).

Murine Treg Isolation and Analysis

Murine splenocytes were stained with monoclonal antibodies to anti-CD3 (145-2C11; BD Bioscience), anti-CD4 (RM4-5; BD Pharmingen), anti-CD25 (PC61; BD Horizon), anti-CD62L (MEL-14; BD Horizon), anti-TNFR2 (TR75-89; BD Pharmingen), anti-CD45.1 (A20.1; eBioscience), anti-TCR VαD (B20.1; BD Bioscience), anti-FoxP3 (MF14; Biolegend), anti-CTLA-4 (UV10-4B9; Biolegend), anti-IL-10 (JES5-16E3; BD Horizon), and Fixable Viability Dye (eBioscience). Interleukin-10 (IL-10) staining was performed using a fixation/permeabilization solution for cytokines (Fixation/Permeabilization Solution Kit with BD GolgiPlug; Biosciences), and FoxP3 cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) straining using the FoxP3 Transcription Factor Staining Buffer Kit (eBioscience), according to manufacturer instructions. Samples were acquired on an LSR-II flow cytometer (Becton Dickinson) and analyzed using Flowjo Software (TreeStar).

Induction of OT-II Cell Proliferation After Adoptive Transfer

OT-II T cells were purified from pooled lymph nodes and spleen of OT-II mice after lysis of the red blood cells and CD4$^+$ T-cell enrichment (Miltenyi Biotech) (purity was 85%–95%). Mice were injected IV with soluble ovalbumin (0.1 mg, A5503; Sigma-Aldrich) and OT-II T cells ($0.5–1.5 \times 10^6$ cells). Five days later, cells from the spleen were stained with anti-CD4, anti-CD45.1, and anti-TCRVα2, and resuspended in buffer containing $1–3 \times 10^4$ blank calibration particles (Becton Dickinson). The total number of live dividing OT-II T cells was calculated from the number of dividing cells relative to the number of beads present in each sample. Control mice received OT-II T cells with PBS alone (without ovalbumin).

Cytokine Levels, Bacteriological Counts, and Histology and Immunohistochemistry Analysis

Cytokines levels in the lungs and spleens were measured by enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (eBioscience). Bacterial counts and histology and immunohistochemistry analysis were determined as previously described in spleen and lung [13]. After scanning histological sections with Nanozoomer 2.0–HT C9600 (Hamamatsu) and capture with the manufacturer's NDP viewer software, lung recruitment of neutrophils was evaluated on histology slides using the Single Integrative Object eXtraction (SIOX) plugin available in Fiji open-source image analysis software (www.fiji.sc). For assessment of edema, pixel ratios between alveolar spaces and tissue areas were determined. For assessment of neutrophil accumulation, ratios of Ly6-G positive pixels to edema pixels were determined. Data were expressed as percent of lung tissue.

Treg depletion, anti-TNFR2 monoclonal antibody treatments, human peripheral blood mononuclear cell (PBMC) sepsis induction, Treg analysis, and RNA sequencing methods are detailed in Supplementary Methods.

Statistical Analysis

Data were plotted using GraphPad prism. Mann-Whitney unpaired test was used for comparisons between 2 groups. A Kruskal-Wallis test was used for multiple comparisons. When

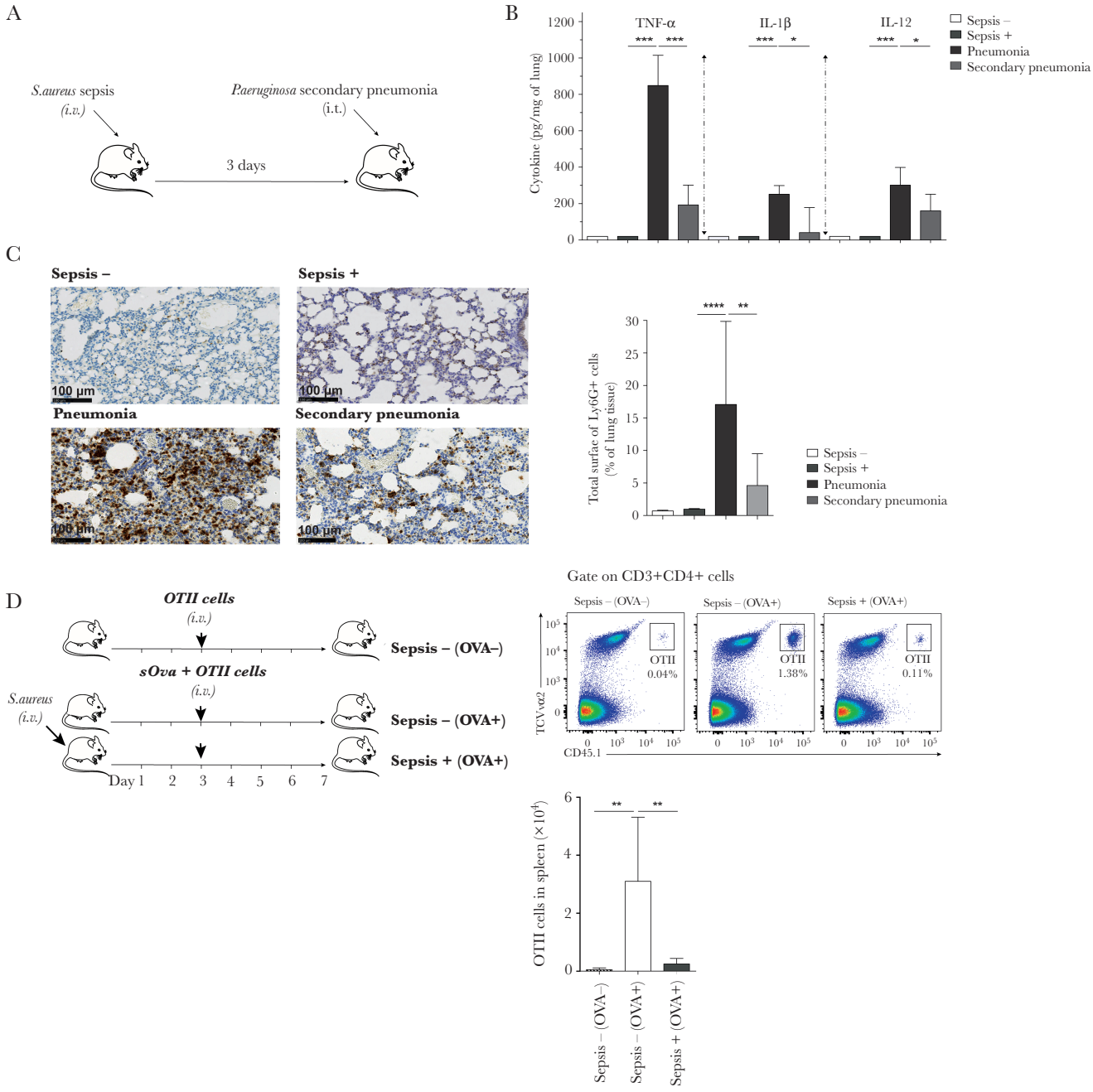

Figure 1. Primary *Staphylococcus aureus* sepsis induces a susceptibility to secondary pneumonia. *A*, Experimental outline of intravenous (IV) injection of *S. aureus* (sepsis) followed 3 days later by intratracheal (IT) injection of *Pseudomonas aeruginosa* (pneumonia). We compared: uninfected mice (sepsis–); after 3 days of *S. aureus* sepsis alone (sepsis+); *P. aeruginosa* pneumonia alone (pneumonia); and pneumonia after 3 days of *S. aureus* sepsis (secondary pneumonia). Samples were taken 72 hours after sepsis or 24 hours after pneumonia, unless otherwise stated. *B*, Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-12 concentrations in lung homogenates. TNF-α was measured 6 hours after onset of pneumonia and IL-1 β and IL-12, 24 hours after pneumonia alone or secondary pneumonia. For control, TNF-α, IL-1β, and IL-12 were also measured 72 hours after sepsis (sepsis+) and in sepsis– mice. *C*, Lung sections were analyzed to quantify neutrophil infiltration (by anti-Ly6-G immunohistochemistry) in sepsis–, 72 hours after sepsis (sepsis+), 24 hours after pneumonia alone (pneumonia), and secondary pneumonia. *D*, Schematic diagram of the experimental transfer of OT-II cells. Uninfected (sepsis–OVA+) and sepsis (sepsis+OVA+) CD45.2$^+$ mice were IV injected with ovalbumin (OVA) and naive OT-II cells (CD3$^+$CD4$^+$CD45.1$^+$ TCRVα2$^+$). For control, uninfected mice were injected with naive OT-II cells (IV) without OVA (sepsis–OVA–). OT-II cell proliferation was assessed in the spleen 5 days after injection of OVA. **P* < .05, ***P* < .01, ****P* < .001. *B–D,* Data are representative of at least 2 independent experiments with at least 5 mice per group. Graphs represent median ± SD.

the difference was significant, a Fisher test was used for intergroup comparison. *P* value < .05 was used for statistical significance.

RESULTS

Sepsis Increases the Susceptibility to Secondary Pneumonia and Induces Immunosuppression

To mimic the frequent clinical scenario of sepsis followed by secondary pneumonia [4], we induced sepsis in mice with an intravenous injection of *S. aureus* followed 3 days later by intratracheal injection of *P. aeruginosa* to induce secondary pneumonia (Figure 1A). In mice with sepsis alone, we observed a spontaneous bacterial clearance (Supplementary Figure 1A) and a systemic inflammatory response in the spleen (Supplementary Figure 1B–D), as reported in septic patients [1, 14]. In order to find out if sepsis alters the lung response to a secondary pneumonia, we compared the host response to a secondary *P. aeruginosa* pneumonia in mice previously infected or not by *S. aureus*. A dramatic weight loss was observed until day 6 during secondary pneumonia, which could not be explained by a higher burden of bacteria or by the additive effect of the 2 hits taken separately [15, 16] (Supplementary Figure 1E–G). The inflammatory response was also impaired during secondary pneumonia compared with pneumonia alone (Figure 1B and 1C and Supplementary Figure 1H). These results prompted us to evaluate if an alteration of the $CD4^+$ T-cell compartment induced by *S. aureus* sepsis could explain the impairment of the host response against secondary pneumonia. We first tested the ability of the immune system to respond in vivo to a newly encountered extracellular antigen after the sepsis initiation, as assessed by the proliferation of $CD4^+$ T cells. For this, we used $CD4^+$ T cells from OT-II $CD45.1^{pos}$ transgenic mice expressing a T-cell receptor that is specific for ovalbumin-derived peptide and assessed OT-II T-cell proliferation in the spleen 5 days after adoptive transfer and ovalbumin immunization of mice on the third day of sepsis. Strikingly, the number of OT-II T cells was dramatically decreased in septic mice infected with *S. aureus* compared with uninfected control mice (Figure 1D). We then questioned if the decreased OT-II proliferation was associated with $CD4^+$ T-cell intrinsic defect. The number of splenic $CD4^+$ T cells was also drastically decreased after *S. aureus* sepsis (Figure 2A), as observed in patients [7]. This series of experiments demonstrated that this model of *S. aureus* sepsis mimicked a clinical scenario with severe direct and indirect impairment of the $CD4^+$ T cells in vivo associated with an altered pulmonary response to secondary pneumonia.

Sepsis Induces Major Treg Activation That Generates a Suppressive Environment

We then assessed the role of Treg in the $CD4^+$ T-cell impairment caused by *S. aureus* sepsis. Despite the important $CD4^+$ T-cell loss induced by sepsis, the Treg number remained unaltered, resulting in an increased percentage of Treg among $CD4^+$ T cells (Figure 2A), and advocating for a specific response to sepsis of Treg among $CD4^+$ T cells.

In order to better characterize the effects of sepsis on Treg, we analyzed the transcriptome of splenic Treg 3 days after the induction of sepsis (additional detail on the method for making these measurements is in Supplementary Methods). Principal component analysis of 16 112 expressed genes revealed that gene expression profiles of uninfected Treg and sepsis Treg were separated by principal component 1 (81% variance explained), suggesting that most gene expression variance was associated with infection rather than experimental variation (principal component 2, 8%) (Supplementary Figure 2A). Therefore we estimated differentially expressed genes and found 492 that were upregulated and 96 downregulated, with an absolute log2-fold change above 1.5 (*Q* value < .05) (Figure 2B). *Gzmb*, *Mki67* (Ki67), and *Havcr2* (TIM-3) were among the most strongly expressed genes in sepsis Treg, suggesting that Treg from septic mice were highly activated compared with controls. To gain a more comprehensive overview of the underlying molecular pathways implicated in the sepsis, these differentially expressed genes were analyzed for gene ontology enrichment. This revealed that sepsis was associated with a transcriptional program driving biological processes, including cell cycle replication and immunological response such as adaptive immune regulation (Supplementary Figure 2B).

The *Irf4* and *Prdm1* (Blimp 1) transcriptional factors, which are required in a nonredundant manner to maintain effector Treg differentiation and functions [17, 18], were also upregulated in sepsis Treg (Figure 2B). Many of the upregulated genes (Figure 2B and Supplementary Table 1) encode for molecules involved in the activation and suppressive function of Treg, suggesting that Treg from septic mice were highly suppressive [19]. We validated the differential expression of 2 important markers of activation (CD62L, CD25) [20, 21] and 2 suppressive mediators (CTLA-4 and IL-10) [22] at a protein level (Figure 2C). Overall, these results suggest that sepsis triggered the maintenance and activation of Treg.

To demonstrate the role of Treg in sepsis-induced immunosuppression, we depleted Treg after the initiation of *S. aureus* sepsis by injection of diphtheria toxin in mice expressing the diphtheria toxin receptor under the control of the FoxP3 gene promotor ($FoxP3^{DTR}$) (Supplementary Figure 2D). Importantly, the depletion of Treg in septic mice restored the expansion of ovalbumin-specific OT-II T cells (Figure 2D) and partially limited $CD4^+$ T-cell loss in infected mice (Supplementary Figure 2D). Altogether, these data demonstrated that during sepsis the early activation of Treg participates in the immunosuppression that increases the susceptibility to secondary pneumonia.

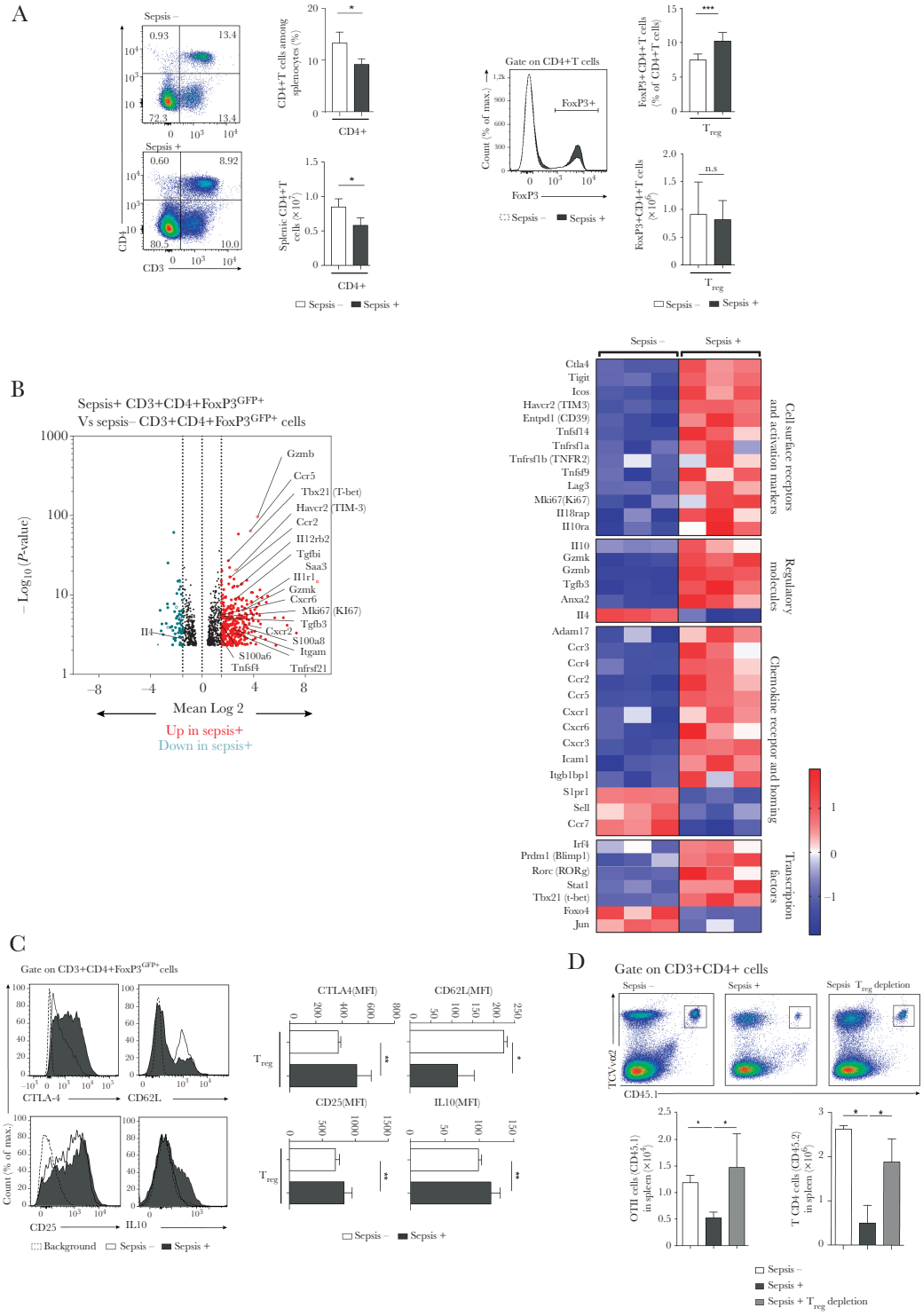

Figure 2. Regulatory T cells (Treg) play a major role in functional alterations of conventional CD4$^+$ T cells. *A*, Splenic CD4$^+$ T-cell proportions and numbers in uninfected (sepsis–) and septic mice 3 days after *Staphylococcus aureus* infection (sepsis+). Splenic Treg (CD3$^+$CD4$^+$FoxP3$^+$) proportions and numbers in uninfected (sepsis–) and septic mice (sepsis+). *B*, Volcano plot of differential gene expression between Treg from sepsis– and sepsis+ mice. Significantly differentially expressed genes (*Q* value < .05) and up- and downregulated genes (log2-fold change >1.5 and < –1.5) in sepsis Treg are shown in red and blue, respectively (left). Heatmap of differentially expressed genes (*Q* value < .05) encoding selected activation markers, regulatory molecules, chemokine receptors, and transcription factors in sepsis+ and sepsis– Treg (right). *C*, Intracellular expression of CTLA-4, interleukin-10 (IL-10), and membrane expression of CD62L, CD25 in Treg from sepsis– and sepsis+ mice. Abbreviation: MFI, mean fluorescence intensity. *D*, Sepsis– and sepsis+ CD45.2$^+$ mice with and without diphtheria toxin (DT)-treated FoxP3-DT receptor (sepsis+ Treg depletion) were IV injected with ovalbumin (OVA) and naive OT-II cells (CD3$^+$CD4$^+$CD45.1$^+$ TCRvα^+). OT-II proliferation was assessed 5 days after OT-II transfer. For Treg depletion, mice were injected IP with DT (0.5 µg first injection and 0.2 µg following injections) administered 12 hours and day 1, 2, 3, 5, and 7 after *S. aureus* sepsis. Number of splenic CD4$^+$CD45.2 T cells at day 8 of sepsis–, sepsis+, and sepsis+ Treg depletion group. **P* < .05, ***P* < .01, ****P* < .001, NS, not significant. *A, C,* and *D*, Data are representative of at least 3 independent experiments with at least 5 mice per group (median ± SD). *B*, Data are representative of 1 experiment with n = 2 treatments in triplicate (sepsis–, sepsis+) and 2 mice were pooled for each treatment.

TNFR2^{pos} Treg Are Highly Activated and May Suppress CD4$^+$ T Cells During Sepsis

While activation of Treg has been described during the late phase of sepsis or chronic inflammation [23], little is known regarding their early activation during the acute inflammatory response of sepsis [24]. The expression of TNFR2 identifies a subset of Treg with the highest suppressive capacity [25, 26], and the transcript of the *TNFR2* gene as well as those of its downstream NF-κB signaling pathway were increased in septic Treg (Figure 2B). We thus hypothesized that the activation of Treg during sepsis was dependent on TNFR2. To examine this hypothesis, we measured the proportion of the TNFR2^{pos} subset among Treg in septic mice and found that the percentage and the number of TNFR2 ^{pos} Treg were increased as compared to uninfected mice (Figure 3A). We observe that TNFR2 expression is overwhelmingly associated with the Treg lymphocyte population in uninfected mice, which is particularly significant in sepsis (Supplementary Figure 3A).

To evaluate whether there is specific gene activation of the TNFR2^{pos} Treg subset, we investigated the transcriptomic differences between splenic TNFR2^{pos} and TNFR2^{neg} Treg 3 days after the onset of sepsis. We found 194 upregulated and 415 downregulated genes in TNFR2^{pos} Treg (>1.5 log2-fold change, Q value < .05; Figure 3B). Interestingly, *Gzmb*, *IL10*, *CCR5*, *Havcr2* (TIM-3), and Tigit genes were upregulated in TNFR2^{pos} Treg. Also, many activation markers, regulatory molecules, chemokine receptors, transcription factors, and antiapoptotic molecules were upregulated in sepsis TNFR2 ^{pos} Treg (Figure 3C). The results of the gene ontology analysis suggest that among Treg, TNFR2^{pos} Treg endorse the majority of effector immunosuppressive functions compared with TNFR2^{neg} Treg (Supplementary Figure 3B). Finally, we validated by flow cytometry the preferential upregulation of CTLA-4 in TNFR2^{pos} Treg (Figure 3D).

TNFR2^{pos} Treg Subset Associated With Cell Cycle and Replication During Sepsis

To investigate how the TNFR2^{pos} Treg contribute to the transcriptomic signature of Treg observed in sepsis (Figure 2), we used the sepsis differentially expressed genes to map each condition on a reduced dimensional space using principal component analysis (Figure 4A). During sepsis, total Treg were closer to TNFR2^{pos} Treg subset than to TNFR2^{neg} cells, suggesting that the TNRF2^{pos} Treg transcriptome is a major contributor to the Treg response. To gain a deeper understanding of the role of TNFR2 on the activation of Treg, we used unsupervised hierarchical clustering of the differentially expressed gene of the sepsis differentially expressed genes in all 4 conditions (Figure 4B) and found that differentially expressed genes can be subdivided in 2 separate clusters. Cluster 1, corresponding to genes activated in sepsis Treg and the TNFR2^{pos} subset, were associated with cell cycle and replication (Figure 4B). Genes in cluster 2, corresponding to those activated in sepsis Treg and in the TNFR2^{neg} subset, drive inflammatory response (Figure 4B). This series of experiments strongly suggests that TNFR2^{pos} Treg display a higher lineage stability, a higher capacity to proliferate, and the TNFR2^{pos} Treg retain the majority of suppressive functions during sepsis.

To demonstrate in vivo the role of TNFR2 in the activation of Treg during sepsis, we infected TNFR2 knock-out mice (TNFR2 KO). Uninfected TNFR2-deficient mice spontaneously display a lower number of splenic CD4$^+$ T cells than wild-type mice (Supplementary Figure 4A). In TNFR2-deficient mice, sepsis does not increase the percentage of Treg among CD4$^+$ T cells (Supplementary Figure 4A). The expression of CTLA4 remained unchanged during sepsis in deficient mice whereas a significant increase was observed in wild-type mice (Figure 4C). Moreover, the expansion of OT-II cells in response to immunization with ovalbumin was impaired in wild-type mice but remained unchanged in TNFR2-deficient mice during sepsis (Figure 4D). Overall, these results demonstrate the role of TNFR2 in the activation of Treg during sepsis.

Therapeutic Use of a Blocking Monoclonal Anti-TNFR2 Antibody

We then questioned if the inhibition of TNFR2 with a blocking monoclonal antibody could decrease the susceptibility to secondary pneumonia. To respond to this question, we treated wild-type mice from day 1 to day 3 of sepsis by IP injection with a blocking monoclonal antibody against TNFR2 (Figure 5A and 5B). The treatment of septic mice with the blocking anti-TNFR2 antibody partially restored the expansion of OT-II cells in response to ovalbumin injected 3 days after the onset of sepsis (Figure 5A and Supplementary Figure 5A). In septic mice treated with the blocking anti-TNFR2 antibody, the lung neutrophil infiltrate was restored during secondary pneumonia [27] (Figure 5B and Supplementary 5B). Overall, this series of experiments shows that a monoclonal antibody blocking TNFR2 has the potential to treat sepsis-induced immunosuppression.

Expression of TNFR2 on Human Treg

We then aimed to verify the extrapolation of our findings in humans. We analyzed PBMCs from healthy donors cultured in vitro with *S. aureus* for 2 hours. Under sepsis condition, the total CD4$^+$ T-cell number was drastically decreased and the number of CD4$^+$FoxP3$^+$ T cells was not altered; however, the percentage of FoxP3$^+$ cells among CD4$^+$ T cells was significantly enhanced (Figure 6A). The expression of CTLA-4 and CD25 were preferentially upregulated in the presence of *S. aureus* in the TNFR2^{pos} Treg subset (Figure 6B). These experimental results in human cells parallel those obtained in our mouse model.

DISCUSSION

Our results can be summarized as follows. Treg are involved early in the immunosuppression observed during sepsis. Among the population of Treg, the TNFR2^{pos} subset appears to play a particular role because a specific blockade or deletion of TNFR2 enhances the

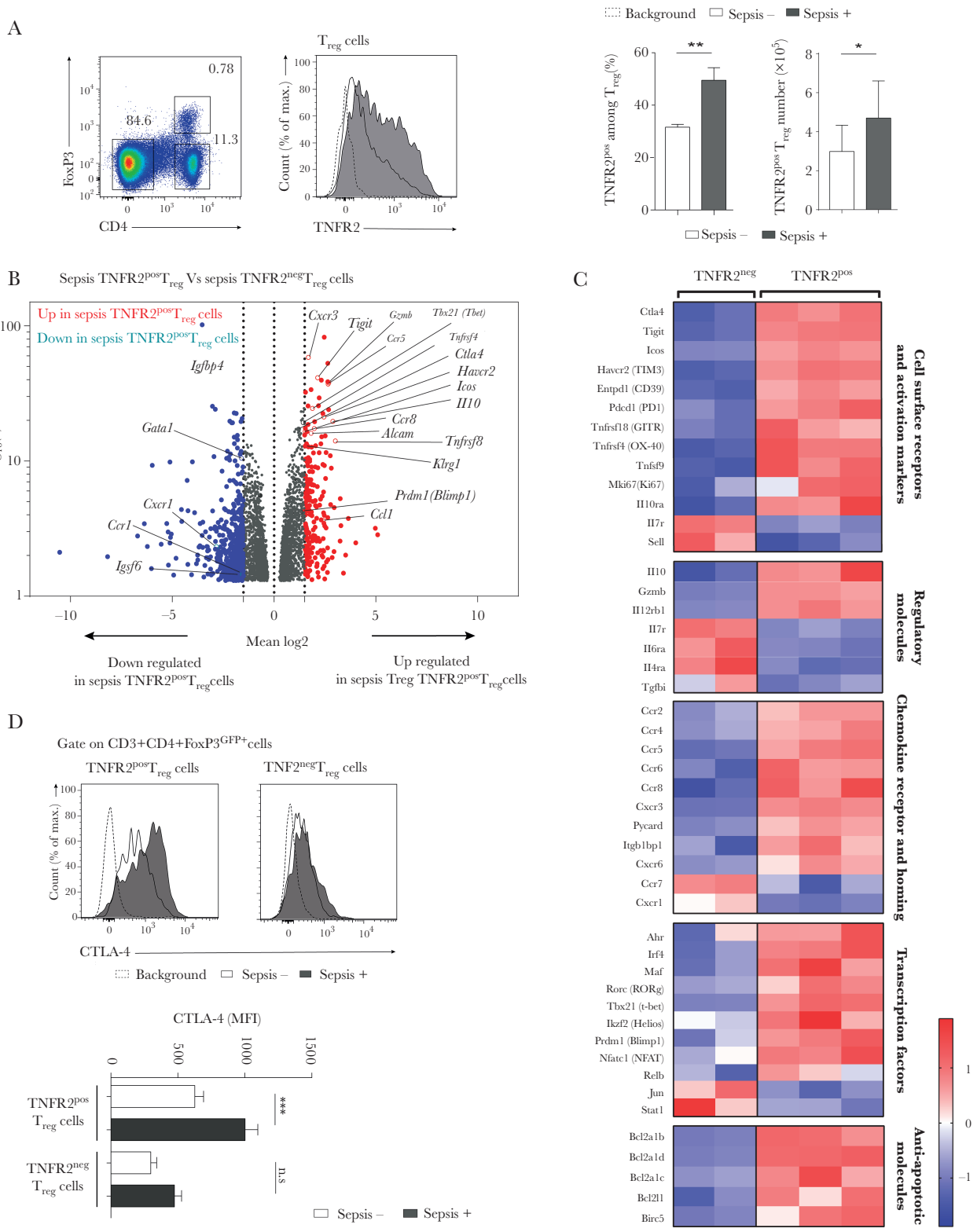

Figure 3. Transcriptome and phenotype analysis of TNFR2^{neg} and TNFR2^{pos} regulatory T cells (Treg) during sepsis. *A*, Splenic TNFR2^{pos} Treg proportions and numbers in uninfected mice (sepsis–) and 3 days after *Staphylococcus aureus* infection (sepsis+). *B*, Volcano plot of differential gene expression between TNFR2^{pos} Treg and TNFR2^{neg} Treg from septic mice. Significantly differentially expressed genes (*Q* value < .05), up- and downregulated genes (log2-fold change >1.5 and < –1.5) in sepsis TNFR2^{pos} Treg are highlighted in red and blue, respectively. *C*, Heatmap of differentially expressed genes (*Q* value < .05) encoding selected activation markers, regulatory molecules, chemokine receptors, transcription factors, and antiapoptotic molecules in TNFR2^{pos} Treg and TNFR2^{neg} Treg from septic mice. *D*, Intracellular expression of CTLA-4 in TNFR2^{pos} Treg and TNFR2^{neg} Treg from uninfected (sepsis–) and septic mice (sepsis+). Abbreviation: MFI, mean fluorescence intensity. **P* < .05, ***P* < .01, ****P* < .001, NS, not significant. *A* and *D*, Data are representative of at least 3 independent experiments with at least 5 mice per group. Graphs represent median ± SD. *B* and *C*, Data are representative of 1 experiment with n = 3 samples per group and 3 mice per sample.

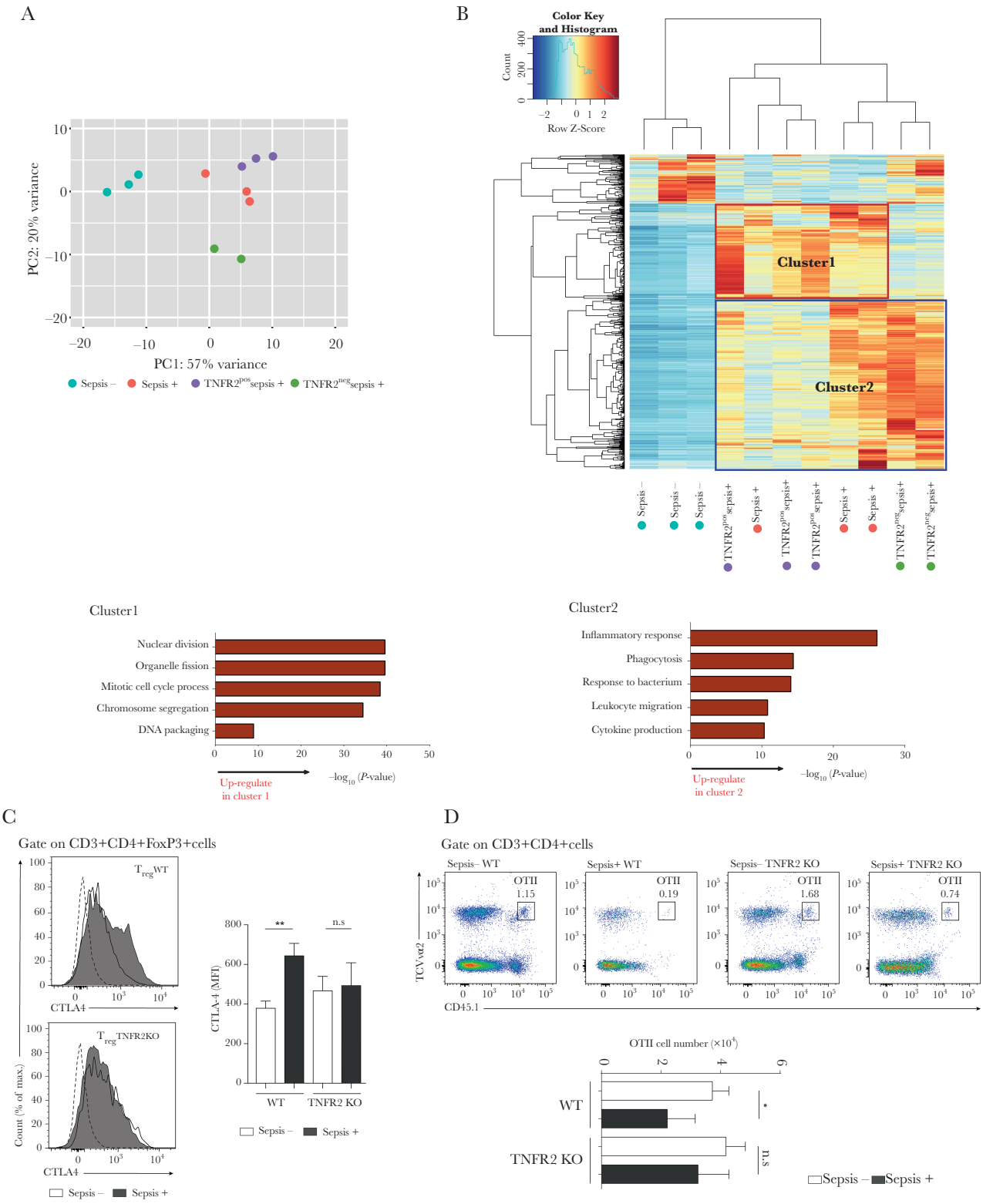

Figure 4. TNFR2^{pos} regulatory T cells (Treg) subset was associated with cell cycle and replication during sepsis. *A*, Principal component analysis of Treg sepsis–, Treg sepsis+, TNFR2^{pos} Treg, and TNFR2^{neg}Treg principal component 1 (PC1) and PC2 show the percentage of variance explained for 15 337 expressed genes. *B*, Heatmap of unsupervised hierarchical clustering of the differentially expressed gene (corrected *Q* value < .05, >1.5 fold) between sepsis+ Treg, sepsis– Treg, TNFR2^{pos} Treg sepsis+, and TNFR2^{neg} Treg sepsis+. Clustering grouped the splenic Treg into 2 gene clusters. Gene ontology analysis (Toppgene) of genes in cluster 1 and cluster 2. Bar charts displays $-\log_{10}$ *P* value. *C*, Intracellular expression of CTLA-4 in Treg was measured from uninfected and sepsis wild-type (WT) mice and in TNFR2 knockout (KO) mice. *D*, In CD45.2^{pos} mice, uninfected (sepsis–) or septic (sepsis+) WT or TNFR2 KO animals were injected (IV) with ovalbumin and OT-II cells (CD3$^+$CD4$^+$CD45.1$^+$ OT-II). OT-II proliferation was assessed 5 days later in the spleen. **P* < .05, ***P* < .01, ****P* < .001, NS, not significant. *C* and *D*, Data are representative of at least 3 independent experiments with at least 5 mice per group. Graphs represent median ± SD. *A* and *B*, Data are representative of 1 experiment with n = 3 samples per group and 3 mice per sample.

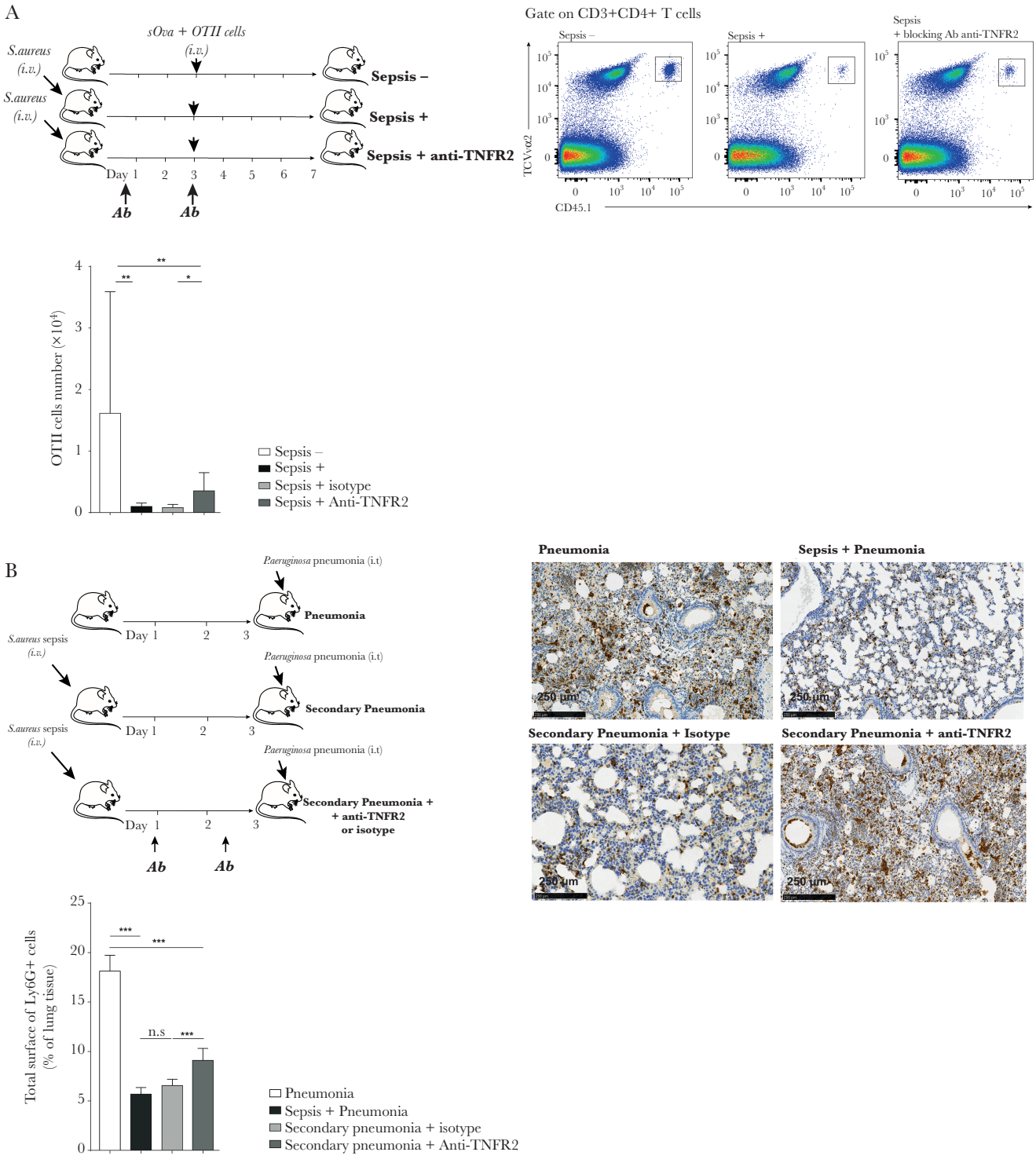

Figure 5. TNFR2 functions during sepsis. *A*, Schematic diagram of the monoclonal anti-TNFR2 blocking antibody treatment and transfer of OT-II cells during *Staphylococcus aureus* sepsis. Septic mice were treated with blocking monoclonal antibody (mAb) anti-TNFR2 (sepsis+ anti-TNFR2) or with isotype control at day 1 and day 3. Mice were then injected IV with ovalbumin (Ova) and OT-II cells. The proliferation of OT-II cells was assessed 5 days later in the spleen. *B*, Schematic diagram of *Pseudomonas aeruginosa* pneumonia alone (pneumonia group), and *P. aeruginosa pneumonia* induced 3 days after *S. aureus* sepsis (secondary pneumonia group) treated with blocking mAb anti-TNFR2 (sepsis+ anti-TNFR2) or with isotype control at day 1 and day 3. Polynuclear infiltration was measured in lung sections collected 24 hours after *P. aeruginosa* pneumonia by anti-Ly6-G immunohistochemistry. **P* < .05, ***P* < .01, ****P* < .001, NS, not significant. Data are representative of 2 independent experiments with at least 5 mice per group. Graphs represent median ± SD.

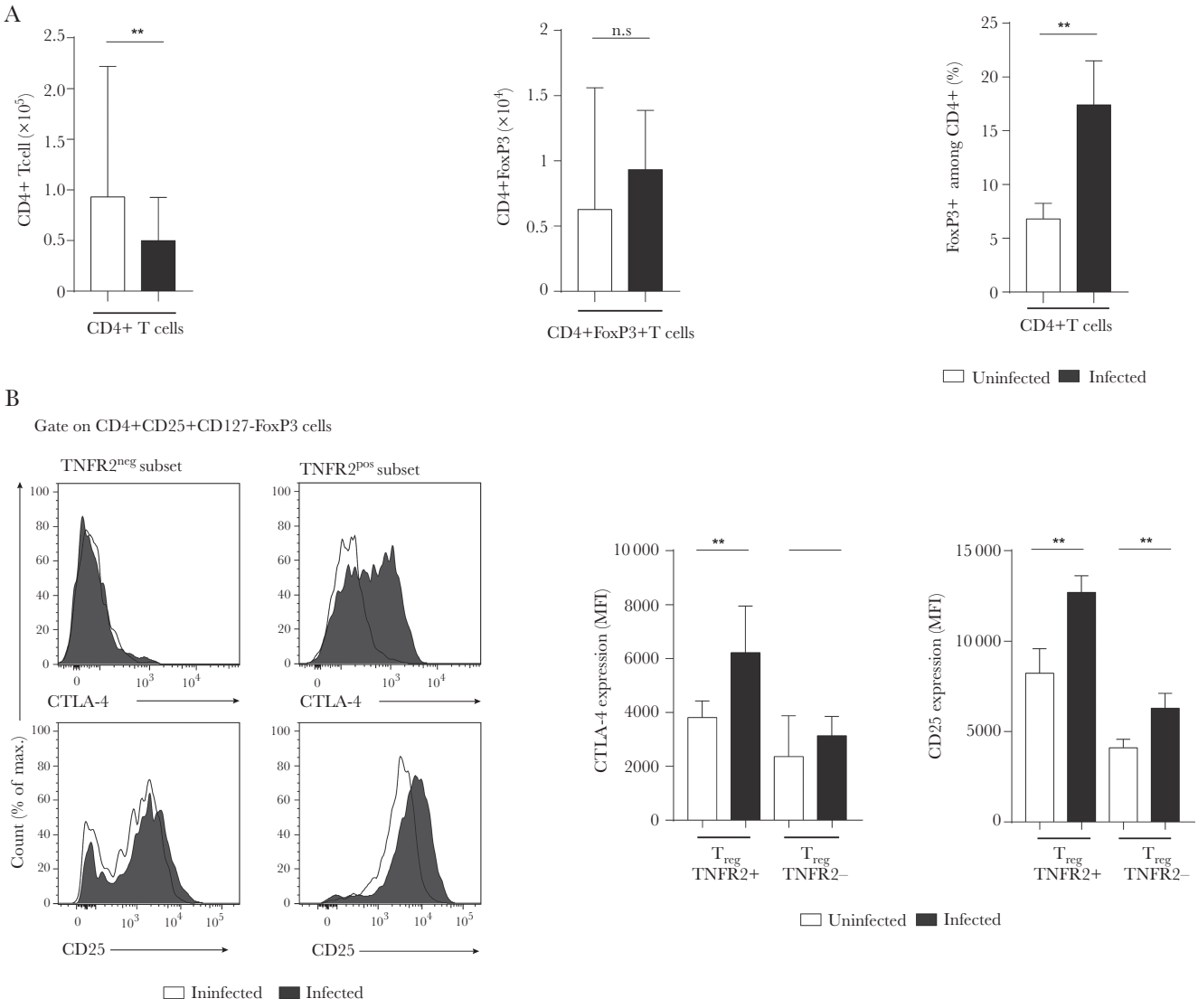

Figure 6. Expression of TNFR2 on human regulatory T cells (Treg). *A*, Number of $CD4^+$ T cells and number and frequency of $FoxP3^+CD4^+$ T cells in peripheral blood mononuclear cells (PBMC) from healthy controls cultured without (uninfected) or with *Staphylococcus aureus* (infected). *B*, Expression of CTLA-4, CD25 among Treg ($CD4^+CD25^+CD127$-$FoxP3^+$) $TFNR2^{pos}$ or $TNFR2^{neg}$ cells in PBMC from healthy controls cultured without (uninfected) or with *S. aureus* (infected). $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, NS, not significant. Data are representative of 10 healthy controls. Graphs represent median ± SD. Abbreviation: MFI, mean fluorescence intensity.

number and functions of $CD4^+$ T cells. Our results obtained in cells from healthy donors parallel those obtained in mice.

We particularly focused on the capacity of the immune system to face secondary bacterial pneumonia, and we developed an original and easy to perform sepsis model in which a significant decrease in the absolute number of $CD4^+$ T cells is observed. Treg are probably important players in the sepsis-induced immunosuppression phenomenon because anergic and suppressive functions are the most important functional properties for these cells, as demonstrated in malaria [24, 28]. In our results, 3 major findings suggest an enhanced Treg function in the septic host: (1) enhanced Treg ratio, (2) restoration of number/proliferation of $CD4^+$ T cells after Treg depletion, and (3) transcriptomic signature of activated Treg confirmed at the protein level by the upregulation of CTLA-4 expression and IL-10 production. The expression of functionally important molecules such as CTLA-4 is restricted to or greatly increased in the subpopulation of Treg with an activated phenotype [17, 19].

Treg undergo further differentiation in the periphery and acquire a fully suppressive effector phenotype characterized by the production of the immunosuppressive cytokine IL-10 [17] and by the downregulation of CD62L [29]. In accordance with these data, we retrieved in Treg a decreased expression of CD62L and increased intracellular expression of IL-10 in sepsis compared with sham-infected animals.

An increased level of TNF-α is a hallmark of severe sepsis, and therefore anti-TNF therapies targeting TNF or TNFR1 were tested in septic shock in an attempt to blunt the initial overwhelming inflammatory response [30, 31]. These studies failed to show any clinical benefit, probably because blocking TNFR1 on effector $CD4^+$ T cells was difficult considering the high rate of apoptosis and T-cell exhaustion. Moreover, the anti-inflammatory effects of these therapies may have aggravated immunosuppression. Oppenheim and his team were the first to demonstrate the particular immunosuppressive capacities of the $TNFR2^{pos}$ Treg population in mice [25]. Blocking TNFR2 with monoclonal antibodies impairs Treg accumulation in tumors [32], suggesting a potential for treatment. Prosurvival genes were significantly upregulated during sepsis in $TNFR2^{pos}$ Treg versus $TNFR2^{neg}$ Treg in our RNA sequencing analysis, and Bcl-2 is a critical target in IL-2 signaling [33], protecting cells from apoptosis. Finally, the TNFR2 subset of Treg is considered to be highly suppressive in chronic human pathologies like cancer and inflammatory diseases [25, 26]. Our data obtained in a mouse model and in humans strongly suggest that these cells participate in immunosuppression induced by sepsis. Alternatively, the increased CD4 T-cell number observed following TNFR2 blockade may be due, at least partly, to reduced cell death because it has been shown that TNFR2 signaling can induce activation-induced cell death in CD4 T cells [34]. Monocytes also express TNFR1 and TNFR2 [35, 36] and therefore we cannot exclude that some of our results may be secondary to the activity of TNFR2 on cells other than Treg.

In this model, we assessed the induction of immunosuppression at an early stage of sepsis (day 3 with persistent bacteremia) because it is the optimal time for a proposed therapeutic intervention in an attempt to avoid a secondary infection caused by immunosuppression. The early detection of immunosuppression (day 4) in patients with sepsis is now well-established in human studies [37, 38] and is the basis of immunotherapy strategies (IL-7, GM-CSF, and anti-PD1 mAb) to overcome postseptic immunosuppression.

In conclusion, during sepsis the early activation of Treg by TNFR2 induces a state of immunosuppression and increases the susceptibility to secondary pneumonia. The restricted expression of TNFR2 on Treg is ideal for therapeutic interventions because targeting this receptor could reduce the toxicity observed with untargeted inhibition of Treg such as anti-CTLA4 [39]. We therefore propose an original and tailored therapy for sepsis patients by blocking Treg with anti-TNFR2 antibodies, with stratification to treat only septic patients with immunosuppression.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Data and materials availability. Data can be accessed upon request from the server of the University of Nantes, France.

References

1. Delano MJ, Ward PA. Sepsis-induced immune dysfunction: can immune therapies reduce mortality? J Clin Invest **2016**; 126:23–31.
2. Vught LA, Klein Klouwenberg PMC, Spitoni C, et al. Incidence, risk factors, and attributable mortality of secondary infections in the intensive care unit after admission for sepsis. JAMA **2016**; 315:1469–79.
3. Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. Lancet Infect Dis **2013**; 13:260–8.
4. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. Nat Rev Immunol **2013**; 13:862–74.
5. Schwulst SJ, Grayson MH, DiPasco PJ, et al. Agonistic monoclonal antibody against CD40 receptor decreases

lymphocyte apoptosis and improves survival in sepsis. J Immunol **2006**; 177:557–65.
6. Boomer JS, To K, Chang KC, et al. Immunosuppression in patients who die of sepsis and multiple organ failure. JAMA **2011**; 306:2594–605.
7. Drewry AM, Samra N, Skrupky LP, Fuller BM, Compton SM, Hotchkiss RS. Persistent lymphopenia after diagnosis of sepsis predicts mortality. Shock **2014**; 42:383–91.
8. Belkaid Y, Piccirillo CA, Mendez S, Shevach EM, Sacks DL. $CD4^+CD25^+$ regulatory T cells control *Leishmania* major persistence and immunity. Nature **2002**; 420:502–7.
9. Qureshi OS, Zheng Y, Nakamura K, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. Science **2011**; 332:600–3.
10. Roquilly A, McWilliam HEG, Jacqueline C, et al. Local modulation of antigen-presenting cell development after resolution of pneumonia induces long-term susceptibility to secondary infections. Immunity **2017**; 47:135–47.e5.
11. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. Immunol Cell Biol **1998**; 76:34–40.
12. Wang Y, Kissenpfennig A, Mingueneau M, et al. Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of $Foxp3^+$ regulatory T cells. J Immunol **2008**; 180:1565–75.
13. Broquet A, Roquilly A, Jacqueline C, Potel G, Caillon J, Asehnoune K. Depletion of natural killer cells increases mice susceptibility in a *Pseudomonas aeruginosa* pneumonia model. Crit Care Med **2014**; 42:e441–50.
14. Shankar-Hari M, Phillips GS, Levy ML, et al; Sepsis Definitions Task Force. Developing a new definition and assessing new clinical criteria for septic shock: for the third international consensus definitions for sepsis and septic shock (Sepsis-3). JAMA **2016**; 315:775–87.
15. Delano MJ, Moldawer LL. The origins of cachexia in acute and chronic inflammatory diseases. Nutr Clin Pract **2006**; 21:68–81.
16. Pugh AM, Auteri NJ, Goetzman HS, Caldwell CC, Nomellini V. A Murine model of persistent inflammation, immune suppression, and catabolism syndrome. Int J Mol Sci. **2017**; 18:1741.
17. Cretney E, Xin A, Shi W, et al. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. Nat Immunol **2011**; 12:304–11.
18. Vasanthakumar A, Liao Y, Teh P, et al. The TNF receptor superfamily-NF-κB axis is critical to maintain effector regulatory T cells in lymphoid and non-lymphoid tissues. Cell Rep **2017**; 20:2906–20.
19. Cretney E, Kallies A, Nutt SL. Differentiation and function of Foxp3(+) effector regulatory T cells. Trends Immunol **2013**; 34:74–80.
20. Kitagawa Y, Sakaguchi S. Molecular control of regulatory T cell development and function. Curr Opin Immunol **2017**; 49:64–70.
21. Levine AG, Arvey A, Jin W, Rudensky AY. Continuous requirement for the TCR in regulatory T cell function. Nat Immunol **2014**; 15:1070–8.
22. Spence A, Klementowicz JE, Bluestone JA, Tang Q. Targeting Treg signaling for the treatment of autoimmune diseases. Curr Opin Immunol **2015**; 37:11–20.
23. Biton J, Khaleghparast Athari S, Thiolat A, et al. In vivo expansion of activated $Foxp3^+$ regulatory T cells and establishment of a type 2 immune response upon IL-33 treatment protect against experimental arthritis. J Immunol **2016**; 197:1708–19.
24. Kurup SP, Obeng-Adjei N, Anthony SM, et al. Regulatory T cells impede acute and long-term immunity to blood-stage malaria through CTLA-4. Nat Med **2017**; 23:1220–5.
25. Chen X, Subleski JJ, Kopf H, Howard OMZ, Männel DN, Oppenheim JJ. Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse $CD4^+CD25^+FoxP3^+$ T regulatory cells: applicability to tumor-infiltrating T regulatory cells. J Immunol **2008**; 180:6467–71.
26. Salomon BL, Leclerc M, Tosello J, Ronin E, Piaggio E, Cohen JL. Tumor necrosis factor α and regulatory T cells in oncoimmunology. Front Immunol **2018**; 9:444.
27. Koh AY, Priebe GP, Ray C, Van Rooijen N, Pier GB. Inescapable need for neutrophils as mediators of cellular innate immunity to acute *Pseudomonas aeruginosa* pneumonia. Infect Immun **2009**; 77:5300–10.
28. Venet F, Chung CS, Monneret G, et al. Regulatory T cell populations in sepsis and trauma. J Leukoc Biol **2008**; 83:523–35.
29. Tomura M, Honda T, Tanizaki H, et al. Activated regulatory T cells are the major T cell type emigrating from the skin during a cutaneous immune response in mice. J Clin Invest **2010**; 120:883–93.
30. Abraham E, Glauser MP, Butler T, et al. p55 Tumor necrosis factor receptor fusion protein in the treatment of patients with severe sepsis and septic shock. A randomized controlled multicenter trial. Ro 45–2081 Study Group. JAMA **1997**; 277:1531–8.
31. Bernard GR, Francois B, Mira JP, et al. Evaluating the efficacy and safety of two doses of the polyclonal anti-tumor necrosis factor-α fragment antibody AZD9773 in adult patients with severe sepsis and/or septic shock: randomized, double-blind, placebo-controlled phase IIb study*. Crit Care Med **2014**; 42:504–11.
32. Chopra M, Riedel SS, Biehl M, et al. Tumor necrosis factor receptor 2-dependent homeostasis of regulatory T

cells as a player in TNF-induced experimental metastasis. Carcinogenesis **2013**; 34:1296–303.
33. Miyazaki T, Liu ZJ, Kawahara A, et al. Three distinct IL-2 signaling pathways mediated by bcl-2, c-myc, and lck cooperate in hematopoietic cell proliferation. Cell **1995**; 81:223–31.
34. Dayer Schneider J, Seibold I, Saxer-Sekulic N, Paredes BE, Saurer L, Mueller C. Lack of TNFR2 expression by CD4(+) T cells exacerbates experimental colitis. Eur J Immunol **2009**; 39:1743–53.
35. Rossol M, Meusch U, Pierer M, et al. Interaction between transmembrane TNF and TNFR1/2 mediates the activation of monocytes by contact with T cells. J Immunol **2007**; 179:4239–48.
36. Venkatesh D, Ernandez T, Rosetti F, et al. Endothelial TNF receptor 2 induces IRF1 transcription factor-dependent interferon-β autocrine signaling to promote monocyte recruitment. Immunity **2013**; 38:1025–37.
37. Leijte GP, Rimmelé T, Kox M, et al. Monocytic HLA-DR expression kinetics in septic shock patients with different pathogens, sites of infection and adverse outcomes. Crit Care **2020**; 24:110.
38. Conway Morris A, Datta D, Shankar-Hari M, et al. Cell-surface signatures of immune dysfunction risk-stratify critically ill patients: INFECT study. Intensive Care Med **2018**; 44:627–35.
39. Raschi E, Mazzarella A, Antonazzo IC, et al. Toxicities with immune checkpoint inhibitors: emerging priorities from disproportionality analysis of the FDA adverse event reporting system. Target Oncol **2019**; 14:205–21.