Immune Unresponsiveness to Secondary Heterologous Bacterial Infection after Sepsis Induction Is TRAIL Dependent

Prajwal Gurung, Deepa Rai, Stephanie A. Condotta, Jeffrey C. Babcock, Vladimir P. Badovinac and Thomas S. Griffith

J Immunol 2011; 187:2148-2154; Prepublished online 25 July 2011;
doi: 10.4049/jimmunol.1101180
http://www.jimmunol.org/content/187/5/2148

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/07/25/jimmunol.1101180.DC1

References
This article cites 59 articles, 32 of which you can access for free at:
http://www.jimmunol.org/content/187/5/2148.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Immune Unresponsiveness to Secondary Heterologous Bacterial Infection after Sepsis Induction Is TRAIL Dependent

Prajwal Gurung,*† Deepa Rai,† Stephanie A. Condotta,‡ Jeffrey C. Babcock,‡ Vladimir P. Badovinac,§‖ and Thomas S. Griffith*§‖

Sepsis is the leading cause of death in intensive care units, and patients who survive the hyperinflammation that develops early during sepsis later display severely compromised immunity. Not only is there apoptosis of lymphoid and myeloid cells during sepsis that depletes these critical cellular components of the immune system, but also the remaining immune cells show decreased function. Using a cecal-ligation and puncture (CLP) model to induce intra-abdominal polymicrobial peritonitis, we recently established a link between the apoptotic cells generated during sepsis and induction of sepsis-induced suppression of delayed-type hypersensitivity. The present study extends this earlier work to include a secondary heterologous bacterial infection (OVA257-expressing Listeria monocytogenes [LM-OVA]) subsequent to sepsis initiation to investigate sepsis-induced alterations in the control of this secondary infection and the associated naive Ag-specific CD8 T cell response. We found that CLP-treated wild-type (WT) mice had a reduced ability to control the LM-OVA infection, which was paralleled by suppressed T cell responses, versus sham-treated WT mice. In contrast, CLP-treated Trail−/− and Dr5−/− mice were better able to control the secondary bacterial infection, and the Ag-specific CD8 T cell response was similar to that seen in sham-treated mice. Importantly, administration of a blocking anti-TRAIL mAb to CLP-treated WT mice was able to restore the ability to control the LM-OVA infection and generate Ag-specific CD8 T cell responses like those seen in sham-treated mice. These data further implicate TRAIL-dependent immune suppression during sepsis and suggest TRAIL neutralization may be a potential therapeutic target to restore cellular immunity in septic patients. The Journal of Immunology, 2011, 187: 2148–2154.
\textbf{monocytogenes}. Murine infection with \textit{L. monocytogenes} is a widely used model for the analysis of cell-mediated immunity to an intracellular bacterial pathogen (24–26). Although both CD4 and CD8 T cells are activated in an Ag-specific manner after \textit{L. monocytogenes} infection (27), CD8 T cells are the most effective mediators of anti-\textit{L. monocytogenes} immunity (28–30). Thus, incorporation of secondary \textit{L. monocytogenes} infection into the CLP model serves as a tool to specifically evaluate sepsis-induced alterations in CD8 T cell immunity to this heterologous bacterial infection. The decision to use \textit{L. monocytogenes} as the secondary infectious agent in this model was further buttressed by the fact that CD8 T cell-mediated immunity against \textit{L. monocytogenes} is TRAIL independent (31), so any changes in the CD8 T cell response to \textit{L. monocytogenes} were predicted to be due to the sepsis-induced immune suppression. The data presented in this study further implicate the prominent role of TRAIL during sepsis-induced immune suppression and suggest the TRAIL/TRAIL receptor pathway as a therapeutic target for restoring T cell-mediated immunity in septic patients.

\section*{Materials and Methods}

\subsection*{Mice}

C57BL/6 (B6) and BALB/c mice were purchased from The National Cancer Institute. \textit{Trail} \textsuperscript{-/-} and \textit{Dr5} \textsuperscript{-/-} B6 mice were obtained from Agenm (Seattle, WA) (32) and Dr. W. El-Diery (University of Pennsylvania, Philadelphia, PA) (33), respectively. \textit{Trail} \textsuperscript{-/-} BALB/c mice were obtained from Dr. Thomas Sayers (National Cancer Institute, Frederick, MD). All mice were housed in the same facility for at least 4 wk, regardless of their source, before use, and animal procedures were performed according to National Institutes of Health guidelines and approved by the University of Iowa Institutional Animal Care and Use Committee. In all in vivo experiments, groups consisted of four or more animals, and experiments were repeated at least two times with similar results before reporting.

\subsection*{Cecal-ligation and puncture}

Sepsis was induced by CLP, as described previously with slight modifications (34). Briefly, mice were anesthetized, and an abdominal incision was made to identify the cecum. The distal one-third of the cecum was ligated with 4–0 silk suture and punctured once using a 25-gauge needle. A small amount of cecal contents was extruded through the perforation. This level of injury was used to create a chronic septic state characterized by the loss of appetite and body weight, ruffled hair, shivering, diarrhea, and/or periorbital eduades and with <5% mortality. The peritoneum was closed with a continuous suture after returning the cecum into the abdomen. The skin was then glued shut with 3M Vetbond (3M Animal Care Products, St. Paul, MN), and 1 ml saline was injected i.p. for resuscitation. For sham-treated mice, all of the steps were performed, except for ligation and puncture of the cecum. In some cases, the CLP-treated mice received 300 μg anti-TRAIL blocking mAb N2B2 (eBioscience) the day after surgery.

\subsection*{Bacterial preparation, infection, and titer}

The attenuated \textit{L. monocytogenes} strains DP-L1942 (attenuated \textit{L. monocytogenes}) and OVA\textsubscript{257}-expressing (attenuated LM-OVA), which are \textit{ActA} deficient, were grown, injected i.v., and quantified as previously described (35). Briefly, the bacteria were grown to log phase (OD\textsubscript{600} 0.06–0.1) in tryptic soy broth media. Bacteria were diluted to 10\textsuperscript{7} CFU in 0.2 ml sterile saline (Hospira, Lake Forest, IL) and injected i.v. Serial dilutions of the same culture used for injections were plated and grown overnight at 37°C and colonies counted to independently determine the actual number of injected bacteria. Spleens and livers were harvested from infected mice on day 3 postinfection and homogenized in 0.2% IGEPAL buffer (Sigma-Aldrich, St. Louis, MO). The homogenized suspension was incubated at room temperature for 30 min, and serial dilutions were plated in streptomycin agar plates. Plates were cultured overnight at 37°C, and bacterial counts were determined.

\subsection*{Flow cytometry}

For staining of surface markers, cells were incubated with fluorochrome-conjugated mAbs at 4°C for 30 min. The cells were then washed with FACS buffer (PBS containing 2% BCS and 0.2% NaN\textsubscript{3}) and then fixed with 2% paraformaldehyde in PBS. mAbs used for surface staining were FITC-CD11a (clone M174; BioLegend), PerCP- or allphyocyanin-CD8 (clone 53-6.7; Biolegend), PE- or PerCP/Cy5.5-CD3 (clone 17A2 for PE and clone 145-C211 for PerCP/Cy5.5; BioLegend), PerCP- or all phyocyanin-CD4 (clone GK1.5; BioLegend), and PE-CD19 (clone 6D5; BioLegend).

For intracellular staining, 10\textsuperscript{6} splenocytes were cultured at 37°C in the presence of OVA\textsubscript{257} and brefeldin A (BD Biosciences) for 6 h. The cells were then washed and stained for surface markers as described above. The cells were then washed once with FACS buffer and fixed with 2% paraformaldehyde in PBS for 20 min at room temperature. After-fixing, cells were washed and resuspended in saponin buffer for 30 min at 4°C. PE- or allphyocyanin–IFN-γ (clone XMG1.2; BioLegend) was added to the cells and incubated for 20 min. Cells were washed and resuspended in FACS buffer and analyzed using a Becton Dickinson FACSCalibur (BD Biosciences).

\section*{Results}

\subsection*{Reduced bacterial clearance and Ag-specific CD8 T cell responses in CLP mice}

The initial hyperinflammatory phase of sepsis is quickly followed by a sustained immunosuppressive state in patients and clinically

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{CLP-treated WT B6 mice demonstrate increased morbidity and susceptibility to LM-OVA infection. A, WT B6 mice underwent sham or CLP surgery. Animal weight was measured daily beginning the day of surgery, and the percent weight loss with respect to the starting weight is shown. B, Sham or CLP surgery was performed on WT B6 mice. On day 2 postsurgery, the mice were infected with 10\textsuperscript{7} CFU attenuated LM-OVA. Animal weight was measured daily beginning the day of surgery, and the percent weight loss with respect to the starting weight is shown. In A and B, the dotted line represents the starting weight of the mice, normalized to 100%. In B, the arrow indicates the time of infection. C, Sham or CLP surgery was performed on WT B6 mice, and the mice were infected with attenuated LM-OVA on day 2 postsurgery as in B. In addition, naive mice that did not undergo any surgery (untreated) were also infected with attenuated LM-OVA infection. The bacterial titer in the spleen and liver were then determined on day 3 postinfection. The dotted line represents the limit of detection (l.o.d.).}
\end{figure}
relevant animal models of sepsis (36, 37), and it has been suggested that this immune suppression is critical to the pathogenesis and subsequent morbidity and mortality in septic patients that acquire secondary infections. Thus, the current study examined the impact of sepsis-induced immunosuppression on the naive CD8 T cell response to the Gram-positive, facultative intracellular bacterium *L. monocytogenes*. Our version of the CLP model of sepsis involves ligation of the distal one third of the cecum and a single puncture to permit release of the cecal contents into the peritoneum. This level of injury reproducibly resulted in the development of many clinical symptoms of sepsis, including a significant loss of weight compared with sham-treated wild-type (WT) B6 mice (Fig. 1A). Importantly, these CLP-treated mice regained all of the lost weight by 7 d after surgery. When we added the secondary infection with attenuated LM-OVA, the sham or untreated WT B6 mice displayed minimal morbidity (as measured by weight loss; Fig. 1B) and were able to control the LM-OVA infection (Fig. 1C). In contrast, CLP-treated WT B6 mice that received the subsequent attenuated LM-OVA infection demonstrated sustained weight loss compared with sham-treated mice that was not regained, and these mice were less able to control the attenuated LM-OVA infection (50- and 1400-fold increase in bacterial titer in the spleen and liver, respectively, compared with untreated or sham-treated mice). These results suggest that the induction of sepsis compromises overall health such that even a secondary heterologous infection with an attenuated bacterial strain cannot be adequately controlled.

Sepsis induces widespread immune cell apoptosis, and, consistent with previous studies (23), we observed a significant reduction in the number of CD8 T cells in WT B6 mice 2 d post-CLP compared with sham-treated mice (Fig. 2A). Similar significant reductions were also seen for CD3+CD4+, CD11c+, and CD19+ cells (Supplemental Fig. 1). We then specifically examined the primary CD8 T cell response 7 d after attenuated LM-OVA infection and found a significant decrease in total number of Ag-experienced [based on CD11a upregulation (38); Fig. 2B] and OVA257-specific CD8 T cells (based on IFN-γ production after in vitro OVA257 restimulation; Fig. 2C, 2D) in the spleens of CLP-treated WT B6 mice compared with sham-treated mice. Because CD8 T cells are essential for the control of many bacterial infections, including *L. monocytogenes*, we have concentrated our analyses on the Ag-specific CD8 T cell response in sham and CLP-treated mice. However, a vigorous Ag-specific CD4 T cell response is generated in B6 mice to residues 190–201 of the *L. monocytogenes* protein listeriolysin O190–201 presented on I-A^b^ (39, 40). Much like that for the Ag-specific CD8 T cell response, there was a significant decrease in total number of listeriolysin O190–201-specific CD4 T cells in the spleens of LM-OVA–infected, CLP-treated WT B6 mice compared with sham-treated WT B6 mice.

**FIGURE 2.** Ag-specific CD8 T cell responses to attenuated LM-OVA are reduced in CLP-treated WT B6 mice. WT B6 mice underwent sham or CLP surgery and were then infected with 10^7 CFU attenuated LM-OVA on day 2 postsurgery. A, Total CD8 T cell numbers in the spleen were determined on day 2 postsurgery (i.e., day of infection). B–D, On day 7 postinfection, the number of Ag-experienced (based on CD11a upregulation) and OVA257-specific CD8 T cells in the spleen was determined. The total number of Ag-experienced CD11ahi CD8 T cells in spleen was determined directly ex vivo by flow cytometry, whereas the number of OVA257-specific CD8 T cells in the spleen was determined by intracellular cytokine staining for IFN-γ after in vitro restimulation with OVA257. C shows representative flow plots measuring the frequency of IFN-γ-producing CD8 T cells with and without OVA257 restimulation in vitro. *p < 0.05 (Student *t* test).

**FIGURE 3.** CLP-treated *Trail*^-/-^ and *Dr5*^-/-^ mice have reduced morbidity after LM-OVA infection and retain the ability to control the LM-OVA infection. Sham or CLP-treated B6 WT, *Trail*^-/-^, and *Dr5*^-/-^ mice were infected with 10^7 CFU attenuated LM-OVA 2 d postsurgery. A–C, Animal weight was measured daily beginning the day of surgery, and the percent weight loss with respect to the starting weight is shown. The dotted line represents the starting weight of the mice, normalized to 100%, and the arrow indicates the time of infection. Arrow points to the time mice were infected with attenuated LM-OVA. D–F, Sham or CLP surgery was performed on WT, *Trail*^-/-^, and *Dr5*^-/-^ B6 mice, and the mice were infected with attenuated LM-OVA on day 2 postsurgery as in A. The bacterial titer in the spleen was then determined on day 3 postinfection. The dotted line represents the limit of detection (I.o.d.).
CLP-treated Trail−/− or Dr5−/− mice retain the ability to control a secondary L. monocytogenes infection and mount CD8 T cell immunity

We recently showed that sepsis-induced apoptosis resulted in the TRAIL-dependent suppression of DTH (23). To determine the extent to which the TRAIL-dependent immune suppression contributed to the sepsis-induced morbidity and inability to control the secondary L. monocytogenes infection, we first examined weight loss in sham- and CLP-treated WT, Trail−/−, and Dr5−/− B6 mice given a secondary infection with attenuated LM-OVA. As in Fig. 1, CLP-treated WT B6 mice showed sustained weight loss compared with sham-treated mice and were unable to control the attenuated LM-OVA infection as well as sham-treated mice (2200-fold increase in L. monocytogenes CFU in the spleen of CLP-treated mice; Fig. 3A, 3D). By comparison, the CLP-treated Trail−/− and Dr5−/− B6 mice were able to recover their body weight back to that of the sham-treated mice (Fig. 3B, 3C) postinfection. Moreover, the splenic LM-OVA burden in CLP-treated Trail−/− and Dr5−/− B6 mice was only slightly increased over sham-treated Trail−/− and Dr5−/− B6 mice (7- and 10-fold, respectively; Fig. 3E, 3F). Data presented in our previous report showed sepsis-induced lymphopenia was TRAIL independent (23), and similar findings were seen in the current study (Fig. 4A). However, there was no decrease in the total number of Ag-experienced CD11ahi (38) or OVA257-specific CD8 T cells in the spleens of CLP-treated Trail−/− and Dr5−/− mice compared with sham-treated mice (Fig. 4B, 4C). Thus, these data suggest that sepsis-induced alterations in morbidity, bacterial control, and CD8 T cell responses following a secondary infection with attenuated LM-OVA require an intact TRAIL/DR5 signaling pathway.

To investigate the extent to which the TRAIL-dependent decrease in ability to control a secondary bacterial infection after sepsis was mouse strain sensitive, we examined the ability of sham- and CLP-treated WT and Trail−/− BALB/c mice to control the secondary L. monocytogenes infection. There was a similar reduction of CD8 and CD4 T cells in CLP-treated WT and Trail−/− BALB/c mice compared with the sham-treated mice (Fig. 5A). Moreover, the CLP-treated WT BALB/c mice had increased splenic LM-OVA titers compared with sham-treated mice, but there were nearly identical LM-OVA titers in the spleens of the sham- and CLP-treated BALB/c Trail−/− CLP mice (Fig. 5B). Thus, these data suggest that there is no strain specificity for the observed TRAIL-dependent alterations in the ability to control a secondary bacterial infection during sepsis.

Therapeutic use of a blocking anti-TRAIL mAb in septic mice decreases weight loss, improves the control of secondary bacterial infection, and restores CD8 T cell responses

The data presented thus far suggest that an intact TRAIL/DR5 pathway correlates with increased morbidity, reduced control of a secondary bacterial infection, and decreased CD8 T cell response in CLP-treated mice. Because the genetic elimination of either TRAIL or DR5 in CLP-treated mice resulted in them behaving similar to sham-treated mice, we determined the extent to which the response of CLP-treated mice to secondary L. monocytogenes infection could be improved through the therapeutic disruption of the TRAIL/DR5 pathway. Thus, CLP-treated WT B6 mice were treated with a blocking anti-TRAIL mAb after the induction of sepsis, but before LM-OVA infection (Fig. 6). Using this approach, the anti-TRAIL mAb-treated mice lost more weight compared with the sham-treated mice, but not as much as the CLP-treated mice (Fig. 6A). However, there was a dramatic reduction in the splenic bacterial burden in the anti-TRAIL mAb-treated mice compared with the CLP-treated mice that did not receive the anti-TRAIL mAb (Fig. 6B). Similarly, the CD8 T cell response was restored in the anti-TRAIL mAb-treated mice (Fig. 6C, 6D). The magnitude of the Ag-specific CD8 T cell response in CLP-treated mice was similar to that seen in CLP-treated mice given an isotype control IgG (data not shown), which is consistent with other reports describing the in vivo use of the anti-TRAIL blocking mAb N2B2 and isotype control Ab (41–43). Together, these results suggest that mAb-mediated disruption of the TRAIL/DR5 pathway had a therapeutic benefit in the septic mice with regard to morbidity, bacterial control, and the CD8 T cell response after a secondary bacterial infection.

Discussion

Sepsis induces widespread immune cell apoptosis that is required for the subsequent immune suppression (23). Consequently, septic patients and animals become very susceptible to subsequent infection that can often result in death (44). In the current study, we used a modified version of the clinically relevant CLP sepsis model. Therapeutic use of a blocking anti-TRAIL mAb in septic mice decreases weight loss, improves the control of secondary bacterial infection, and restores CD8 T cell responses.
model to include a secondary heterologous bacterial infection to study the role of TRAIL in sepsis-induced alterations in bacterial clearance and CD8 T cell immunity. Our results provide further evidence to suggest that TRAIL is a key molecular component in the establishment of sepsis-induced immune suppression. In this particular case, sepsis induced TRAIL-dependent immune suppression that limited the host’s ability to control a secondary bacterial infection and reduced the bacterial Ag-specific CD8 T cell response. Our data also suggest that TRAIL may be a potential therapeutic target during sepsis, because neutralization of TRAIL in septic mice restored the ability of these mice to clear the bacterial infection and mount a normal bacterial Ag-specific CD8 T cell response.

The CLP mouse model is considered the gold standard of sepsis models because it reproduces the pathophysiology of polymicrobial sepsis and intra-abdominal peritonitis seen in humans (34). Early after CLP, mice show many of the symptoms of sepsis that are seen in septic humans, such as hypothermia, tachycardia, and proinflammatory cytokine production. Depending on the severity of the sepsis induced (and the experimental interest of the investigator), death may occur within the first 48 h, or the mice will survive and develop immune suppression. This flexibility in disease severity and ability to study acute or chronic sepsis is simultaneously one of the biggest advantages and disadvantages of the CLP model. We reproducibly have a >95% animal survival rate in CLP-treated mice, which is similar to that reported by others examining the chronic effects of sepsis on the responsiveness of the immune system. A number of reports have combined secondary infection with CLP, and it is clear that sepsis alters the immune response to the infectious pathogen in the preclinical models much like that seen in the clinical situation. For example, CLP-treated mice are highly susceptible to pulmonary Pseudomonas aeruginosa infection and demonstrate increased mortality compared with sham-treated mice (2, 45, 46). One weakness in using P. aeruginosa as the secondary infections agent is that specific T cell responses are not as easily followed as they are when L. monocytogenes is used. The vast amount of information gathered over recent years regarding the T cell response to L. monocytogenes is one reason why we chose to use this bacterial pathogen in our system. L. monocytogenes normally induces a robust CD8 T cell response (25), so the observation that CLP-treated WT mice had a reduced ability to control a secondary attenuated L. monocytogenes infection and reduced CD8 T cell responses compared with sham-treated WT mice or CLP-treated Trail−/− or Dr5−/− mice was very intriguing. Interestingly, a recent report by Delano et al. (46) showed that CLP mice were more resistant to virulent L. monocytogenes infection when compared with sham-treated mice, resulting in increased survival. These data are in direct contrast with the data we show in this report. Possible explanations for these differences, compared with our data, include (but are not limited to): 1) use of attenuated versus virulent L. monocytogenes; 2) day of infection (day 2 versus day 3 postsurgery); 3) infectious dose (10^7 CFU attenuated L. monocytogenes versus 10^9 CFU virulent L. monocytogenes); 4) day of assessment of bacterial titer (day 3 versus day 5 postsurgery); and 5) severity of sepsis induction (<5% versus ~15% mortality). Attenuated and virulent L. monocytogenes elicits very distinct immune responses (47). Attenuated L. monocytogenes is replication...
Despite all of the studies that have been performed to understand the pathogenesis of sepsis, therapeutic targets still remain ill-defined, especially for immunosuppressed sepsis patients that are susceptible to secondary infections. Recent studies have shown that therapies that block apoptosis of lymphocyte populations, either through administration of IL-7, IL-15, or anti-PD1 mAb, result in increased survival after sepsis induction in mice (55–57). Although these findings are exciting, these reports did not investigate the health of the immune system in regard to the ability to control subsequent heterologous infections. We included the use of a blocking anti-TRAIL mAb in the present series of experiments, not only as a complementary way to demonstrate the importance of TRAIL in the sepsis-induced unresponsiveness to a secondary heterologous bacterial infection, but also to test the extent to which therapeutic disruption of the TRAIL/DR5 pathway would have any benefits during sepsis. We hypothesized the anti-TRAIL mAb would inhibit the function of the immunosuppressive TRAIL-expressing CD8 T~reg~ cells generated during sepsis (23), and the data showing the restored ability to control the LM-OVA infection and CD8 T cell responses would suggest this occurred. It is also possible that the anti-TRAIL mAb was having an effect outside of the immune system. For example, apoptosis of epithelial cells in the small and large intestine is increased in animals and human suffering from sepsis and is related to a poor prognosis (37, 56, 58, 59). Although the data presented in Fig. 6 investigated the potential impact of the anti-TRAIL mAb on immune system responses to a secondary heterologous bacterial infection after sepsis, we cannot exclude the possibility that the anti-TRAIL mAb was also inhibiting the death of cells outside of the immune system. Thus, anti-TRAIL mAb blockade of intestinal epithelial cell apoptosis may have contributed to the increased weight gain in this group of CLP-treated mice. Further investigation, which would also include analyses of CLP-treated Trail<sup>−/−</sup> and Dr5<sup>−/−</sup> mice, is needed to determine the likelihood of this. Regardless, the data presented in this study suggest TRAIL could be a potential therapeutic target in sepsis to help decrease the susceptibility to potentially life-threatening secondary heterologous infections.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1. **CLP surgery results in a significant decrease in the number of CD4+ T cells, CD11c+ cells, and CD19+ cells in the spleen.** WT B6 mice underwent sham or CLP surgery. The total number of CD3+CD4+, CD11c+, and CD19+ cells in the spleen were determined 48 h after surgery. *p < 0.05 (Student’s t-test).
Supplemental Figure 2. Suppressed Ag-specific CD4 T cell response in *L. monocytogenes*-infected, CLP-treated WT B6 mice. WT B6 mice underwent sham or CLP surgery. 48 h later, the mice were infected with 10^7 CFU att LM-OVA i.v. Spleens were harvested on d 7 p.i., and the frequency and number of IFNγ-producing CD4 T cells were determined after *in vitro* stimulation with MHC II (I-Ab)-restricted epitope derived from the *L. monocytogenes* protein listerolysin O (LLO)190-201. * p < 0.05 (Student’s t-test).