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Immune responses in relation to the type and time of thermal injury: An experimental study

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ABSTRACT

Background: Thermal injuries are followed by a complex immune response, but the relationship between the severity of burn injury and the time exposure to the thermal injury on the extent of the immune response is still not known.
Objective: This study focuses on characterising the effect of temperature and time exposure on the post-burn immune response.
Methods: We used 120 C57BL/6 male mice divided equally in 5 burn groups and one sham operated group (groups A–E and sham). Ten mice per group were sacrificed at 24 and 48 h after burn injury and whole blood was collected; specimens of liver, lung, spleen, kidney and bowel were excised. Apoptosis and TREM-1 expression on circulating blood cells were measured. Splenocytes were isolated and stimulated for cytokine production; the rate of apoptosis of splenocytes was also measured.
Results: Production of IL-17 from splenocytes of mice group D was enhanced. Considerable effects were shown on the apoptosis of circulating lymphocytes and of spleen cells. The apoptotic rates varied between groups and also evolved after 24 and 48 h. To examine the origin of this differential response, quantitative bacterial cultures of liver, lung and kidney were made but no differences were observed compared with sham-operated animals.
Limitations: This study was based on an experimental murine model.
Conclusion: There is a unique response for each type of injury depending on the temperature of the thermal source and the exposure time.

Introduction

Thermal injury induces a remarkable change in physiological parameters resulting in cachexia, hypermetabolism, inflammation and immune dysfunction [1–3]. The high incidence of morbidity and mortality following major burns can often be attributed to immune dysfunction and wound healing complications. These conditions increase the patient's susceptibility to infection leading to systemic inflammatory response syndrome (SIRS) and multiple organ failure. Animal models have been developed over the past years to study the effects of burn injury on the physiological and immunological parameters. Previous studies using murine models have reported the suppression of cell-mediated immune responses after burn injury, as well as susceptibility to septic complications and high mortality [4–7].

However, few data are available on the immune responses after thermal injury. In particular, the impact of the type and time of thermal exposure have never been described. The aim of this study was to systematically evaluate the impact of the

Abbreviations: IFNγ, interferon-gamma; IL, interleukin; TNFα, tumour necrosis factor-alpha; TREM-1, triggering receptor expressed on myeloid cells-1.

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temperature and of the time of the exposure to the thermal source on the immune system, using a well-established murine thermal injury model.

Animals and methods

Animals

The experimental protocol was approved by the Veterinary Directorate of the Prefecture of Athens, according to Greek legislation in conformance with the 160/91 Directive Council of the EU (K/4897/16-07-2009). This experiment used 120 C57BL/6 male mice (18–22 g; 8–10 weeks of age). The mice were given one week to acclimatize in the animal facility before the study began. Mice were fed standard chow (type 4rf 18) and were allowed water *ad libitum*. They were kept in an isolated room with a controlled temperature of 24 °C and a day–night cycle of 8am–8pm.

Experimental design

Mice received a scald burn as described elsewhere [8,9]. Briefly, mice were anaesthetized by intramuscular injection of ketamine/xylazine and the dorsal surface was shaved. The animal was placed in a custom insulated mould exposing 20% of their total body surface area (TBSA) along the dorsum based on Meeh's formula for TBSA of a 20 g mouse: $A = KW^{2/3}$, where A is area in cm^2, $K = 8{:}95$ and W = body weight in grams [10].

Animals were randomly assigned to either a sham treatment group or a burn group ($n = 20$ per group). Two mice from each group were studied per day of experiment. All mice were subjected to thermal injury of various intensity and time duration, as described below:

- Group Sham: no burn injury; control group
- Group A: with a scaled burn for 60 s at 60 °C;
- Group B: with a scaled burn for 45 s at 60 °C and then for 45 s at 4 °C;
- Group C: with a scaled burn for 15 s at 75 °C;
- Group D: with a scalded burn for 5 s at 90 °C; and
- Group E: with a scalded burn for 45 s at 4 °C and then for 45 s at 60 °C.

Group B injury establishes a partial thickness burn as proved by Besner et al. [11]. Groups C and D burn injuries can be considered as full thickness burns by Carter et al. [12]. The groups A and E are considered as intermediate thermal injuries. After the injury all mice were resuscitated with 1 ml of Ringer's lactate administered by intraperitoneal injection and returned to their cages. The cages were placed on a heating pad for 2 h until the mice were fully awake, at which time they were returned to the animal facility. Until sacrifice, mice were receiving rectal suppositories of paracetamol to reduce suffering.

Ten mice per group were sacrificed at 24 and 48 h after burn injury. Sacrifice was done by inhalation of diethyl ether followed by ketamine injection, five times as much as the initial dose. At sacrifice, one midline abdominal incision was performed under aseptic conditions. After peritoneal cavity was entered, intestines were displaced to the left and blood was sampled from the lower vena cava under aseptic conditions. Blood was collected into heparin-coated tubes for flow cytometry. Using separate sterile instruments, specimens of liver, right lung, spleen, and right kidney were excised and put into separate sterile containers. Spleens were used for splenocyte isolation and the remaining tissues were used for quantitative cultures.

Laboratory analysis

Tissues were weighted, homogenized and quantitatively cultured as described previously [13]. The number of viable cells was expressed by its log 10 value in cfu/g.

Splenocytes were isolated after gentle squeezing of the spleen through a sterile filter (250 mm, 12 cm × 13 cm, Alter Chem Co, Athens, Greece). After counting in a Neubauer chamber, isolated splenocytes were stimulated for the production of tumour necrosis factor-alpha (TNFα), interleukin 10 (IL-10), interleukin 17 (IL-17) and of interferon-gamma (IFNγ) as previously described [13]. The other half of splenocytes were used to determine the rate of apoptosis as described below. Measured cytokines were selected as an expression of macrophage activation (TNFα), of Th1 responses (IFNγ), of Th2 response (IL-10) and of Th17 response (IL-17).

Within a cell suspension containing 1×10^6 splenocytes red blood cells were lysed (VersaLyse Solution, Immunotech, Marseille, France). After centrifugation at $300 \times g$ for 5 min, the cells were washed and reconstituted with ANNEXIN-binding buffer (Immunotech, Marseille, France). Cells were incubated for 15 min at 4 °C with ANNEXIN-V at the fluorochrome fluorescein isothiocyanate (FITC, emission 525 nm, Immunotech) and propidium iodide (PI, emission 613 nm, Invitrogen, OR, USA). The stained cells were analysed after running through an EPICS XL/MSL flow cytometer (Beckman Coulter Co., Miami, FL) with separate gating for lymphocyte and macrophage based on their characteristic forward and side scattering. Cells staining positive for ANNEXIN-V and negative for PI were considered apoptotic; unstained cells were used as controls.

A volume of 0.3 ml of whole blood was incubated with anti-TREM-1 monoclonal antibody at the fluorochrome phycoerythrin (PE, emission 575 nm, R&D Systems, Minneapolis, USA). After incubation for 45 min at 4 °C red blood cells were lysed and fixed with 1.6% formalin. White blood cells were washed with PBS (pH 7.2) (Biochrom), reconstituted in 0.5 ml PBS and analysed through the cytometer with gating for granulocytes and monocytes based on their characteristic forward and side scattering. IgG2a (R&D) isotype controls were used for each sample. Results were expressed as mean fluorescence intensity (MFI) on cells.

Statistical analysis

Results were expressed as means ± SE. Comparisons between groups were done by the Kruskall–Wallis test. Any value of P below 0.05 after adjustment by Bonferroni for multiple comparisons was considered significant.

Results

Thermal injury was accompanied by considerable down-regulation for the production of TNFα from splenocytes of groups A, B, D and E. Additionally, production of IL-17 from group D was increased significantly 48 h after thermal exposure. However, no effect was shown by thermal injury on the production of IL-10 and of IFNγ (Fig. 1). Similarly to TNFα release from splenocytes, the level of expression of the pro-inflammatory receptor TREM-1 on circulating monocytes was increased (Fig. 2) in animals of group B.

Considerable effects of burn injury were shown on the rate of apoptosis of circulating lymphocytes and of spleen cells. More precisely, apoptosis of circulating lymphocytes of group C was decreased at 24 h and increased after 48 h; apoptosis of lymphocytes of the spleen of groups C and D decreased after 24 h; and apoptosis of macrophages of the spleen of groups B and C increased at 24 h (Fig. 3).