Macrophage response in experimental third-degree skin burns treated with allograft. Histological and immunohistochemical study

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Abstract
Macrophages are some of the innate immune cells with a central role in inflammatory and immune responses. Studies in the last 20 years have shown that these cells have a particular influence in the reparative processes also. Our aim in this study was to evaluate the macrophage response in third-degree skin burns treated with allograft in an experimental model. Macrophages were specifically highlighted by immunohistochemical staining with anti-CD68 antibody. In the first evolutive part of the reparatory process, macrophages rapidly increased both numerically and as a relative area with about 300%, and then decreased progressively along with the granulation tissue maturation. Macrophage overall response curve was similar in animals treated with allograft and in the control group (untreated), which leads us to believe that the allograft does not induce a more ample immune response that could be regarded as pathological.

Keywords: allograft, skin burns, macrophages, CD68, granulation tissue.

Introduction
Skin burns are one of the most common injuries encountered in clinical practice worldwide. Only in U.S., the American Burn Association stated that in 2011 there were about 450,000 burned patients who required medical treatment, representing an increase of 340% compared to 1995. Of these 45,000 required hospitalization and 24,750 had to be treated in specialized care centers for the burned [1].

The skin is an organ essential for maintaining overall body homeostasis. Therefore, restoring skin integrity and maintaining skin homeostasis following various injuries is a vital process [2]. Healing of cutaneous third-degree burns is very complex, requiring a complex and dynamic interaction between epithelial cells (keratinocytes), normal connective cells present in the remaining tissues (fibroblasts, endothelial cells) and immune cells [3, 4]. The repair process is a sequential one: inflammatory reactions at the site of aggression, cellular and tissue debris removal, formation of connective and epithelial tissue, and finally, structural and functional maturation of these tissues [5]. The repair process of the cutaneous organ aims to restore the barrier function of the [6].

At present, it is considered that macrophages play a critical role in inflammatory and immune responses [7]. They derive from stem cells from bone marrow and reach the bloodstream as monocytes. Under physiological conditions, and especially in inflammatory conditions, monocytes cross the capillary wall and migrate into tissues, where they differentiate into macrophages. It seems that in reparative processes, macrophages orchestrate the whole repair process, acting both as phagocytes, eliminating cellular debris and tissue from the wound, but also as a major source of growth factors [8].

In this study, we aimed to analyze the response of macrophages in experimentally induced third-degree skin burns in animals during the healing process by applying skin allograft compared with macrophages in the spontaneous healing reaction.

Materials and Methods
The study was conducted on two batches of 35 adult common Wistar rats, weighing between 280 and 310 g. The animals were kept before and during the course of the experiment in the Animal Facility of the University of Medicine and Pharmacy of Craiova, Romania, under standard light, temperature and humidity conditions, having permanent access to food and water (ad libitum). Before the experiment, the animals received general anesthesia by intramuscular injection of Ketamine
hydrochloride (Ketalar®, Parke-Davis), each 85 mg/kg and Xylazine hydrochloride (Rompun®, Bayer), 6 mg/kg. The hair was removed from an area of around 4–5 cm² from the upper portion of the dorsal region of each animal, on which a special cone-shaped metal device made of stainless steel, weighing 350 g and with a diameter of 1 cm, equipped with a control thermometer, heated to 100°C in a pot of boiling water, was applied for five seconds. After producing the burn, each animal was bandaged using a dry bandage applied on the wound. The evolution of the burned wound and animal welfare were monitored daily.

A first group of 35 animals was left to heal spontaneously. The animals were randomly divided into seven subgroups of five animals each. Every three days (at 3, 6, 9, 12, 15, 18, and 21 days), under general anesthesia, the lesion and 2–3 mm perilesional tissue were harvested, from each subset of five animals in order to properly assess the dynamic of the macrophage response within the burned wound during spontaneous healing. Then, each animal underwent surgical suture of the wound.

After 24 hours from the infliction of the burn under general anesthesia, from each animal in the second batch the burnt area was removed followed by insertion of a skin allograft. Skin allografts were collected from a group of rats of the same breed. Under anesthesia with Ketamine hydrochloride and Xylazine hydrochloride, after removing the hair from the back of the rat, skin fragments were cut using a 3×2 cm from donor animals were harvested, using a Acculan (Aesculap®) type dermatome, fitted with a depth adjusting system in the range of 0.1–1 mm. Skin fragments were kept in saline solution heated to 37°C for a few minutes and perforated for drainage of possible fluid accumulation, then were applied on the receiving animal. Allograft was sutured with absorbable thread along the edges of the wound, and then a moistened bandage was applied and fixed with adhesive tape. Animals were monitored daily under standard light, heat and food conditions. For the study, the animals of this group were also divided into seven subgroups of five animals each. Again, every three days following the completion of the procedure (at 3, 6, 9, 12, 15, 18, and 21 days), under general anesthesia, the allograft areas were harvested from each subset of five animals.


**Histological study**

Fragments from skin burns sampled from both animals with spontaneous healing and animals treated with allograft were fixed in 10% neutral formalin for 72 hours at room temperature (21–23°C) and embedded in paraffin wax. Sections were cut using a rotary microtome (Microm HM350) equipped with a waterfall based section transfer system (STS, Microm). Four-micrometer thick sections were then stained using Hematoxylin–Eosin and Goldner–Szekely trichrome.

**Immunohistochemical study**

For the immunohistochemical study, 3-µm thick sections were cut using the same equipment and collected on poly-L-Lysine coated slides, dried in a thermostat at 37°C for 24 hours in order to obtain a perfect adhesion of the biological material to the surface of the histological slide. For single immunohistochemistry, following deparaffinization and hydration, after antigen retrieval, sections were cooled down to room temperature and were incubated for 30 minutes in a 1% hydrogen peroxide solution. The sections were next washed in PBS, followed by a blocking step of 30 minutes in 2% skin milk. Next, the slides were incubated with the primary antibodies overnight at 4°C, and the next day, the signal was amplified for 30 minutes using a peroxidase polymer-based secondary detection system (EnVision, Dako).

The signal was detected with 3,3′-diaminobenzidine (DAB) (Dako) and the slides were coverslipped in DPX (Fluka) after Hematoxylin counterstaining.

The antibody used for highlighting the macrophage activity was the mouse anti-rat CD68 antibody (AbD Serotec, clone ED1, code MCA341GA, 1:200 dilution).

The sections were imaged with a Nikon Eclipse 55i microscope (Nikon, Apidrag, Romania) equipped with a 5-megapixel cooled CCD camera.

Images were captured and archived using a Nikon frame grabber and the Image ProPlus 7 AMS software (Media Cybernetics Inc., Buckinghamshire, UK). Statistical analysis of data was performed using the ANOVA test for global comparison of several data categories, the Student t-test for paired data, and the Pearson correlation coefficient for assessing the interdependence of two groups of numerical values. The software packages used were Microsoft Excel and SPSS ver. 16.

**Results**

**Qualitative assessment of the macrophage response in burns treated with allograft compared to those with spontaneous healing**

Three days after allografting, CD68-positive macrophages, developed in small numbers at the periphery of the burned wound, with mainly perivascular disposition, in the early granulation tissue at the boundary between the normal skin and the lesional area. We also noticed a focal accumulation of macrophages around areas of interstitial edema in the depth of the wound. Macrophages had approximately 20–25 µm, heterogeneous finely granular cytoplasm indicating the presence of a relatively small amount of lysosomes in the cytoplasm (Figure 1).

In the contact area between the allograft and the remaining connective tissue after excision of the burned skin, we noted the presence of small amounts of polymorphonuclear neutrophils and rare lymphocytes. We can say that at this time point, both the local...
inflammatory response and the regenerative response of the connective tissue had a moderate intensity. In the group of animals with spontaneous healing (control group), the inflammatory reaction was less intense, more diffuse, without focal accumulations of macrophages (Figure 2).

At six days after allografting, in the depth and on the edges of the wound, a young granulation tissue developed, composed of numerous fibroblast-type cells, abundant intercellular matrix and fine collagen fibers. The inflammatory response within the wound intensified, being present throughout the entire area subjected to thermal aggression and treated by allografting. At the junction between the allograft and the connective tissue of the burned area, we noted the presence of a large number of polymorphonuclear neutrophils and rare macrophages (Figures 3–6). In contrast, a higher density of macrophages was observed in the granulation tissue in the depth of the wound (Figure 7). Also, in the contact area between the allograft and the granulation tissue we often revealed the presence of an amorphous, slightly acidophilic material, probably represented by extravasated plasma proteins, cellular debris and polymorphonuclear lysates (Figures 3 and 5).

On the histological samples from animals with spontaneous healing (control group), both the inflammatory response and granulation tissue appeared as having a more homogeneous distribution within the lesion but less intense than in the allograft group.

At nine days after allografting, the granulation tissue reached the surface of the burned wound, filling the heat-induced lesion. Macrophage numbers were greatly increased (Figure 8), with the highest density being observed at the boundary between the allograft and the granulation tissue (Figure 9). The allograft was mostly disorganized, fragmented, containing remaining hair follicles. Around allograft debris and hair follicles, numerous macrophages were observed arranged in groups of various sizes, highly reactive to CD68, indicating an increase in the number of lysosomes and phagocytic activity.

At the contact area between the base of the allograft and granulation tissue, we noticed a polymorphous cellularity, consisting mainly of polymorphonuclear neutrophils with a band arrangement, some being morphologically intact others partially lysed, along with numerous macrophages arranged in either a diffuse pattern or a patchy one. Macrophages had increased dimensions, with abundant phagocytic intracytoplasmic material. This entire cellular population at the surface of the wound, at the boundary between the allograft and the granulation tissue, was included in a slightly acidophilic background material, edematous and with cellular debris. The intense response of polymorphonuclear leukocytes and macrophages also manifested sometimes as microabscesses (clusters of polymorphonuclear cells surrounded by a layer of macrophages); in other microscopic fields, we noticed that phagocytosis of the microabscess content led to their partial cystic transformation (Figures 10 and 11).

In the depth of the granulation tissue, macrophages were unevenly dispersed, being more numerous in areas with young granulation tissue and less numerous in its deep portion where the maturation of the granulation tissue was more intense. At the edge of the wound, reepithelization was obvious.

In the control group, the inflammation and regenerative process appeared more intense also in the superficial aspect of the burned wound. In contrast, the macrophage and polymorphonuclear neutrophil reaction was weaker than in the allograft group we did not notice the development of microabscesses (Figure 12).

On the histological samples from 12 days after allografting, we found small remaining allograft fragments, heavily infiltrated with polymorphonuclear neutrophils, and an increased number of macrophages in the superficial granulation tissue, as well as a decreased number in the deep area, where they appeared in a diffuse pattern. In this batch, we found not only perivascular CD68-positive macrophages but also within neoinformation vessels (Figure 13). This may suggest that certain cytokines are able to stimulate intravascular monocytes to acquire macrophage properties, before crossing the vascular wall into the connective tissue. At this time point, the restoration of the surface epithelium was intense with keratinocytes progressing from the wound edge towards its center, underneath allograft debris infiltrated by polymorphonuclear neutrophils and over the immature granulation tissue. In the control group at this time, the density of macrophages appeared somewhat more intense and homogeneous than in the allograft group.

At 15 days after allografting, the histological and immunohistochemical study of tissue fragments allowed us to note that most of the allograft was destroyed, with increasingly smaller allograft fragments identified at the surface of the granulation tissue. Overall, macrophage response appeared to be weaker than in the previous groups (Figure 14), most macrophages being identified in the superficial wound, with only few macrophages in the deep portion, where the granulation tissue was becoming more and more mature because of collagen fiber densification. Polymorphonuclear neutrophils appeared as a narrow band at the contact between allograft debris and granulation tissue. Also, cellular assessment of the allograft area emphasized the presence of rare multinucleated giant cells, considered as part of the foreign body reaction to allograft fragments (Figure 15).

Macrophage response in the control group was similar to that of macrophages in the allograft group but the intensity of the reaction appeared slightly reduced.

On histological and immunohistochemical slides from tissues sampled at 18 and 21 days, we have seen a gradual reduction in the inflammatory response in both the allograft group (Figure 16) and in the group of animals with spontaneous healing. The highest density of inflammatory cells, including macrophages, was still at the surface of the lesion where the granulation tissue was a young one, and was extremely low in the deep tissue of the skin where the granulation tissue was mature.

We also noted that at 21 days, even in areas where the
surface epithelium was fully restored, in dermal tissue a significant number of CD68-positive macrophages were preserved, which suggests that dermal remodeling process continues.

Figure 1 – Young granulation tissue at the wound edge with reduced inflammatory reaction, three days after allografting. Macrophages, specifically highlighted by anti-CD68 antibody present in the granulation tissue, but their number is relatively small (CD68 immunolabeling, ×400).

Figure 2 – Picture of granulation tissue of burned wound from an animal in the control group, three days after the injury: emergence of a small number of macrophages (CD68 immunolabeling, ×400).

Figure 3 – Overview of burned wound to six days after allografting. At the contact between the allograft and granulation tissue is noted the development of an acute inflammatory infiltrate composed mainly of polymorphonuclear neutrophils disposed as a “band” overlying an amorphous acidophilic material. The young granulation tissue occupies the middle and the deep region of the wound (HE stain, ×100).

Figure 4 – Microscopic appearance of the burned wound surface treated with allograft, six days after grafting. We noted the emergence of numerous polymorphonuclear neutrophils at the limit between the allograft and young granulation tissue (Goldner–Szekely trichrome stain, ×200).
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Figure 5 – Amorphous acidophilic material with numerous intact polymorphonuclear neutrophils or partially lysed and disposed between the allograft and the granulation tissue, observed at six days after allografting (HE stain, ×400).

Figure 6 – Macrophage response at six days after allografting from the surface of the lesion. Immediately below the allograft, the inflammatory reaction is dominated by the presence of polymorphonuclear neutrophils, while macrophages can be seen in the depth of the wound (CD68 immunolabeling, ×400).

Figure 7 – Numerous macrophages in the deep region of the burned wound, mainly perivascular, six days after allografting (CD68 immunolabeling, ×400).

Figure 8 – Microscopic image of macrophage response nine days after allografting with both increased number and volume of macrophages. The cytoplasm is heterogeneous, vacuolar, due to the endocyted material. The intensely positive reaction to CD68 betrays an increase for cytoplasmic lysosomes (CD68 immunolabeling, ×400).

Figure 9 – Cluster of macrophages at the boundary allograft and granulation tissue (CD68 immunolabeling, ×400).
Figure 10 – Microscopic image of a microabscess with incipient cystic transformation observed in the boundary area between allograft and granulation tissue (CD68 immunolabeling, ×100).

Figure 11 – Detail from the previous image with macrophage reaction within the microabscess (CD68 immunolabeling, ×400).

Figure 12 – Immunohistochemical appearance of macrophage response at nine days after the burn, in one animal from the control group: a significantly smaller number of macrophages compared to the allograft group (CD68 immunolabeling, ×400).

Figure 13 – Immunohistochemical appearance of the burned wound at 12 after allografting: mostly perivascular disposition of macrophages and also within angiogenic capillaries (CD68 immunolabeling, ×400).

Figure 14 – Macrophage response at 15 days after allografting. We notice fewer macrophages compared to previous batches (CD68 immunolabeling, ×400).

Figure 15 – Multinucleated giant cells seen at 15 days after allografting (CD68 immunolabeling, ×400).
Quantitative assessment of the macrophage response in allograft-treated burns compared with spontaneous healed ones

In addition to the qualitative evaluation of the distribution of CD68-positive macrophages in burns treated with allograft and those with spontaneous healing, we performed an assessment of the maximum density of macrophages using the “hot-spot” method. Using this technique, we evaluated both the number of macrophages per surface unit (mm$^2$) and the surface occupied by them, in areas with high cellular density.

Thus, the maximum density of macrophages in both groups of animals increased progressively and intensely from day 3 to day 9 (during this time the number of macrophages per surface unit increased by almost 300%), followed by a slow number decrease until day 21 (Figure 17). A similar trend throughout the experiment was also seen in the relative area occupied by macrophages (Figure 18). It was also noted that there is a good correlation between the number of macrophages and their relative area throughout the experiment (Figure 19).

Figure 17 – Variation in the number of macrophages in areas of spontaneous healing was parallel to areas with allografting, without significant differences between them. There was actually a strong correlation between the two sets of values ($r=0.886$). Overall, there was a statistically significant difference between the number of macrophages for spontaneous healing $[F(6.21)=4.2, p<0.001]$ and allograft $[F(6.21)=26.61, p<0.001]$.

Figure 18 – Variation in the area of macrophages in the cases with spontaneous healing was parallel to those with allografting, without significant differences between them, except the 3d and 18d time points (Student t-test, $p<0.0.5$). There was actually a good correlation between the two sets of values ($r=0.622$). Overall, there is a statistically significant difference between the values for CD68 area for spontaneous healing $[F(6.21)=12.85, p<0.001]$ and allograft $[F(6.21)=10.03, p<0.001]$. 
Discussion

The skin is the largest organ of the human body. It has many functions, including those to protect the body against pathogens and hydroelectrolyte loss [9].

In severe burns, when there is interested an area more than 20% of the body surface, hydroelectrolyte losses are massive, worsening clinical condition of the patient and morbidity [10, 11].

In extensive and deep burns, in order to reduce hydroelectrolyte losses and to prevent local infections, skin graft surgery is necessary. Best graft for healing losses are massive, worsening clinical condition of the patient and morbidity, patient and morbidity [10, 11].

In our study, we observed that local inflammatory reaction essential cells were represented by polymorphonuclear neutrophils and macrophages. They originated from the blood vessels in the periphery or depth of the burned wound. According to some authors (Lucas T et al., 2010), the influx of macrophages plays a crucial role in tissue repair [16]. Others consider that “key cells” in after-burn inflammatory response are polymorphonuclear leukocytes, mastocytes and endothelial cells [13].

What is very well stated by most researchers is that the after-burn skin integrity recovery process is very complex and depends on the depth, area and location of the lesion. We noted that remaining connective cells are involved in skin integrity restoration, especially fibroblasts, immune cells and endothelial cells. The first part of the repair reaction was dominated by immune cells. In the first 9–12 days since thermal aggression, in the burned wound appeared a major influx of polymorphonuclear neutrophils and macrophages, both in allograft treated animals group and in animals control group, then their number has been gradually reduced as granulation tissue has matured. Macrophage number explosively increased by about 300% from three to nine days after thermal aggression. We believe this reaction of the monocyte–macrophage cells system is due to chemotactic factors release in the wound, produced by residual local cells or by polymorphonuclear neutrophils but also by some substances derived from connective tissue degradation during thermal aggression. Main function of tissue macrophages is phagocytosis, these cells being able to endocyte and phagocyte pathogens, dead cells, cellular debris and various extracellular matrix components.

Based on their functional properties, at least two subpopulations of mononuclear phagocytes have been described (M1 and M2) [17]. The M1 subpopulations of cells predominantly secrete pro-inflammatory mediators that trigger and amplify inflammatory responses, whereas the M2 subpopulation cells mainly produce anti-inflammatory mediators taking part in reducing inflammatory responses while also playing an important role in wound healing and angiogenesis [18, 19]. The two cell subpopulations have several distinct surface molecules. However, tissue macrophages seem to be very plastic cells with the ability to move from a functional subpopulation to another, depending on the stimuli received [20]. According to some researchers [21, 22], in the wound healing process, most of these activated macrophages have a M2 phenotype.

In our study, CD68 marking of macrophages revealed only their phagocytosis ability, as it is known that CD68 is a membrane glycoprotein located on lysosomes and endosomes [23]. Increasing intensity of immunohistochemical reaction to CD68, the increase in size of C68 positive cells and vacuolar cytoplasm increases in the phagocytosis process of cellular and tissue debris resulting from skin exposure to high temperatures. It was also noted that in the initial phase of the development process of wound repair, both in the group of animals to which we applied allograft and the group with spontaneous healing, CD68-positive cells appeared predominantly perivascular and even intravenously. This can be explained by the fact that the glycoprotein CD68, unlike other markers of monocyte–macrophage line is present in both tissue macrophages and mono-
cytes in blood [24, 25]. In addition, it is possible that certain cytokines or other soluble factors stimulate monocytes to macrophages transformation even when they are intravascularly. Other authors [5] have also noted that in the healing process of wounds, most macrophages were perivascular.

According to some authors, in humans, as in all species of mammals, wound healing can be divided into three consecutive phases: inflammation, granulation tissue formation and remodeling. The transition from one stage to another would depend on the maturation and differentiation of primary cell populations involved in repairing: keratinocytes, fibroblasts and macrophages [26–28]. In our study, we observed that the local inflammatory process and granulation tissue formation begins concurrently. However, in the early days, an intense inflammatory reaction dominated. As the formation and maturation of granulation tissue, the reaction of macrophages decreased in intensity. Similar data were communicated by other authors who, like us, using animal models have shown that the number of macrophages increases in the first part of tissue repair, reaching a peak during the formation of granulation tissue and decreases progressively during the cell maturation phase [29].

Regarding the relationship between the response of neutrophils and of the macrophages, it was noted that in animals treated with allograft, neutrophil response was very strong, being most often identified as a dense band of cells arranged on the edge of granulation tissue and the allograft while macrophages were more numerous between neutrophils and granulation tissue. This microscopic was maintained until about 15 days of application allograft. According to some authors [16], neutrophils are present in the wound only in the early stage of wound healing, while macrophages persist in all stages of tissue repair.

In recent years, numerous studies have highlighted the importance of the innate immune response in inflammatory processes and in the repair of skin lesions [30]. Thus, macrophages in skin wounds remove cellular and tissue debris, preventing microbial infection and, through the growth factors they excrete, have a beneficial effect on tissue regeneration. However, due to the release of proinflammatory mediators and cytotoxic substances in large quantities, macrophage activity may prevent the tissue repair process [16]. Other relatively recent studies on animal models have shown that wound healing was impaired if a small number of macrophages was present in the wound [31, 32].

We believe, as do other authors [4], that so far the phenotypic and functional characteristics of macrophages participating in the healing of skin burns are not known.

## Conclusions

In the first part of the healing process of third degree skin burns, macrophages grew intensely, both numerically and as a relative area, then their number and relative area decreased progressively as the tissue matured structurally and functionally. Overall response of macrophages in third degree skin burns in rats showed a similar curve in animals treated with allograft as well as in the control group, which makes us believe that allograft does not induce a more important immune response, which would be considered pathological.

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