ORIGINAL ARTICLE / ÖZGÜN MAKALE

Turkish Journal of Immunology - Open Access
www.turkishimmunology.org

Turk J Immunol 2020; 8(2):57–64

The Effect of *in vivo* Macrophage Depletion on Skin Allograft Rejection in Wild-type and CD8 Knockout Mice

Sağlıklı ve CD8 Geni İptal Edilmiş Farelerde Deri Allograftının Reddi Üzerine Makrofajların *in vivo* Etkisi

Abdel-Rahman YOUSSEF

Abstract

Introduction: Macrophages are a well-recognized cells of the cellular infiltrate within allograft rejection, but their role has not yet been fully addressed. This study was aimed to examine the effect of macrophage depletion on skin allograft rejection.

Materials and Methods: Macrophages were depleted in recipient mice by injection of clodronate liposomes before and after skin transplantation till the day of rejection. Skin allograft rejection in C57BL/6 and CD8 knockout (KO) mice across full or class II major histocompatibility complex (MHC) antigens or multiple minor mismatches was compared.

Results: In fully MHC-mismatched grafts, clodronate liposomes treatment prolonged BALB/c skin graft survival to 12.0±0.3 days in C57BL/6 mice (p=0.0351) and to14.0±0.3 days in CD8 KO mice (p=0.0007) versus untreated control. In multiple minor mismatched BALB/B skin grafts, clodronate liposomes treatment prolonged graft survival to 13.2±0.4 days in C57BL/6 mice (p=0.0055) and to 14.4±0.4 days in CD8 KO mice (p=0.0042). However, clodronate liposomes treatment did not improve class II mismatched bm12 skin grafts survival time either in C57BL/6 or CD8 KO mice Macrophages in rejecting grafts were markedly reduced in clodronate liposomes-treated mice in comparison with control.

Conclusion: Clodronate liposomes prolonged skin allograft survival. Hence, macrophages may play an important but not essential role in skin allograft rejection.

Keywords: Skin, allograft rejection, macrophages depletion

Öz

Giriş: Makrofajlar, allogreftlerin reddedilmesinde görevi olduğu iyi bilinen ve greft etrafındaki infiltratta bulunan hücrelerdir, ancak, buradaki görevleri tam olarak anlaşılamamıştır. Bu çalışmadaki amaç, cilt grefti reddinde, makrofajları yok etmenin rolünü araştırmaktır.

Gereç ve Yöntemler: Çalışmada, alıcı farelerde makrofajları yok etmek için cilt grefti reddedilinceye kadar cilt naklinden önce ve sonra klodronat lipozomları verildi. Sadece sınıf II doku uyumluluk kompleksi (DUK) olmayan veya bir den fazla minör uyumsuzluğu olan C57BL/6 ve CD8 geni iptal edilmiş farelerde deri allograft rejeksiyonu karşılaştırıldı.

Bulgular: Tam DUK uyumsuzluğu olan C57/BL6 farelerdeki greftlerde klodronat lipozomları deri grefti yaşam süresini 12.0±0.3 güne, (P=0.0351), CD8 İE farelerde ise 14±0.3 güne (p=0.0007) çıkardı. Birden fazla minör doku uyumsuzluğu bulunan BALB/B farelerde ise klodronat lipozomları C57/BL6 farelerinde deri grefti yaşam süresini 13.2±0.4 güne, (P=0.0055), CD8 İE farelerde ise 14.4±0.4 güne (p=0.0042) artırdı. Bununla birlikte, klodronat lipozomları, ne C57/BL6 ne de CD8 geni İE farelerde II.Sınıf DUK uyumsuzluğu olan bm12 deri greftlerinin yaşam süresini değiştirmedi. Lipozom verilmiş farelere nakledilmiş ve doku reddine uğramış greftlerde makrofajların belirgin olarak azalmış olduğu saptandı.

Sonuç: Klodronat lipozomları, deri greftlerinin yaşam sürelerini artırır. Böylece, makrofajların deri greftlerinin reddinde önemli olan ancak vazgeçilmez olmayan bir rolleri olduğunu belirtmek mümkündür.

Anahtar Sözcükler: Cilt, allogreft reddi, makrofaj azaltma

[1]Faculty of Dentistry, Umm Al-Qura University, Basic and clinical sceiences, Makkah, Saudi Arabia
[2]Faculty of Medicine, Suez Canal University, Microbiology, Ismailia, Egypt
[3]University of Bristol, School of Cellular & Molecular Medicine, Bristol, United Kingdom

Correspondence:
Abdel-Rahman YOUSSEF
Faculty of Dentistry, Umm Al-Qura University, Basic and clinical sceiences, Makkah, Saudi Arabia
E-mail: amyoussef@uqu.edu.sa

Received: May 29, 2020
Accepted: Jul 20, 2020

https://doi.org/10.25002/tji.2020.1266

Available online at
http://www.turkishimmunology.org

Introduction

Acute allograft rejection is triggered by an immune response against the graft.[1] The tissue grafted and the degree of antigenic mismatch between the donor and the recipient are important determinants of the speed with which allografts are rejected.[2,3] Two major immunological mechanisms contribute to allograft rejection: the early nonspecific innate response and the late donor-specific adaptive response mediated by recipient T cells.[1]

Donor or recipient antigen presenting cells (APCs), mainly dendritic cells present the alloantigens to recipient T cells and activate them.[1] These activated T cells migrate into the graft where they activate granulocytes and macrophages that have infiltrated the graft caused by inflammatory stimuli.[1]

Although allograft rejection is T cell-dependent, other immune cells play a part in the rejection process.[4] It has been shown that the lack of $CD4^+$ T cells allowed permanent survival of fully MHC-mismatched heart grafts.[4] Furthermore, adoptive transfer of $CD4^+$ T cells from CD8 knockout (KO) mice confirmed that $CD4^+$ T cells alone were sufficient to induce rejection of MHC class I– or class II-disparate skin grafts[5] or heart allograft.[6] Moreover, both cytolytic and noncytolytic $CD4^+$ T cells were capable of promoting rapid acute cardiac allograft rejection.[7] On the contrary, the absence of B cell did not prolong skin and heart allografts.[8,9] Taken together, it likely that effective allograft rejection could occur in the absence of T cell cytotoxicity pathways and B cells, suggesting that other effector pathways, such as delayed-type hypersensitivity (DTH) responses of macrophages, may be critical for allograft rejection.

Macrophages are a well-recognized component of the cellular infiltrate in acute allograft rejections.[10,11] Macrophage influx into the graft site starts from the second day of transplantation till the day of rejection.[10] Histopathologic studies of skin allograft rejection have shown infiltration of macrophages (55–65%), granulocytes (20–25%), and lymphocytes (15–20%).[10] Macrophages can participate in the process of graft rejection in different ways.[1] Macrophages are capable of recruitment of lymphocytes into the graft site through the production of IL-1, TNF-α[12], and CC chemokines[13], or activation of T cell by antigen presentation and production of costimulatory molecules.[1] Macrophages can also work as effector cells in DTH immune response through the production of a variety of intracellular mediators such as TNF-α, nitric oxide (NO) and reactive oxygen intermediates that might contribute to macrophages-mediated apoptosis[14] and local inflammatory reaction.[15]

Various methods for suppression of macrophage function have been used.[16] Compounds such as silica, carrageenan, dextran sulfate, and gadolinium chloride can block phagocytosis, but they can activate macrophage directly and induce production of pro-inflammatory cytokine (TNF-α and IL-1) and/or NO.[16] Moreover, silica can affect T cells and natural killer cells.[16] A selective method to eliminate macrophages using clodronate liposomes has been described by van Rooijen and Sanders.[17] Liposomes are artificially prepared spheres consisting of concentric phospholipid bilayers separated by aqueous compartment. They are formed spontaneously when phospholipid (phosphatidylcholine) is dispersed in water.[17] Clodronate liposomes have shown an inhibitory effect on TNF-α and NO production by macrophages, therefore, the application of liposomes as a phagocytosis blocking agent offers the advantage of minimizing the side effects on cytokine production and secretion.[16]

When macrophages ingest the liposomes by phagocytosis, the phospholipid bilayers of the liposomes are disrupted under influence of lysosomal phospholipases and the intracellular released clodronate induces apoptosis.[18] Free clodronate is not a toxic drug in itself and has an extremely short half-life but does not cross the cell membrane.[19,20] Clodronate liposomes treatment had no observable effects on neutrophils[21] or lymphocytes[22] or dendritic cells.[23]

Since macrophages are present in nearly every organ of the body and have important immunoregulatory functions, their elimination may affect the process of graft rejection.[9] Short-term depletion of macrophage by clodronate liposomes has delayed cellular infiltration and rejection of fetal pig pancreas xenograft in non-obese diabetic mice (NOD).[24] In addition, $CD4^+$ T cell-activated macrophages were capable of rejecting an islet cell xenograft without further signals from $CD4^+$ T cells in NOD– SCID (severe combined immunodeficiency) mice, but macrophages sensitized to pig islet grafts were not able to reject mouse islet allografts.[25] Furthermore, subconjunctival administration of clodronate liposomes after orthotopic corneal allotransplantation prevented graft rejection beyond 100 days in rats.[26]

In the present study, clodronate liposomes were used to investigate the effect of *in vivo* macrophage depletion on skin allograft rejection in wild-type C57BL/6 and CD8 KO mice across full or class II major histocompatibility complex (MHC) antigens or multiple minor mismatches.

Materials and Methods

This work was conducted in the facilities of the School of Cellular and Molecular Medicine, University of Bristol, UK.

Mice

BALB/c (H-2^d), C57BL/6 (H-2^b) and B6. C-H-2bm12 (H-2^b) mice were bred at the University of Bristol SPF animal facility and BALB/B (H-2^b) mice were purchased from Harlan (UK). CD8 KO mice of C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and bred in the same facility. Male animals of 6–10 weeks of age were used. All experimental mice were housed in conventional conditions and fed water and standard animal chow ad libitum. All animal experiments complied with UK Home office regulations and the principles of laboratory animal care were followed throughout.

Liposome preparation and in vivo depletion of macrophages

Clodronate liposomes and control liposomes containing PBS only were purchased from the clodronate liposomes Organization (Vrije Universiteit, The Netherlands). Liposomes were prepared at a concentration of 1 mg/ml. Macrophages were depleted by intraperitoneal injection of clodronate liposomes at a dose of 100 µL/10 g body weight (BW), one and three days before skin transplantation then 50 µL/10 g BW weekly till the day of rejection. Additional dose 50 µL/10 g BW was injected subcutaneously one day before skin transplantation then weekly after transplantation to deplete macrophages in draining lymph nodes. Control (untreated) mice received PBS-liposomes

Skin grafts

C57BL/6 mice (n=36) and CD8 KO mice (n=35) received skin grafts from BALB/c (major and minor histocompatibility antigen (HA) disparity; full mismatch), BALB/B (multiple minor HA disparity; minor mismatch) or bm12 (single MHC class II disparity; single mismatch). The skin was grafted onto the lateral thoracic wall according to the technique of Billingham and Medawar[27] and secured in place by paraffin gauze and plaster of Paris cast. Casts were removed at day 7 and grafts scored daily. The day of rejection was taken as the first day when >90% necrosis was seen.

Immunohistochemistry (IHC)

The spleens and skin were collected, surrounded by optimal cutting temperature (OCT) compound and immediately frozen in liquid nitrogen and stored at -70°C. Cryosections (5 µm thickness) were cut at -20°C and fixed in dehydrated acetone for 10 minutes then washed in PBS for 5 minutes. Subsequent incubations of the sections were done with 5-minutes washing in PBS twice between each step. All endogenous biotin, biotin receptors, and avidin binding sites present in tissues were blocked with DAKO biotin blocking systems (DAKO Cambridgeshire, UK). Endogenous peroxidase enzyme activities were blocked with 0.3% H_2O_2 for 15 minutes and non-specific antibody binding was blocked with 5% rabbit serum (DAKO Cambridgeshire, UK) for 10 minutes. Macrophages stained with antibodies against F4/80 antigen (rat IgG2b; Serotec, Kidlington, UK). Biotinylated rabbit anti-rat IgG antibody (Vector Laboratories, Inc., Burlingame, CA) was utilized as a secondary antibody. Rat IgG2b (Serotec, Kidlington, UK) was used as a control antibody. After the application of horseradish peroxidase (HRP)-conjugated streptavidin (DAKO Cambridgeshire, UK) for 30 minutes, peroxidase reaction was developed by 3.3'diaminobenzidine (DAB) substrate chromogen (DAKO Cambridgeshire, UK) for 10 minutes to form a visible insoluble dark brown precipitate. The nuclei were light counter-stained with hematoxylin and the slides were mounted in glycerin gelatin. Semi-quantitative analysis of F4/80 IHC stains was performed using ImageJ software (National Institute of Health, USA).

Data analysis

Kaplan-Meier plots for graft survival were generated and analyzed using GraphPad Prism 7 software (GraphPad Software Inc, San Diego). Graft survival is given as mean ± SEM. The log-rank test was used to compare the survival distributions between two groups and calculate the p-value. Differences were considered to be statistically significant at $p<0.05$.

Results

Specific macrophage depletion in clodronate liposomes treated mice

F4/80$^+$ macrophages in the normal spleen were present mainly in the red pulp (Figure 1b). One day after clodronate liposomes treatment, F4/80$^+$ splenic macrophages were markedly depleted (Figure 1c) and only very few were left 2 days after clodronate liposomes treatment (Figure 1d). By day 7, F4/80$^+$ splenic macrophages were still considerably depleted (Figure 1e), but marked recovery was observed by day 10 (Figure 1f). The recovery of macrophages on

Turk J Immunol 2020; 8(2):57–64

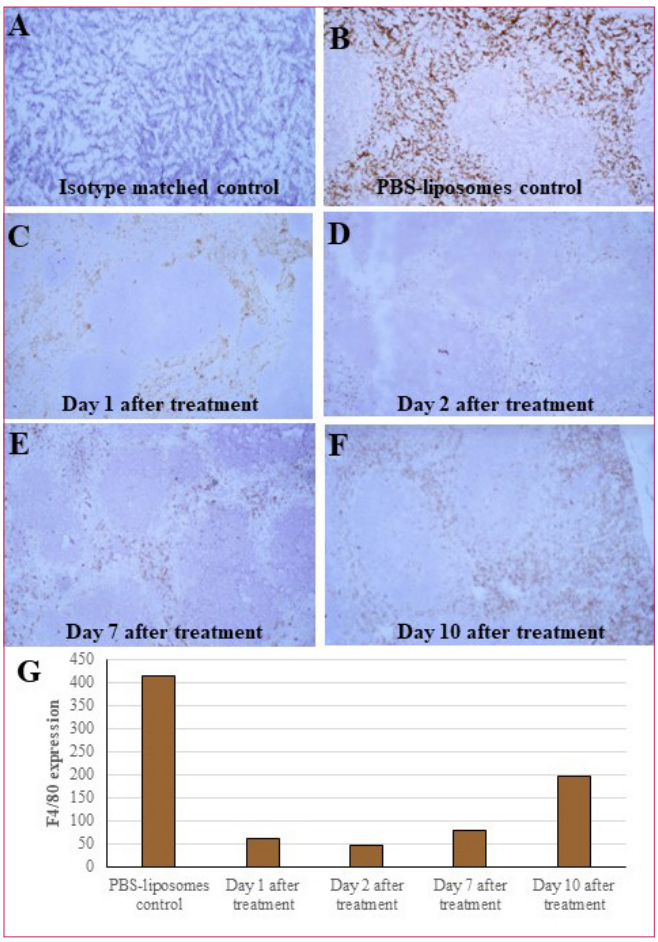


Figure 1. a–g. F4/80 expressions in the spleen after treatment with clodronate liposomes or PBS-liposomes (control). Isotype matched control staining is shown in (a). Spleens were assessed in normal mice before treatment (b), and at day 1 (c), day 2 (d), day 7 (e) and day 10 (f) after clodronate liposomes treatment. Semi-quantitative analysis of IHC was performed using imageJ software (g) (magnification ×100).

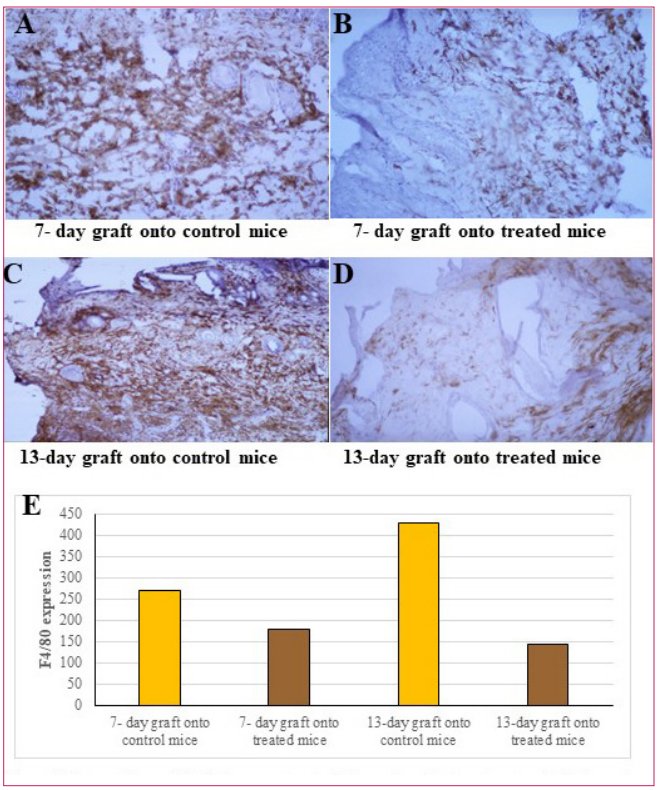


Figure 2. a–e. Comparison of F4/80 expressions in skin grafts after treatment with clodronate liposomes or PBS-liposomes control. BALB/B skin was grafted onto either clodronate liposomes treated– or PBS-liposomes control C57BL/6 mice (multiple minor mismatched). The skin grafts were assessed on day 7 (a, b) and on day 13 which is the rejection day (c, d). In control mice, macrophages infiltration was intense and distributed throughout the dermis and epidermis whereas in clodronate liposome-treated mice the infiltration is markedly reduced. Semi-quantitative analysis of IHC was performed using imageJ software (e) (magnification ×200).

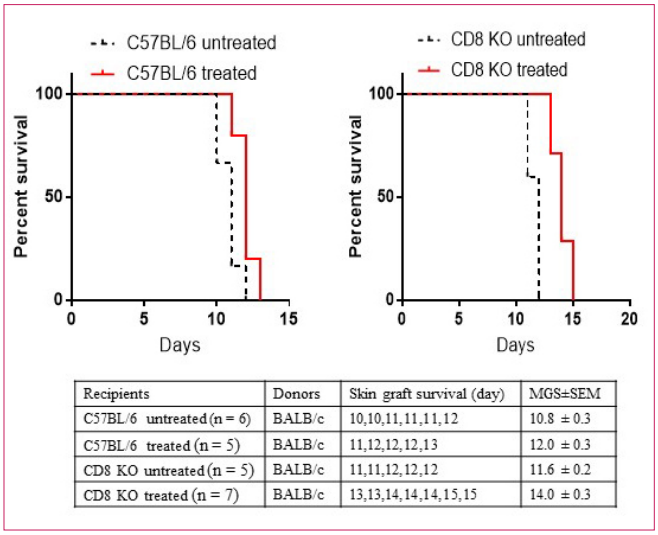
days 1, 2, 7, and 10 after clodronate liposomes treatment was semi-quantified in Figure 1g.

Macrophage infiltration in skin allografts

F4/80 expressions in minor mismatched skin grafts after treatment with clodronate liposomes or PBS-liposomes control were compared (Figure 2). Seven days after transplantation, control mice showed marked infiltration by macrophage which were distributed throughout the dermis and epidermis (Figure 2a) whereas, in mice treated with clodronate liposomes, macrophage infiltration was markedly reduced and mainly seen in the dermis (Figure 2b). On day13 (rejection day), the skin grafts were heavily infiltrated by macrophages, which were distributed throughout all skin layers and among necrotic tissue (Figure 2c). However, in clodronate liposomes treated mice, macrophage infiltration was markedly reduced and observed throughout the dermis and epidermis (Figure 2d). F4/80 expressions in skin grafts after treatment with clodronate liposomes were semi-quantified compared with control (Figure 2e).

Effect of macrophage depletion on the survival of fully MHC-mismatched skin graft

The survival of fully MHC-mismatched skin allografts is shown in Figure 3. C57BL/6 mice rejected the BALB/c grafts after 10.8±0.3 days, but clodronate liposomes treatment prolonged graft survival to 12±0.3 days ($p=0.0351$). In CD8 KO mice, fully MHC-mismatched skin allografts survived for 11.6±0.2; but, clodronate liposomes treatment prolonged graft survival to 14±0.3 ($p=0.0007$).

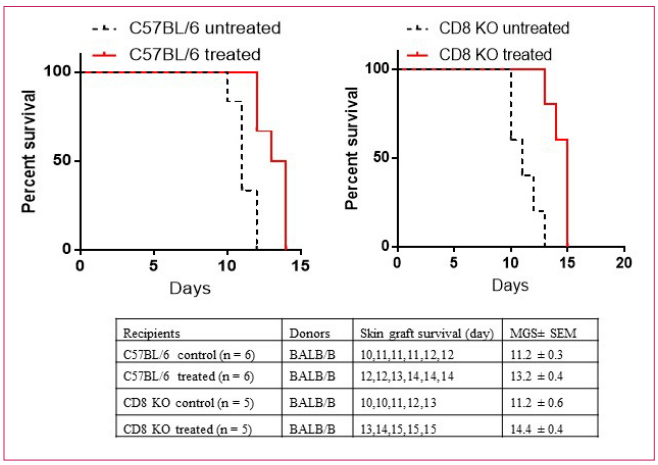


Recipients	Donors	Skin graft survival (day)	MGS±SEM
C57BL/6 untreated (n = 6)	BALB/c	10,10,11,11,11,12	10.8 ± 0.3
C57BL/6 treated (n = 5)	BALB/c	11,12,12,12,13	12.0 ± 0.3
CD8 KO untreated (n = 5)	BALB/c	11,11,12,12,12	11.6 ± 0.2
CD8 KO treated (n = 7)	BALB/c	13,13,14,14,14,15,15	14.0 ± 0.3

Figure 3. Effect of macrophage depletion on survival of fully MHC–mismatched skin graft. BALB/c skin was grafted onto C57BL/6 and CD8 KO mice which were treated with clodronate liposomes or PBS-liposomes. Graft survival was monitored daily. The day of rejection was taken as the first day when >90% necrosis was seen (MGS, mean graft survival; SEM, standard error of mean; p=0.0351 compared with PBS-liposomes-treated control C57BL/6 mice; p=0.007 compared with PBS-liposomes-treated control CD8 KO mice).

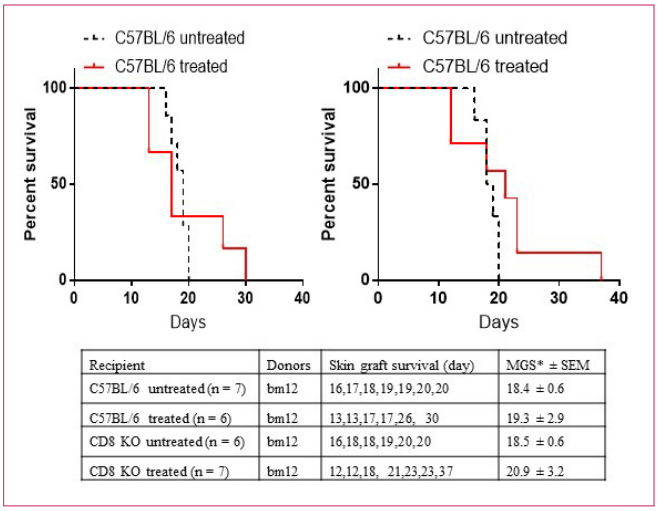
Effect of macrophage depletion on the survival of minor HA mismatched skin graft

The graft survival of minor mismatched skin grafts is shown in Fig. 4. C57BL/6 mice rejected the BLAB/B grafts after 11.2±0.3 days, but clodronate liposomes treatment prolonged graft survival to 13.2±0.4 days (p=0.0055). In CD8 KO mice, minor mismatched skin allografts survived for 11.2±0.6 days; but, clodronate liposomes treatment prolonged graft survival to14.4±0.4 days (p=0.0042).



Recipients	Donors	Skin graft survival (day)	MGS± SEM
C57BL/6 control (n = 6)	BALB/B	10,11,11,11,12,12	11.2 ± 0.3
C57BL/6 treated (n = 6)	BALB/B	12,12,13,14,14,14	13.2 ± 0.4
CD8 KO control (n = 5)	BALB/B	10,10,11,12,13	11.2 ± 0.6
CD8 KO treated (n = 5)	BALB/B	13,14,15,15,15	14.4 ± 0.4

Figure 4. Effect of macrophage depletion on survival of multiple minor HA mismatched skin graft. BALB/B skin was grafted onto C57BL/6 and CD8 KO mice which were treated with clodronate liposomes or PBS-liposomes (control). Graft survival was monitored daily. The day of rejection was taken as the first day when >90% necrosis was seen (MGS, mean graft survival; SEM, standard error of mean; p=0.0055 compared with PBS-liposomes-treated control C57BL/6 mice; p=0.0042 compared with PBS-liposomes-treated control CD8 KO mice).



Recipient	Donors	Skin graft survival (day)	MGS* ± SEM
C57BL/6 untreated (n = 7)	bm12	16,17,18,19,19,20,20	18.4 ± 0.6
C57BL/6 treated (n = 6)	bm12	13,13,17,17,26, 30	19.3 ± 2.9
CD8 KO untreated (n = 6)	bm12	16,18,18,19,20,20	18.5 ± 0.6
CD8 KO treated (n = 7)	bm12	12,12,18, 21,23,23,37	20.9 ± 3.2

Figure 5. Effect of macrophage depletion on survival of single MHC class II antigen mismatched skin graft. bm12 skin was grafted onto C57BL/6 and CD8 KO mice which were treated with clodronate liposomes or PBS-liposomes. Graft survival was monitored daily. The day of rejection was taken as the first day when >90% necrosis was seen. No significant difference between clodronate liposomes treated mice and control was found (p>0.05) (MGS, mean graft survival; SEM, standard error of mean; p=0.7641 compared with PBS-liposomes-treated control C57BL/6 mice; p=0.1587 compared with PBS-liposomes-treated control CD8 KO mice).

Effect of macrophage depletion on the survival of single MHC class II antigen mismatched bm12 skin graft

As can be seen in Fig. 5, clodronate liposomes treatment did not improve class II mismatched (bm12 onto C57BL/6) skin allografts survival time either in C57BL/6 mice (19.3±2.9 days) or in CD8 KO mice (20.9±3.2 days) compared with control (18.4±0.6 and 18.5±0.6 days respectively).

Discussion

It is difficult to study the role of macrophages in the graft rejection process *in vivo* due to a lack of methods that specifically deplete or inactivate them. However, a van Rooijen developed a method for selective suppression of macrophage function based on the liposomes mediated intracellular delivery of dichloromethylene diphosphonate (Cl2MDP or clodronate).[23] It is believed that clodronate liposomes injected intraperitoneally may affect higher

numbers of macrophages than if administered by other routes. Peritoneal macrophages are primarily affected by intraperitoneal injection. However, lymph drains from the peritoneal cavity that offers a large absorbing surface from which the liposomes enter the circulation rapidly through the lymph nodes in the abdominal cavity and, as a consequence, liver and splenic macrophages will also be affected.[28]

In this study, clodronate liposomes intraperitoneal injection of C57BL/6 mice resulted in depletion of splenic macrophages after 2 days with partial recovery after 7 days and considerable recovery after 10 days. This is consistent with previous studies in murine models.[24,29] To achieve long-term macrophage depletion, clodronate liposomes were injected every week till the day of rejection.

In the current experiments, clodronate liposomes were injected intraperitoneally on day 0 and day 2, and skin grafts were performed on day 3. By this time, clodronate liposomes depleted tissue macrophages especially in the peritoneum, spleen, and liver and thereby reduced the total number and recruitment to the site of the graft. In the present work, clodronate liposomes reduced macrophage infiltration of skin grafts. However, during graft rejection, macrophage infiltration increased concordant with splenic macrophage recovery and it was observed in the dermis and epidermis, but it was still markedly reduced in comparison with the controls. These results may be explained by the effect on the circulating monocytes as the numbers of peripheral blood monocytes were sharply decreased 2 days after clodronate treatment.[24,30] In addition, peripheral blood monocytes may be delivered to the liver and spleen of clodronate liposomes treated mice to compensate for the depleted macrophages. Another possibility is that clodronate liposomes may enter the graft site from the local damaged endothelium and locally depleted macrophages after they have infiltrated.

Macrophages are present in all types of allografts and their numbers increased steadily as rejection progress.[31] Macrophages are associated with the skin[10] and heart[11] allograft rejection, but it is not known if this association has a significant effect on the grafted tissue and the mechanisms involved in graft rejection.

In the present study, the survival of MHC full and minor mismatched skin grafts was improved in C57BL/6 and CD8 KO mice treated with clodronate liposomes compared with control mice and correlated with reduced graft macrophage infiltration suggesting that macrophages may play a role in skin allograft rejection. However, macrophage depletion treatment prolonged graft survival by only 2–3 days, suggesting that macrophages are not major effector cells in skin allograft rejection.

During the course of graft rejection, the contribution of macrophages early as a non-specific mechanism or late as effector cells in DTH response is still unclear. Survival of liver transplants was improved significantly by treatment of donor rats with gadolinium chloride 24 hours before storage in the liver to inactivate the Kupffer cells[32], this suggests the participation of macrophage early in the graft rejection process. On the other hand, Yamamoto et al.[10] claimed that the major effector cells mediating allografted skin rejection are allograft-induced macrophages and not T cells. Previous studies suggested that allospecific DTH response alone is sufficient to mediate graft rejection. $CD4^+$ T helper cell 1 (Th1) cells can recruit and activate macrophages within the allograft, leading to DTH alloreaction[33] through the production of IFN-γ.[34] The depletion of macrophages results in a decrease in expression of macrophage-derived cytokines, IL-12, IL-1, and TNF-α and a decrease in the Th1 immune response.[35] Therefore, the decrease in the Th1 immune response along with the downregulation of macrophage-derived soluble mediators may play a role in the prolongation of skin graft survival in clodronate liposomes treated mice.

In CD8 KO mice, cytotoxic T lymphocyte (CTL) pathway promoting skin allograft rejection, could not be excluded in the present study. A subset of $CD4^+$ T cells has been reported to have a cytotoxic function.[36] $CD4^+$ CTL preferentially lyse their target via the interaction between Fas and Fas ligand on the allogenic targets.[37] In the skin, keratinocytes are known to express Fas in the basal state and may become sensitive to Fas ligand-mediated apoptosis induced by alloreactive $CD4^+$ cytotoxic T cells.[38]

It is important to stress that in bm12 to C57BL/6 strain combination, MHC class II mismatch (3 amino acids within the Ia MHC class II antigen) is recognized only by $CD4^+$ T cells. Macrophage depletion did not improve class II mismatched skin allografts survival time either in C57BL/6 or in CD8 KO mice and there was no significant difference between graft survival time in wild-type and CD8 KO mice. $CD4^+$T cells may display direct cytotoxicity toward cells expressing MHC class II alloantigens.[39] However, recent studies raise the possibility of the CD4-

dependent pathway of skin allograft rejection is mediated by IL-5-activated eosinophils.[40,41] Eosinophils can be activated by Th2 cytokines (IL-4 and IL-5) and produce several toxic molecules such as the neurotoxin, eosinophil cationic protein, eosinophil peroxidase, and major basic protein, that may damage the allograft.[42]

This study has some limitations. Although clodronate liposomes treatment markedly reduced macrophage infiltrate in skin allograft, it did not deplete macrophage completely. Hence it is difficult to assess the role of macrophages in allograft rejection accurately. Even if clodronate liposomes depleted macrophages completely, the rate of repopulation must be considered.

In conclusion, clodronate liposomes markedly reduced graft infiltration by macrophage and prolonged the survival of skin allografts. These data demonstrated that macrophages may play an important but not essential role in skin allograft rejection.

Ethics Committee Approval: All animal experiments complied with UK Home office regulations and the principles of laboratory animal care were followed throughout.

Conflict of interest: None

Acknowledgments: This work was funded by an Egyptian Government fellowship to Youssef AR.

References

1. Moreau A, Varey E, Anegon I, Cuturi MC. Effector mechanisms of rejection. Cold Spring Harb Perspect Med 2013;3:a015461. [Crossref]
2. Howard JC, Butcher GW. The Mechanism of Graft Rejection and the Concept of Antigenic Strength. Scand J Immunol 1981;14:687-91. [Crossref]
3. Zhang Z, Zhu L, Quan D, Garcia B, Ozcay N, Duff J, et al. Pattern of liver, kidney, heart, and intestine allograft rejection in different mouse strain combinations. Transplantation 1996;62:1267-72. [Crossref]
4. Youssef AR, Otley C, Mathieson PW, Smith RM. Role of $CD4^+$ and $CD8^+$ T cells in murine skin and heart allograft rejection across different antigenic desparities. Transpl Immunol 2004;13:297-304. [Crossref]
5. Dalloul AH, Ngo K, Fung-Leung WP. CD4-negative cytotoxic T cells with a T cell receptor α/β intermediate expression in CD8-deficient mice. Eur J Immunol 1996;26:213-8. [Crossref]
6. Krieger NR, Yin DP, Fathman CG. CD4+ but not CD8+ cells are essential for allorejection. J Exp Med 1996;184:2013-8. [Crossref]
7. VanBuskirk AM, Wakely ME, Orosz CG. Acute rejection of cardiac allografts by noncytolytic $CD4^+$ T cell populations. Transplantation 1996;62:300-2. [Crossref]
8. Cerny A, Ramseier H, Bazin H, Zinkernagel RM. Unimpaired first-set and second-set skin graft rejection in agammaglobulinemic mice. Transplantation 1988;45:1111-2. [Crossref]
9. Alexander DZ, Pearson TC, Hendrix R, Ritchie SC, Larsen CP. Analysis of effector mechanisms in murine cardiac allograft rejection. Transpl Immunol 1996;4:46-8. [Crossref]
10. Yamamoto N, Einaga-Naito K, Kuriyama M, Kawada Y, Yoshida R. Cellular basis of skin allograft rejection in mice: specific lysis of allogeneic skin components by non-T cells. Transplantation 1998;65:818-25. [Crossref]
11. Christmas SE, MacPherson GG. The role of mononuclear phagocytes in cardiac allograft rejection in the rat. Cell Immunol 1982;69:271-80. [Crossref]
12. Mantovani A, Dejana E. Cytokines as communication signals between leukocytes and endothelial cells. Immunol Today 1989;10:370-5. [Crossref]
13. Rollins BJ. Chemokines. Blood 1997;90:909–28. [Crossref]
14. Kobayashi K, Kaneda K, Kasama T. Immunopathogenesis of delayed-type hypersensitivity. Microsc Res Tech 2001;53:241-5. [Crossref]
15. Justiz Vaillant AA, Zulfiqar H, Ramphul K. Delayed Hypersensitivity Reactions. Treasure Island (FL): Stat Pearls Publishing; 2020. https://www.ncbi.nlm.nih.gov/books/NBK519023/
16. van Rooijen N, Sanders A. Elimination, blocking, and activation of macrophages: three of a kind? J Leukoc Biol 1997;62:702-9. [Crossref]
17. Van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994;174:83-93. [Crossref]
18. van Rooijen N, Sanders A, van den Berg TK. Apoptosis of macrophages induced by liposome-mediated intracellular delivery of clodronate and propamidine. J Immunol Methods 1996;193:93-9. [Crossref]
19. van Rooijen N, Hendrikx E. Liposomes for specific depletion of macrophages from organs and tissues. Methods Mol Biol 2010;605:189-203. [Crossref]
20. Van Rooijen N, Sanders A. Kupffer cell depletion by liposome-delivered drugs: comparative activity of intracellular clodronate, propamidine, and ethylenediaminetetraacetic acid. Hepatology 1996;23:1239-43. [Crossref]
21. Qian Q, Jutila MA, Van Rooijen N, Cutler JE. Elimination of mouse splenic macrophages correlates with increased susceptibility to experimental disseminated candidiasis. J Immunol 1994;152:5000-8. https://www.jimmunol.org/content/152/10/5000
22. Claassen I, Van Rooijen N, Claassen E. A new method for removal of mononuclear phagocytes from heterogeneous cell populations in vitro, using the liposome-mediated macrophage "suicide" technique. J Immunol Methods 1990;134:153-61. [Crossref]
23. van Rooijen N, Kors N, Kraal G. Macrophage subset repopulation in the spleen: differential kinetics after liposome-mediated elimination. J Leukoc Biol 1989;45:97-104. [Crossref]
24. Fox A, Koulmanda M, Mandel TE, van Rooijen N, Harrison LC. Evidence that macrophages are required for T-cell infiltration and rejection of fetal pig pancreas xenografts in nonobese diabetic mice. Transplantation 1998;66:1407–16. [Crossref]
25. Yi S, Hawthorne WJ, Lehnert AM, Ha H, Wong JKW, van Rooijen N, et al. T Cell-Activated Macrophages Are Capable of Both Recognition and Rejection of Pancreatic Islet Xenografts. J Immunol 2003;170:2750–8. [Crossref]

26. Slegers TP, Torres PF, Broersma L, van Rooijen N, van Rij G, van der Gaag R. Effect of macrophage depletion on immune effector mechanisms during corneal allograft rejection in rats. Invest Ophthalmol Vis Sci 2000;41:2239–47. https://iovs.arvojournals.org/article.aspx?articleid=2123049
27. Billingham RE, Medawar PB. The Technique of Free Skin Grafting in Mammals. J Exp Biol 1951;28:385–402. https://jeb.biologists.org/content/jexbio/28/3/385.full.pdf
28. Van Rooijen N, Bakker J, Sanders A. Transient suppression of macrophage functions by liposome-encapsulated drugs. Trends Biotechnol 1997;15:178–85. [Crossref]
29. Van Rooijen N, Kors N, Ende M v. d., Dijkstra CD. Depletion and repopulation of macrophages in spleen and liver of rat after intravenous treatment with liposome-encapsulated dichloromethylene diphosphonate. Cell Tissue Res 1990;260:215–22. [Crossref]
30. Huitinga I, Damoiseaux JGMC, van Rooijen N, Döpp EA, Dijkstra CD. Liposome Mediated Affection of Monocytes. Immunobiology 1992;185:11–9. [Crossref]
31. Mayer TG, Bhan AK, Winn HJ. Immunohistochemical analyses of skin graft rejection in mice: Kinetics of lymphocyte infiltration in grafts of limited immunogenetic disparity. Transplantation 1988;46:890–8. [Crossref]
32. Savier E, Lemasters JJ, Thurman RG. Kupffer cells participate in rejection following liver transplantation in the rat. Transpl Int 1994;7:183–6. [Crossref]
33. Sirak J, Orosz CG, Wakely E, Vanbuskirk AM. Alloreactive delayed-type hypersensitivity in graft recipients: Complexity of responses and divergence from acute rejection. Transplantation 1997;63:1300–7. [Crossref]
34. Mark Doherty T. T-cell regulation of macrophage function. Curr Opin Immunol 1995;7:400–4. [Crossref]
35. Jun HS, Yoon CS, Zbytnuik L, Van Rooijen N, Yoon JW. The role of macrophages in T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Exp Med 1999;189:347–58. [Crossref]
36. Doymaz MZ, Foster CM, Destephano D, Rouse BT. MHC II-restricted, CD4+ cytotoxic T lymphocytes specific for herpes simplex virus-1: Implications for the development of herpetic stromal keratitis in mice. Clin Immunol Immunopathol 1991;61:398–409. [Crossref]
37. Hahn S, Gehri R, Erb P. Mechanism and Biological Significance of CD4-mediated Cytotoxicity. Immunol Rev 1995;146:57–79. [Crossref]
38. Gutierrez-Steil C, Wrone-Smith T, Sun X, Krueger JG, Coven T, Nickoloff BJ. Sunlight-induced basal cell carcinoma tumor cells and ultraviolet-B- irradiated psoriatic plaques express Fas ligand (CD95L). J Clin Invest 1998;101:33–9. [Crossref]
39. Rosenberg AS, Singer A. Cellular basis of skin allograft rejection: An in vivo model of immune-mediated tissue destruction. Annu Rev Immunol 1992;10:333–60. [Crossref]
40. Martinez OM, Ascher NL, Ferrell L, Villanueva J, Lake J, Roberts JP, Krams SM. Evidence for a nonclassical pathway of graft rejection involving interleukin 5 and eosinophils. Transplantation 1993;55:909–18. [Crossref]
41. Le Moine A, Surquin M, Demoor FX, Noël JC, Nahori MA, Pretolani M, et al. IL-5 mediates eosinophilic rejection of MHC class II-disparate skin allografts in mice. J Immunol 1999;163:3778–84. https://www.jimmunol.org/content/163/7/3778.abstract
42. Rothenberg ME. Eosinophilia. N Engl J Med 1998;338:1592–600. [Crossref]