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The Effect of Atorvastatin (Lipitor) on the Duration of Survival of Allogeneic Skin Graft and the Growth of B16F10 Melanoma Cells in Mice

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Authors' contributions

This work was carried out in collaboration between all authors. Author NZ performed the experiments and statistical analysis for his Master of Sciences, wrote the draft of the manuscript and assisted in preparing the revised manuscript. Author FER supported the study as a research assistant and did bench work. Author NSAA supported the study as a research assistant and did the serum cholesterol determinations. Author AMA is the PI who designed the study, and revised the first and second draft of the manuscript. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: To evaluate the immunomodulatory effect of using non-cholesterol lowering dose of atorvastatin (AS) on skin allograft survival and on tumor growth in mice.
Study Design: Experimental Study.
Place and Duration of Study: Department of Experimental Pathology, Immunology and Microbiology, Faculty of Medicine, American University of Beirut; 2011-2012.
Methodology: BALB/c mice were transplanted with skin allografts from C57BL/6 mice and given either AS alone or in combination with immunosuppressive agents. Average survival days of skin allografts were recorded and serum levels of interleukin-1β (IL-1β) and interferon-γ (IFN-γ) were quantified. BALB/c mice and C57BL/6 mice were challenged intraperitoneally with B16F10 melanoma cancer cells (cancer cell line syngeneic to C57BL/6 mice) and were then treated with AS. They were observed

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regularly for tumor growth.
Results: The results indicated that in transplant mice AS given alone or in combination with immunosuppressive agents prolonged allograft survival time through non-cholesterol lowering mechanisms in spite of a non-significant change in serum cytokine levels. Furthermore, AS treatment enhanced tumor growth in C57BL/6 mice and promoted tumor growth in BALB/C mice.
Conclusion: It can be speculated that AS down expresses TLR and modifies MHC presentation resulting in hindering the generation of an innate and adaptive immune response.

Keywords: Immunosuppression; skin transplantation; cancer; statins; cytokines.

1. INTRODUCTION

Statins are widely used clinically for the prevention of primary and secondary cardiovascular diseases owing to their potent plasma cholesterol reduction properties [1]. Statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), the enzyme that catalyzes the rate limiting step in the biosynthesis of cholesterol. Inhibition of HMG-CoA reductase by statins prevents the production of cholesterol and of other downstream intermediates including geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) that are involved in post-translational modification of essential proteins including nuclear lamins, Rho, Ras and Rac GTPases [2]. These proteins serve crucial functions in cell migration, differentiation and proliferation [3].
Extensive studies on the non-cholesterol lowering effects of statins have generated convincing evidence that statins possess pleiotropic effects and can alter numerous aspects of key biological systems including the immune system [4]. Hot et. al. reported that simvastatin or rosuvastatin inhibited the pro-inflammatory effects of interleukin-17 (IL-17) and tumor necrosis factor-α (TNF-α) on endothelial cells [5]. The two cytokines have been implicated in the pathogenesis of some autoimmune diseases including rheumatoid arthritis and psoriasis. Earlier we reported that atorvastatin (AS) suppressed interferon-γ (IFN-γ), interleukin-4 (IL-4) and antibody levels, in mice immunized with egg albumin [6] Moreover, it has been observed that acute rejection episodes were less, and duration of kidney graft survival was longer in recipients that were given statins in addition to conventional immunosuppressive therapy [7]. Statins have been suggested as potential therapeutic agents to treat a wide range of immune-related diseases including autoimmune diseases, cancer, asthma and graft rejection [8,9,10].

The experiments described in the present study were performed to test whether AS which is the most widely prescribed drug for the treatment of hypercholesterolemia and the most potent statin with a high lipophilic tendency prolongs survival of allogeneic skin graft and affects tumor growth in mice.

2. MATERIALS AND METHODS

2.1 Atorvastatin and Immunosuppressive Agents

AS (Lipitor, Pfizer, New York, NY, USA) tablets were pulverized and suspended in phosphate buffered saline (PBS). Cyclosporin A (CSA) suspension (Sandimmune, Novartis, Basel, Switzerland) was diluted in olive oil. Prednisone (P, Cortancyl, Sonafi Aventis,

Paris, France) was dissolved in PBS. Mycophenolate mofetil (MMF, Cellcept, Roche, Basel, Swtizerland) was dissolved in PBS. The amount injected intraperitoneally in a volume of 0.3ml when given alone or 0.1ml when given in combination is given in Table 1. A dose of 40 mg/kg of AS was used because in a previous study this dose was determined to be the minimum dose that affected the immune response to egg albumin in mice [6]. Pulverized AS tablets rather than pure AS were used because the idea was to simulate as much as possible the use of AS clinically.

Table 1. Treatment of BALB/c mice that received a skin transplant obtained from C57BL/6 mice

Group	Treatment
1	PBS
2	Oil
3	Prednisone (P), 20mg/kg
4	Atorvastatin (AS), 40mg/kg
5	Mycophenolate mofetil (MMF), 100mg/kg
6	Cyclosporin A (CSA), 20mg/kg
7	P, 10mg/kg + MMF, 50mg/kg + CSA, 10mg/kg
8	AS, 20mg/kg + P, 10mg/kg + MMF, 50mg/kg + CSA, 10mg/kg

**intraperitoneal injections were given to BALB/c mice 2 days prior to, and every other day after the C57BL/6 skin was transplanted.*

2.2 Animals

BALB/c and C57BL/6 mice aged 6 – 8 weeks old were obtained from the animal care facility at the American University of Beirut Faculty of Medicine. Animal rooms were maintained at 21 ± 1ºC, 50% ± 10% humidity, and 12-hour light/dark cycle. Commercial rodent ration and water were freely available. Animals were acclimatized for at least 1 week before experimentation. All animal handling procedures were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Faculty of Medicine at the American University of Beirut.

2.3 Effect of AS on Skin Allograft Survival

In the case of the effect of AS in comparison with established immunosuppressive agents on the survival of skin transplants, 8 groups of BALB/c mice were used, each group consisting of 9 mice. They were treated as indicated in Table 1. Pharmacological agents were tested alone or in combination. Intraperitoneal injections were given to BALB/c mice 2 days prior to, and every other day after the C57BL/6 skin was transplanted. On days 5 and 10 post transplantation and on rejection day, 3 mice from each group were terminally anesthetized with a 0.1ml of a mixture of 0.075ml ketamine (Rotexmedica Trittau, Germany) (final concentration 37.5mg/ml) and 0.025ml xylazine (Interchemie, Castenray, Holland) (final concentration 5mg/ml). Next, mice were exsanguinated by cardiac puncture. Death following blood collection was ensured by heart incision or by giving an overdose of the anaesthetic. Blood from each group was pooled; the serum was separated and used to determine interleukin-1β (IL-1β) and IFN-γ levels by Enzyme-Linked Immunosorbant Assay (ELISA).

2.4 Total Cholesterol Levels in Normocholesterolemic Mice Receiving AS

Ten BALB/c mice were used for testing for the effect of AS on cholesterol levels. Five mice received daily injection of AS or PBS for a period of 5 days. On Day 5, blood was collected by cardiac puncture; serum was separated and used to determine cholesterol levels.

Cholesterol levels were determined by an automated analyzer (Cobas Integra 400+, Roche, Basel, Switzerland).

2.5 Transplantation

The skin transplantation procedure described by McFarland and Rosenberg [11] was adopted with some modifications. In brief, a fully mismatched allogeneic skin transplantation procedure was performed two days after the first injection was given. Recipient and donor mice were anesthetized with a mixture 0.025ml of xylazine and 0.075ml ketamine for the procedure. Hair of the skin to be transplanted was trimmed with a clipper. Skin allografts of 2 x 1 cm (full thickness) from black C57BL/6 mice were grafted onto the back of BALB/c white mice. A Tegaderm I.V Film Dressings; (http://www.nu-careproducts.co.uk/dressings2.htm) was used to cover the graft. For best results, an additional gauge tape was wrapped around the mouse. Transplanted mice were then put on a warm-pad until they woke up. Dressing was removed and the allograft was observed daily. Signs of graft rejection included hair loss, scar formation, shrinkage and necrosis of graft. Graft rejection was defined as >80 percent destruction of the allograft. On the other hand growth of hair on the graft was indicative of graft survival.

2.6 ELISA

Serum IL-1β and IFN-γ levels were determined by ELISA using single analyte ELISArray IL-1β kit and single analyte ELISArray IFN-γ kits (SABiosciences, Fredrick, MD, USA). The procedure described by the manufacturer was followed. The technique relies on the standard quantitative sandwich based ELISA to measure the amount of IL-1β or IFN-γ in serum.

2.7 B16F10 Melanoma Cells

B16F10 melanoma cells are syngeneic to C57BL/6 mice [12,13]. They were maintained as monolayers *in vitro* in RPMI-1640 containing 1% L-Glutamine and Hepes buffer supplemented with 1% penicillin-streptomycin and 10% heat inactivated fetal bovine serum [14,15]. A 0.3 ml suspension containing $2.5x10^5$cells was injected. . Preliminary studies were performed to determine the appropriate cell concentration.

2.8 Effect of AS on Growth of B16F10 Melanoma Cells in C57BL/6 and BALB/c Mice

Two groups of BALB/c mice and 2 groups of C57BL/6 mice were used. One group of each strain was given a daily intranperitoneal (i.p.) injection of 0.3ml of PBS and the other group of each strain was given a daily ip injection of AS (40mg/kg). One week after the first injection, each mouse in both groups was challenged with 0.3ml of the tumor cell suspension. Treatment was sustained on daily basis following tumor challenge. Overall health and activity of mice were assessed by regular observation and weighing. Tumor

growth was evaluated by palpitation and later ascertained upon dissection. Unfortunately the tumor grew in solid rather than ascites form otherwise the growth of tumor could have been expressed as cells/ml.

2.9 Statistical Analysis of Data

Whenever applicable data were expressed as Mean±SD. Comparison between two groups for total cholesterol levels was performed using student-t test. Graft survival was evaluated by generating kaplan–meier survival curves. P-values <0.05 were considered statistically significant.

3. RESULTS

3.1 Cholesterol levels

Serum cholesterol levels were determined; results are shown in Table 2. Average level of cholesterol in mice receiving AS (93.6mg/dl) was lower than the group receiving only PBS (100.6 mg/dl). P value (0.526) indicates no significant difference.

Table 2. Serum cholesterol levels of mice treated with AS or PBS daily for a period of 5 days

Group	Total cholesterol levels of individual mouse (mg/dl)					Average (mg/dl)
Control (PBS)	102	97	83	134	87	100.6±20.2
AS	98	91	83	112	84	93.6±11.9

3.2 Graft Survival

It was noted that in all treated groups there was a prolongation of graft survival of 7 to 13.5 days as compared to the controls. There was an average prolongation of graft survival of 7 (±3.5) days in the group that received AS alone compared to the control group (P value>0.05). Fig. 1 is a comparison of the appearance of the skin grafts in a mouse that was treated with AS and a mouse treated with PBS. Note the growth of hair on the graft transplanted onto the AS-treated mouse. Moreover, when AS was added to triple therapy (P + CSA + MMF) graft survival was prolonged an additional 3.5 (±0.7) days when compared to the triple therapy alone (Figs. 2 and 3).

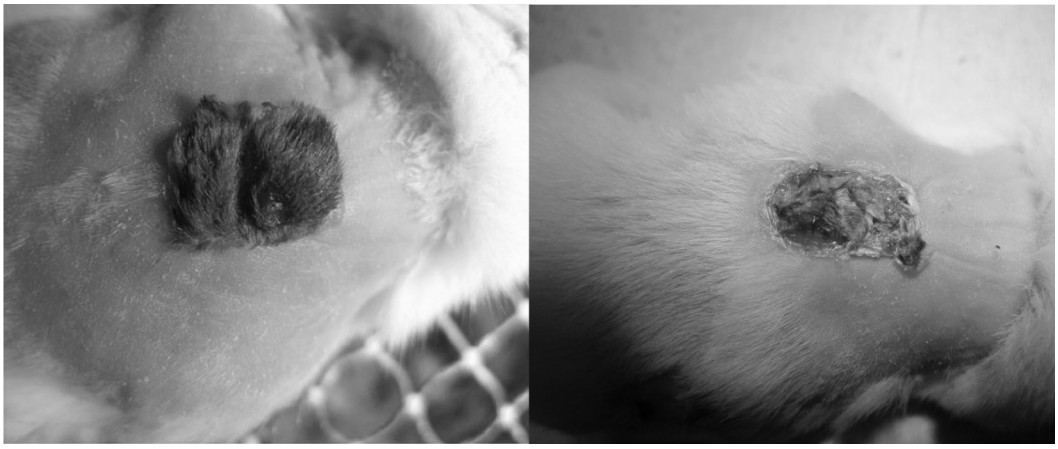

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Fig. 1. Macroscopic evaluation of skin allografts. BALB/c mice were transplanted with full-thickness C57BL/6 mice skin grafts. Skin grafts were assessed by visual and tactile inspection until necrosis, defined as >80 percent destruction of the allograft. (A) Shows transplanted skin of mouse treated intraperitoneally with AS alone (40mg/kg) every other day starting two days prior to transplantation. Picture was taken 13 days post-transplantation. Minor signs of necrosis could be noticed including mild scar formation and shrinkage. (B) Shows transplanted skin of mouse treated intraperitoneally with PBS. Picture was taken on day 9 post-transplantation; signs of rejection including extensive necrosis, shrinkage and hair loss are noticed

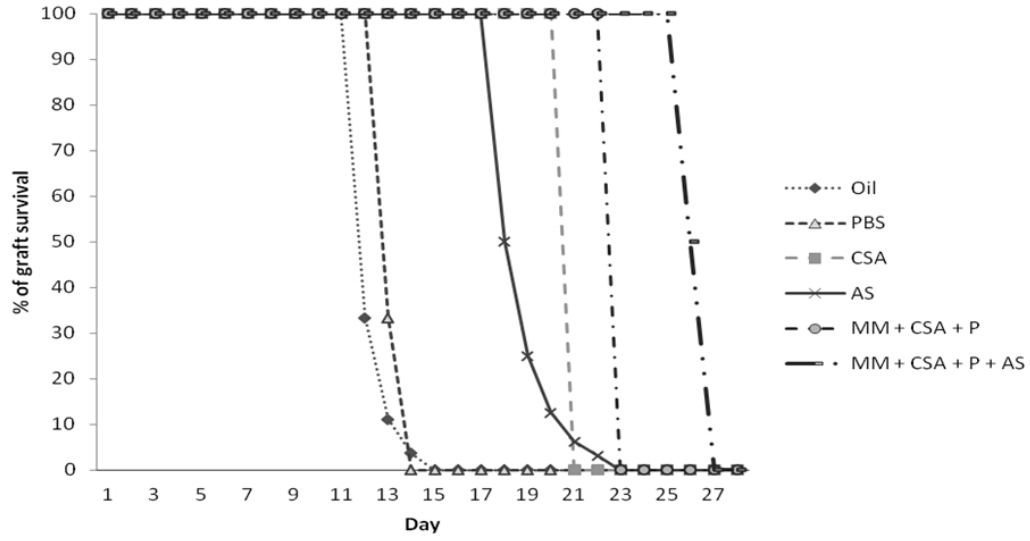

Fig. 2. Kaplan–Meier survival curve. Graft survival rate was prolonged with AS treatment. AS alone increased survival rate as compared to the control groups (oil and PBS). In addition, when AS was given along with the triple therapy further graft survival was observed compared to the triple therapy alone and the control groups

AS = Atorvastatin; CSA = Cyclosporin A; P = Prednisone; MMF = Mycophenolate Mofetil.

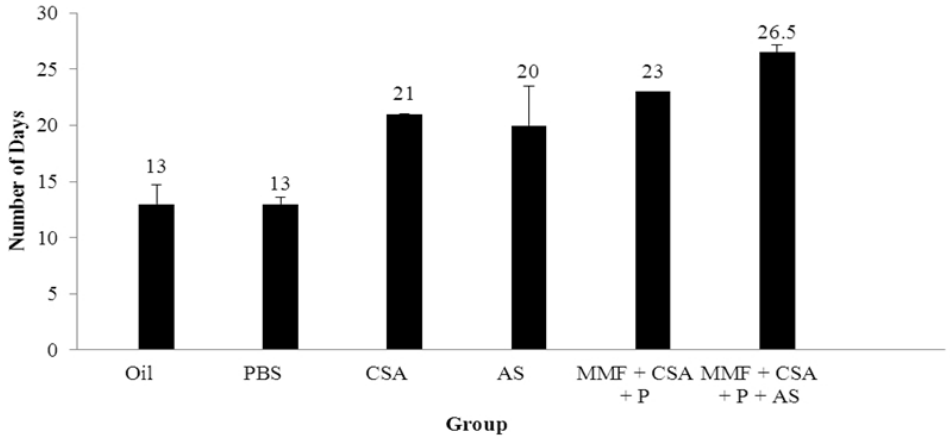

Fig. 3. AS prolonged skin allograft survival in BALB/c mice. Recipients in the AS group showed increased average allograft survival time compared with the control (PBS) group. The average survival days of the skin allografts in the group treated with AS and triple therapy was increased compared to the group receiving the triple therapy alone. Data are shown as the mean±SD. Small sample size in the triple therapy group (without AS) hampered statistical analysis

AS = Atorvastatin; CSA = Cyclosporin A; P = Prednisone; MMF = Mycophenolate Mofetil.

3.3 Serum IL-1β and IFN-γ Levels

There was no significant difference in serum levels of IL-1β between treated and control groups at any time point. Similarly, expression of IFN-γ did not vary significantly compared to the control groups for all analyzed time points (Figs. 4 and 5).

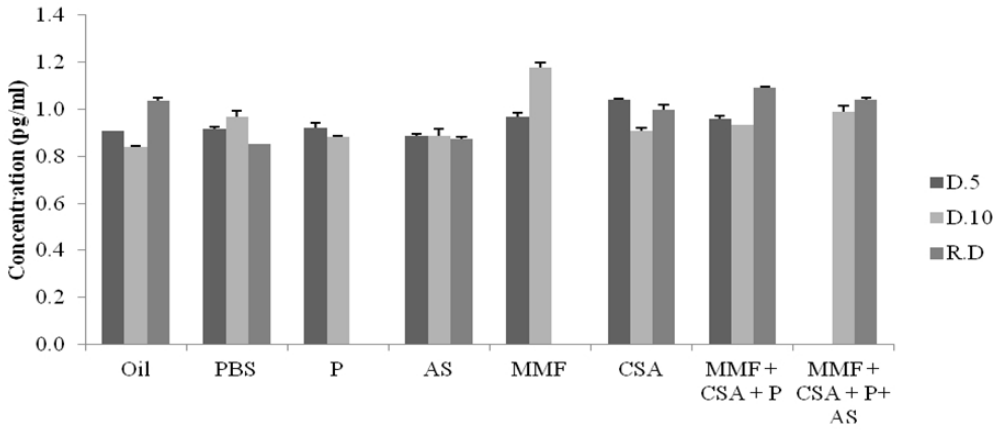

Fig. 4. Serum levels of IL-1β in the different groups of mice as detected by ELISA at 5 and 10 (D) days post transplantation and on rejection day. Data are shown as the mean±SD of results from duplicate samples. Mice in P and MMF group did not survive to rejection day

RD = rejection day; AS = Atorvastatin; CSA = Cyclosporin A; P = Prednisone; MMF = Mofetil Mycophenolate.

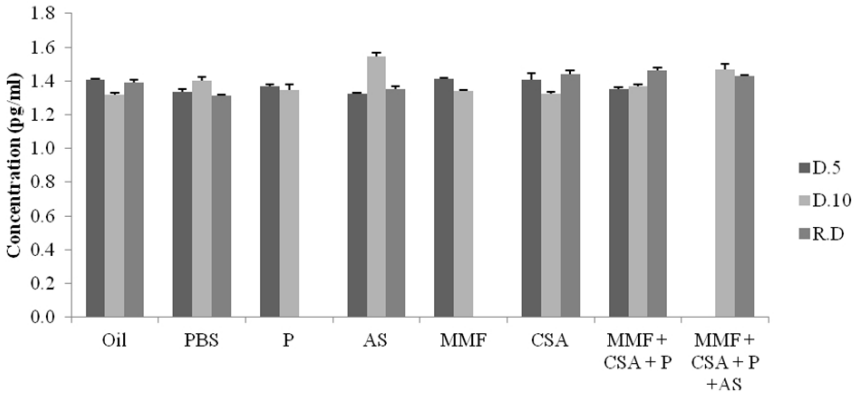

Fig. 5. Serum levels of INF-γ in the different groups of mice as detected by ELISA at 5 and 10 (D) days post transplantation and on rejection day. Data are shown as the mean±SD of results from duplicate samples. Mice in P and MMF group did not survive to rejection day

RD = rejection day; AS = Atorvastatin; CSA = Cyclosporin A; P = Prednisone; MMF = Mofetil Mycophenolate.

3.4 Effect of AS on Growth of the B16F10 Melanoma

There was an increase in body weight of BALB/c mice treated with AS as well as in the size of their abdomens (Fig. 6). Dissection revealed an abnormally large mass in the lower abdomen since the tumor cells grew as solid mass. However, the increase in abdomen size in surviving BALB/c mice subsided after 18 days post-tumor challenge. On the other hand, growth of tumor on day 16 post-tumor challenge in the AS-treated C57BL/6 mice was apparent when compared to the C57BL/6 mice that were not treated with AS.

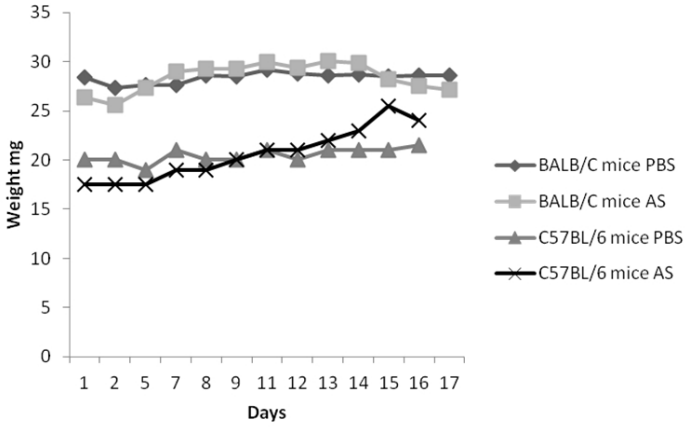

Fig. 6. Average weight of BALB/c mice and C57BL/6 mice after tumor challenge. Groups were given i.p. injections of either the vehicle alone or the assigned treatment (AS). BALB/c mice receiving AS had more weight increase as compared to the PBS group suggestive of further growth of the tumor. Day 16 post tumor growth shows a peak increase of body weight of C57BL/6 mice receiving AS treatment as compared to the PBS group

AS = Atorvastatin

4. DISCUSSION

The introduction of immunosuppressive agents was a breakthrough in organ transplantation and has increased one year allograft survival rates by more than 80% [16]. However, it has been reported that they disrupt proper functioning of organs and they have been associated with severe side effects such as, increased frequencies of opportunistic infections, nephrotoxicity and cancer, when given in therapeutic doses [17]. In practice, one of the reasons combination therapy is used is to be able to reduce the dose of each immunosuppressive agent employed; resulting in a decrease in toxic effects. Based on this practice, in this study doses of each immunosuppressive agent was decreased when used in combination.

Pulverized Lipitor tablets rather than pure AS were used to simulate as much as possible human therapy. However, the route of administration differed. Whereas the oral route of administration is used for human subjects, AS and immunosuppressive agents were injected intraperitoneally to mice. Attempts to administer medications orally to mice met with difficulties.

Statins, or HMG-CoA reductase inhibitors, are a family of drugs that are commonly used to treat hypercholesterolemia and to prevent primary and secondary cardiovascular diseases. Evidence is growing that suggests that statins have multifactorial cholesterol-independent immunomodulatory properties that extend to include beneficial effects in conditions such as organ transplantation, rheumatoid arthritis, multiple sclerosis and renal diseases [18,1,19] Statins are well tolerated and easy to administer making them a good choice for treatment in many pathological condition.

Allograft recognition can be direct or indirect [20]. In the former case which occurs early post-transplantation, donors Antigen Processing/Presenting Cells (APC) migrate from the graft to secondary lymphoid tissue where they activate host T helpers and T cytotoxic cells. The activated T cytotoxic cells then migrate to the graft and destroy it. In the latter case which occurs later post-transplantation, donor antigens are processed by recipient APC which in turn activate T helper and T cytotoxic cells in the secondary lymphoid tissue. Again, the T cytotoxic cells get access to the graft and destroy it. In both cases Major Histocompatability Complex (MHC) molecules expressed on the surface of APC are involved.

This study provides evidence for modulation of the immune system by AS by cholesterol-independent mechanisms. The observation that AS does not appear to alter serum cholesterol levels is consistent with other studies showing that statins do not lower significantly cholesterol levels in normocholesterolemic mice [21,22].

Several studies have shown that statins may possess immunomodulating properties including effects associated with pro-inflammatory cell migration, proliferation, differentiation, cytokine release (e.g., IFN-γ and interleukin-2) and antigen presentation [23,24,25]. These reported effects have mostly been attributed to the statins' ability to reduce prenylation [4]. Nonetheless, to the best of our knowledge, no published studies have considered the effect of AS on skin allograft survival in mice. We observed that at a dose of 40mg/kg, treatment of AS when given as a monotherapy resulted in a significant increase in mean survival time of skin allografts compared to controls, in the absence of any hypocholesterolemic effect. In a previous study we tested the effect of different doses of AS on anti-egg albumin antibody, IL-4 and IFN-γ production in mice immunized with egg albumin. A minimum effective dose of 40

mg/kg was determined [6]. In this study, the same dose of AS in mice receiving skin allografts or mice challenged with tumor cells was used. When AS was given in combination with immunosuppressive agents, in particular CSA, a prolongation of skin graft survival was observed. CSA inhibits the CYP3A4 complex in the liver which metabolizes AS [26]. By doing so, CSA would mediate the potentiation of AS by increasing its bioavailability.

The increase in mean survival time was not associated with a reduction in serum pro-inflammatory cytokine production. Similarly, immunosuppressive monotherapy and combination therapy did not result in a significant change in the expression level of serum IFN-γ and IL-1β albeit a net increase in the mean survival time of skin allografts was observed. There are at least two explanations supporting these results: 1- The variation in the expression level of these two cytokines may be significant in the site of transplantation and not in serum. Monocytes mature into macrophages when they reach their target organ and IL-1β is a cytokine that is mostly expressed by activated macrophages and endothelium cells. It is likely that IL-1β may be expressed only locally and that any change in serum may not be detectable. 2- The selected time points to study the expression level of serum IFN-γ and IL-1β may not correspond with the actual physiological changes. When the immune response is triggered during allograft rejection, some pro-inflammatory cytokines are expressed early after transplantation and are mainly secreted by cells of the innate immune system while others are expressed later during the immunological response and contribute to the development and the maintenance of the adaptive immune response. IL-1β is secreted by macrophages which are involved in the early response and IFN-γ is required for early events including the activation of macrophages and T-cytotoxic cells [27]. Therefore, the analysis of serum IFN-γ and IL-1β expression level at earlier time points using more sensitive techniques might have been more appropriate. On the other hand statins have been reported to reduce Toll-Like Receptor-4 (TLR-4) expression via inhibition of protein geranylgeranylation and farnesylation. TLR-4 and other Pattern Recognition Receptors (PRR) are needed to generate the production of cytokines in an inflammatory response. Their down-expression might explain why cytokine levels were unaltered [28,29]. Another factor that might explain why cytokine levels were not different than controls is the report that indicates that statins modify the induction of MHC expression (via inhibition of protein geranylgeranylation and farnesylation [30]. In their modified form they probably are not capable of presenting antigens to T-lymphocytes that normally would be activated to produce cytokines.

However, these results are not in agreement with previous studies showing that immunosuppressive treatment [31,32,33] in murine models of skin transplant could inhibit IFN-γ secretion over a similar period of time.

An association between immunosuppressive therapy and cancer have been reported [34]. Some tumors can be considered as allografts since they possess foreign antigens and are supposedly rejected by an active immune system. The C57BL/6 tumor cell line (B16F10) can grow only in C57BL/6 black mice. This tumor cannot cross histocompatibility barriers and grow in other strains of mice. Our preliminary experiments indicated that the C57BL/6 tumor cells grew in BALB/c mice treated with AS. AS-treated BALB/c mice challenged intraperitoneally with tumor cells had extended abdomens, and dissection of one mouse revealed a large mass. In other AS-treated BALB/c mice belonging to the same group tumors started to regress at 18 day post-tumor challenge. Perhaps the effect of AS was overrun by the active immune system. In agreement with the hypothesis that AS suppresses the immune system, tumor growth rate was enhanced by AS treatment compared to PBS-treatment in C57BL/6 mice. It is also worth noting a paradox. A number of anti-tumor agents

used clinically are also immunosuppressive agents. This might apply to AS because some reports indicate the anti-tumor potential of statins [1].

5. CONCLUSION

In conclusion, it appears that AS may increase the number of survival days of skin allografts and promote tumor growth in murine models. The results of the serum pro-inflammatory cytokine analysis of transplant mice do not provide conclusive evidence about the mechanisms behind the immunomodulatory effects of statins. Nonetheless, the prevention of acute rejection suggests that statins therapy may at least down-regulate the innate and adaptive immune response, thus allowing better survival of the skin allografts. The observed findings that AS allowed tumor growth across histocompatibility barriers tend to support this assumption. Based on previous reports [28,29,35,36]. It can be speculated that AS down expresses TLR and modifies MHC molecules resulting in hindering the generation of an innate and adaptive immune response.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Smaldone C, Brugaletta S, Pazzano V, Liuzzo G. Immunomodulator activity of 3-hydroxy-3-methilglutaryl-CoA inhibitors. Curr Med Chem Cardiovasc Hematol Agents. 2009;7(4):279-294.
2. Zhou Q, Liao JK. Pleiotropic effects of statins - Basic research and clinical perspectives. Circ J. 2010;74(5):818-826.
3. Kwak B, Mulhaupt F, Myit S, Mach F. Statins as a newly recognized type of immunomodulator. Nat Med. 2000;6(12):1399-1402.
4. Greenwood J, Steinman L, Zamvil SS. Statin therapy and autoimmune disease: From protein prenylation to immunomodulation. Nat Rev Immunol. 2006;6(5):358-370.

5. Hot A, Lavocat F, Lenief V, Miossec P. Simvastatin inhibits the pro-inflammatory and pro-thrombotic effects of IL-17 and TNF-α on endothelial cells. Ann Rheum Dis. 2012.
6. El-Haibi C, Rahal E, Khauli RB, Abdelnoor AM. Effect of atorvastatin on antibody, interleukin-4 and gamma-interferon production in mice immunized with egg albumin. Immunopharmacol Immunotoxicol. 2006;28(3):459-470.
7. Rahal EA, Chakhtoura M, Dargham RA, Khauli RB, Medawar W, Abdelnoor AM. The impact of prophylactic antiviral agents and statin administration on graft longevity in kidney allograft recipients. Immunopharmacol Immunotoxicol. 2012;34(5):763-767.
8. Kotyla P. The role of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors (statins) in modern rheumatology. Ther Adv Musculoskelet Dis. 2010;2(5):257-69.
9. Poynter JN, Gruber SB, Higgins PDR, Almog R, Bonner JD, Rennert HS, et. al. Statins and the risk of colorectal cancer. N Engl J Med. 2005;352(21):2184-2192.
10. Zhu T, Zhang W, Wang D-, Huang N-, Bo H, Deng W, et. al. Rosuvastatin attenuates mucus secretion in a murine model of chronic asthma by inhibiting the gamma-aminobutyric acid type A receptor. Chin Med J. 2012;125(8):1457-14.
11. McFarland HI, Rosenberg AS. Skin allograft rejection. Curr Protoc Immunol. 2009(SUPPL. 84):4.4.1-4.4.13.
12. Bevaart L, Jansen MJ, van Vugt MJ, Verbeek JS, van de Winkel JG, Leusen JH. The high-affinity IgG receptor, FcgammaRI, plays a central role in antibody therapy of experimental melanoma. Cancer Res. 2006;66(3):1261-1264.
13. Dua P, Ingle A, Gude RP. Suramin augments the antitumor and antimetastatic activity of pentoxifylline in B16F10 melanoma. Int J Cancer. 2007;121(7):1600-1608.
14. Wack C, Kirst A, Becker JC, Lutz WK, Brocker EB, Fischer WH. Chemoimmunotherapy for melanoma with dacarbazine and 2,4-dinitrochlorobenzene elicits a specific T cell-dependent immune response. Cancer Immunol Immunother. 2002;51(8):431-439.
15. el-Sabban ME, Pauli BU. Cytoplasmic dye transfer between metastatic tumor cells and vascular endothelium. J Cell Biol. 1991;115(5):1375-1382.
16. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The registry of the International Society for Heart and Lung Transplantation: Eighteenth Official Report - 2001. J Heart Lung Transplant. 2001;20(8):805-815.
17. Suzuki J-, Koga N, Kosuge H, Ogawa M, Haraguchi G, Maejima Y, et. al. Pitavastatin suppresses acute and chronic rejection in murine cardiac allografts. Transplantation. 2007;83(8):1093-1097.
18. Raggatt LJ, Partridge NC. HMG-CoA reductase inhibitors as immunomodulators: Potential use in transplant rejection. Drugs. 2002;62(15):2185-2191.
19. Paraskevas KI, Tzovaras AA, Briana DD, Mikhailidis DP. Emerging indications for statins: A pluripotent family of agents with several potential applications. Curr Pharm Des. 2007;13(35):3622-3636.
20. Celli S, Albert ML, Bousso P. Visualizing the innate and adaptive immune responses underlying allograft rejection by two-photon microscopy. Nat Med. 2011;17(6):744-749.
21. Shimizu K, Aikawa M, Takayama K, Libby P, Mitchell RN. Direct Anti-Inflammatory Mechanisms Contribute to Attenuation of Experimental Allograft Arteriosclerosis by Statins. Circulation. 2003;108(17):2113-2120.

22. Suzuki J-, Koga N, Kosuge H, Ogawa M, Haraguchi G, Maejima Y, et al. Pitavastatin suppresses acute and chronic rejection in murine cardiac allografts. Transplantation. 2007;83(8):1093-1097.
23. Weber C, Erl W, Weber KSC, Weber PC. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b- dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. J Am Coll Cardiol. 1997;30(5):1212-1219.
24. Youssef S, Stüve O, Patarroyo JO, Ruiz PJ, Radosevich JL, Mi Hur E, Bravo M, Mitchell DJ, Sobel RA, Steinman L, Zamvil SS. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. Nature. 2002;420(6911):78-84.
25. Aktas O, Waiczies S, Smorodchenko A, Dörr J, Seeger B, Prozorovski T, Sallach S, Endres M, Brocke S, Nitsch R, Zipp F. Treatment of relapsing paralysis in experimental encephalomyelitis by targeting Th1 cells through atorvastatin. J Exp Med. 2003;197(6):725-733.
26. Lennernäs H. Clinical pharmacokinetics of atorvastatin. Clin Pharmacokinet. 2003;42(13):1141-1160.
27. Janeway CA Jr., Travers P, Walport M, Mark J Shlomchik. Immunobiology New York: Garland Science; 2001.
28. Katsargyris A, Klonaris C, Tsiodras S, Bastounis E, Giannopoulos A, Theocharis S. Statin treatment is associated with reduced toll-like receptor 4 immunohistochemical expression on carotid atherosclerotic plaques: A novel effect of statins. Vascular. 2011;19(6):320-326.
29. Methe H, Kim J-, Kofler S, Nabauer M, Weis M. Statins decrease Toll-like receptor 4 expression and downstream signaling in human CD14+ monocytes. Arterioscler Thromb Vasc Biol. 2005;25(7):1439-1445.
30. Mach F. Statins as immunomodulators. Transpl Immunol. 2002;9(2-4):197-200.
31. Zheng H, Du G, Zhao R, Tang B, Zhu N. Enhanced allogeneic skin-graft survival using sCD95L, sCD152, interleukin 10 and transforming growth factor-β in combination, and comparison with ciclosporin. Clin Exp Dermatol. 2009;34(6):721-727.
32. Gong F, Shen Y, Zhang Q, Sun Y, Tang J, Tao F, et al. Obaculactone suppresses Th1 effector cell function through down-regulation of T-bet and prolongs skin graft survival in mice. Biochem Pharmacol. 2010;80(2):218-225.
33. Gong Q, Deng D, Ding J, Wang C, Bian Z, Ye Z, et al. Trichosanthin, an Extract of Trichosanthes kirilowii, Effectively Prevents Acute Rejection of Major Histocompatibility Complex-Mismatched Mouse Skin Allograft. Transplant Proc. 2008;40(10):3714-3718.
34. Penn I, Starzl TE. Immunosuppression and cancer. Transplant Proc. 1973;5(1):943-947.
35. Kuipers HF, Biesta PJ, Groothuis TA, Neefjes JJ, Mommaas AM, Van Den Elsen PJ. Statins affect cell-surface expression of major histocompatibility complex class II molecules by disrupting cholesterol-containing microdomains. Hum Immunol. 2005;66(6):653-665.

36. Ghittoni R, Patrussi L, Pirozzi K, Pellegrini M, Lazzerini PE, Capecchi PL, Pasini FL, Baldari CT. Simvastatin inhibits T-cell activation by selectively impairing the function of Ras superfamily GTPases. FASEB Journal. 2005;19(6):605-607.

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