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Kinetics of mast cell migration during transplantation tolerance

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ABSTRACT

Background: After inflammatory stimulus, mast cells (MC) migrate to secondary lymphoid organs contributing to adaptive immune response. There is growing evidence that MC also contribute to transplant tolerance, but little is known about MC kinetics in the setting of transplant tolerance and rejection. Likewise it has been demonstrated that complement split products, which are known to act as chemoattractants for MC, are necessary for transplant tolerance.
Methods: Naive skin and lymph nodes, skin grafts and draining lymph nodes from wild type and complement deficient mice treated with a tolerogenic protocol were analyzed.
Results: Early after tolerance induction MC leave the graft and migrate to the draining lymph nodes. After this initial efflux, MC reappear in tolerant skin grafts in numbers exceeding that of naive skin. MC density in draining lymph nodes obtained from tolerant mice also increased post transplant. There was no difference in MC density, migration and degranulation status between wild type and complement deficient mice implicating that chemotaxis is not disturbed in complement deficient mice.
Conclusion: This study gives detailed insight in kinetics of MC migration during transplant tolerance induction and rejection providing further evidence for a role of MC in transplant tolerance.

1. Introduction

Recent studies have shown that mast cells (MC) play an important role in innate and adaptive inflammatory responses [1]. Beside their well described role in allograft rejection [2] MC are also necessary in the establishment of peripheral tolerance after skin and solid organ transplantation [3–6]. The functional need for MC during the initiation phase of tolerance was demonstrated in a murine skin graft model [7] and a heterotopic heart transplant model [8]. Recently it has been proposed that MC act via direct interaction with T-regulatory cells [7, 9–12], but the mechanisms of MC mediated transplant tolerance are not understood.

Secondary lymphoid organs are essential for initiating the immune response to microbial antigens. After inflammatory stimulus MC migrate to the draining lymph nodes where they interact with B and T lymphocytes [13–16]. In transplantation, secondary lymphoid organs play an important role in rejection response and tolerance induction [17]. However migration of MC between donor graft and secondary lymphoid organs received little attention. This study investigates the kinetics of MC migration into and out of the donor organ and draining lymph nodes during tolerance induction and rejection.

It has been shown that the complement system is important for the induction and maintenance of tolerance [18–20]. This effect is mediated via complement split product iC3b binding on complement receptor 3 on antigen presenting cells. Recent publications demonstrated complement component 3 (C3) and complement component 3a receptor (C3aR) to be crucial for HY specific transplant tolerance induction [21, 22]. However the underlining mechanisms for this complement dependant tolerance are not known. The complement system is able to activate MC via C3 split products [23] and complement component 3a (C3a) is a strong chemoatractant for MC [24]. We hypothesized that one of the mechanisms that account for complement dependant transplant tolerance is migration of MC from the graft to the secondary lymphoid organs via C3 split product induced chemotaxis. To test this hypothesis we analyzed MC migration in a murine model of complement dependant tolerance.

2. Materials and methods

2.1. Mice

Animals were kept in specific pathogen free animal facilities and were used between the age of 8 and 12 weeks in accordance with the Animals (Scientific Procedures) Act 1986. C57BL/6 mice were purchased from Harlan Limited (UK) and Charles River (MA, USA). Homozygous

Abbreviations: C3, complement component 3; C3a, complement component 3a; C3aR, complement component 3a receptor; MC, mast cell; WT, wild type.

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$C3^{-/-}$ and $C3aR^{-/-}$ C57BL/6 mice were kind gifts from Drs. M Carroll and Bao Lu, respectively (Harvard Medical School).

2.2. Skin transplantation and definition of rejection

Skin transplantation was performed as previously described [25], except that full thickness trunk skin, was used instead of tail skin. Rejection was defined by >90% macroscopic necrosis of the skin. Mice were checked daily.

2.3. Donor lymphocyte infusion protocol

For donor lymphocyte preparation male spleens were harvested, mashed through a 40 μm cell strainer and washed in phosphate buffered saline (Oxoid, UK). Red blood cells were removed using an ammonium chloride-based lysing reagent (BD Pharm Lyse; BD Pharmingen, USA) according to the manufacturer's instructions. After washing cells in phosphate buffered saline thirty five million cells were injected into recipients via the tail vein immediately before skin transplantation.

2.4. Histology

Frozen tissue samples were cut into 8 μm sections. MC granule stain was performed with Toluidine-blue. Slides were fixed in acetone/methanol (Sigma, MO, US) 1:1 solution for 5 min and stained for 5 min with Toluidine-blue staining solution (0.5% w/v Toluidine-blue in 0.5 N hydrogen chloride acid; Sigma). Slides were analyzed by a Diplan microscope (Leitz, Germany) and a DXM1200DF digital camera (Nikon, Japan), using Lucia G software (Nikon). Positive cells were counted in at least 20 random high power fields (×400) of each sample by an observer blinded to the experimental conditions.

2.5. Immunohistochemistry

For immunohistochemistry standard methods were used [26]. Slides were incubated with a rat IgG2A anti-mouse stem cell factor R/c-kit monoclonal antibody (clone 180627; R&D Systems, UK) and visualized with a biotinylated goat anti-rat polyclonal antibody (Pharmingen, CA, US) followed by a streptavidin-horse radish peroxidase conjugate (Pharmingen) and Vector NovaRed Substrate kit (Vector Laboratories) used according to the manufacturer's protocol. Sections were analyzed as described above.

2.6. Statistical analysis

Kaplan Meier analysis was applied to calculate graft survival. Numbers of MC are displayed as median per mm^2. Mann Whitney *U* Test was used to compare density of MC between groups. All tests were two-sided, with a 5% type I error. Statistical calculations were performed using SPSS for Windows, version 17.0 (SPSS Inc., USA).

3. Results

3.1. MC in tolerant skin grafts

First we analyzed MC density in naive trunk skin from male C57BL/6 wild type (WT) mice. As shown in Figs. 1 and 2a, MC granule stain with Toluidine-blue revealed an average of 41 MC per mm^2. For analysis of MC density in tolerant skin grafts male WT trunk skin was transplanted to female WT recipients in combination with a donor lymphocytes infusion. This protocol leads to specific tolerance induction towards the male HY antigen and thus to indefinite graft survival whereas without donor lymphocyte infusion skin grafts are chronically rejected [27,28]. At day 7 after transplantation 10 MC per mm^2 were detectable in the skin grafts harvested from tolerant mice, which was significantly less compared to naive skin ($p = 0.001$). Similar results were obtained at day 7 in skin grafts from rejecting controls (18 MC/mm^2, $p = 0.08$), whereas syngeneic skin graft controls had MC numbers comparable to naive skin (37 MC/mm^2, $p = 0.4$). These data demonstrate that MC density within minor mismatched skin grafts is reduced compared to naive skin, irrespective of the graft outcome.

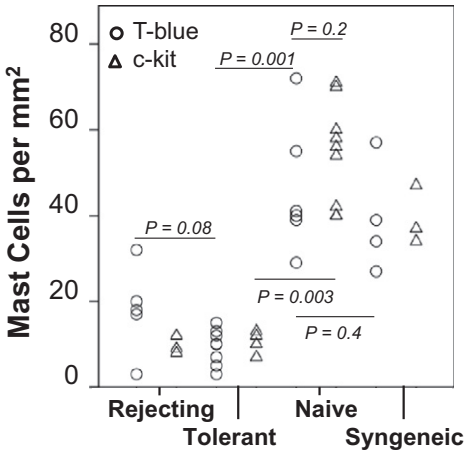

Fig. 1. MC granule and surface stain of naive skin and skin grafts from WT mice. Female mice received male trunk skin after infusion of 35×10^6 male splenocytes. Rejecting and syngeneic controls did not receive donor splenocyte infusions. Skin grafts were harvested at day 7 after transplantation and analyzed by means of Toluidine-blue granule stain (open circle) and c-kit surface stain (open triangle). (A) Numbers of MC calculated within skin grafts obtained from donor lymphocyte induced tolerant mice did not differ from those obtained from rejecting controls. In contrast MC density within skin grafts obtained from syngeneic controls was significantly higher compared to tolerant grafts and did not differ from numbers obtained from naive skin. There was no significant difference between MC granule and surface stain. Dot plots represent groups of three to nine animals.

In order to characterize the kinetics of MC in donor specific tolerance in more detail, we analyzed donor skin grafts from mice treated with donor lymphocyte infusion over time (Figs. 3, 2b). From day 14 post transplantation MC density in tolerant skin grafts began to increase, exceeding numbers obtained from naive skin at day 50 (87 MC/mm^2, $p = 0.02$) and at day 100 (73 MC/mm^2, $p = 0.04$). In contrast there was no significant difference in MC density between syngeneic skin graft controls at day 100 and naive skin (65 MC/mm^2, $p = 0.1$). Taken together these results demonstrate that after an initial reduction in MC density in tolerant grafts, cell numbers rise over time exceeding those detected in naive skin.

It has been shown that MC escape granule stain after complete degranulation [29,30]. Therefore we performed a MC surface stain with c-kit to analyze the total MC density independently of the degranulation status (Figs. 1, 2a). Within naive skin 56 MC per mm^2 were counted which was not significantly different compared to the Toluidine-blue granule stain ($p = 0.2$). Similar to Toluidine-blue stains, skin grafts harvested from tolerant mice stained with c-kit showed markedly reduced MC density at day 7 after transplantation compared to naive skin (11 MC per mm^2, $p = 0.003$). Surface stain in rejecting and syngeneic controls showed also similar results compared to those obtained from Toluidine-blue stain and there was no significant difference in MC density between tolerant skin grafts and rejecting controls ($p = 0.9$). This suggests that the observed reduction in MC density early after transplantation is not due to MC degranulation and that there is no difference in the amount of MC degranulation between tolerant and rejecting grafts.

3.2. MC in draining lymph nodes of skin grafts

It has been shown that MC traffic from the local site of inflammation to secondary lymphoid organs [13–16]. Therefore, we investigated if the observed reduction in MC density in the skin grafts early after transplantation was the result of MC migration to the draining lymph nodes. We found an average of 10 MC per mm^2 detected by Toluidine-blue stain in draining lymph nodes from tolerant mice at day 7 after skin graft transplantation, which is significantly more compared to numbers in lymph nodes harvested from naive mice (1.4 MC/mm^2, $p = 0.008$; Figs. 4, 2c). There was no difference compared to lymph nodes harvested from rejecting controls (8 MC/mm^2, $p = 0.9$), but there were less MC in the draining lymph nodes harvested from syngeneic controls (3 MC/mm^2, $p = 0.03$; Figs. 5, 2d). A longitudinal analysis revealed that from day 14 after transplantation MC numbers within draining lymph nodes from tolerant mice further rose with a maximum at day 100 post transplantation (80 MC/mm^2; Fig. 5). The expansion of MC in draining lymph nodes from tolerant mice was predominantly in the sub capsular region, suggesting an influx of MC via the afferent lymphatics (Fig. 5). In contrast MC numbers in draining lymph nodes from syngeneic controls did not increase in the same way over time (19 MC/mm^2; Fig. 5).

3.3. MC in $C3^{-/-}$ and $C3aR^{-/-}$ skin grafts

Having established that MC migrate from skin grafts into draining lymph nodes early after transplantation, and re-accumulate in tolerant skin grafts with time, we investigated whether factors known to influence MC migration would affect this process. Therefore we