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1 **Accelerated Corneal Graft Rejection in Baby Rats**

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7

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1 **Abbreviations**

2 APC – Antigen Presenting Cell

3 NK Cell – Natural Killer Cell

1 **Abstract**

2

3 Background

4 Penetrating keratoplasty in infants has a very poor outcome compared to adults. It is of intrinsic
5 interest to gain insight into the still unknown immunological mechanisms of graft failure because
6 any form of uncorrected corneal opacity leads to amblyopia.

7 Methods

8 Allogeneic keratoplasty was performed between Lewis and Fisher rats. The recipients' ages were
9 10 and 3 weeks, respectively. All experiments were controlled syngeneically. Survival rates were
10 calculated and cellular infiltrates analyzed histologically.

11 Results

12 Median graft survival times were 15 days in old recipients and 9 days in young recipients
13 ($p < 0.01$). We noted fewer infiltrating cells in the younger rats than in the older ones on the day of
14 rejection. Despite the fact that T cells dominated we detected significantly more NK in young
15 recipients at all time points after transplantation when compared to old recipients.

16 Conclusions

17 We established an animal model that shows similar rejection kinetics as in children, i.e. corneal
18 graft failure occurs sooner in young rats. Already little infiltration was sufficient to reject a
19 corneal allograft. The dominance of infiltrating NK cells and the vigorous rejection process
20 suggest an involvement of the innate immune system in this process.

1 **Introduction**

2 Keratoplasty can restore corneal clarity in most cases. This is especially important in early
3 childhood during which any form of uncorrected corneal opacity or refractive error leads to
4 life-long poor vision (amblyopia). However, while adults show a very low rate of corneal
5 graft rejection in standard situations of only 10%, corneal graft failure occurs in up to 85% of
6 transplanted children under 3 years of age [1-3]. Astonishingly, this is observed with no sign
7 of preexisting ocular surface inflammation, as only seen otherwise in high risk corneal beds.
8 Here infection, chemical burn, immunological disorders such as ocular pemphigoid, perforation
9 or re-transplantation lead to an elevated rejection rate. Thus, circumstances other than a pre-
10 inflamed eye seem to be responsible for graft failure in infant humans. It is of great interest to
11 gain insight into the underlying mechanisms to prevent these children from becoming amblyopic.
12 Various animal models revealed evidence that CD4⁺ T cells play a predominant role during
13 graft rejection in low and high risk corneal beds [4-5]. These animal models vary in their
14 dynamics and strength of immune reactions but reproduce the clinical results with respect to
15 corneal graft survival. In short, Streilein and others demonstrated that the excellent survival
16 rates following keratoplasty are a consequence of the immune privileged status of the cornea
17 and the eye itself [6-7].

18 Our group proposed an animal model for keratoplasty in baby rats to address this problem in
19 the young. However, nothing had yet been reported on the rejection kinetics or mechanisms
20 underlying graft failure in these young animals [8]. We used this model to elucidate the
21 factors that might lead to immunological graft rejection in young rats.

1 **Materials and Methods**

2 Animals and Anesthesia

3 Inbred female Fisher (Rt1^{lv1}) and Lewis (Rt1^l) rats (Charles River, Germany) were used as donors
4 and recipients. All animals were treated in accordance to the ARVO Statement for the Use of
5 Animals in Ophthalmic and Vision Research. Rats were anesthetized with a short inhalation of
6 isofluran followed by an intraperitoneal injection of a mixture of ketamine, xylazine and atropine,
7 respectively.

8 Corneal transplantation

9 Orthotopic penetrating keratoplasties were performed as described previously [8-9]. Briefly,
10 Fisher donor buttons (2.5mm) were obtained and the animals were killed thereafter. Recipients
11 were anesthetized and the central cornea was removed using a 2mm trephine. The graft was fixed
12 with 8 interrupted sutures (11.0 Ethilon, Ethicon, Germany). Finally, a blepharoraphy was
13 applied. To exclude any potential difference in the graft's immunogenicity due to its size and the
14 vicinity to peripheral antigen presenting cells (APC) in young recipients, the protocol was
15 modified for 10 week old recipients and a corneal button with a diameter of 3.5mm was
16 orthotopically transplanted.

17 Clinical graft assessment and rejection kinetics

18 All grafts were daily examined by two investigators for signs of opacity according to an
19 international score [9, 19]. Grafts with technical difficulties such as cataract, infection, loss of the
20 anterior chamber or massive hyphema were excluded. The time point of rejection was identified
21 as an opacity score of 4 (complete opacity).

22 Groups

23 Only 10-week-old donor buttons were used considering the fact that in humans most of the grafts
24 are derived from adults. Group 1 consisted of 10-week-old Fisher donor and Lewis recipients. In

1 group 2, a 10-week-old Fisher cornea was used and grafted to a 3-week-old Lewis recipient.

2 Groups 3 and 4 resembled the syngeneic controls. Each group consisted of 12 recipients.

3 Immunohistochemistry

4 Cryosections of four bulbi per group were taken and fixed in acetone. Primary mouse-anti-rat

5 antibodies were applied and a biotinylated rabbit anti-mouse secondary antibody was used to

6 bind a streptavidine alkaline phosphatase. After incubation with a substrate (alkaline-

7 phosphatase-kit1, Vector, USA), sections were counterstained with Mayer's hematoxylin.

8 Positively stained cells were counted within three squares in the grafted corneal stroma and

9 calculated as mean cellular infiltrate per mm^2 .

10 Antibodies

11 Primary antibodies used for immunohistochemistry were anti-CD4 (clone W3/25), anti-CD8

12 (clone OX-8), anti-CD161 (clone 10/78), anti-CD163 (clone ED2), anti-dendritic cells (clone

13 OX-62) and anti-CD25 (clone Ox-39). A secondary biotinylated polyclonal rabbit-anti-mouse

14 immunoglobulin and streptavidine alkaline phosphatase were obtained from DAKO, Germany.

15 Statistics

16 The time interval from corneal transplantation to rejection was analyzed using the

17 Kaplan–Meier method and the groups compared using the log-rank test. The densities of

18 infiltrating immune cells were compared statistically using the T-test.

19

1 **Results**

2 1) Complete rejection occurs earlier in young than in old recipients

3 All of group 1's grafts failed after a median rejection time of 15 days. Group 2's transplants were
4 rejected after a median period of only 9 days (fig.1a). The difference in the survival rates between
5 groups 1 and 2 proved to be statistically highly significant ($p<0.01$). None of the syngeneic
6 controls rejected. We transplanted in 10 week old Lewis rats a 3.5mm corneal button to a 3.0mm
7 corneal bed to rule out that the observed difference in graft survival was a consequence of the
8 diverse ratio between donor and recipient corneal tissue and of the different distance of the graft's
9 margin to the limbal APC between young and old animals, respectively. As apparent in fig.1b,
10 we saw no statistical difference, regardless of whether a 2.5mm (group 1) or a 3.5mm corneal
11 button (group 1a) had been grafted.

12

13 2) The time point of complete rejection coincides with a lower cellular infiltrate in young 14 recipients

15 On the median days of graft failure (days 9 and 15) we analyzed infiltrating mononuclear cells on
16 HE-stainings. A moderate cellular infiltrate was detected in both syngeneic control groups (fig.2).
17 Yet we also observed a remarkable infiltration of leukocytes in both allogeneic groups 1 and 2.
18 However, the density of cells within the graft proved to be statistically significantly higher in the
19 group 1 animals on the day of rejection (day 15) when compared to group 2 at the equivalent time
20 point (day 9) ($p<0.0001$).

21

22 3) Accumulation of cells of the adaptive immunity in the graft

23 Infiltrating cells were stained in rejected corneas. Calculations of infiltrating $CD4^+$ and $CD8^+$ T
24 cells, activated $CD25^+$ cells and $Ox-62^+$ dendritic cells are shown in figs.3a-d. No significant

1 cellular infiltration was seen in the syngeneic controls. Hardly any cells were stained positively
2 on day 9 in group 1's grafts, whereas we detected a clear infiltrate in the transplants of group 2.
3 This difference was statistically significant ($p < 0.01$). However, by day 15 all cell types had
4 increased, that no significant differences in cell numbers were detectable between groups 1 and 2
5 any more. No further increase in cellular infiltration was observed in both groups thereafter. On
6 the respective median days of rejection (i.e. day 9 for young and day 15 for old recipients) $CD4^+$,
7 $CD8^+$ and $Ox-62^+$ cells infiltrated statistically significantly stronger in group 1's animals
8 ($p < 0.01$), whereas no significant difference was observed for $CD25^+$ cells.

9

10 4) Infiltration of cells of the innate immune system

11 Infiltrating $CD163^+$ macrophages and $CD161^+$ NK/NKT cells were stained on the median time
12 points of rejection (days 9 and 15). Both cell types were low in cell numbers on day 9 in group 2,
13 whereas nearly no infiltration was visible in group 1 ($p < 0.01$). $CD163^+$ macrophages
14 accumulated in both groups that no statistical difference was visible on day 15 any more (fig.4b).
15 On the respective days of rejection (i.e. day 9 for group 2 and day 15 for group 1) statistically
16 significantly more $CD163^+$ macrophages infiltrated in old recipients when compared to young
17 animals ($p < 0.01$). $CD161^+$ NK/NKT cells increased on day 15 in groups 1 and 2, but dominated
18 statistically significantly in group 2's animals when compared to the group 1 ($p < 0.01$) (fig.4c).
19 No statistical difference could be seen for infiltrating $CD161^+$ cells when comparing the median
20 time points of rejection of the respective group (i.e. day 9 for group 2 and day 15 for group 1).

1 **Discussion**

2 It is known from humans that graft failure in children under 3 years of age occurs more
3 frequently than in adults [1-3]. It is thus imperative to improve graft survival, because any form
4 of opacification or refractive error of the cornea leads to amblyopia. In order to be able to
5 specifically treat children following penetrating keratoplasty, it is important to better understand
6 the mechanisms that lead to immunological graft failure. Our group recently suggested a rat
7 model for corneal transplantation that enabled us to study the rejection of corneal allografts in
8 young individuals [8]. However, nothing so far was reported on the dynamics of graft rejection in
9 young individuals.

10 Kinetics of corneal allograft failure

11 Fig.1 illustrates that rejection kinetics appeared to differ between 3- and 10-week-old recipients.
12 Rejection did appear significantly earlier in 3-week-old than in 10-week-old recipients (day 9 vs.
13 day 15). Corneal antigen presenting cells (APCs) play an important role during immunological
14 corneal allograft failure and are located mostly in the periphery nearby the limbus [10-12]. The
15 diameter of the 10-week-old rat cornea is significantly larger than that of a 3-week-old animal.
16 Therefore, the margin of a corneal graft gets closer to these corneal APC in a young recipient and
17 the increased rejection could be seen as a consequence of an earlier onset of an allo-recognition
18 by these APCs. To exclude this, the graft's diameter was elevated in old recipients. However, the
19 median graft survival time was not changed in our model (fig.1b) and thus the vicinity of the
20 graft to the peripheral APCs did not influence the rejection process. Our findings are in contrast
21 to common clinical observations where a larger graft correlates with a higher incidence for
22 rejection [13]. In our model a single minor mismatch induces a devastating allograft response
23 within the first 15 postoperative days. In this short period graft size does not seem to be a risk
24 factor and might be overrun by the aggressive immune response in this model.

1 A stronger lymphangiogenesis has been reported in 8-week-old when compared to 15-months-old
2 mice and was suggested to influence a stronger corneal allograft failure in young animals [14].
3 However, this rather reflects a reduced corneal lymphangiogenesis in aged animals. It would be
4 necessary to analyze the density of lymphatics in 3-week-old compared to 8-week-old after an
5 inflammatory stimulus to understand their role during the elevated allograft rejection in our
6 model. However, lymphangiogenesis influences corneal allograft rejection and it certainly might
7 be an important factor for corneal allograft rejection in the baby rat model.

8 Corneal graft failure coincides with low immune infiltration

9 Astonishingly, the rejected grafts of young recipients presented fewer infiltrating cells than did
10 these of the older recipients at the time of immunological graft failure (fig.2). Considering these
11 data together with our observation that graft failure in young animals occurred more vigorously,
12 we wondered what rendered those few infiltrating immune cells within the transplant so potent
13 that they could lead to graft failure. Several cell types have been reported to contribute to corneal
14 allograft rejection [7]. However, the major player turned out to be CD4⁺ T cells [5]. Together
15 with the knowledge that the T cell system develops within early childhood [15-16], one possible
16 explanation is that a specific T cell response in young recipients is missing. We compared the T
17 cell pool of 3-and 10-week-old Lewis rats and observed no differences in the numbers of various
18 T cell populations, nor in the CD4/8 T cell ratio in blood, spleen and lymphnode (not shown).
19 Thus, a fully-developed T cell system is probable.

20 Characterization of the infiltrate within a corneal allograft

21 We were able to characterize the immune response leading to allograft failure in greater detail
22 by staining for a variety of leukocyte populations such as CD4⁺ and CD8⁺ T cells, CD25⁺ cells,
23 CD163⁺ macrophages and Ox-62⁺ dendritic cells on the median time points of rejection (days 9
24 and 15). In 10-week-old rats hardly any of these cells infiltrated whereas a significant cellular

1 infiltrate was observed in 3-week-old recipients on day 9. (figs.3+4). An accumulation of all
2 of these cells was observed until day15 in both groups without an additional increase thereafter.
3 This demonstrates that an unspecific additional inflammation was present in 3-week-old
4 recipients even after they had experienced complete graft failure. However, other factors more
5 unspecific than T cells might have contributed to complete opacity in young recipients. It is
6 known, that NK cells play a pivotal role in compensating for an underdeveloped T cell system in
7 young individuals [17-18]. However, aside from similar T cell numbers in 3- and 10-week-old
8 Lewis rats, we noted no differences in the numbers of $CD3^+CD161^+$ NK and $CD3^+CD161^+$ NKT
9 cells between the two recipient groups in blood, spleen and lymphnode (not shown).

10 Differences in infiltrating NK/NKT cells

11 Histological staining on postoperative day 9 revealed a statistically significant dominance of
12 infiltrating $CD161^+$ NK/NKT cells in young versus old recipients. Although we were able to also
13 demonstrate an accumulation of $CD161^+$ cells on day 15 in both groups, this did not equalize the
14 cell numbers. In fact, $CD161^+$ NK/NKT cells were still statistically significantly higher in cell
15 numbers on postoperative day 15 in young recipient rats (fig. 4c). However, unlike T cells,
16 $CD161^+$ cells infiltrated to a similar extend on the actual days of rejection for the respective
17 groups (i.e. day 9 for the 3-week-old recipients and day 15 for the 10-week-old recipients). We
18 therefore hypothesize that NK/NKT cells in young recipients have a stronger potency to reject a
19 corneal graft, which might be due to the cells themselves or to age-dependent changes in the
20 suppressive ocular environment between young and old rats. This led us to the conclusion that
21 $CD161^+$ cells might be a key factor during corneal allograft failure in 3-week-old recipients.
22 It has been reported that NK cells play a role during corneal graft rejection in rats [19] and that
23 NKT cells contribute to the induction of anterior chamber associated immune deviation (ACAID)
24 [20]. Both NK as NKT cells contribute to the innate immune system and may be key to immune

1 mediated pathologies in young individuals. Depletion experiments [21] of either of the two
2 CD161⁺ cell populations, or transplantation into nude rats lacking T and B cells but owning a
3 competent NK cell system [22] would give better insight into the role of infiltration NK and NKT
4 cells, respectively. Furthermore, additional analyses of the aqueous humor [23] or cytokines
5 within a rejected graft [24] would lead to better understanding of the processes and of the cells
6 that contribute to graft failure in infants.

7 Considering these data together, we have demonstrated for the first time an animal model of
8 keratoplasty that mimics the faster rejection of corneal allografts in human infants. In so doing,
9 we made investigation of an allogeneic immune reaction after keratoplasty in infants possible.

10 Although an underdeveloped T cell system in our model seems to be unlikely, we demonstrated
11 that CD161⁺ NK/NKT cells may be key players in corneal allograft rejection in young
12 individuals. Even if additional congenital or structural abnormalities count as poor prognostic
13 factors in humans infants regarding corneal allograft rejection [25], our observation may
14 ultimately lead to a better understanding of the mechanisms of graft failure in human infants and
15 provide new treatment strategies for its prevention.

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1 **Fig. 1:** a) Kaplan-Meier analyses of graft survival. Animals of group 1 (10 week
2 old recipients) are indicated by 1, those of group 2 (3 week old recipients) by 2. b)
3 Median survival times were compared in 10 week old recipients using grafts of
4 two different diameters (i.e. 2.5mm onto a 2.0mm corneal bed and 3.5mm onto a
5 3.0mm corneal bed, respectively). No significant difference in median survival
6 times could be observed between rejection of a corneal transplant with a diameter
7 of 2.5mm (1) and 3.5mm (1a) ($p>0.1$).

1 **Fig.2:** Mean cellular infiltration per mm² within the graft on the expected days of rejection
2 (i.e. days 15 and 9). Animals of group 1 (10 week old recipients) are indicated by 1, those of
3 group 2 (3 week old recipients) by 2. The corresponding syngenic controls are indicated as
4 syn.1 and 2, respectively. Cellular infiltration on day 9 in group 2 was statistically
5 significantly lower than in group 1 on day 15 ($p < 0,0001$).

1 **Fig. 3:** Mean cellular infiltration was calculated from 4 individual corneal transplants on the
2 expected days of rejection. Animals of group 1 (10 week old recipients) are indicated by 1,
3 those of group 2 (3 week old recipients) by 2. The corresponding syngenic controls are
4 indicated as syn.1 and 2, respectively. a) CD4⁺ T cells, b) CD8⁺ T cells, c) CD25⁺ cells and
5 Ox-62⁺ dendritic cells. All stained cell types infiltrated statistically significantly stronger in
6 group 2 compared to 1 on day9 (p<0.01, respectively). No statistical difference was observed
7 between those groups on day15. The infiltration of CD4⁺, CD8⁺ and Ox-62⁺ cells was
8 statistically significantly elevated in animals of group 1 on the median rejection time point
9 (day15) when compared to group 2 on its median rejection time point (day9) (p<0.01),
10 whereas no statistical difference was observed for CD25⁺ cells.

1 **Fig. 4:** Mean cellular infiltration of CD163⁺ macrophages and CD161⁺ NK/NKT cells was
2 calculated from 4 individual corneal transplants on the expected days of rejection. Animals of
3 group 1 (10 week old recipients) are indicated by 1, those of group 2 (3 week old recipients)
4 by 2. The corresponding syngenic controls are indicated as syn.1 and 2, respectively. Both
5 cell types infiltrated statistically significantly stronger in group 2 compared to 1 on day9
6 ($p < 0.01$, respectively). No statistical difference was observed for CD163⁺ between those
7 groups on day15, whereas CD161⁺ cells showed a stronger infiltration in group 2 ($p < 0.01$).
8 The infiltration of CD163⁺ cells was statistically significantly elevated in animals of group 1
9 on the median rejection time point (day15) when compared to group 2 on its median rejection
10 time point (day9) ($p < 0.01$), whereas no statistical difference was observed for CD161⁺ cells.

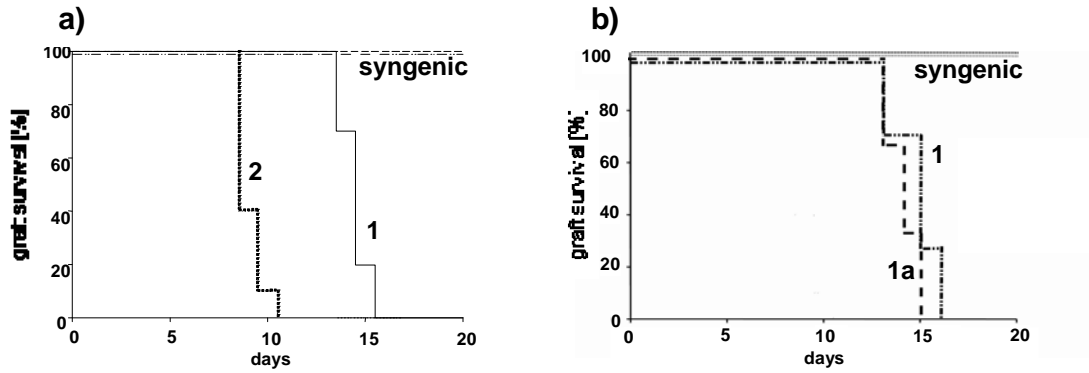


Fig. 1: a) Kaplan-Meier analyses of graft survival. Animals of group 1 (10 week old recipients) are indicated by 1, those of group 2 (3 week old recipients) by 2. b) Median survival times were compared in 10 week old recipients using grafts of two different diameters (i.e. 2.5mm onto a 2.0mm corneal bed and 3.5mm onto a 3.0mm corneal bed, respectively). No significant difference in median survival times could be observed between rejection of a corneal transplant with a diameter of 2.5mm (1) and 3.5mm (1a) ($p > 0.1$).

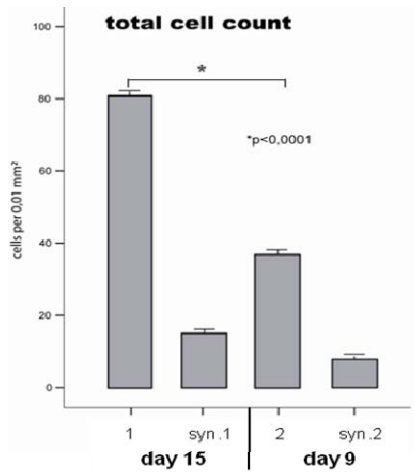


Fig.2: Mean cellular infiltration per mm² within the graft on the expected days of rejection (i.e. days 15 and 9). Animals of group 1 (10 week old recipients) are indicated by 1, those of group 2 (3 week old recipients) by 2. The corresponding syngenic controls are indicated as syn.1 and 2, respectively. Cellular infiltration on day 9 in group 2 was statistically significantly lower than in group 1 on day 15 ($p<0,0001$).

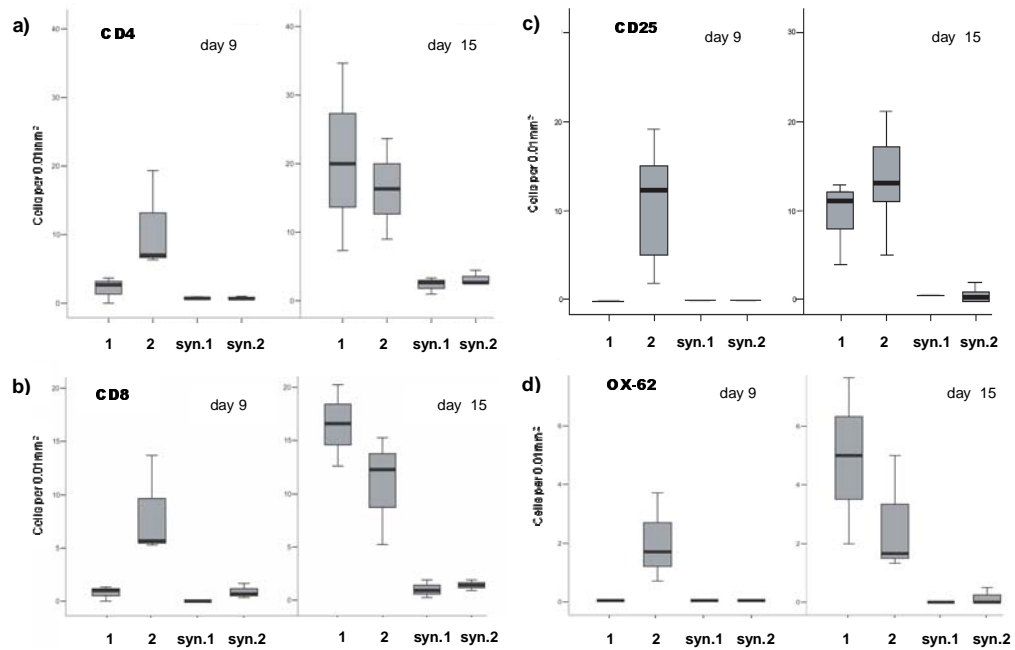


Fig. 3: Mean cellular infiltration was calculated from 4 individual corneal transplants on the expected days of rejection. Animals of group 1 (10 week old recipients) are indicated by 1, those of group 2 (3 week old recipients) by 2. The corresponding syngenic controls are indicated as syn.1 and 2, respectively. a) CD4⁺ T cells, b) CD8⁺ T cells, c) CD25⁺ cells and Ox-62⁺ dendritic cells. All stained cell types infiltrated statistically significantly stronger in group 2 compared to 1 on day9 ($p < 0.01$, respectively). No statistical difference was observed between those groups on day15. The infiltration of CD4⁺, CD8⁺ and Ox-62⁺ cells was statistically significantly elevated in animals of group 1 on the median rejection time point (day15) when compared to group 2 on its median rejection time point (day9) ($p < 0.01$), whereas no statistical difference was observed for CD25⁺ cells.

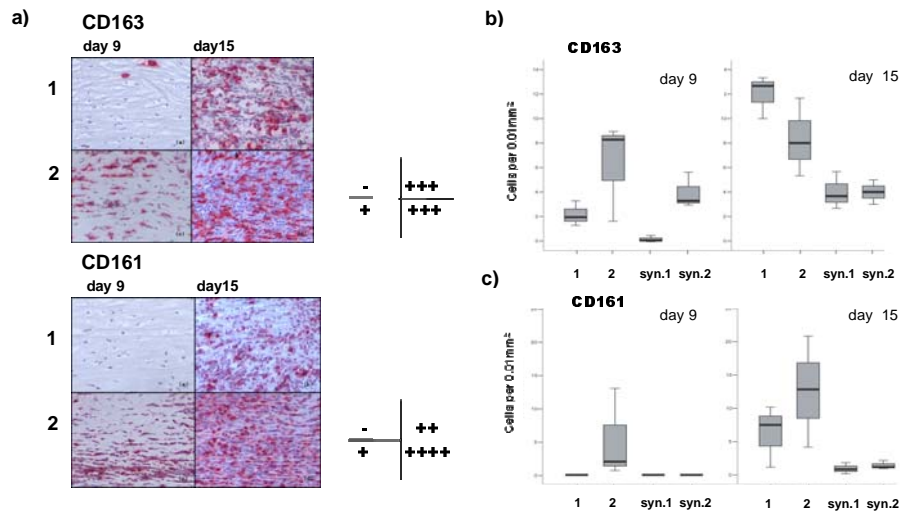


Fig. 4: Mean cellular infiltration of CD163⁺ macrophages and CD161⁺ NK/NKT cells was calculated from 4 individual corneal transplants on the expected days of rejection. Animals of group 1 (10 week old recipients) are indicated by 1, those of group 2 (3 week old recipients) by 2. The corresponding syngenic controls are indicated as syn.1 and 2, respectively. Both cell types infiltrated statistically significantly stronger in group 2 compared to 1 on day9 ($p < 0.01$, respectively). No statistical difference was observed for CD163⁺ between those groups on day15, whereas CD161⁺ cells showed a stronger infiltration in group 2 ($p < 0.01$). The infiltration of CD163⁺ cells was statistically significantly elevated in animals of group 1 on the median rejection time point (day15) when compared to group 2 on its median rejection time point (day9) ($p < 0.01$), whereas no statistical difference was observed for CD161⁺ cells.