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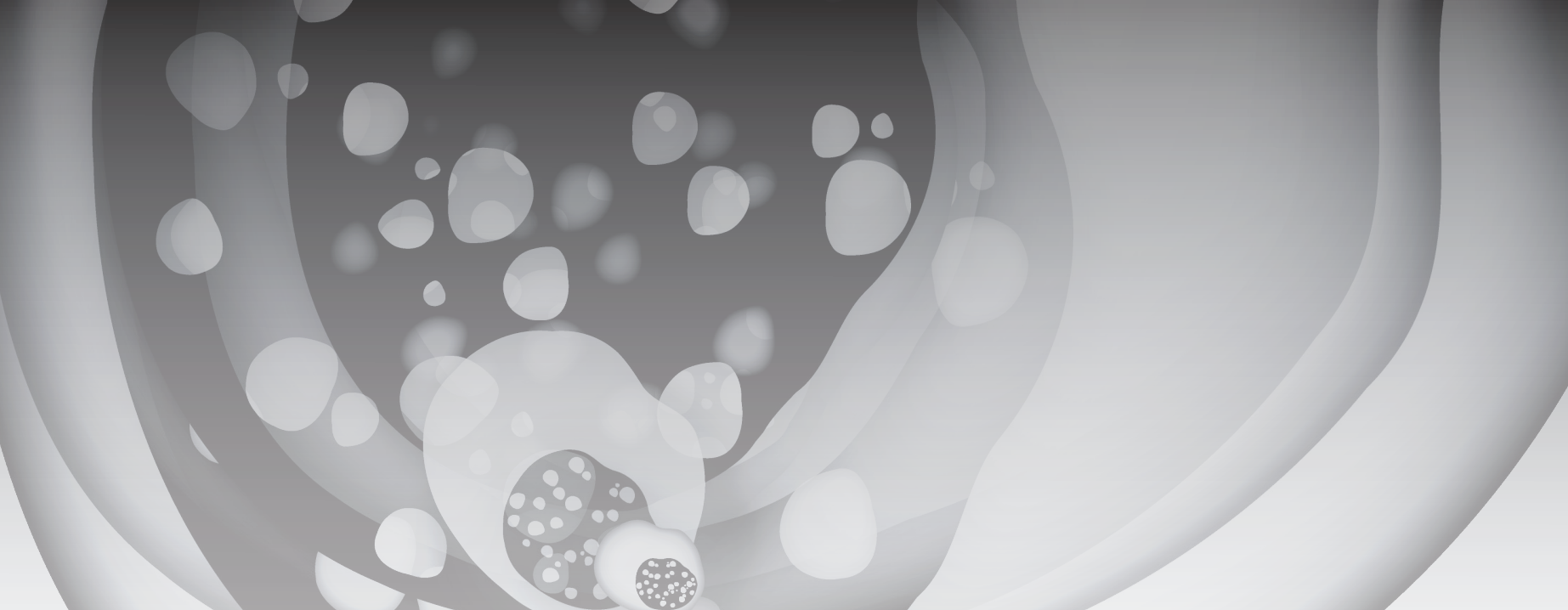
Title: Minor histocompatibility antigen specific cytotoxic and regulatory immune responses in health and disease
Issue Date: 2015-01-20



CHAPTER VII

General discussion

In this thesis several minor H antigen specific cytotoxic and regulatory immune responses are studied in health and disease. In line with previous research of the group of prof. Goulmy we identified minor H antigen specific responses induced by the physiological setting of pregnancy or after transplantation.

In the first three chapters pregnancy related induction of minor H antigen specific responses are studied. Since our results indicate a wider range of responses than earlier anticipated, it is questioned whether pregnancy related minor H antigenic responses influence stem cell transplantation (SCT) outcome.

Minor H antigen specific responses in Umbilical Cord Blood

The use of umbilical cord blood (UCB) has increased over the years as stem cell source for transplantation[1]. Cord blood has several advantages and disadvantages compared to peripheral blood stem cells (PBSC) or bone marrow (BM) as discussed in the introduction of this thesis. Although it is believed that cord blood in general is less antigen experienced than adult blood[2, 3] in chapter II we show that UCB is far from naïve. Before it has been shown that UCB contains specific T cells directed against maternal antigens and viruses[4-6]. Here we show that UCB also contains specific T cells directed against non-maternal antigens, in this case HY. We assume that these T cells are directed against microchimeric cells present in the mother. The latter cells can be derived from an older brother, a phenomenon known as transmaternal cell trafficking. After isolation and clonal expansion of the T cells we tested the cytotoxic activity of UCB derived cells. In vitro analyses showed two types of functional antigen specific cytotoxic T cells (CTL); i.e. those recognizing the natural ligand and those recognizing peptide loaded target cells only. The latter difference between the two types of CTL could be explained by differential TCR avidity for its ligand as well as being indicative for functionally different types of T cell clones i.e. CTL versus Tregulator (Treg) cells. Before we have tested *ex-vivo* derived T cell lines with different tetramer staining intensity. Tetramer bright staining intensity corresponded with a high avidity TCR recognizing the natural ligand[7]. T cells derived from a tetramer^{dim} staining population had a regulatory phenotype. These data are reviewed in ref 8[8]. In these studies tetramer staining intensity, which is associated with TCR affinity, correlated with the functional avidity of the T cells to lyse the natural antigen. Nevertheless within the tetramer dim staining population cytotoxic T cells could be isolated. In our cord blood samples we did not separately isolate T cells based on the level of tetramer staining intensity. After expanding isolated T cells, the obtained T cell clones showed different levels of tetramer staining intensity, which was not correlated with TCR avidity to recognize the natural ligand or peptide loaded target cells. Therefore

in this study we could not relate TCR affinity, reflected by tetramer staining with TCR avidity and a strong cytotoxic response as displayed by the clones recognizing the natural ligand. Whether the T cells with low TCR avidity, meaning only peptide loaded target cell recognition, are indeed functionally different T cell *in vivo*, remains to be seen.

Since UCB derived T cells have been educated in the tolerogenic environment of pregnancy it is plausible that cord blood contains many Tregs[9]. This is supported by various studies in which tolerance is shown, which is probably induced during pregnancy. Firstly, tolerance against maternal antigens have been shown in healthy adult men[10]. Secondly, tolerance against an older sibling has been suggested, since patients transplanted with a younger sibling donor have better outcome[11]. Furthermore tolerance against antimicrobial antigens like Malaria can be induced by prenatal exposure[12].

In our study not only peptide specific T cells but also HY-tetramer positive T cell clones, which do not lyse the antigen upon recognition, have been isolated. By the lack of reliable *in vitro* regulatory tests and MHC class I Treg specific markers we were not able to label these T cells as regulatory T cells. The majority of the HY-specific T cell clones we isolated from our UCB samples displayed this phenotype; clear tetramer staining without interferon gamma production, proliferation or cytotoxicity upon antigen recognition, either natural ligand or peptide loaded target cells (data not shown). Whether these T cells are immature and therefore lack co-stimulatory molecules, are anergic T cells or are indeed Treg remains subject of further research. We only studied antigen specific T cells against HY as a proof of principle. Probably similar responses can be found directed against virtually any antigen present in the mother which can travel over the placenta. This means that UCB is not naïve, but might contain predominantly regulatory T cell responses, explaining the lower incidence of Graft versus Host Disease (GvHD) after UCB transplantation and the fact that less stringent HLA matching is acceptable[13]. Additionally, when the HLA mismatch between UCB and donor is identical to a non inherited maternal HLA antigen, outcome is better compared to a HLA-mismatch which is not shared by the mother[14]. This does further support the finding of (reglulatory) antigen specific responses already present in UCB.

In utero priming of T cells influences the immune repertoire of adult stem cell transplantation donors

Antigen specific T cells which have been identified in UCB might reflect the presence of similar T cells in the adult immune repertoire. It is likely that these T cells play an important role in the earlier described birth order effect in transplantation. With

the birth order effect, the influence of transmaternal cell trafficking on the immune system in HLA-identical sibling transplantation is described. It is hypothesized that there is a difference in transplantation outcome between sibling donors which are younger than the patient and sibling donors that are older than the patient. This was first described in a study by Bucher et al. In that study transplantation outcome was better when a younger sibling donor was used[11]. This can be explained by in utero exposure of the younger sibling to antigens of the older sibling. Since this first study, conflicting results have been published regarding the effect of birth order in several transplantation settings[11, 15, 16]. In chapter III we questioned whether gender of the donor and/or of the recipient is involved in the birth order effect. For this we analyzed a group of 311 HLA-identical sibling transplantations. Interestingly, the birth order effect was only significantly present in adult female donor/female recipient SCT pairs. A plausible explanation for this finding might be a "multiple hit" immunization. The first priming of T cells against sibling antigens occurs in utero trough transmaternal cell trafficking in a tolerogenic environment. In healthy male donors these antigen specific T cells directed against sibling antigens will usually not be reactivated during life. In healthy fertile female donors these T cells can be reactivated during pregnancy. When the fetus shares antigens with the older sibling(s) of the mother, antigen specific T cells can be reactivated in again the tolerogenic environment of pregnancy. This might lead to antigen specific regulatory T cells. It remains questionable why we only observed this effect in transplantations between sisters and not in female to male transplantations. This difference might be explained by the strong immunogenic effect of HY. HY is a broadly expressed minor H antigen and different peptides of the Y gene can be presented in many different HLA molecules, probably many more than is known until now. It can be reasoned that these responses are too diverse and too strong to be completely regulated, although many women harbor HY specific regulatory T cells.

Unfortunately we were not informed about the parity of the donors. Therefore we can only hypothesize about the mechanisms involved. Whether successive exposure to the antigen is the key to the beneficial effect of birth order remains to be seen. It would be very interesting in future research to compare nulliparous (preferably sexually not active) donors with parous donors with female offspring and parous donors with male or mixed offspring to further test the above described hypothesis.

The complex influence of pregnancy on the immune status of women

Parous female donors are frequently avoided as possible stem cell donors. But the evidence that the sensitized immune status of women after pregnancy might have a negative effect on transplantation outcome is scarce. Sensitization can be analyzed

in vitro before transplantation by testing cytotoxic responses of the possible donor against the recipient. Since sensitization is a known risk factor for acute GvHD, these donors are preferably not selected. Reviewing large studies on the impact of donor gender and parity on transplantation outcome[17] a negative effect on the incidence of chronic GvHD of female donors in male recipients and of parous female donors in female recipients is shown. There was no influence of gender on acute GvHD, relapse, overall survival or transplant related mortality. Additionally, a large retrospective study on allogeneic HSCT procedures performed in the US showed that donor age is the only non-HLA factor affecting overall and disease-free survival[18].

In chapter IV we show that pregnancy indeed does not solely lead to sensitization. We clearly show that a substantial number of women display a more regulatory T cell phenotype instead of a sensitized phenotype. This maternal tolerance might also play a role in haplo-identical transplantation, in which mothers as donors have better outcome than fathers[19]. Unfortunately currently there is no reliable *in vitro* assay to identify possible tolerant donors, with a low risk of GvHD. A cumbersome method which can be used to determine donors' pre-transplant tolerant state is the trans vivo delayed type hypersensitivity assay (tvDTH). In this mouse model human PBMC are injected in the footpad of the mouse in combination with a recall antigen and a specific antigen to test antigen specific tolerance. Whenever there is tolerance the recall response will be reduced when the antigen for which the person is tolerant is present[20]. Earlier we have used the tvDTH assay to test minor H antigen specific tolerance after renal transplantation[21] and in healthy donors to test minor H antigen specific cytotoxic or tolerant responses[10]. In the current study we have used this assay to test HY specific tolerance in healthy female donors, from which detailed family and obstetric history was obtained. HY-specific tolerance was present in many nulliparous and parous women. The presence of tolerance was independent of the gender of the offspring and whether the donors had an older brother or not. Especially nulliparous women had a predominant tolerogenic phenotype. Women can be exposed to male antigens in many ways, so probably not only pregnancy can induce a male specific immune reaction. Apart from the transmaternal cell flow as discussed above and possible missed abortions, heterosexually active women are frequently exposed to male antigens in the mucosa. It is known that mucosal exposure to antigens can lead to tolerance as is used in oral tolerance induction protocols[22]. Furthermore tolerance towards paternal antigens (e.g. HY) is induced before pregnancy by coitus and probably plays an important role in successful conception[23]. Whether tolerant women become sensitized during or after pregnancy is unknown, but probably some of these women will, explaining the difference we have found between nulliparous and parous women. That this sensitization is (mainly) directed against male antigens

is supported by the fact that secondary recurrent miscarriages are frequently preceded by male pregnancy[24]. Extensive research is needed to determine the exact influence of pregnancy on the female immune system. Questions which still need to be addressed are, e.g.: What is the influence of subsequent pregnancies? What is the influence of (early) spontaneous abortions? What happens in time; is there a difference in the immunological phenotype of women early (women with young children) or late (women with adult offspring) after pregnancy. What is the influence of (type of) sexual intercourse. Although (some of) these questions are difficult to be answered, it is important to acquire a better understanding of the immune status of potential female donors.

We are all born chimera

The presence of circulating fetal cells in mothers (microchimerism) during and after pregnancy is well established[25, 26]. Nevertheless, nulliparous women carry male microchimeric cells as well as is shown by others[27-29] and in chapter IV. Vanished male twins[30], (un)known miscarriages of male fetuses[31], male leukocytes present in semen entering the female's circulation[32, 33], and transmaternal passage of cells derived from older brothers[11] have been suggested as possible sources of male microchimerism. Most of these explanations are educated guesses based on indirect evidence. In chapter III, we identified male microchimerism in different leukocyte subsets in female UCB which can only be explained by transmaternal passage. Whether these cells are derived from previous pregnancies or have survived in these mothers from birth onwards, has not been studied in detail yet. Whether these microchimeric cells will persist throughout life is uncertain, but they probably will in many children, since (maternal) microchimeric cells have been found in pediatric tissue[34] and in healthy adult males[30].

In this thesis (chapter IV) we have tried to relate the presence of microchimeric cells in women to a sensitized or tolerant phenotype. We hypothesized that the presence of microchimeric cells in peripheral blood would reflect a tolerant state of that woman. If microchimeric cells lead to cytotoxic responses (a sensitized phenotype) it will in the end kill the microchimeric cells. Persistence of the microchimeric cells implies tolerance of these cells (a tolerant phenotype). Nevertheless the immune system is much more complex than that. There is a delicate balance between cytotoxic and regulatory T cells, which is clearly not solely reflected by the presence of microchimeric cells in peripheral blood. It could be that the presence of microchimeric cells in different organs, bone marrow or lymph nodes much better reflects a tolerant phenotype, as has been shown in mice35. Material from these sights cannot be routinely obtained from healthy donors. This implies that either reliable readout systems for

tolerance or surrogate markers corresponding with a tolerant phenotype should be identified.

Our findings of microchimerism in UCB and nulliparous donors have led us to propose that all humans are born as microchimera's. Future microchimerism studies should identify the full microchimeric repertoire within an individual which we expect to comprise maternal, fetal, fraternal, grandmaternal et cetera material.

Today research regarding microchimerism is a hot topic. Chimeric cells can be found in virtually any organ. Many researchers have tried to relate microchimerism to clinical conditions like auto-immune disease and cancer. This has led to conflicting results, from which it is difficult to draw the right conclusions. Chimeric cells are frequently present in affected organs. Until now it is not clear whether the presence of these cells is the cause or consequence of the disease. Are they involved in the inflammatory responses, either as cytotoxic cells, or as the antigen against which the 'host' is reacting, or are they regenerating damaged tissue? It is plausible that all these mechanisms are involved in different stages of the disease.

Minor H antigens might not be clinically relevant in HLA identical Kidney transplantation

In chapter V the role of minor H antigens in HLA identical sibling renal transplantation is studied. This international multicenter study was initiated as a case control study to compare long term (>10 years) survivors of HLA-identical sibling renal transplantation with patients experiencing acute or chronic graft rejection. Along the inclusion of patient data it became clear that our initial set up had failed. Most of our partners had included all of their HLA-identical transplantations, independent of outcome and period of follow up. In our cohort of 444 transplantations 36 patients experienced a rejection episode, of which only 8 resulted in graft loss. In these patients we could not identify minor H antigens related to rejection. From this we can conclude that outcome after HLA-identical sibling renal transplantation is very good and is scarcely complicated with graft rejection. Therewith it can be questioned what the clinical relevance of possible minor H antigen mismatches in this small subgroup of renal transplantations is. Earlier, a large study of 158 652 non-related cadaver transplants showed increased graft loss in female recipients with male donors after multivariate analyses taking other known confounding factors, such as original disease, donor and recipient age, number of HLA-mismatches and preformed panel-reactive antibodies, into account. This increased graft loss was seen after 1 year and between 2 and 10 years; indicating that in both acute and chronic rejection HY plays a role[36]. In a smaller cohort the presence of de novo antibodies directed against the HY genes RPS4Y1 and/or DDX3Y is described in female patients transplanted with male grafts

which correlated with acute rejection[37]. Little data are available on the role of autosomally encoded minor H antigens in renal allografting. One single center study reported on the detrimental effect of HA-1 mismatching and chronic allograft nephropathy[38]. In a larger cohort HLA-A, -B, -DRB1 matched renal transplantations were analyzed for the influence of HA-1, HA-2, HA-3, HA-8, HB-1, ACC-1 and UGT2B17 mismatches[39]. In this study no influence of the latter minor H antigen mismatches in 5-year death-censored graft survival was observed. Therefore from our own results and that of earlier studies we can conclude that the role of minor H antigen mismatches in graft rejection is probably small. Nevertheless the role of minor H antigen mismatches is possibly more important in graft tolerance. Successful tapering of immunosuppressive medication in HLA-identical but minor H antigen mismatched renal transplant recipients has been performed[40]. The authors concluded that tapering of medication could be executed despite minor H antigen mismatches between donor and recipient, indicating that minor H antigens are irrelevant for rejection of the renal allograft. Alternatively, one could speculate that minor H antigen mismatches may be beneficial for maintenance of the graft thereby facilitating withdrawal of immunosuppressive agents. This is supported by a study, which has been performed in our group in collaboration with the group of prof. Burlingham. Here immunosuppressive drugs were successfully stopped in a woman, who had been transplanted with her HLA-identical, minor H antigen HA-1 mismatched, sister. Both $CD8^{pos}$ minor H antigen-specific T regulator and cytotoxic T effector cells co-existed in the presence of minor H antigen presenting microchimeric cells [21]. Interestingly, this patient was not only HA-1 mismatched with her renal graft but also with her offspring and mother. Therefore tolerance might already have been induced during pregnancy, leading to reactivation of tolerance after transplantation. Nevertheless HA-1 is a hematopoietic restricted minor H antigen and therefore it is questionable whether transplantation tolerance has been induced by HA-1 specific Treg itself. Although HA-1 expressing hematopoietic cells can initially be present in the graft, renal epithelial cells do not express HA-1. Therefore tolerance of the renal graft might not be mediated through HA-1 specific Tregs, but the presence of these Treg might reflect the tolerant state of the recipient and might have contributed to the graft tolerance by creating a tolerant environment.

In conclusion, whenever there is a role of minor H antigens in renal transplantation, the role is probably minor. Therefore future research must on the one hand further focus on identification of renal specific (epithelial) markers which might play a role in graft rejection and/or acceptance. On the other hand identification of patients with good allograft acceptance in whom immunosuppressive drugs can be tapered is very important[40-42], especially concerning the complications associated with these drugs[43].

The unique T cell receptor of HA-1 specific T cells

As described before HA-1 specific cytotoxic T cells have a restricted T cell receptor (TCR) usage. All thus far described HA-1 specific T cells share the same TCR Vbeta, namely TRBV7-9 (according to the current nomenclature). In this thesis cytotoxic and tolerogenic responses after pregnancy have been studied. Although we have mainly focused at responses against the HY antigen as a proof of principle, similarly HA-1 specific responses are induced[4, 10, 44]. In chapter VI we questioned whether HA-1 specific tolerogenic T cells used the same TCR as cytotoxic T cells. Therewith being able to speculate about the origin of these Treg. Until now it is not clear whether antigen specific Treg induced by pregnancy are CTL which have been changed into Treg by cytokines and tolerogenic co-stimulation in the environment of pregnancy, originate from the same precursor cells as CTL, or are a distinct entity. In the group we studied, we confirmed that HA-1 tetramer^{pos} T cell clones that lyse HA-1 natural ligand expressing target cells in vitro, all share the restricted TCR Vbeta TRBV7-9 indeed. Furthermore we showed that not only HA-1 specific T cells that recognize the natural ligand, but also T cell clones that specifically lyse exogenous HA-1 peptide loaded target cells use the restricted TCR Vbeta TRBV7-9. Additionally none of the HA-1 tetramer staining T cell clones that do not lyse natural or peptide loaded HA-1 expressing cells share the TRBV7-9. What the in vivo function of these different T cells is remains speculation. It is expected that the high avidity CTL clones, e.g. recognizing the natural ligand, are most potent in vivo and thus relevant for the Graft versus Leukemia reactivity[7, 45].Nonetheless it remains questionable what the in vivo difference is between natural ligand recognizing T cells and peptide specific T cells. As described above peptide specific T cells might be more regulatory T cells than cytotoxic T cells. Therewith implying that functionally different T cells have the same restricted TCR VBeta usage. This would be a unique finding, which might interfere with current clinical studies. It has been illustrated over the years that the minor H antigen HA-1 is an unique antigen. The hematopoietic restriction of the antigen makes it a very good target for additional immunotherapy for hematological malignancies. Several different studies have been performed in order to enhance the strong anti-leukemic effect of HA-1. Initially graft versus leukemia was achieved by donor lymphocyte infusions after HA-1 mismatched bone marrow transplantation. Nowadays more antigen specific targeted immunotherapy is under study. This can, amongst others, be accomplished by T cell receptor (TCR) transfer. HA-1 is a perfect candidate for these type of studies since the restricted TCR usage of HA-1 specific T cells. Our current findings suggest that with the this method not only T cells with a high anti-leukemic potential will be generated, but possibly also T cells only recognizing the peptide, which may be Treg. Therewith allowing relapse to occur.

Furthermore since tetramer staining does not solely isolate CTL, speculations have been made to use TRBV7-9 monoclonal antibodies additionally. Currently TRBV7-9 specific monoclonal antibodies are not available, but are under study. Therefore in the future it might be possible to isolate HA-1 specific T cells using a TRBV7-9 antibody in combination with HA-1 tetramer staining. It seems attractive to give the obtained cells directly to patients as antigen specific donor lymphocyte infusion. Until CTL can be clearly distinguished from Treg these therapies are not eligible for patients. Thorough *in vitro* functional analyses should be performed of both primarily isolated T cells and of T cells in which TRBV7-9 is used for TCR transfer for adoptive T cell therapy.

Future directions

In order to translate the above described research into clinical practice further research is needed. Herein a few aspects are crucial.

1) Antigen specific CD8 Treg culture protocols and reliable *in vitro* read out systems should be developed.
2) Clinical studies are needed in which donor pre-transplant sensitization and/or tolerance regarding several minor H antigens are studied in relation to transplantation outcome.
3) To be better informed about (female) donors pre-transplant immune status donor centers are encouraged to gather information about the family (birth order) and pregnancy history of the donor.
4) Antigen specific responses present in cord blood should be further studied, mainly in relation to double cord blood transplantation in order to improve outcome of these transplants.
5) Renal specific antigens should be identified which can be involved in either kidney graft rejection or acceptance.

In conclusion, more research is needed in order to be able to identify the perfect donor in relation to the specific background of the patient.

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