# THE IMPORTANCE OF DONOR MICROFLORA IN LATE-ONSET GRAFT VERSUS HOST DISEASE

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## SUMMARY

Late-onset Graft versus Host Disease (LO-GvHD) may occur in lethally irradiated mice after allogeneic bone marrow transplantation. In contrast to acute GvHD, which is caused by T-cells, late onset GvHD is effected by the presence of intestinal microflora in the recipient during the first 40 days after transplantation. LO-GvHD does not occur in germfree or totally decontaminated animals. Based on the results of fundamental studies this review discusses the possible mechanism of LO-GvHD which is characterised by wasting disease and hypoplasia of lymphoid organs. In order to induce LO-GvHD engrafted bone marrow cells need the presence of intestinal microflora in the recipient. In normal situations the autochthonous (self) intestinal microflora, which predominantly consists of anaerobes, induces immuno-tolerance but not immune reactivity within the host. Depending on the degree of similarity with self microflora, non-self i.e. allochthonous microflora may induce immune reactivity instead. Immuno-tolerance can be enhanced by intra peritoneal challenge with autochthonous microflora but not or only little with allochthonous microflora. This review discusses the increase of LO-GvHD in gnotobiotic C3H recipients associated with microflora of C57Bl donors. LO-GvHD was found to the highest level when C57Bl donors in turn had been challenged with their own microflora mice 10 days before bone-marrow harvesting. In conclusion a) BM cells carry "memory" for MF antigens, b) autochthonous-(like) microflora antigens predominantly induce tolerance i.e. are non-immunogenic and c) Recipients colonised with Donor-like microflora are at increased risk for LO-GvHD.

## INTRODUCTION

Allogeneic Bone Marrow Transplantation (BMT) following aggressive (sublethal) chemo and or radio-therapy has become a standard procedure in the treatment of certain haematological malignancies. However, regardless optimal donor selection, Graft versus Host Disease (GvHD) may occur as a serious complication after allogeneic BMT. Despite the fact that there are many causes and definitions for GvHD, it is generally circumscribed as immuno-competent, host incompatible, cells i.e. bone marrow, spleen, and/or T-cells that attack lymphoid and epithelial tissues of the host (recipient). Meanwhile, the recipient should be unable to counter-attack the engrafted cells.

Several factors have been found to contribute to GvHD:

a) Major Histocompatibility Complex (MHC) difference between donor and recipient
b) the type of cells engrafted e.g. purified T-cells, spleen cells in combination with BM cells
c) the number of cells engrafted
d) the intestinal microflora

Several precautions are generally taken in order to prevent GvHD:
a) donor and recipient are matched as much as possible according their MHC antigens; i.e. HLA in man and H-2 in mice
b) the bone marrow graft is depleted from T-cells if present
c) patients are totally decontaminated and nursed under a germfree conditional regimen
d) patients receive short or long-term GvHD immunosuppressive therapy

Due to these additional regimens GvHD has decreased and is no longer a serious threat after allogeneic BMT. Total decontamination, and thus the need for strict barrier nursing, is not only effective in preventing non-viral infections but also GvHD (*van der Waaij* and *Heidt*, 1986; *Beelen* et al., 1992). However, some negative aspects still remain; e.g. strict barrier nursing procedures are laborious and therefore very expensive, and implicate strong psychological stress for the patient. Whereas immunosuppressive therapy is effective against GvHD, patients treated as such are prone to viral infections and tumour development.

One of the questions worth investigating is why recipient intestinal microflora in a way induces or aggravates GvHD. As noted, GvHD can be mitigated by total decontamination. However, if the question can be answered how the microflora is instrumental in GvHD, total decontamination combined with expensive high care and germfree strict-barrier nursing would no longer be necessary. In this way the duration of GvHD suppressive therapy might be shortened, if still necessary, implicating a lower risk for viral infections to occur. This may additionally implicate maintenance of the potentially beneficial immuno-stimulating effects by components of the gut microflora as well as a better control of colonisation of the intestinal tract by potential pathogenic bacteria and fungi.

## HISTORY

In the late sixties and early seventies Van Bekkum et al. found a strong correlation between intestinal microflora (I-MF) and GvHD in mice (*van Bekkum* et al., 1974; *van Bekkum* and *Knaan*, 1977). MF-associated GvHD, at first named 'secondary disease', was found when lethally irradiated conventional inbred mice were engrafted with normal, T-cell negative, BM (T⁻BM) from H-2 incompatible (allochthonous) donors. MF associated GvHD was found to differ from acute GvHD, found after engraftment of BM in combination with spleen or T-cells (T⁺BM). Important differences, which were seen between T⁻BM and T⁺BM engrafted mice, were:
a) mortality in T⁺BM recipients was 100% whereas only a part of the recipient group died when engrafted T⁻BM
b) mice engrafted with T⁺BM died within 21 days whereas T⁻BM mice died between 28 until 100 days after BMT
c) signs and symptoms of GvHD were totally absent in germfree or totally decontaminated T⁻BM engrafted mice
d) mortality was delayed but still 100% in germfree or totally decontaminated recipients engrafted with T⁺BM.

Because of the delayed occurrence, MF associated GvHD is named Late Onset GvHD (LO-GvHD). LO-GvHD in mice is characterised histologically by aplasia of lymphoid organs e.g. thymus, spleen and lymph nodes and destruction of epithelial tissues e.g. skin, and gut. Clinically, mice suffering from LO-GvHD show diarrhoea, progressive wasting, and a ruffled fur. In contrast to T-cell mediated acute GvHD, LO-GvHD is not always fatal. Additional investigations have shown that total mitigation of LO-GvHD remains when mice are maintained bacteria-free i.e. germfree until 40 days after BMT by which time they can be conventionalised without adverse effects (*van Bekkum* and *Knaan*, 1977). This window phase of 40 days found in mice may exist in man as well. These observations have been one of the reasons why patients nowadays receive total decontamination initially after BMT for a restricted period of several weeks. Indeed, there is increasing evidence that total decontamination reduces GvHD in man (*Beelen* et al, 1992; *Heidt*, 1989; *Vossen* et al., 1990).

Despite the fact that elimination of potential pathogenic bacteria e.g. *Enterobacteriaceae* and *Pseudomonadaceae* during the leukopenic phase has been found to reduce the number of infections, septic periods, and subsequently death, no direct correlation has been found between LO-GvHD and these bacteria as infectious agents (*Veenendaal* et al., 1988; *Vossen* et al., 1990). Instead, it has been postulated that bacteria in the intestinal tract, particularly Gram-negative rods, induce antibodies that cross react with tissue antigens and subsequently cause LO-GvHD (*van Bekkum* and *Knaan*, 1977; *van der Waaij* and *Heidt*, 1986). However, there is ample evidence that Gram-negative rods e.g. *Enterobacteriaceae* are of minor importance if any in the pathogenesis of LO-GvHD (*Heidt*, 1989;

*Veenendaal* et al., 1988; 1990; *Vossen* et al., 1990; *Veenendaal*, 1995) and if so their role may be limited to a non-specific immuno-modulation by lipopolysaccharide (LPS) i.e. endotoxin (*Moore* et al., 1987a; 1987b).

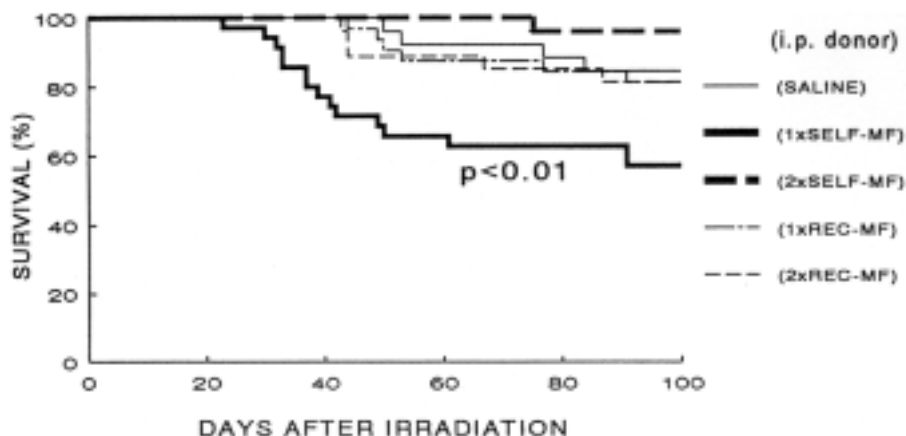The conditions needed for LO-GvHD to occur are fourfold:
a) recipients should either carry a conventional or a diverse but well developed SPF microflora,
b) should receive lethal irradiation prior to BMT,
c) BMT should be carried out over a total MHC (H-2) barrier, and
d) some sort of interaction has to take place between intestinal microflora antigens and engrafted BM cells e.g. NK-cells or B-cells.

In mice, the latter (d) apparently takes place during the window phase lasting until 40 days after BMT causing LO-GvHD.

The window phase gives proof that I-MF plays a central role in the pathogenesis of LO-GvHD. Thus MF associated LO-GvHD in fact appears to be Graft versus Microbial Flora Disease. An important feature of LO-GvHD is the absence of proper T-cell function in combination with dysplasia of the thymus. This finding has similarities to thymusless nude mice. In fact nude mice also suffer from runting disease if they are maintained under conventional conditions. There is a main difference, however, between nude mice and LO-GvHD mice. Whereas nude mice already have a deficient T-cell system from the start, T-cell deficiency develops during LO-GvHD after BMT. This difference is subject for discussion. The critical window phase of 40 days, during which mice are prone to suffer from LO-GvHD, generates many questions e.g.:
a) What type of interaction takes place between donor BM-cells and recipient-MF which eventually leads to LO-GvHD
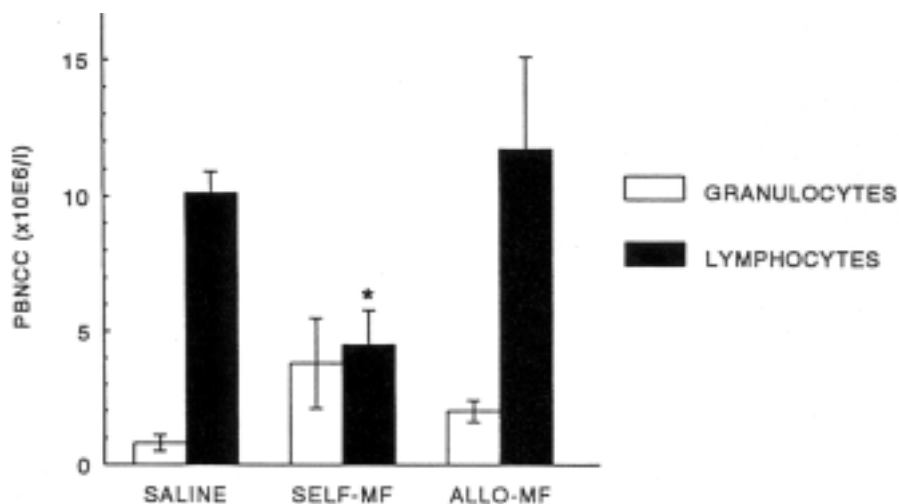
**Figure 1**: Survival rates of lethally irradiated (9 Gy) C3H/He (H-2k) recipients engrafted with $10^7$ nucleated BM cells from C57Bl/6J (H-2b) donors 19-100 days after BMT. C57Bl Donors had been pretreated by i.p. injection either with saline, washed faecal flora of their own (SELF-MF) or C3 recipients (RECIP-MF). I.p. injection was performed on day -10 for single injection and day -38 and -10 for repeated injection. The number of recipients at day 19 were: group A (2x saline) n=26, group B (1x SELF-MF) n=35, group C (2x SELF-MF) n=25, group. D (1x RECIP-MF) n=32, and group E (2x RECIP-MF) n=26. Mortality in group B (1x SELF-MF) was significantly the highest (p<0.01) compared to all other groups (Kaplan-Meier analysis).

b) Is there any evidence for donor BM-cells with specific affinity for (components of) the recipient-MF

c) Is there any correlation between composition of the donor-MF, the recipient-MF, and LO-GvHD

d) Is (LO-)GvHD generated by some sort of key component in the recipient-MF

e) Should an immunological classification system of intestinal microflora be developed to explain, predict, and make it possible to prevent LO-GvHD in the future

## DONOR MICROFLORA AND LO-GvHD

Experiments in mice have shown a close interaction between intestinal (microflora) antigens and the BM compartment (*Alley* et al., 1986; *Veenendaal*, 1995). Even microflora modulation, induced by oral treatment with small spectrum antibiotics as used for selective decontamination, has been found to influence the composition of the BM (*Goris* et al., 1985; 1986). Additionally, an enhancing effect on LO-GvHD in C3H recipient mice has been observed when C57Bl donors are given selective decontamination for two weeks prior to BMT (*Veenendaal* et al., 1988). This result leads to the postulation that engrafted BM cells carry certain 'memory' for MF-antigens, present in the recipient, which is activated by oral treatment with non-absorbable small spectrum antibiotics. It seems most likely that this effect is caused by bacterial antigens, which could for example be released in the intestinal lumen during antibiotic treatment upon bacterial disintegration, pass through the intestinal mucous and after some time reach the circulation. Experiments in which

**Figure 2**: Peripheral blood nucleated cell count (PBNCC); granulocytes and lymphocytes in C57Bl/6J mice 10 days after i.p. injection with 0.5 ml washed faeces from the B6-strain (SELF-MF) (n=8) or C3H/He mice (ALLO-MF) (n=7). Control donors (n=7) were injected with saline only. *: p<0.05
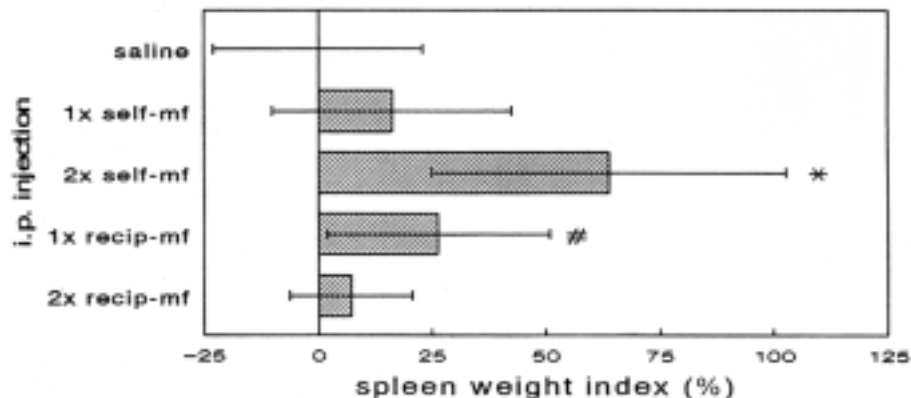
C57Bl donors were challenged intra peritoneal with either their own MF or that of the future C3H recipients showed that LO-GvHD increased significantly when BM donors received one *single* intra-peritoneal (i.p.) injection with their own MF (SELF-MF) 10 days before BMT (*Veenendaal*, 1995). In some way these animals were given an artificial form of enhanced translocation of autochthonous microflora. This effect disappeared when donors were i.p. injected *twice* with SELF-MF respectively 38 and 10 days before BMT.

LO-GvHD was found at the lowest level in this group. Parenteral challenge of BM donor animals with recipient-MF (NON-SELF-MF) surprisingly did not influence LO-GvHD compared to saline injected control donors (Figure 1). Thus LO-GvHD in these studies was not only affected by the recipient MF. Instead, the donor MF appeared to be even more important. This effect was found in a mouse model in which LO-GvHD was present at a low baseline level giving a mortality rate of approximately 20%.

## INTERACTIONS BETWEEN IMMUNE SYSTEM AND MICROFLORA

Different functional changes not only occur in the BM upon parenteral challenge with SELF-MF antigens compared to NON-SELF-MF but also in the peripheral blood and the spleen. Figure 2 shows that a single i.p. injection of C57Bl mice with SELF-MF decreased the number of lymphocytes in favour of granulocytes as compared to i.p. injection with NON-SELF-MF from C3H mice. Figure 3 shows that spleen enlargement in mice may occur at a higher level after a single i.p. injection with NON-SELF-MF as compared to SELF-MF. On the other hand the spleen size is normalised or not effected anymore after

**Figure 3**: Spleen weight index in C57Bl/6J mice i.p. injected either with saline (n=7), 1x SELF-MF (n=13), 1x RECIP-MF (n=5), 2x SELF-MF (n=12), or 2x RECIP-MF (n=10). I.p. injection was performed on day -10 for single injection and day -38 and -10 for repeated injection. *: p<0.01 compared to all other groups; #: p<0.05 compared to saline injected animals.

repeated i.p. injection with NON-SELF-MF, whereas the spleen size further increases after repeated i.p. injection with SELF-MF. I.p. injection with SELF-MF and NON-SELF-MF both increase the level of total IgM in serum whereas the level of IgA and IgG in serum remains unaffected (*Veenendaal*, 1995). The same has been found for MF-specific IgM antibodies. However, MF specific IgM antibodies has been found at a significantly higher lever against SELF-MF as compared to NON-SELF-MF originated from C3H mice.

These data implicate an immunological difference between SELF-MF and NON-SELF-MF in the mouse i.e. C57Bl (B6) and C3H (C3) mice. I.p. challenge with either microflora does not induce IgG or IgA seroconversion in B6 mice. Instead, IgM may be the most important regulating immunoglobulin in the immune response against microflora antigens.

Since no difference was found in the number of viable aerobic bacteria present in either MF (Table 1), the bacterial fraction, which has to be held responsible for the different reactions described above, must be looked for within the composition of the highly concentrated anaerobes. Micromorphological examination revealed a difference in this fraction in either MF. This indirectly indicates that LO-GvHD might be associated with differences in the level of (immuno-)regulation between "SELF" (autochthonous) MF and "NON-SELF" (allochthonous) MF; particularly concerning the highly concentrated obligate anaerobes. Herein SELF-MF antigens predominantly may induce immuno-tolerance. When these bacteria 'attack' the host outset their normal habitat e.g. extra-intestinal more primitive immune reactions are the only defence against it. This explains why spleen enlargement further increased upon a second i.p. challenge with SELF-MF but not with NON-SELF-MF.

Immuno-tolerance apparently is important for the symbiosis between the host and its MF. Bacteria may only be able to survive within the intestinal lumen when there is no specific (mucosal) immunity. Lack of mucosal immunity may either be due to a gap in the immune repertoire or due to active suppression by antigen specific suppresser

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**Table 1**: Analysis of pooled faeces from B6 and C3 Mice

| Microorganism | B6 (n=5) | C3 (n=5) |
|---|---|---|
| *E. coli* (API:5144552) | $1.6 \times 10^3$ | $2.0 \times 10^3$ |
| *Prot. mirabilis* (API:0536000) | $2.0 \times 10^4$ | $2.5 \times 10^4$ |
| *Enterococcus* spp. | $5.0 \times 10^4$ | $3.0 \times 10^4$ |
| *Staph. aureus* | $1.0 \times 10^4$ | $1.5 \times 10^4$ |
| *Bacillus* spp.* | ND** | ND |
| obligate anaerobic bacteria*** | $10^{11}$ | $10^{11}$ |

Data represent concentrations in microorganisms/g. faeces

*: Qualitative aerobic culturing only

**: Microscopic analysis by eye revealed a morphological difference between highly concentrated obligate anaerobic fractions in B6 and C3 faeces.
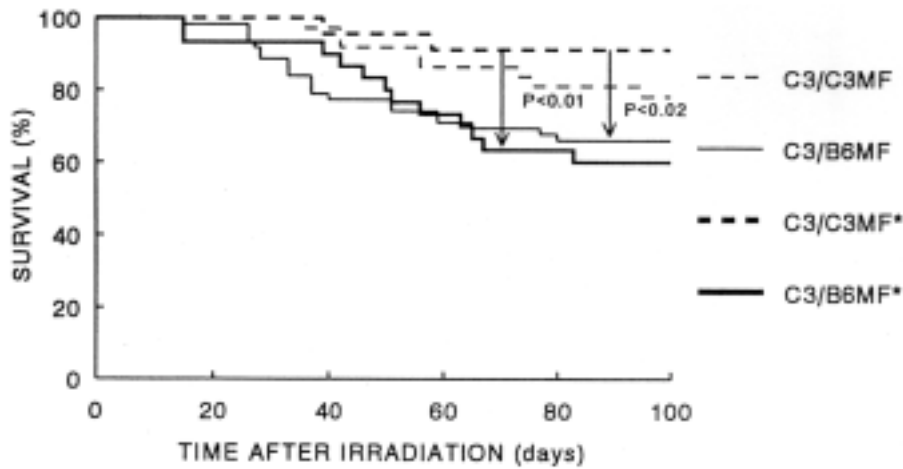
***: ND=not determined

cells. By selecting bacteria that are, or become, immunologically inert, the host will prevent itself from undesirable immune reactions; e.g. antibodies which fix complement and/or cross-react with host type antigens and cause respectively inflammation or autoreactivity.

General suppression of specific immunity against SELF-MF is likely to be compensated by stimulation of the innate immune system i.e. granulocytosis and monocytosis. In the absence of IgG and IgA, the isolated IgM antibody response upon i.p. challenge may indicate the absence of SELF-MF antigen specific Th2-cell mediated immunity. On the other hand the existence and increase of IgM antibodies may also point at the presence of an idiotype anti-idiotype network which physiologically regulates MF-antigens.

It remains speculative whether, and if so how, induction of immuno-tolerance exists or more likely is enhanced inside the BM compartment upon i.p. challenge with SELF-MF. Possibly during live the host and it's MF live in symbiosis. For reasons of necessary immunological peace with SELF-MF the host only generates an innate (primitive) immune reaction, e.g. granulocytes and macrophages and/or IgM, together with a non-T-cell mediated systemic immuno-suppression e.g. by natural killer or suppresser (NK) cells. These reactions may constantly take place upon physiological translocation of SELF-MF antigens. "Tolerance" mediated by NK cells may either be local in the gut associated lymphoid tissue (GALT) or systemic e.g. at the bone marrow level. In the latter, large granular lymphocytes (LGLs) may function as such. During live, MF and immune system thus are in an equilibrium of "tolerance". However, if during (artificially) enhanced translocation of MF, e.g. by i.p. challenge, MF-antigens "escape" from the local immune defence by the GALT and will be presented at peripheral sites e.g. spleen and bone marrow. When this happens the innate defence (granulopoiesis and myelopoiesis) is stimulated as well as suppression (=tolerance). Subsequently a new equilibrium is formed which supplies adequate defence without systemic stimulation e.g. of BM upon a second artificial enhanced translocation. If true, this explains why repeated i.p. injection of donors with SELF-MF did not change or even slightly mitigated LO-GvHD in C3 recipients shown in Figure 1.

MF that generates immuno-tolerance over a wide spectrum of MF antigens, like the SELF-MF in B6 mice, may be

**Figure 4**: Survival of irradiated (9 Gy) gnotobiotic C3H/He (C3) (H-2k) recipients engrafted with BM from C57Bl/6J (B6) (H-2b) donors. Recipients were the first generation offspring ex-germfree mice associated with murine microflora either from SPF-C3 mice (C3/C3MF) or SPF-B6 mice (C3/B6MF). B6 donors were i.p. injected either with their own B6-MF (i.p./B6) or with saline (-/B6) 10 days prior to BMT. Numbers/group: C3/C3MF (n=37); C3/B6MF (n=62); C3/C3MF* (n=23); C3/B6MF* (n=30). *: B6 donor i.p. with B6MF.

an important tool to predict LO-GvHD. Herein lymphocyte reduction 10 days after a single i.p. challenge with SELF-MF may implicate induction of immuno-suppression or enhancement of existing tolerance. It seems plausible that BM, harvested from B6 mice at this point, increases LO-GvHD in C3 recipients by inducing immuno-suppression; i.e. hypoplasia of lymphoid tissues. This reaction will then be enhanced by immuno-tolerant MF-antigens in the recipient.

As mentioned earlier, LO-GvHD can only occur when engrafted BM-cells face the presence of MF in the recipient. Theoretically, engrafted BM-cells may interact with remaining but not dividing host immune cells with memory for recipient MF-antigens during proliferation and differentiation which takes place during repopulating of the host. However, this may not be very likely since LO-GvHD is prevented by total decontamination of the recipient even if it is started only shortly before BMT.

Based on the findings discussed, it can be postulated that LO-GvHD is to be expected predominantly in a) recipients carrying a donor-like microflora and b) if the donor's MF has recently been modified e.g. by an infectious disease and antibiotic treatment.

## MATCHING DONOR AND RECIPIENT MICROBIAL FLORA AND LO-GvHD

We have found that LO-GvHD increases when C3 recipients carry a B6 donor like MF (*Veenedaal* et al., 1990; *Veenendaal*, 1995). The recipients used in this study were the first generation offspring from ex-germfree C3 mice associated either with original C3 or B6-MF and were maintained in separate 'germfree' isolators. The results shown in Figure 4 strongly suggest that recipients, which carry an intestinal microflora which is (partly) homologous

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to that of the donor, are at increased risk for LO-GvHD. Since the homologous MF used in this study was original donor type, the donor and particularly its BM may have contained memory for immuno-tolerance against this MF. Again, this "memory" might have been activated to a higher level by a single i.p. injection of donor mice with SELF-MF 10 days before BM harvesting. If this assumption is correct, this feature is of key importance for LO-GvHD to develop.

The question remains, however, which type of BM cells, with apparent immuno-suppressive memory for MF antigens, are involved in the pathogenesis of LO-GvHD. Part of the mechanism that induces LO-GvHD may be an interaction with recipient-MF which has great similarity with the type of immuno-regulation of self-MF antigens.

Natural-Killer (NK) and -Suppresser (NS) cells play a profound immuno-regulating role in the bone marrow. Moreover, NK as well as NK-like so-called Large Granular Lymphocytes in bone marrow have been described to play a role in the immunosuppressive phase of GvHD, like LO-GvHD (*Guillen* et al., 1986; *Imamura* et al., 1988). A second and certainly not less important function of NK cells concerns their role in the mucosal immune system (*Brandtzaeg* et al., 1988; 1990). Besides NK or NK-like cells, which are likely to regulate the BM response against intestinal microflora antigens, a regulatory role of anti-idiotypic antibodies i.e. an idiotype anti-idiotype B-cell network should not be excluded on the forehand. However, so far no evidence is available that suggests such a regulatory mechanism in LO-GvHD.

## PERSPECTIVES IN MICROFLORA ASSOCIATED GvHD

Despite evidence that genetic differences between donor and recipient are important, LO-GvHD predominantly appears to be caused by the composition of the microflora of both. The precise mechanism of this, in fact Graft versus Microbial Flora, phenomenon remains an enigma. More research is needed to confirm the postulation that NK cells or large granular lymphocytes (LGL) in the bone marrow are indeed instrumental in the development of LO-GvHD. Isolation of NK cells or LGLs from the bone marrow may enable *in vitro* stimulation experiments with related and unrelated whole MFs or with fractions of these. Investigating delayed type hypersensitivity (DTH) responses against MF or MF-fractions by NK-cells may be an alternative for measuring differences in cellular reactivity against MFs of donor and recipient.

B6 donor mice injected with SELF-MF 10 days prior to BMT and C3 gno-tobiotic recipient mice associated with the B6 donor MF may serve as a suitable animal model for *in vivo* experiments. This model may also serve for adoptive transfer studies and for kinetic studies with regard to the development of GvH reactions in lymphoid organs like thymus, spleen and bone marrow, as well as non-lymphoid tissue e.g. intestines, and skin. The model may also be used for time/dose finding studies in which not only donors but also recipients are parenterally challenged with different doses of microflora at different times respectively before and after BMT. Cytokines e.g. IFNgamma, and TNF alpha should then be measured during the follow-up after BMT and GvHD in order to collect additional information on the possible subsets of cells, e.g. NK cells and macrophages, that might be involved in the ontogeny of GvHD.

Finally, a simplified classification of

intestinal microflora into related and non-related is functional for discussing LO-GvHD in inbred mice. However, this simplification is difficult to transpose to clinical BMT in which individual patients each may carry a different intestinal microflora (*Apperloo-Renkema* et al., 1992; *Jansen* et al., 1993; *Meijer-Severs* and *van Santen*, 1986). *Apperloo-Renkema* et al. (1992) demonstrated a new technique of microflora analysis which combines quantitative indirect immunofluorescence with digital image analysis. This so called immuno(micro)morphometri-cal analysis on stool specimens combined with serum, sampled from donors and recipients before and after BMT, may be of great future interest with regard to either predicting risks or monitoring development of GvHD after clinical BMT. An alternative for immuno(micro)morphometry, may be analysis of faeces and serum by using the fluorescence activated cell sorter, a method described by *van der Waaij* and colleagues (1994). Immuno(micro)morphometry as well as FACS analysis of faeces and serum are easy to apply in human BMT.

## ACKNOWLEDGEMENTS

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