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ORIGINAL ARTICLE

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Interferon-γ is not involved in the intestinal manifestations of the acute murine semiallogenic graft-versus-host disease

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Abstract *Background:* The acute murine semiallogenic graft-versus-host disease (GvHD) is known to be associated with Th1 cytokines secreting lymphocytes in the spleen and lymph nodes. However, whether this cytokine secretion pattern is also involved in the intestinal manifestations of acute GvHD (crypt hyperplasia and villous atrophy) is not known, so far. *Methods:* We first investigated the secretion of interleukin (IL) 4 (indicative of Th2-type differentiation) and interferon (IFN) γ (Th1-type differentiation) by splenic and by small bowel lamina propria lymphocytes. In addition, animals were treated with neutralizing antibodies to IL-4 or IL-12. The effect of this treatment on the intestinal morphology was examined. Second, we also investigated the effect of donor-derived IFN-γ by using donor lymphocytes from IFN-γ knock-out animals. Third, animals were treated with the fusion protein OX40-Ig which interferes with the OX40-OX40L interaction and thereby inhibits the intestinal manifestations of acute GvHD. *Results:* We found that, whereas splenic lymphocytes secrete an excess of IFN-γ, lymphocytes of the intestinal lamina propria secrete less IFN-γ and IL-4 than control animals. When OX40-Ig is administered to animals with acute GvHD, the intestinal histology normalizes as well as the secretion of IFN-γ and IL-4, indicating that the intestinal morphology is not affected by the secretion of IFN-γ by lamina propria lymphocytes. The treatment of animals suffering from acute GvHD with anti-IL-4 and anti-IL-12, which blocks the differentiation of IFN-γ secreting T-lymphocytes, did not significantly affect the development of crypt hyperplasia or villous atrophy. Furthermore, donor lymphocytes of IFN-γ knock-out animals also induced the intestinal manifestations of acute GvHD. *Conclusions:* These findings indicate that IFN-γ is not crucial for the development of crypt hyperplasia and villous atrophy in the murine semiallogenic GvHD.

Keywords Interferon-γ · Graft-versus-host disease · Interleukin-12 · Interleukin-4 · Villous atrophy · Crypt hyperplasia

Introduction

Many new insights into the physiology of numerous immune mechanisms have accompanied the concept, first postulated by Coffman and Mosmann [1], of the differentiation of pathways of $CD4^+$ T helper cells into the Th1-type [secretion predominantly of interferon (IFN) γ, tumor necrosis factor (TNF) α secretion] and the Th2-type [secretion of interleukins (ILs) 4, 5, 10]. The possibility of actively inducing a shift from a Th1 to a Th2 type of response or vice versa has opened many new therapeutic aspects in the management of infectious dis-

eases [2], tolerance induction, vaccine therapy, autoimmune disease, and transplant rejection (for review see [3]). For example, in an experimental model for a Th1-mediated inflammatory bowel disease (trinitrobenzene sulfonic acid induced colitis) the disease was cured by treatment with anti-IL-12 or anti-CD40L [4, 5], which both induce a switch from a Th1 to a Th2 type of response. Similar results have been obtained in multiple other animal models for autoimmune diseases [3]. Furthermore, it has been demonstrated in the murine acute semiallogenic graft-versus-host disease (GvHD) that a Th1 type of cytokine secretion prevails in the spleen and lymph nodes [6]. After the treatment with anti-IL-12 a switch to a Th2 type of immune response was induced, and the systemic manifestations of GvHD were prevented [7]. However, the acute semiallogenic GvHD is a well established animal model for the investigation of T cell mediated intestinal crypt hyperplasia and villous atrophy [8]. The intestinal cytokine secretion pattern that is potentially involved in these manifestations has not been studied so far, although it has been speculated that a Th1 type of cytokine secretion would prevail. In addition, a study by Mowat [9] demonstrated that treatment of GvHD animals with an monoclonal anti-IFNγ antibody prevents the intestinal manifestations of this disease. However, the actual intestinal cytokine secretion pattern was not investigated.

Therefore we investigated the intestinal cytokine secretion pattern in this animal model. Furthermore, we studied the effect of an induced switch from a potential Th1 to a Th2 type of response and vice versa by the administration of anti-IL-4 and anti-IL-12 monoclonal antibodies to the development of crypt hyperplasia and villous atrophy. In another set of experiments we studied the effect of a treatment with the OX40-Ig fusion protein, which has been was shown to inhibit the intestinal manifestation of GvHD [10] on the intestinal cytokine secretion pattern in order to investigate whether this inhibitory effect of OX40-Ig is associated with a switch in the differentiation pathways of $CD4^+$ T helper cells.

Materials and methods

Animals, cell preparations, and induction of acute semiallogenic graft-versus-host disease

C57BL/6, DBA2, and the F1 generation of these two mouse strains (B6D2F1) were raised and kept under standard conditions in the animal facility of the University Hospital of Kiel, Germany. To induce semiallogenic GvHD a slightly modified procedure as described by Guy-Grand and Vassalli [11] was performed. To prepare donor lymphocytes, spleen and mesenterial lymph nodes of C57BL/6 or of IFN-γ knock-out mice, respectively, were removed and pressed through a cell filter (40-µm pore size). Red blood cells were subsequently lysed by a hypotonic lysing buffer (ACK buffer, Böhringer Ingelheim, Germany). The resulting lymphocytes (80×10^6 cells/animal) were transferred to 8- to 14-week-old irradiated (7.5 Gy) B6D2F1 mice of the same sex by intraperitoneal injection. The small bowel was removed and frozen in liquid nitrogen or fixed in 10% phosphate-buffered (pH 7.4) formalin for further analysis. Control animals consisted of irradiated B6D2F1 mice which were transferred with the same amount of syngeneic (B6D2F1) cells.

Treatment regimens of recipient animals

After the induction of GvHD one group of recipient B6D2F1 mice were treated with anti-IL-12 monoclonal antibody (C17.5, kindly provided by Dr. Trinchieri, Philadelphia, Pa., USA; 500 µg or 1 mg on days 0 and 2), anti-IL-4 (11B11, kindly provided by Dr. R.L Seder; 500 µg or 1 mg on days 0 and 2) or the OX40-Ig fusion protein (200 µg per day intraperitoneally on days 0–4; kindly provided by David Calderhead [12]) or human IgG as control. The animals were killed, and organs were removed as described above. Each group consisted of at least five animals.

Detection of mucosal atrophy

Formalin-fixed jejunum specimens were embedded in paraffin and cut in 6- to 8-µm sections. To detect mucosal atrophy some slides were stained with hematoxylin and eosin, and the villous height and crypt depth were measured using a graded ocular.

Preparation of lamina propria lymphocytes

Lamina propria lymphocytes were isolated as described previously [5]. In brief, the small bowel of treated or untreated animals was prepared 6 days after the induction of GvHD and cut longitudinally. These slices were incubated in Hank's balanced salt solution (without calcium and magnesium) containing EDTA and dithiothreitol for 30 min in a shaking water bath at 37°C. Subsequently the intestines were incubated for 2×15 min in RPMI 1640 containing collagenase (Boehringer Mannheim, Mannheim, Germany) and DNase I (0.1 mg/ml; Boehringer Mannheim) in a shaking waterbath at 37°C. Afterwards the resulting cell suspension was passed through cotton wool and separated using a Percoll (Pharmacia, Uppsala, Sweden) gradient of 40% and 80%. The cells of the interface were collected and incubated on six-well plates coated with anti-CD3 (1 µg/ml) and anti-CD28 (1 µg/ml; Pharmingen, San Diego, Calif., USA) for 2 days. The supernatants were then collected and stored at –20°C for further analysis.

Cytokine ELISA

The determination of the secreted IFN-γ and IL-4 was performed using the antibodies and the protocol as described by Pharmingen.

Statistical analysis

Statistical analysis was performed using the analysis of variance *t* test for paired subgroups.

Results

IL-4 and IFN-γ secretion by splenic and lamina propria lymphocytes

Six days after the induction of acute semiallogenic GvHD or after the transfer of syngeneic lymphocytes

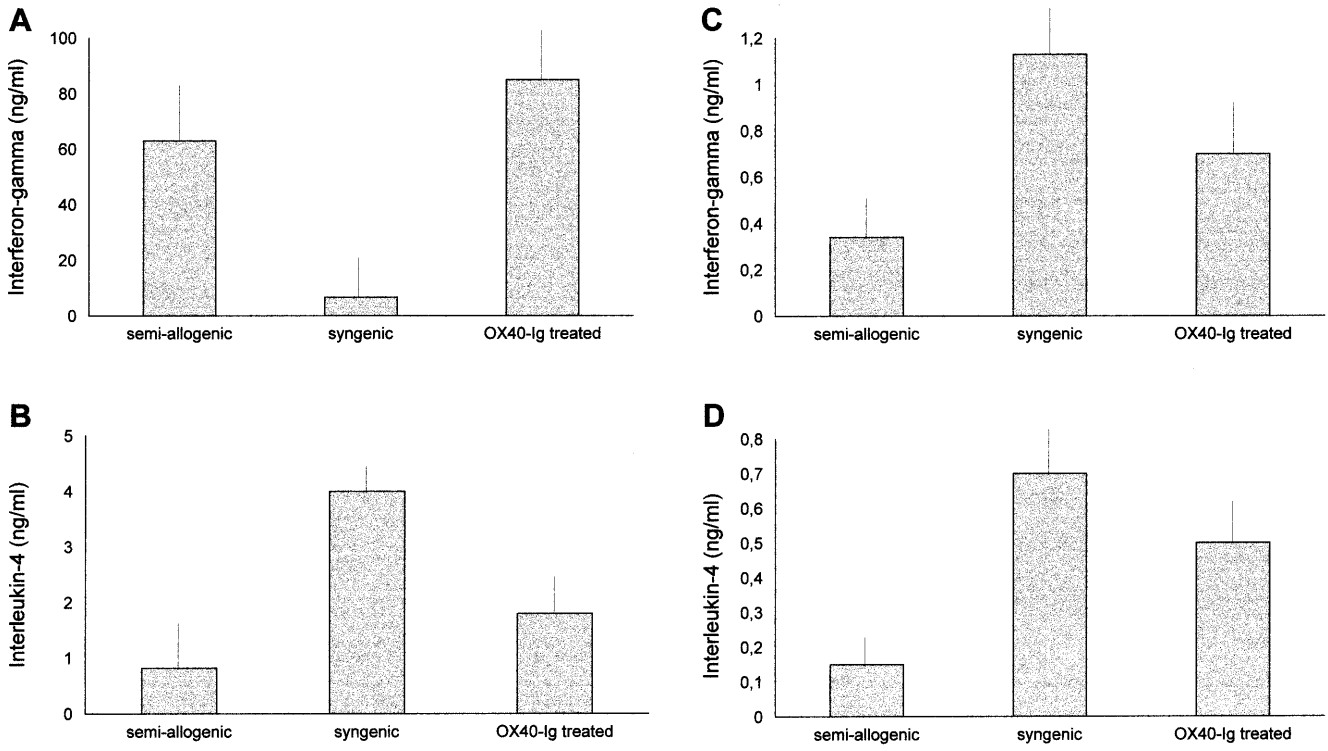

Fig. 1 Lymphocytes of the spleen (**A**,**B**) or of the lamina propria of the small bowel (**C**,**D**) from semiallogenic GvHD animals, from syngeneic control animals, and from OX40-Ig treated GvHD animals were prepared as described in the text and incubated on anti-CD3/anti-CD28 coated plates for 48 h. Interferon-γ (**A**,**C**) or IL-4 (**B**,**D**) secretion was determined by enzyme-linked immunosorbent assay. The depicted data are from one of five representative, independent experiments (mean +SD)

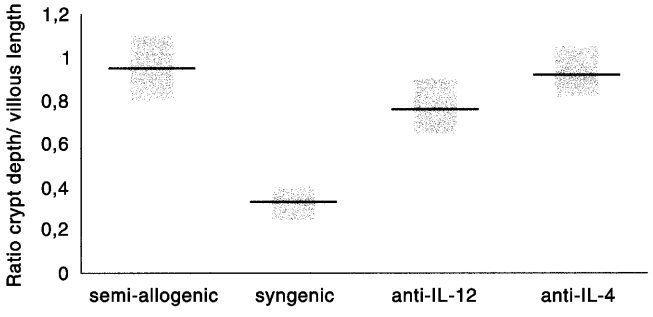

Fig. 2 Villous length and crypt depth of syngeneic control animals, of untreated semiallogenic GvHD animals and of anti-IL-12 (0.5 mg) or anti-IL-4 (0.5 mg) treated GvHD animals were determined on hematoxylin and eosin stained paraffin sections using a graded ocular. Each group consisted of at least five animals. The depicted data are the mean (±SD) of the ratio of crypt depth/villous length. There was no difference in the outcome of the animals treated with 0.5 mg or with 1 mg of the respective antibodies on days 0 and 2 (data not shown)

(control animals) the intestinal manifestations of acute GvHD are characterized by infiltrating $CD4^+$ T cells, crypt hyperplasia, and villous atrophy as previously described [10]. Lymphocytes of the spleens and of the lamina propria of the small bowel were prepared and incubated for 2 days on anti-CD3 plus anti-CD28 coated six-well plates. As depicted in Fig. 1, splenocytes of GvHD animals secrete significantly more IFN-γ than IL-4 (Th1 type of differentiation) than the syngeneic control animals which exhibit a more Th2 type of cytokine secretion, for example, they predominantly secrete IL-4. Treatment with OX40-Ig, which inhibits the development of crypt hyperplasia and villous atrophy in this animal model [10] did not significantly change the cytokine secretion of those animals that were transferred with semiallogenic lymphocytes. In contrast, as also shown in Fig. 1, lamina propria lymphocytes of GvHD animals secrete neither IL-4 nor IFN-γ. Furthermore, when the animals are treated with OX40-Ig, the cytokine secretion pattern of syngeneic lamina propria lymphocytes is almost resumed.

Effect of treatment with monoclonal antibodies anti-IL-4 and anti-IL-12

To evaluate whether a Th1 or Th2 cytokine secretion pattern is crucial for the establishment of the intestinal manifestations of acute GvHD we treated one group of animals after the induction of acute GvHD with an anti-IL-4 and another group with an anti-IL-12 monoclonal

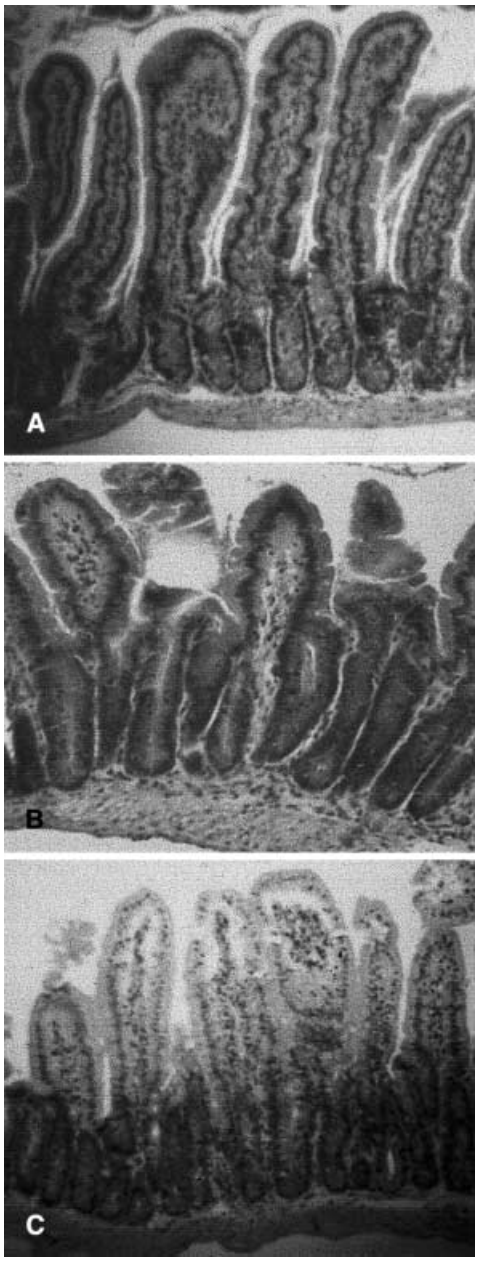

Fig. 3 Irradiated recipient animals were either transferred with 80×106 syngeneic lymphocytes (**A**), semiallogenic wild-type C57BL/6 cells (**B**), or lymphocytes from interferon-γ knock-out mice on a C57BL/6 background (**C**). On day 6 after the cell transfer the animals were killed and hematoxylin and eosin stained paraffin section of the jejunum were made as described in the text. The depicted photomicrographs demonstrate representative sections of four independent experiments

antibody. Anti-IL-4 inhibits the differentiation of Th2-type T cells while anti-IL-12 prevents the priming of Th1 type of cytokine secreting cells. On day 6 we killed these animals and examined formalin-fixed, paraffin-embedded sections of the jejunum for the manifestations of GvHD (crypt hyperplasia and villous atrophy). As demonstrated in Fig. 2, these antibody treatments did not cause significant changes concerning crypt hyperplasia and villous atrophy in the GvHD animals (as confirmed by the analysis of variance *t* test).

Transfer of semiallogenic donor lymphocytes from IFN-γ knock-out mice

When donor lymphocytes from IFN-γ knock-out mice (BL/6 background) were prepared and transferred into B6D2F1 recipient mice, the intestinal architecture of GvHD animals was unaltered by the use of IFN-γ deficient mice as donor animals. Figure 3 demonstrates that donor-derived IFN-γ does not seem to be crucial for the development of crypt hyperplasia and villous atrophy in the recipient mice.

Discussion

The present study suggests that IFN-γ is not crucial for the establishment of the intestinal manifestations of acute GvHD in mice. This hypothesis is based on four lines of evidence. First, lamina propria lymphocytes of GvHD animals produce less and not more IFN-γ than control animals. In addition, the treatment with OX40-Ig, which prevents the intestinal damage of GvHD in this model [10], is associated with greater IFN-γ production than in untreated GvHD animals. Third, the administration of anti-IL-12, which inhibits the differentiation of Th1-T cells, does not result in a significant amelioration of intestinal morphology. Finally, the administration of donor lymphocytes from IFN-γ knock-out animals also leads to significant crypt hyperplasia and villous atrophy. These data are in accordance with a previous study using IFN-γ receptor knock-out mice as small bowel donor animals an allotransplantation model. Intestinal allografts from these animals were rejected in the same way as allografts from wild-type mice [13]. Another previous study using IFN-γ knock-out mice as donors did not investigate their effect on the intestinal damage [14]. However, the present data are in contrast to an earlier study by Mowat [9] who demonstrated that antibodies to IFN-γ prevent the modest intestinal damage taking place during acute GvHD in *nonirradiated* B6D2F1 host animals. Furthermore, the present study to some extent contradicts the dogma that acute GvHD is mainly associated and – even more clearly – a product of a Th1 type of T-helper cell differentiation [6, 15].

The recent literature on the involvement of cytokines in acute GvHD, however, does not support the view that a Th1 type of differentiation is deleterious in this pathological clinical and experimental condition. In contrast, numerous studies have shown that IFN-γ, or IL-12 via

IFN-γ, prevents GvHD in murine bone marrow transplant models. This somewhat surprising effect has been reproduced by many different laboratories [16, 17, 18, 19, 20, 21, 22, 23]. How IFN-γ mediates its "suppressor" effects is not quite clear; increased NO production induced by IFN-γ may be one mechanism [24]. However, it is important to note that the same systemic immunologically mediated disease has different types of T cell differentiation patterns in different organs. This may also indicate that a simple therapeutic strategy in human GvHD based on the Th1-Th2 concept will not be able to implement in the nearest future.

The intestinal damage of the murine acute semiallogenic GvHD largely resembles the histological changes in the human small intestine in disease entities such as celiac disease (gluten-sensitive enteropathy). Therefore this animal model was used to study possible pathogenetic mechanisms of this immune-mediated human disease. Whether the results of the present study shed any new light on the role of cytokines in human celiac disease, however, can so far be doubted, in our opinion. The published data on the cytokine expression by lamina propria lymphocytes in celiac disease are not at all consistent [25], but the more compelling evidence points to upregulated IFN-γ production in the intestines of patients with celiac disease [26, 27, 28, 29]. Furthermore, IFN- γ is the first cytokine to be upregulated after an in vivo gluten challenge in gluten-sensitive individuals [30]. In addition, anti-IFN-γ treatment in organ cultures of gluten-sensitive patients in vitro inhibits the intestinal damage induced by gluten [31].

In conclusion, the present study suggests that IFN-γ is not important for the intestinal damage in the murine acute semiallogenic GvHD. These data are in accordance with those of previous studies demonstrating an even beneficial effect of IFN-γ in allogenic bone marrow transplant models. However, in this specific point this animal model probably does not mimic the clinical condition of human celiac disease, which seems to be affected more by IFN-γ secreting lymphocytes.

References

1. Mosmann, TR, Coffman RL (1989) Th1 and Th2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7:145–173
2. Finkelman FD, Donohue T, Goldhill J, Sullivan CA, Morris SC, Madden KB, Gause WC, Urban JC (1997) Cytokine regulation against parasitic gastrointestinal nematodes. Annu Rev Immunol 15:505–533
3. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH (1998) The interleukin-12/interleukin-12-receptor system: role in normal an pathologic immune responses. Annu Rev Immunol 16:495–521
4. Stüber ER, Strober W, Neurath MF (1996) Blocking the CD40L-CD40 interaction in vivo specifically inhibits the priming of Th1-T cells. J Exp Med 183:693–698
5. Neurath MF, Fuss I, Kelsall B, Stüber ER, Strober W (1995) Antibodies to IL-12 abrogate established experimental colitis in mice. J Exp Med 182:1281–1290
6. Ferrara JLM (1998) The cytokine modulation of acute graft-versus-host disease. Bone marrow transplantation 21 [Suppl 3]:S13–S15
7. Williamson E, Garside P, Bradley JA, Mowat AM (1996) IL-12 is a central mediator of acute graft-versus-host disease in mice. J Immunol 157:689–699
8. Marsh MN (1992) Gluten, major histocompatibility complex, and the small intestine. Gastroenterology 102:330–354
9. Mowat AM (1989) Antibodies to IFN-γ prevent immunologically mediated intestinal damage in murine graft-versus-host reaction. Immunology 68:18–23
10. Stüber ER, Freier A, Marinescu D, Fölsch UR (1998) Involvement of OX40-OX40L interactions in the intestinal manifestation of the murine acute Graft-versus-Host disease. Gastroenterology 115:1205–1215
11. Guy-Grand D, Vassalli P (1986) Gut injury in mouse graft-versus-host reaction. J Clin Invest 77:1584–1595
12. Calderhead DM, Buhlmann JE, van der Eertwegh AJM, Claassen E, Noelle RJ, Fell HP (1993) Cloning of mouse Ox40: a T cell activation marker that may mediate T-B interactions. J Immunol 151:5261–5271
13. Veitch AM, Higgins LM, MacDonald TT, Farthing MJG (1998) Lack of evidence for interferon-gamma as a mediator in a graft-rejection model of enteropathy. Gastroenterology 114:G4535
14. Ellison CA, Fischer JM, HayGlass KT, Gartner JG (1998) Murine graft-versus-host disease in an F1-hybrid model using IFN-gamma gene knockout donors. J Immunol 161:631–640
15. Ferrara JLM, Krenger W (1998) Graft-versus-host disease: the influence of type 1 and type 2 cytokines. Transfus Med Rev 12:1–17
16. Yang YG, Dey B, Sergio JJ, Sykes M (1997) Interleukin-12 prevents severe acute graft-versus-host disease (GVHD) and GVHD-associated immune dysfunction in a fully major histocompatibility complex haplotype mismatched murine bone marrow transplantation model. Transplantation 64:1343–1352
17. Sykes M, Szot GL, Nguyen PL, Pearson DA (1995) Interleukin-12 inhibits murine graft-versus-host disease. Blood 86:2429–2438
18. Brok HP, Vossen JM, Heidt PJ (1997) Interferon-gamma-mediated prevention of graft-versus-host disease: development of immune competent and allotolerant T cells in chimeric mice. Bone Marrow Transplant 19:601–606
19. Konieczny BT, Dai Z, Elwood ET, Saleem S, Linsley PS, Baddoura FK, Larsen CP, Pearson TC, Lakkis FG (1998) IFN-gamma is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. J Immunol 160:2059–2064
20. Brok HP, Heidt PJ, Meide PH van der, Zurcher C, Vossen JM (1993) Interferon-gamma prevents graft-versus-host disease after allogenic bone marrow transplantation in mice. J Immunol 151:6451–6459

21. Yang YG, Dey BR, Sergio JJ, Pearson DA, Sykes M (1998) Donor-derived interferon γ is required for inhibition of acute graft-versus-host disease by interleukin-12. J Clin Invest 102:2126–2135
22. Okubo T, Hagiwara E, Ohno S, Tsuji T, Ihata A, Ueda A, Aoki I, Okuda K, Miyazaki J, Ishigatsubo Y (1999) Administration of an IL-12-encoding plasmid prevents the development of chronic graft-versus-host disease (GVHD). J Immunol 162:4013–4017
23. Murphy WJ, Welniak LA, Taub DD, Wiltrout RH, Taylor PA, Vallera DA, Kopf M, Young H, Longo DL, Blazar BR (1998) Differential effects of the absence of interferon-gamma and IL-4 in acute graft-versus-host disease after allogenic bone marrow transplantation in mice. J Clin Invest 102:1742–1748
24. Krenger W, Falzarano G, Delmonte J, Snyder KM, Byon JC, Ferrara JL (1996) Interferon-gamma suppresses T-cell proliferation to mitogen via the nitric oxide pathway during experimental graft-versus-host disease. Blood 88:1113–1121
25. Dawoud A, Nakshabendi I, Foulis A, Mowat AM (1992) Immunohistochemical anlaysis of mucosal gamma-interferon production in coeliac disease. Gut 33:1482–1486
26. Beckett CG, Dell'Olio D, Kontakou M, Przemioslo RT, Rosen-Bronson S, Ciclitira PJ (1996) Analysis of interleukin-4 and interleukin-10 and their association with the lymphocytic infiltrate in the small intestine of patients with coeliac disease. Gut 39:818–823
27. Nilsen EM, Lundin KEA, Krajci P, Scott H, Sollid M, Brandtzaeg P (1995) Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon γ. Gut 37:766–776
28. Kontakou M, Sturgess RP, Przemioslo RT, Limb GA, Nelufer JM, Ciclitira PJ (1994) Detection of interferon gamma mRNA in the mucosa of patients with coeliac disease by in situ hybridisation. Gut 35:1037–1041
29. Breese EJ, Kumar P, Farthing MJG, MacDonald TT (1994) Interleukin-2 and interferon-γ producing cells in the lamina propria in celiac disease. Dig Dis Sci 39:2243
30. Kontakou M, Przemioslo RT, Sturgess RP, Limb GA, Ellis HJ, Day P, Ciclitira PJ (1995) Cytokine mRNA expression in the mucosa of treated coeliac patients after wheat peptide challenge. Gut 37:53–57
31. Przemioslo RT, Lundin KEA, Sollid LM, Nelufer J, Ciclitira PJ (1995) Histological changes in small bowel mucosa induced by gliadin sensitive T lymphocytes can be blocked by anti-interferon γ antibody. Gut 36:874–879