4. DISCUSSION

4.1 Autoimmune Diseases in GM-CSF- and GM-CSF/IL-3-Deficient Mice

My studies indicate that GM-CSF and GM-CSF/IL-3 deficiency in mice on the C57Bl/6, but not on the Balb/c background, greatly enhances the susceptibility for the development of autoimmune diseases.

Autoimmune diseases have a complex pathology, which is caused by the combined, eventually stochastic effects of genetic and environmental factors.

The most potent genetic influence on susceptibility to autoimmune diseases is maintained by genes of the MHC locus, but the influence of non-MHC susceptibility genes have gained growing attention within the last few years (88). The genes for the cytokines, GM-CSF and IL-3, are both outside of the MHC locus, and closely related to each other (35). The fact, however, that I observed autoimmune diseases in cytokine-deficient mice on the C57Bl/6, but not on the Balb/c background, suggests a crucial influence for additional genes, other than the non-MHC genes, GM-CSF and IL-3, in the development of the disease.

One of the established mechanisms underlying autoimmune diseases involves the impaired phagocytosis of apoptotic cells by macrophages (95). Cells undergoing apoptosis normally express cell internal molecules, such as DNA and histones, on their surface (151). During a pathologically impaired phagocytosis, these molecules may have the chance to get recognized by other cells of the immune system and, thus, induce autoimmune responses (152, 153). By measuring the capacity of peritoneal macrophages of WT, GM-CSF- and GM-CSF/IL-3-deficient mice to ingest apoptotic cells, I found a markedly compromised phagocytic activity of the macrophages of GM-CSF- and GM-CSF/IL-3-deficient mice compared to WT controls (Figure 3.1.2) (8).

C1q complement factor deficiency was shown some time ago as a cause for SLE (154). It is thought that C1q, by binding to apoptotic cells and microorganisms, serves as

an opsonizing agent for macrophages (155). Since most of my GM-CSF- and GM-CSF/IL-3-deficient mice generated autoantibodies against C1q, the resulting effective C1q deficiency in these mice may further impair the phagocytosis of apoptotic cells and contribute to the development of autoimmune diseases.

Interestingly, SLE was attenuated in GM-CSF/IFN-γ- and GM-CSF/IL-3/IFN-γ-deficient mice, although these mice showed phagocytic defects similar to GM-CSF/IL-3-deficient mice (8). Lymphoid aggregates in these mice were absent from the renal pelvis, and the immune-mediated glomerulonephritis was mild and resembled to a nephritis. Moreover, anti-dsDNA autoantibodies, rheumatoid factors, and anti-C1q reactivity were reduced (Figure 3.1.1 C and D). Taken together, these results reveal a critical role for IFN-γ in the onset of SLE, and they are in accordance with earlier observations for the requirement of IFN-γ for full blown autoimmune diseases (156, 157).

DCs, the most important APCs, are thought to play a crucial role in maintaining self-tolerance (56, 158). Abnormal DC populations were recently observed in patients with autoimmune diabetes (159), and impaired responses of DCs from NOD mice to GM-CSF have been described by several groups (160, 161). Conversely, administration of IL-3 to young NOD mice delayed the onset of diabetes and significantly reduced the incidence of the development of the disease in these mice (162). GM-CSF and IL-3 are both known to stimulate the growth, and differentiation, of DCs from hematopoietic precursors (56). Surprisingly, mice rendered deficient in GM-CSF and IL-3 maintain normal numbers of spleen and lymph node DCs (55). These mice, however, are markedly compromised in the priming of contact hypersensitivity reactions, suggesting a defect in DC function, as earlier elucidated in our laboratory (55). Defects in the function of DCs, caused by the combined deficiency of GM-CSF and IL-3, likely enhance the susceptibility for the development of autoimmune diabetes, since an autoimmune diabetes could only be found in my aged GM-CSF/IL-3 doubly deficient, but not in the aged single cytokine-deficient mice.

Antigen-processing DCs are capable to directly expand $CD25^+$ $CD4^+$ regulatory T cells (138). Immunologic self-tolerance in the periphery can be maintained by these regulatory T cells, which constitute 5-10% of the peripheral $CD4^+$ T cells, and deficiency of $CD25^+$ $CD4^+$ regulatory T cells has been linked to the development of several

autoimmune disorders (163, 164). A classical feature of $CD25^+$ $CD4^+$ regulatory T cells is that they are anergic upon TCR-mediated stimulation and suppress the activation and proliferation of $CD25^-$ $CD4^+$ and $CD8^+$ T cells (165). Expression of CTLA-4 on the $CD25^+$ $CD4^+$ regulatory T cells is mandatory for their suppressive activity (166). Although I did not find differences in the total numbers of $CD25^+$ $CD4^+$ regulatory T cells, or in the expression of their co-stimulatory molecules CD28 and CTLA-4, between diabetic GM-CSF/IL-3-deficient and WT mice, I could not rule out an intrinsic functional defect in these cells.

In addition to the role in the activation of the suppressive $CD25^+$ $CD4^+$ T cells, CTLA-4 seems to be responsible for elevating the threshold of TCR signaling with other T cells (167). Similar to the findings in the transgenic NOD diabetes model, application of anti-CTLA-4 mAb in 12-days-old GM-CSF/IL-3-deficient mice induced diabetes in greater than 70% of the injected mice within 3 weeks, whereas IgG isotype-matched control treated doubly deficient mice did not develop diabetes over a period of 3 months of observation (Figure 3.1.4) (130). Analogous to the data obtained with the NOD transgenic mice, the time frame of the anti-CTLA-4 treatment was important (130). Injection of anti-CTLA-4 mAb into GM-CSF/IL-3-deficient mice that were older than 3 weeks did not have any effect on diabetes development. A possible explanation for the time-limited effect of the anti-CTLA-4 treatment could be that DCs present the relevant β cell antigens to $CD25^+$ $CD4^+$ regulatory T cells within the first 3 weeks after birth. Since CTLA-4, as a co-stimulatory molecule, seems to be absolutely required for this antigen presentation (166), blocking the interaction between CTLA-4 on the regulatory T cells, or its ligands CD80 and CD86 on the APCs, may prevent the activation of the $CD25^+$ $CD4^+$ suppressor T cells, and finally lead to uncontrolled recognition of self-antigen by $CD25^-$ $CD4^+$ and $CD8^+$ T cells. The fact that I could not induce diabetes by anti-CTLA-4 mAb treatment of WT controls indicates that additional, as yet unknown, control mechanisms, possibly at the level of DCs, may be involved. We hypothesize that these control mechanisms may be absent in the functionally defective DCs of GM-CSF/IL-3-deficient mice, and may play an essential role for the spontaneous development of autoimmune diabetes in the aged GM-CSF/IL-3-deficient mice.

Chronic inflammatory processes on the basis of a compromised immune response against pathogens, as previously described to occur in GM-CSF/IL-3-deficient mice, likely promote the development of autoimmune diseases (55). One possible mechanism to induce autoimmune diseases is infection related loss of T cells following dysregulated homeostatic proliferation of the remaining T cells (102). It was also reported that stimulation of DCs by infectious agents via Toll-like receptors could play an important role for the induction of autoimmunity (104). Moreover, inflammatory processes stimulate IFN-γ production by lymphocytes involved in innate, and adaptive immunity such as NK cells and subsets of T cells (17). IFN-γ was shown to be essential for the destruction of β cells, and, thus, development of autoimmune diabetes (157). Although our GM-CSF/IL-3-deficient mice that, additionally, lack IFN-γ often suffered from serious infections (8), they did not develop diabetes, and their pancreatic islets rarely showed lymphocytic aggregates. Thus, it would be interesting to determine whether GM-CSF/IL-3-deficient mice will develop autoimmune diabetes by eliminating the inflammatory compound under continuous administration of anti-microbial, or anti-inflammatory drugs (currently under investigation, G. Dranoff).

Taken together, my studies indicate that deficiencies of GM-CSF, or GM-CSF and IL-3, greatly enhance the susceptibility to development of autoimmune diseases by the complex interactions of: genetic factors, defective antigen presentation, impaired phagocytosis of apoptotic cells, and chronic inflammatory processes as a result of a compromised immune response against pathogens.

4.2 GM-CSF/IFN-γ- and GM-CSF/IL-3/IFN-γ-Deficient Mice Develop Cancer, But Not Under Anti-Infectious Or Anti-Inflammatory Therapy: Inconsistency in the Cancer Immune Surveillance Theory

A high proportion of GM-CSF/IFN-γ- and GM-CSF/IL-3/IFN-γ-deficient mice began to develop lymphomas (mostly of B cell type), and, to a lesser extent, solid tumors, at the age of about 8 months. As potential causative events, I could characterize precursor lesions in B cell lymphomagenesis. Although secondary lymphoid tissues of 2-3-months-old mice did not show evidence of atypical hyperplasia, or clonal proliferation, GCs in these mice were markedly enlarged. Activated B cells of GM-CSF/IFN-γ- and GM-CSF/IL-3/IFN-γ-deficient mice turned out to be resistant to fas-(CD95)-mediated killing (Figure 3.6.1) (8). As I found, the defect in fas-mediated apoptosis was B cell extrinsic and caused by the combined deficiency of IFN-γ and TNF-α (8). In this regard, it was earlier described that alveolar macrophages of GM-CSF-deficient mice disclosed a greatly reduced TNF-α production upon stimulation compared to alveolar macrophages of WT mice (168). The fact, however, that I could not find a defect in fas-mediated apoptosis of B cells of GM-CSF singly deficient mice, suggests that, besides the lack of IFN-γ, additional, as yet unknown, mechanisms contribute to the fas resistance of B cells of GM-CSF/IFN-γ- and GM-CSF/IL-3/IFN-γ-deficient mice (8). The fas-fas ligand system is essential in GC B cell homeostasis and aged fas-deficient mice are known to develop B cell lymphomas (150, 169). I propose that in GCs of the GM-CSF/IFN-γ- and GM-CSF/IL-3/IFN-γ-deficient mice, B cell survival is enhanced by a combination of their fas resistance and the impaired phagocytosis of apoptotic cells (that was similar to the impaired phagocytosis observed in GM-CSF- and GM-CSF/IL-3-deficient mice) (8, 169). Thus, in the setting of chronic antigenic stimulation by inflammatory processes, this expanded pool of B cells likely acquires an increased risk for transforming mutations due to rare errors in somatic hypermutation (170).

My observation that anti-microbial therapy inhibits both lymphomagenesis and solid tumor formation is consistent with clinical reports that a variety of infectious agents are linked with human B cell lymphomas and solid neoplasms, including carcinomas of the: stomach, liver, biliary tract, colon, and bladder (8, 171, 172). Thus, my model may prove useful for clarifying whether microbes function as co-factors that drive target cell proliferation and/or mediate target cell transformation.

In all the lymphomas analyzed, the pro-inflammatory enzyme COX-2 was highly expressed (Figure 3.4.1 B). Besides its pro-inflammatory effects, COX-2 has recently been shown to enhance cancer susceptibility, by induction of metalloproteinase and VEGF (144, 173). The suppression of tumor formation with the COX inhibitor sulindac, as observed in my mice, was first described in a colorectal cancer model (120). My tumor model could also help to distinguish between the pro-inflammatory effects of the COX enzymes and other COX-mediated metabolic changes to enhance cancer susceptibility.

Taken together, my results confirm that the complex interaction of infectious/pro-inflammatory agents and defective immune homeostasis is a critical determinant of cancer development.

My findings that anti-infectious, or anti-inflammatory, treatment nearly completely suppressed tumor formation triggered by the combined absence of GM-CSF and IFN-γ, without influencing the fas resistance and phagocytic defect, contradict the theory of Schreiber et al. of a T cell-mediated IFN-γ-based Cancer Immune Surveillance Mechanism (19, 112). This theory's origin derives from studies showing that mice lacking IFN-γ, IFN-γ receptor (IFN-γ-R), or IFN-γ-producing cell types, have increased tumor susceptibility to the chemical carcinogen MCA (19, 112). Their idea got further support by the observations of Smyth et al. (116). His group monitored spontaneous tumor formation in WT versus IFN-γ-deficient mice and found that 50% of the IFN-γ-deficient mice spontaneously developed disseminated lymphomas, whereas no tumors could be detected in WT controls (116). IFN-γ- and IFN-γ-R-deficient mice were also impaired in the rejection of transplanted tumors (174-176). Moreover, blocking endogenous IFN-γ by neutralizing antibodies inhibited rejection of transplanted tumors (113). In contrast, tumor transplants transfected to secrete IFN-γ were rejected (177, 178).

Based on all these observations, Schreiber et al. postulated that T cell secreted IFN-γ is acting to enhance the recognition, and finally destruction, of transformed cells by the immune system (19). According to the concept of Burnet and Thomas that the immune system can recognize, and destroy, nascent transformed cells, Schreiber et al. redefined the observed, IFN-γ related phenomenon as "Cancer Immune Surveillance", and indicated that it may function as a component of a more general process they termed "Cancer Immunoediting" (109, 112, 179). Briefly, "Cancer Immunoediting" includes host-protecting and tumor-sculpting actions of the immune system that not only may prevent but also shape neoplastic diseases (112). The idea of host-protection is mainly based upon the wide-known fact that immunosuppressed individuals have a higher incidence of developing cancer than immunocompetent individuals (112). Tumor-sculpting means imprinting of tumors by their immunologic environment in which they form (112). It is based on experiments showing that chemically induced tumors, formed in the absence of an intact immune system, are more immunogenic than tumors that arise in immunocompetent hosts (112). For example, when chemically induced tumors isolated from WT, or RAG-deficient mice, were transplanted into RAG-deficient recipients, they all grew with similar kinetics, indicating that there were no inherent growth differences between tumors raised in the presence, or absence, of an intact immune system (118). In addition, 17 out of 17 tumors originally isolated form WT mice were capable of establishing progressively growing cancer when transplanted into naïve immunocompetent hosts. In contrast, 8 of 20 tumors, originally generated in RAG-deficient mice, were rejected when transplanted into immunocompetent hosts, indicating that tumors formed in the absence of an intact immune system are, as a group, more immunogenic than tumors that arise in immunocompetent hosts (112, 118).

Over the past few years, however, there has remained controversy about the existence of the Cancer Immune Surveillance and Cancer Immunoediting Mechanisms as they have been postulated by Schreiber et al. (180, 181). Indeed, with the exception of experiments using mice defective in IFN-γ signaling, that show an increased tumor susceptibility to MCA, all further experiments supporting their theory were based on tumor transplantation experiments (180). It is unlikely, however, that rejection of transplanted tumor cells, typically mediated by T cells, and inhibition of MCA-induced

carcinogenesis, involve the same mechanism (180). Furthermore, it is suspicious that regardless of which IFN-γ producers (T cell subpopulations, or all the T cells, NK, or NKT cells) are lacking, mice have the same increased susceptibility to MCA (112). This indicates that either all of these cells participate actively in tumor elimination, or steady state IFN-γ concentrations in the mice lacking these cells are different from their WT littermates (180).

The fact that tumor formation in my compound IFN-γ-deficient mice could be completely suppressed by treating them with anti-microbicidal agents and, to a high extent, by treating them with anti-inflammatory agents, clearly contradicts the idea that an absence of an IFN-γ-dependent Immune Surveillance is the main cause for tumor development in my system. If tumors on the background of an IFN-γ deficiency would, indeed, arise solely due to a lack of Immune Surveillance (as postulated by Schreiber et al.) (112), it would not be explicable that: firstly, the treatment with antibiotics, or anti-inflammatory agents, of my compound IFN-γ-deficient mice led to such a dramatic decrease in tumor formation, and, secondly, I found, in contradiction to the report of Smyth et al. (116), only one single lymphoma in my cohort of 41 aged IFN-γ -deficient mice (these IFN-γ-deficient mice were between 14 and 27-months-old at autopsy) (8). My results rather indicate that the major role of IFN-γ in tumor prevention may be due to its role in promoting host resistance to microbial agents (22, 27, 182), since there is, as already mentioned above, compelling evidence that chronic inflammation, or infection, increases the risk of neoplasia (183). In agreement with this idea, my thesis suggests that the high incidence of lymphomas in IFN-γ -deficient mice reported by Smyth et al. reflects, at least in part, the involvement of an infectious agent not present in our mouse colony (8, 116). Although Schreiber et al. claim that their aged mice used in their studies were maintained on the same broad-spectrum antibiotic used in my study, evidence for such an antibiotic treatment is missing from their cited publications (118, 181).

A further argument against the idea of an IFN-γ-mediated T cell-dependent Immune Surveillance was recently given by the observations of Blankenstein and Qin. They re-investigated MCA-induced carcinogenesis using RAG-deficient mice (180). This time, WT and RAG-deficient mice were obtained from the same colony. Surprisingly,

and in contradiction to earlier reports, both RAG-deficient mice, and WT controls, developed tumors at similar frequencies and the T cell-competent group showed only a minor delay (118, 180). Thus, in contrast to IFN-γ-R-deficient animals (119), mice lacking only IFN-γ expressing T and NKT cells (whereas other IFN-γ producing cells, such as NK cells, remain intact) do not show a substantially increased susceptibility to MCA (180).

Although there is, to my mind, insufficient evidence for the existence of a Cancer Immune Surveillance Model as it is proposed (112), IFN-γ may play, nevertheless, important roles in the defense against tumors.

Blankenstein et al. explained the increased susceptibility of IFN-γ-deficient mice to MCA as being due to the lack of an IFN-γ-R-dependent foreign body reaction that normally encapsulates MCA (119). They found that in the course of a tissue repair response, MCA was encapsulated, and remained life-long in tumor-free mice within microscopic scars (119). Their results suggested that fibroblasts, in the vicinity of MCA, deposited an extracellular matrix that protected the local tissue from mutations (119). This mechanism seemed to be impaired in IFN-γ-R-deficient mice and the diffusion of MCA from the injection site measured was significantly higher in these mice than in WT controls (119). Interestingly, GM-CSF also has been shown to promote and accelerate scar formation and wound healing (184).

Taken together, the occurrence of serious infections, and spontaneous tumor formation, in mice lacking GM-CSF and IFN-γ suggests a synergistic effect of the two cytokines, not only in the prevention of infectious-related processes, but, more generally, in the protection against potentially cancerogenic tissue damages induced by the environment.