1. INTRODUCTION

1.1 Cytokines in Autoimmune Diseases and Cancer

Cytokines are secreted, or membrane-bound, proteins that regulate the: growth, differentiation, and activation of immune cells (1). As a result, dysregulation of cytokine production, or action, is thought to play an important role in the development of diseases, such as autoimmune disorders and cancer (1, 2).

Autoimmune diseases, which affect approximately 5% of the population, and disproportionately befall woman, comprise a heterogeneous group of poorly understood disorders (3). In general, these illnesses are characterized by an abnormal lymphocyte activation, but non-lymphoid cells, especially Antigen-Presenting Cells (APCs), such as macrophages and Dendritic Cells (DCs), are thought to contribute critically to disease pathogenesis (4).

The likelihood of developing invasive cancer during a lifespan is 43.5% for the male and 38.5% for the female population in the U.S.A. (5). Although alterations in tumor suppressors and oncogenes underlie the cell-autonomous defects that are characteristic of cancer, tumors arise and progress within a microenvironment that is replete with healthy, non-transformed cells (1). Crosstalk between non-mutated and neoplastic cells is increasingly recognized to influence various stages of carcinogenesis (6). Thus, an important factor that likely proves decisive in moulding the host reaction against cancer is the mixture of cytokines that is produced in its microenvironment (7).

In the following thesis I show that mice deficient of the cytokine Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) and mice doubly deficient of GM-CSF and Interleukin-3 (IL-3), develop a Systemic Lupus Erythematosus (SLE)-like disorder, associated with impaired phagocytosis of apoptotic cells (8). The majority of these doubly deficient mice, additionally, develop a mild autoimmune diabetes. Concurrent deficiency of the cytokine Interferon (IFN)-γ attenuates the autoimmune

diseases, but promotes the formation of diverse hematologic and solid neoplasms, within a background of persistent infection and inflammation (8).

1.2 IFN-γ

IFN-γ is a cytokine that plays many critical roles in promoting both protective immune responses and immunopathologic processes (9-11). Since the initial description of its anti-viral activity, the understanding of the molecular biology and physiology of IFN-γ has greatly increased (12). IFN-γ is produced by several cell types (e. g. T, B, Natural Killer (NK) cells, Natural Killer T (NKT) cells, macrophages, and mast cells) upon activation with immune and inflammatory stimuli (13-16). During the early phase (4-96 hours) of a developing immune response, however, NK cells are the major source of IFN-γ (17, 18). Later (after 96 hours), during the adaptive phase of an immune response, IFN-γ is mainly produced by $CD4^+$ and $CD8^+$ T cells (17, 18). IFN-γ exerts its biological activity by interacting with the IFN-γ receptor, that is ubiquitously expressed on nearly all cells (11). Functionally active IFN-γ receptors consist of two distinctive subunits: a 90 kDa receptor α chain (IFNGR1), and a 62 kDa receptor β chain (IFNGR2) (19). IFN-γ responses in cells result from the ligand-induced coupling of the activated IFN-γ receptor complexes to components of the JAK-STAT signaling pathway (11, 20, 21). IFN-γ signaling mainly requires three specific JAK-STAT pathway components: the protein tyrosine kinases, JAK1 and JAK2, and the transcription factor Stat1 (21-25).

By generating mice that lack: IFN-γ, either of the IFN-γ receptor subunits, or any of the three JAK-STAT signaling proteins, it could be demonstrated that disruption of the IFN-γ signaling pathway resulted in the ablation of innate immunity, rendering the host highly susceptible to infection by a variety of microbial pathogens and certain viruses (21-24, 26, 27). These findings have been generalized to humans by the discovery of individuals with inactivating mutations in the IFN-γ receptor complex who die early in

life from uncontrolled mycobacterial infections (28-30). Thus, the physiological role of IFN-γ in promoting host resistance to infectious organisms is unequivocal.

1.3 GM-CSF and IL-3

GM-CSF and IL-3 stimulate the: proliferation, differentiation, and activation of hematopoietic cells in many similar ways (31). These overlapping functions reflect, at least in part, the shared use of the β_c subunit for receptor signaling (32). Signaling events in response to GM-CSF or IL-3 include: the activation of JAK2 and, consecutively, the activation of transcription factor Stat5, the Ras pathway (including: Vav, Shc, Raf and Mitogen Activated Protein Kinase (MAPK)), the Src family kinases Fyn and Lyn, Fps/Fes, Phosphaditylinositol-3 Kinase p85, Protein Kinase C, calcium ion flux, and inositol phosphate mobilization (33, 34). The proximity of GM-CSF and IL-3 genomic sequences on mouse chromosome 11 and on human chromosome 5 underscores their close relationship and suggests that these cytokines may have evolved from ancient gene duplication (35).

Notwithstanding these similarities, mice rendered singly deficient in either GM-CSF or IL-3 manifest distinct phenotypes. Animals deficient in GM-CSF display normal steady-state hematopoiesis, but develop a lung disease resembling *Pulmonary Alveolar Proteinosis* (PAP) (36, 37). The pathogenesis of PAP involves a reduction in surfactant clearance by defective alveolar macrophages (38-40). Alveolar macrophages in these mice show reduced: cell adhesion, phagocytosis, pathogen killing, and mannose- and Toll-like receptor expression. Moreover, the transcription factor Pu.1 is markedly reduced in these macrophages (41). Conversely, retrovirus-mediated expression of Pu.1 completely restored the defective macrophage functions (41). Intriguingly, most humans with PAP harbor high titers of neutralizing anti-GM-CSF antibodies or, less commonly, mutations in the β_c receptor subunit (42, 43).

GM-CSF-deficient mice also show compromised: antigen-specific IgG and cytotoxic T-cell responses, IFN-γ production, and general phagocytic defects (44-47).

Taken together, these immune defects confer increased susceptibility to *Listeria monocytogenes*, group B streptococcus, and *Pneumocystis carinii*, but partial protection against: endotoxin challenge and collagen-induced arthritis (48-52).

Although IL-3-deficient mice, similarly to GM-CSF-deficient mice, display intact steady state hematopoiesis, unlike GM-CSF-deficient animals, they maintain normal pulmonary homeostasis (53). Mice deficient in IL-3 mount attenuated mast cell and basophil responses to parasite infection, that result in compromised worm expulsion (54). They also show partial reductions in contact hypersensitivity reactions to haptens applied epicutaneously (53).

Surprisingly, mice doubly deficient in GM-CSF and IL-3 maintain normal numbers of hematopoietic cells too, but they show an increased number of circulating eosinophils (55). Earlier studies underscored the striking abilities of GM-CSF and IL-3 to stimulate the growth and differentiation of DCs from hematopoietic precursors (56).

Since we did not find abnormal numbers of DCs in our GM-CSF/IL-3-deficient mice, we evaluated DC function in these mice by their ability to develop contact hypersensitivity (55). Contact hypersensitivity is a form of a delayed-type hypersensitivity reaction (type IV hypersensitivity reaction) in which hapten-protein conjugates are presented by cutaneous DCs, following their migration to regional lymphnodes, to hapten-specific $CD4^+$ and $CD8^+$ T lymphocytes (57-59). Indeed, GM-CSF/IL-3-deficient mice developed a markedly weaker contact hypersensitivity reaction to haptens applied epicutaneously than mice deficient in either factor alone suggesting a functional defect of DCs in the compound knock out mice (55).

Transgenic mice, that locally overproduce GM-CSF in the stomach, spontaneously developed an autoimmune gastritis, demonstrating that GM-CSF overproduction is sufficient to break tolerance and to initiate autoimmunity (60).

Vaccination of WT mice with irradiated B16 mouse melanoma cells engineered to secrete GM-CSF (GM-B16 vaccine), or, to a lesser extent IL-3, stimulates tumor destruction and leads to potent, specific, and long-lasting anti-tumor immunity (61). Both, $CD4^+$ and $CD8^+$ T cells, are required for this anti-tumor response (61). Also, injection of tumor cells expressing GM-CSF results in a dramatic increase in $CD11^+$ DCs in the spleen and tumor infiltrate (62). GM-CSF stimulated DCs were $CD8\alpha^-$ and

expressed high levels of the co-stimulatory molecules CD80 (B7-1) and CD1d, revealing a critical role of these molecules in enhancing the function of DCs (62).

1.4 Cytotoxic T Lymphocyte-Associated Antigen-4

Research over the past decade has established a two signal model of T cell activation to account for the fact that T cell activation requires not only stimulation via the T cell antigen receptor (TCR) by antigen-peptide MHC complexes, but also an additional co-stimulatory signal (63). The additional co-stimulatory signal is provided by: members of the CD28 receptor family (CD28, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), or ICOS) on the T cells, and by members of the B7 ligand family (e. g. CD80, CD86 (B7-2), and B7-H3) on the APCs (64-67). CTLA-4 binds CD80 and CD86 with much higher affinity than the CD28 receptor does (68). Although some initial experiments suggested a positive role of CTLA-4 in co-stimulating T cells, there is now growing evidence that CTLA-4 has an inhibitory effect on T cell stimulation (69-73). Recently, it could be shown that CTLA-4 was also involved in the control of a special subset of T cells, the $CD25^+$ $CD4^+$ regulatory T cells (74). CTLA-4-deficient mice suffer from a rampant lymphoproliferative disorder characterized by polyclonal T cell proliferation and early lethality, providing compelling support for a critical role for CTLA-4 in the down-regulation of T cell responses (75-77). Whereas CD28, the most important co-stimulatory receptor, is constitutively expressed on the $CD4^+$ and $CD8^+$ T cell surface, CTLA-4 expression is upregulated in these cells after stimulation, and reaches a maximum after 2-3 days (70). Only $CD25^+$ $CD4^+$ regulatory T cells have been reported to constitutively express CTLA-4 (74).

Since the initiation of the T cell response to tumors seems to require the presentation of tumor-derived antigens on the cell surface of professional APCs, such as DCs, with the capacity to provide co-stimulation, it was hypothesized that temporary removal of CTLA-4-mediated inhibition could lead to enhancement of anti-tumor responses (66, 78). Indeed, administration of antibodies that block CTLA-4 interactions

resulted in rejection of tumors such as: prostatic carcinoma, lymphoma, renal carcinoma, and colon carcinoma (79-82). However, CTLA-4 blockade was not effective as a single agent against poorly immunogenic tumors such as B16 melanoma (83, 84). Similarly, the GM-B16 vaccine showed only weak effects against pre-existing tumors, although it was very effective in inducing prophylactic immunity to the B16 melanoma. If used in combination with anti-CTLA-4, however, a strong synergism was induced resulting in the complete rejection of B16 melanoma (84).

1.5 Mechanisms Leading to Autoimmune Diseases

Autoimmune diseases are thought to result from a failure in the mechanisms of the immune system that establish and maintain non-responsiveness, or tolerance to self (85). A striking feature of human autoimmune diseases is their female predominance (86). In human SLE, for example, this predominance approaches 90% of all the cases, especially within the 15-64-years-old age group (87). These data strongly suggest a role for female sex hormones in lupus pathogenesis, and it has been thought that these hormones may act as modulators of disease expression (86). It has also been known for a long time that autoimmune diseases often cluster in the same families, indicating a genetic predisposition (88). In this regard, particular haplotypes of the Major Histocompatibility Complex (MHC) are strongly associated with an elevated susceptibility for autoimmune diseases (89). Genes outside the MHC locus, however, were shown to contribute to autoimmune diseases as well (88).

Several mechanisms underlying enhanced susceptibility to autoimmune diseases have been identified. For example, polymorphism of CTLA-4 was recently recognized as a risk factor for some autoimmune disorders such as autoimmune diabetes (90). Complete absence of $CD25^+$ $CD4^+$ regulatory T cells causes a wide range of autoimmune disorders (91, 92). Defects in DC maturation have been described in the Non-Obese Diabetic (NOD) mouse (93).

Scott et al. reported that mice carrying mutations in the Mer tyrosine kinase had impairments in phagocytosis of apoptotic cells, and consecutively developed anti-double stranded DNA (anti-dsDNA) autoantibodies (94). Their findings directly provided *in vivo* evidence for the concept that defects in clearance of apoptotic cells may underlie systemic autoimmunity (95).

Mice deficient in the *src*-family kinase Lyn, an inhibitory component of the B cell receptor (BCR) signaling pathway, developed a severe syndrome resembling SLE (96). Similarly, overexpression of B Cell Activating Factor (BAFF) led to a SLE-like disorder, and has been observed in human suffering from SLE and other autoimmune diseases (97, 98). BAFF induces the survival of a subset of immature splenic B cells, referred to as translational Type 2 (T2) B cells (99). BAFF allows T2 B cells to survive and differentiate into mature B cells in response to signals through the BCR (99). Since immature transitional B cells are targets for negative selection, a feature thought to promote self-tolerance, excessive BAFF-mediated survival of immature B cells may contribute to the maturation of autoreactive B cells (99, 100). New findings, however, suggest that BAFF directly promotes the survival of peripheral autoreactive B cells by protecting them from BCR-induced death signals (101).

Lymphopenia, as often seen with illness and stress, has recently been described as a risk factor for the development of autoimmune diseases (102). As a cause, a dysregulated homeostatic proliferation of T cells could be identified (102). Homeostatic proliferation is a mechanism that maintains T cell numbers at a steady state in lymphoid organs, and also drives their expansion following T cell loss. It is tightly regulated by both the available space in lymphoid organs and engagement of specific growth factors (103).

Microbial infections have been directly linked to autoimmune diseases. It was recently reported that Toll-like receptor stimulation of DCs by various microbial products was sufficient to induce autoimmune myocarditis in an *in vivo* model, where DCs were previously loaded with a heart-specific self peptide (104).

1.6 The Cancer Immune Surveillance Theory

Paul Ehrlich first hypothesized that the immune system could repress a potentially "overwhelming frequency" of carcinomas (105). In the 1950s, the work of Medawar could clarify the critical role for cellular components of the immune system in mediating allograft rejection (106). It became clear, that the underlying mechanism of the destruction of transplanted tumors derived from non-inbred mouse strains by the immune system was one of allograft rejection rather than tumor-specific rejection (106). As soon as inbred strains were available, the idea, that tumors were immunologically distinguishable from normal cells, could be critically tested. The fact that mice could be immunized against syngeneic transplants of tumors induced by chemical carcinogens, or viruses, established the existence of tumor-specific antigens (107). It finally led to the formulation of the hypothesis of "cancer cell recognition and destruction by the immune system" ("Burnet's hypothesis") by Burnet and Thomas (108, 109). The hypothesis states that the immune system can recognize and destroy tumor cells due to their possession of new antigenic features (108). This concept was later abandoned since studies with chemically induced tumors, or spontaneous tumor formation, in inbred mice lacking T cells and Wild Type (WT) mice were inconclusive (110, 111).

Since 1994, however, the concept of Burnet and Thomas has undergone a renaissance, mainly for two reasons: firstly, endogenous production of IFN-γ was shown to protect the host against the growth of transplanted tumors and the formation of chemically induced tumors (19, 112). Using tumor transplantation approaches, researchers found that immunogenic fibrosarcomas grew faster and more efficiently in WT mice treated with neutralizing monoclonal antibodies specific for IFN-γ than in mice without treatment (113). Mice lacking sensitivity to either IFN-γ, or components of its pathway (i. e. IFNGR1/2 or Stat1), developed tumors more rapidly and with greater frequency than WT mice when challenged with different doses of the chemical carcinogen 3-methylcholanthrene (MCA) (19). Moreover, doubly mutant mice lacking the tumor suppressor p53 and the IFNGR1 subunit of the IFN-γ receptor formed a wider spectrum of tumors as compared with IFN-γ-sensitive mice lacking only p53 (19). The

second key finding was the observation that perforin-deficient mice were more prone to MCA-induced tumor formation compared to their WT counterparts (114). Perforin is a component of the cytolytic granules of cytotoxic T cells and NK cells. It is important in mediating lymphocyte-dependent killing of infected cells and tumor cells (115). After challenge with MCA, perforin-deficient mice developed significantly more tumors compared with perforin-sufficient mice (116). Similar results were obtained by MCA injection into RAG-2-deficient mice that fail to rearrange lymphocyte antigen receptors, and thus completely lack T, B, and NKT cells (117, 118).

All these observations led to the formulation of the "Cancer Immune Surveillance Theory" stating that the unmanipulated immune system is capable of recognizing and eliminating primary tumors, and that T lymphocytes and IFN-γ are important in this process (Figure 1.6.1) (112).

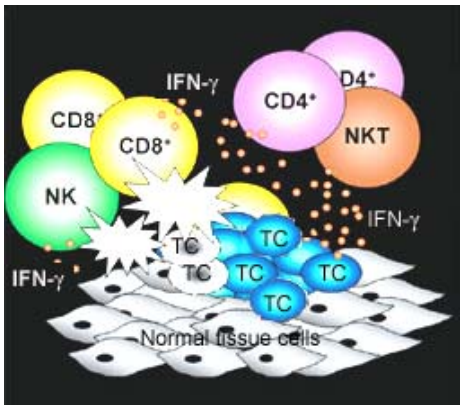

Figure 1.6.1 Concept of the "Cancer Immune Surveillance Theory". Transformed cells (TC) get recognized by cells of the immune system such as NK cells, NKT cells, and T cells. These cells begin to secrete IFN-γ that: firstly, is acting to enhance the recognition of the transformed cells by the cells of the immune system (e. g. by inducing in the transformed cells proteins that are involved in antigen processing/presentation), and secondly, further activates cells of the immune system such as macrophages and cytotoxic $CD8^+$ T cells, which participate in tumor cell destruction (19).

1.7 Experimental System and Hypothesis

As earlier reported by Glenn Dranoff, vaccination with irradiated tumor cells engineered to secrete GM-CSF, or, to a lesser extent IL-3, stimulates potent, specific, and long-lasting anti-tumor immunity (61). This observation let us hypothesize that the endogenous production of these cytokines may function in tumor suppression. To test our hypothesis, we generated: GM-CSF- (36), IL-3- (53), and GM-CSF/IL-3- (55) deficient mice.

We also wondered, whether IFN-γ deficiency, which enhances susceptibility to chemical carcinogenesis (19, 114, 119), favors spontaneous tumor formation in the absence of GM-CSF and IL-3. Moreover, mice lacking GM-CSF, IL-3, and IFN-γ served to test the hypothesis that endogenously produced IFN-γ forms the basis of a T cell-dependent tumor surveillance system that controls the development of spontaneously arising tumors in mice (19).

Since GM-CSF/IL-3-deficient mice are susceptible to infections and, as previously reported, have a defect in DC function (55), I was also interested whether these mice would develop autoimmune disorders.

Further, treatment of infection and inflammation in all these mice with antibiotics or anti-inflammatory agents should help to clarify the influence of infection and inflammation on the development of diseases.

GM-CSF- (36), IL-3- (53), GM-CSF/IL-3- (55), and IFN-γ- (26) deficient mice were backcrossed at least nine generations onto the C57Bl/6 strain. Homozygous doubly and triply deficient mice were obtained by intercrossing. I also kept cohorts of WT C57Bl/6 mice, which were obtained from the cytokine knock out breeder pairs, and cohorts of GM-CSF- and GM-CSF/IL-3-deficient mice, and WT mice, on a pure Balb/c background.