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Interleukin-10 Induces Apoptosis in Developing Mast Cells via a Mitochondrial, STAT3-dependent Pathway

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Interleukin-10 Induces Apoptosis in Developing Mast Cells via a Mitochondrial, STAT3-dependent Pathway

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By
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ABSTRACT

INTERLEUKIN-10 INDUCES APOPTOSIS IN DEVELOPING MAST CELLS VIA A MITOCHONDRIAL, STAT3-DEPENDENT PATHWAY

By Daniel Paul Bailey

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Director: John J. Ryan, Ph.D., Associate Professor, Department of Biology

Objective.

The aim of this study was to determine the effects of interleukin-10 on mast cell development from bone marrow progenitors.

Materials and Methods.

Unseparated mouse bone marrow cells were cultured in IL-3+SCF, giving rise to mast cells and monocytes/macrophages. The addition of IL-10, and the use of Signal Transducer and Activator of Transcription (STAT)3-deficient bone marrow cells were employed to measure the effects of IL-10 and STAT3 expression on cell viability, proliferation, and differentiation. Bax-deficient and Bcl-2 transgenic bone marrow cells were used to determine the importance of the mitochondria in IL-10-mediated effects.

Overview.

Mast cells arise from hematopoietic stem cells and continue development in either connective tissue or mucosa. Th2 cytokines have been implicated in the regulation of mast cell development and subsequent function. Mast cells have also been shown to be essential players in many Th2 immune responses.

In the following study we investigate the effects of the Th2 cytokine IL-10 on mast cell development from isolated bone marrow progenitors. The addition of IL-10 to whole murine bone marrow greatly reduced cell numbers and altered the phenotype of the developing progenitor cells. The reduction in cell numbers was due to apoptosis, as judged by DNA fragmentation and caspase activation. The apoptosis observed included alteration in mitochondrial membrane potential. Furthermore, apoptosis could be reduced by the overexpression of Bcl-2 or by ablating p53 expression. Utilizing a flox/cre system we found that IL-10 mediated apoptosis required expression of Stat-3, since Stat-3 deficient bone marrow cells did not undergo apoptosis in response to IL-10. In this study we also observed significant alterations in the mast cell growth factor receptors IL-3R and c-kit. The loss of these growth factor receptors may explain the apoptosis induced by IL-10. These data demonstrate the potent regulatory capabilities of Th2 cytokines on mast cells, a central effector in the Th2 response.

Introduction

History of Mast Cells

The mast cell was first characterized by Paul Ehrlich in June of 1878. The nomenclature mast cell is derived from the German name originally given to this highly granulated cell "mastzelle" which means well-fed cell (1). Mast cells are rounded, mononuclear cells that are heavily granulated. The granules found within a mast cell appear purple when stained with aniline blue dyes, as a result of the reaction of the dye with highly-acidic heparin contained within the granules (2). Mast cells are derived from pluripotent CD34+ bone marrow progenitors, and upon exiting the bone marrow complete their development in mucosal and connective tissue through a process requiring cytokine-regulated trafficking, proliferation, and differentiation (3).

The mast cell has been shown to be involved in hypersensitivity reactions, autoimmune diseases and innate immunity. Murine mast cells are used to investigate multiple diseases processes, although there are differences between murine and human mast cells. Murine mast cells require IL-3 to differentiate and survive in vitro while human mast cells require IL-6 (4). The cytokine stem cell factor (SCF) is critical for mast cell development and survival in both mice and humans.

Mast cells are divided into two subsets based on their expression of proteases, in both humans and rodents. In humans, MC_{TC} contain tryptase, chymase, carboypeptidase and cathepsin G (5). MC_T express only tryptase. MC_{TC} are mostly found in the skin and mucosa, while MC_T are mainly observed in alveoli and epithelium of the intestines and airway. In rodents, they are classified as connective tissue mast cells (CTMC) and

mucosal mast cells (MMC). CTMC express mast cell specific protease (MMCP)-5,6, while MMC express MMCP-1-4 (6).

Th2 CELLS

Everyday the human body is insulted by a barrage of harmful pathogens and allergens. In order to protect us against this onslaught the immunological response has evolved into two distinct arms to recognize and respond to microorgansims and non-self antigen. These are the innate and adaptive immune responses. The innate arm is the first to encounter foreign invasion and responds in a non-specific fashion to antigenic stimuli by producing multiple agents to kill or neutralize the foreign agent. For the most part induction of the innate response is sufficient to clear intrusive agents from the body. However, in the case that the noxiuos agent is not controlled by the innate response, the adaptive response is also engaged.

At the forefront of this attack lie T lymphocytes that orchestrate the complex balance of immunity. Within the T lymphocyte population, CD4+ T helper-2 (Th2) cells are critical effector cells necessary for regulating the humoral immune response to allergens and pathogens. This subset of CD4+ effector T cells develop from naïve CD4+ cells during the initial response to antigen in the peripheral lymphoid tissue. Upon antigen presentation via dendritic cells in the lymph node and subsequent T cell activation in the presence of interleukin (IL)-4, the naïve CD4+ T cell population will develop into IL-4,-5,-6,-10, and -13 producing Th2 cells. The diverse array of cytokines produced by Th2 cells regulates the immune response so that the eliciting agent is

neutralized while maintaining a balance of pro-inflammatory and anti-inflammatory measures (7).

WHAT ARE Th2 CYTOKINES?

Th2 cytokines are predominantly produced by activated CD4+ T cells but can also be produced by other cells, including mast cells and macrophages. The Th2 cytokine group is made up of the hematopoietin, and IL-10 families of cytokines. These cytokines have both pro-inflammatory and anti-inflammatory activities. Th2 cytokines not only play an important role in allergic disease and autoimmune disorders but they are also important for cell development and maturation. The striking importance of balancing these activities is most obvious in disorders where the immune system is dysfunctional, such as allergic and autoimmune disease.

IL-4

Interleukin (IL)-4 is produced by Th2 cells (8), mast cells (9), and basophils (10). It stimulates proliferation of activated B cells (8), mast cells, and T cells (11). On B cells, IL-4 elicits expression of MHC class II (12), CD23 (13), IgE and IgG1 (14). IL-4 also downregulates production of pro-inflammatory cytokines from macrophages, while increasing antigen-presenting functions (Reviewed in 15, 16). Of special importance, IL-4 induces Th2 development, while inhibiting Th1 development (10, 17). These activities make IL-4 central to the Th2 response, with some dampening effects on cellular immunity.

A role for Th2 cytokines such as IL-4 in atopic diseases is attributed to its promoting IgE production and Th2 development, although the genetic predisposition for mounting an unbalanced Th2 response is incompletely understood. In addition to asthma, IL-4 may be tied to other inflammatory diseases in which the Th2 response is dysregulated. For a review of the roles of IL-4 in allergy, see (18, 19). For example, chronic IL-4 production elicits a lupus-like inflammatory disease in transgenic mice (20).

IL-4 signals by binding to the IL-4Rα, in combination with the common gamma chain, or in some cases with IL-13Rα1. Ligand binding conveys cellular signals through a number of pathways, including IRS1/2, phosphatidylinositol 3'-kinase (PI3-K), Ras-MAPK, and the Jak-Stat pathway (Reviewed in 21). As shown by genetically targeted mice, the 94kDa transcription factor Stat6 is necessary for IL-4-mediated Th2 differentiation and IgE production (22-24). Stat6 is critical to IL-4 function, as illustrated by the similarities between animals made genetically deficient in IL-4 or Stat6 (8, 10, 25, 26). Thus, Stat6 is considered to be an essential mediator of IL-4 responses.

IL-10

Interleukin (IL)-10 was initially identified as cytokine synthesis inhibitory factor (27), a potent Th2-derived factor that decreases Th1 cell activation and cytokine secretion. These activities were shown to be partly indirect, through the ability of IL-10 to dampen monocyte/macrophage antigen presentation, via reduced expression of co-stimulatory molecules, MHC II antigens, and cytokines (28). IL-10 is produced by Th2 cells, Th0 cells, $CD45Rb^{low}$ regulatory T cells, monocyte/macrophages, keratinocytes, some B cells, and mast cells (Reviewed in 27). The importance of IL-10 was perhaps

best demonstrated through the creation of IL-10-deficient mice, which develop a Th1-mediated enterocolitis and have potent Th1 responses (29). The inhibitory effects of IL-10 have best been shown in the regulation of the inflammatory activities of macrophages and dendritic cells [Reviewed in 30 Moore].

The evolutionary importance of IL-10 has been made clear by the identification of IL-10 homologues expressed by Epstein-Barr virus, equine herpes virus 2, pox ORF virus, and cytomegalovirus (30-35). Further, an entire family of IL-10-related cytokines, including IL-19, IL-20, IL-22, IL-24, and IL-26 has recently been identified, greatly increasing the focus on this regulatory factor (reviewed in 36).

IL-10 signaling has been studied in some depth. Its heterodimeric, interferon-like receptor is widely expressed on hematopoietic cells and other lineages, offering many potential targets for IL-10 action. While IL-10 receptor signaling can involve phosphatidylinositol 3'-kinase, ribosomal S6 kinase, and members of the AP-1 family (reviewed in 28), much focus has been on the role of the Jak-Stat signaling system. IL-10 is known to employ Jak1 and Tyk2 to activate Stat3, with activation of Stat1 and Stat5 in some systems. Stat3 has been shown to be the most important IL-10 signaling pathway, required for most IL-10 functions and able to directly mimic some IL-10 activities (37-42).

Few cytokines have a stronger link to disease outcomes than IL-10. In clinical trials, IL-10 treatment reduces the symptoms associated with Crohn's disease, rheumatoid arthritis, psoriasis, and hepatitis C infection (43-52). Productive immunotherapy for allergic disease correlates with increased IL-10 production (53-57), and mutations in the IL-10 promoter are linked to allergy incidence (58). In animal models, IL-10 has also

been shown to be a potent immunomodulator. Summarizing these studies, IL-10 treatment mitigates the severity of pathology in experimental allergic/autoimmune encephalomyelitis (EAE) (59-61), non-obese diabetes (62), inflammatory arthritis (63-69), airway hyperresponsiveness/allergy, and inflammatory bowel disease (IBD) (70). These data are supported by the spontaneous IBD that IL-10-deficient mice develop 29). Further, anti-IL-10 treatment worsens EAE incidence and severity, and protective therapy with IFNγ may induce IL-10 synthesis (71, 72). Thus, IL-10 has been shown in a broad range of studies to possess clinically relevant inhibitory abilities.

There are exceptions to the protective role of IL-10. Owing to its B cell stimulatory capabilities, IL-10 may worsen systemic lupus erythematosus (SLE). In this regard, IL-10 levels positively correlate with disease, and anti-IL-10 therapy decreases autoantibody production in some mouse models. Perhaps most importantly, a recent clinical trial found that anti-IL-10 treatment significantly improved symptoms in 5 of 6 patient (73). Despite these findings, there is also support for a beneficial role for IL-10 in SLE. For example, in a Fas-deficient model of Lupus, IL-10 deficiency worsened disease (74), and IL-10 decreased Th2-type autoantibody formation (75). It appears that the true role of IL-10 in SLE may be partly determined by the mechanisms leading to clinical disease, particularly with reference to Th1- versus Th2-mediated responses. On balance, IL-10 signaling has clear links to inflammatory disease, and inducing or inhibiting this signaling is an area of considerable clinical interest

MAST CELLS

Mast cells were first recognized for their importance in parasite clearance. In contrast to this protective role, the mast cell is best characterized for its role as an effector cell of allergic disease. Recently the importance of mast cells to adaptive immunity has expanded. The mast cell response has been shown to be essential for resistance to bacterial infection and involved in the inflammatory responses characteristic of several clinical autoimmune conditions including rheumatoid arthritis(76-78), sjogren syndrome (79), systemic sclerosis (80), multiple sclerosis (81), thyroid disease (82), chronic urticaria (83, 84), pemphigus (85), and atherosclerosis (86). Mast cell deficiency also greatly diminishes inflammation in animal models of multiple sclerosis and heart disease (87, 88). These data support the contention that mast cells participate in both T helper cell (Th)1 and Th2 responses. Given this contribution, the means by which cytokines alter the mast cell response is an area of research that may yield clinically important tools for treating inflammatory disease.

MAST CELL DEVELOPMENT

Mast cells are derived from pluripotent hematopoietic stem cells found in the bone marrow. Recently, Galli et.al. described a cell population in adult mouse bone marrow characterized as Lin(-)c-Kit(+)Sca-1(-)-Ly6c(-)-FcepsilonRIalpha(-)-CD27(-)beta7(+)-t1ST2(+), that gives rise only to mast cells in culture (89). This population was able to reconstitute the mast cell compartment when transferred into c-kit mutant mast cell-deficient mice. Mast cell committed precursors exit the bone marrow and complete their development in connective and mucosal tissues. In these locations

mast cells are positioned to function as sentinel cells of host defense. The process of mast cell development is driven by the activities of IL-3 and stem cell factor (SCF), two well-characterized mast cell growth and differentiation factors (3). With the broadening importance of mast cells to immune function and pathology, mast cell development, maturation, and function are key areas of interest. Defining the factors that regulate mast cell recruitment to peripheral tissues, maintenance of mast populations, and removal of non-essential, or aging mast cells needs further investigation.

MAST CELL ACTIVATION AND FUNCTION

Activation of mast cells results in the release of a wide array of biologically active preformed and induced compounds that can result in leukocyte chemotaxis, smooth muscle contraction in bronchial and gastrointestinal tracts, increased vascular permeability and mucus production by goblet cells (90, 91). Mast cell activation is often achieved via the cross-linking and aggregation of IgE receptors by specific antigen (90, 91). Mast cells may also be activated via non-IgE signals including direct interaction with pathogenic bacteria (92, 93), or bacterial products such as enterotoxin B (94), or cholera toxin (92, 95), and components of the complement cascade (96). Compounds released by mast cells include preformed vasoactive amines such as histamine and serotonin, proteoglycans, proteolytic enzymes and cytokines such as tumor necrosis factor (TNF)-a, and interleukin (IL)-16 (97). In addition, mast cells can be stimulated to make three classes of arachidonic acid-derived lipid mediators, including prostaglandin D2 (PGD2), leukotrienes (LTC4, LTD4, and LTE4), platelet activating factor (PAF), as well as multiple cytokines including IL-1,3,4,5,6,8,9,10,12,13,16,18, GM-CSF, TNF-a,

lymphotoxin, macrophage inhibitory protein (MIP)1-a, MIP-1b, TCA-3, leukemia inhibitory factor (LIF), transforming growth factor (TGF)-b, and nerve growth factor (NGF) (97). Due to the vast array of mediators produced by mast cells and their frequent intersection with the Th2 response, it is important to gain a better understanding of how this interaction contributes to the homeostatic regulation of the mast cell population.

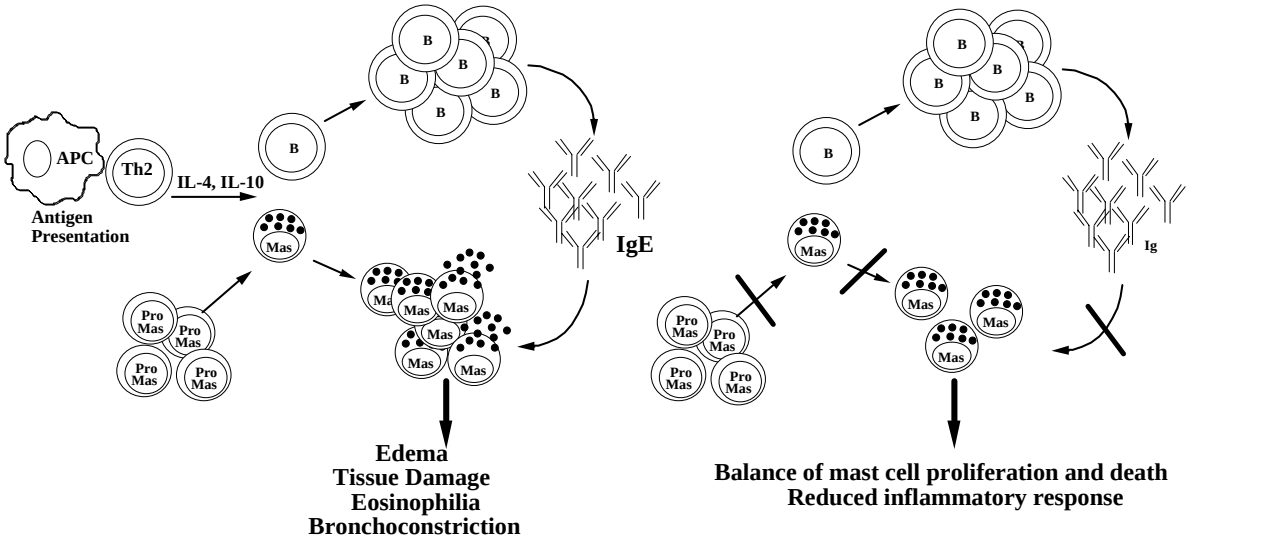
EFFECTS OF IL-4 AND IL-10 ON MAST CELLS

Many studies find correlations between serum IL-10 and on-going immune responses or disease progression. For example, serum IL-10 levels are elevated for at least 5 days during septicemia, with some patients demonstrating up to 2800pg/ml (98, 99). Colorectal cancer, breast cancer, B cell lymphoma, and hepatocellular carcinoma patients all show elevated serum IL-10 (100-103) . This is also true in chronic hepatitis, myocardial infarction, Graves disease, and systemic sclerosis (104-107). In related studies using an animal model, Fred Finkelman's group has shown that IL-4 concentrations exceeding 10ng/ml are detected following anti-CD3 injection (108), and that serum IL-4 concentrations remain elevated for more than 10 days during parasite infections (109). Each of these studies demonstrates that local inflammation can increase serum cytokine concentrations, possibly affecting cells at considerable distance from the site of origin. This is important to understanding mast cell homeostasis, since systemic cytokines could be affecting mast cells throughout their otogeny. This is the rationale for our investigations into how Th2 cytokines alter mast cell development, as well as the function and survival of differentiated mast cells. Mast cell responses are well-established as a central aspect of chronic inflammatory disease. Our group is focused on

understanding the mast cell “life cycle” - how its development, function, and survival are controlled, and the consequences of losing this balance. Mirroring classic models, we have formed a hypothesis of cytokine-mediated mast cell homeostasis, in which the signaling molecules both elicit and limit the mast cell response (Figure 1). The immune responses in which mast cells function are frequently under the control of Th2 lymphocytes. Our laboratory has investigated the control of mast cell homeostasis through feedback regulation by the Th2 cytokines IL-4 and IL-10, which are important in eliciting mast cell–mediated inflammation.

IL-4 is a mast cell growth factor (11), and induces FcεRI expression on developing human mast cells (110, 111). These and other activities argue for the pro-atopic nature of IL-4 in mast cell biology. But in support of an anti-inflammatory role for IL-4 we have found that IL-4 decreases mast cell Kit and FcεRI expression, inhibits IgE-mediated activation, and induces death of mast cell precursors (112-114). We find that Stat6 activation is both necessary and sufficient to elicit many of these responses (112-114). Other groups have reported IL-4-induced apoptosis of human mast cells (115 Oskeritzain) and have employed our assays to demonstrate inhibitory effects of IL-4 on mast cells (112, 116, 117). Central in these assays is the element of timing: IL-4 has no inhibitory effects on mast cells until 3 days of stimulation. Our hypothesis is that this timing frames an "inflammatory window" during which mast cells serve their protective roles in immunity. Following this is an "inhibitory window" wherein IL-4 reduces mast cell activation, causes arrest and apoptosis of mature cells, and finally kills developing precursors (Model of Mast Cell Inflammatory Response).

Model of Mast Cell Inflammatory Response



Pro-Inflammatory

Days 0-3
Mast cell activation and ensuing inflammation

Anti-Inflammatory

Post Day 3
Inhibition of mast cell-activating receptors
Death of mature mast cells and bone marrow progenitors

On mast cells, we found that IL-10, like IL-4, inhibits expression and/or function of the high affinity receptor for IgE, FcεRI, and Kit (112-114, 118-120). IL-10 serves as a potent inducer of apoptosis in developing mast cells through a STAT-3 dependent pathway. In these studies we observed IL-10 inhibited mast cell survival through downregulation of IL-3 and SCF receptors, both critical for mast cell survival and proliferation. We have also found that combined signaling with IL-4 and IL-10 induces apoptosis of differentiated mast cells (10, 119).

SUPPORT FOR CYTOKINE-MEDIATED MAST CELL HOMEOSTASIS

Results from other laboratories support our theory that Th2 cytokines regulate mast cell homeostasis. For example, IL-4-knockout mice have increased basal peritoneal mast cell numbers, implying defective homeostasis (121). A detailed series of studies with intestinal pathogens also offers support. Immunity to helminthe infections is accomplished by a time-limited Th2 response accompanied by mast cell hyperplasia in the intestine. This immune response appears to be limited partly by mast cell apoptosis, as recently published (122). However, Stat6-deficient mice, which lack many IL-4 responses, have a five-fold greater mast cell hyperplasia during such infections (123). Mirroring this, we have found that Stat6-deficient mast cells do not undergo IL-4+IL-10-induced apoptosis (112-114, 116).

Studies of disease-linked polymorphisms provide additional theoretical support for the notion that cytokines regulate mast cell biology. Mast cells are central effectors of allergic inflammation. Allergy-related polymorphisms of the IL-10 promoter, the IL-4 promoter, and the IL-4 receptor alpha chain have been noted (54, 124-129). It is possible

that such genetic changes may prevent Th2-mediated mast cell regulation, though these effects are unknown. Lastly, chronic mast cell hyperplasia is most notable in mast cell neoplasms that involve gain-of-function mutations in Kit (130-132). Cell lines bearing these mutations do not undergo IL-4+IL-10-induced apoptosis (119), and fit a description of homeostatic dysregulation, like all neoplasms.

Loss of homeostatic constraints lies at the root of all chronic diseases. If Th2 cytokines dampen the mast cell response, our theory predicts that sustained Th2 cytokine production should normally limit mast cell-mediated inflammation. The fact that this does not appear to happen in diseases like asthma could be used to argue against our theory. However, *we believe that loss of mast cell homeostasis may be part of the disease etiology*, such that chronic disease represents a departure from the constraints maintained in healthy subjects. Thus our theory will shed light both on normal physiology as well as pathophysiology.

Materials and Methods

Culture System

Bone marrow cells were extracted from the femurs of C57BL/6x129 mice ("wild type"; from Taconic Farms,Germantown, NY), STAT3 flox/-; lysMcre mice (133, 134), Bax-deficient mice, p53-deficient mice (Jackson Labs, Bar Harbor, ME), or H2K-Bcl-2 transgenic mice (135). Cells were cultured at $5x10^5$ cells/ml in complete RPMI medium (cRPMI), consisting of RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (all from Biofluids, Rockville, MD) for 21 days. Cultures contained IL-3 (5 ng/ml) + SCF (50 ng/ml), and IL-10 at the indicated concentrations (purchased from R&D Systems, Minneapolis). During the culture period, cells were fed every 4-7 days by removing half of the culture contents with or without scraping as indicated. An equal volume of new medium and cytokines was replaced into the cultures. Importantly, in scraped cultures that are there was no selection against adherent cells (predominantly macrophages) as is frequently the case for mast cell development assays. As such, these cultures gave rise to a mixture of monocyte/macrophages and mast cells. Prior to harvesting samples for analysis, cultures were scraped, so that adherent cells were included in all assessments. The efficacy of scraping was determined by examining cultures with an inverted microscope, which revealed that nearly all adherent cells were removed from the plate surface. By comparison, cultures that were passaged to remove adherent cells developed into virtually pure (>95%) mast cells, based on histochemical analysis and flow cytometry staining for FcεRI and Kit.

Flow Cytometry Staining

Samples were obtained by removing a 200μl sample after scraping. Samples were washed with phosphate-buffered saline (PBS) containing 3% fetal calf serum (FCS) and 0.1% sodium azide (FACS Buffer), then incubated with 10μl rat anti-mouse FcγRII/FcγRIII ascites (clone 2.4G2) to prevent non-specific interaction of antibodies with IgG receptors. Cells were then stained with FITC-labeled IgG (Southern Biotech. Inc., Birmingham, AL), PE-labeled IgG (BD Biosciences Co., San Diego, CA), PE-labeled anti-Mac-1 (Southern Biotech), FITC-labeled anti-T1/ST2 (Morwell Diagnostics, Zurich, Switzerland), PE-labeled anti-Kit (BD Biosciences), FITC-labeled anti-IgE (Southern Biotech), FITC-labeled anti-CD13 (BD Biosciences), or PE-labeled anti-$\beta_{IL\text{-}3}/\beta_c$ (BD Biosciences).

Assessment of Live Cell Numbers

To compare the relative number of live cells in each culture condition, samples were prepared as described for flow cytometry staining, using 200μl of cells removed from each culture. Samples were analyzed for 45 seconds (time resolution 0.1 seconds) on a BD FACscan (BD Biosciences). Live cell gating was accomplished by forward and side scatter parameters, the efficacy of which was confirmed by propidium iodide exclusion. Live cell numbers in experimental conditions were compared to cells cultured in IL-3+SCF (control conditions) to determine a percent change relative to this control group. We found this method to be more objective and consistent than similar assessments of cell numbers such as trypan blue exclusion.

Propidium Iodide Analysis of Apoptosis

To detect sub-diploid DNA, a 200µl sample was removed from scraped cultures and washed twice with PBS. Samples were fixed in an ethanol solution and stained with propidium iodide in the presence of RNase A as described previously (119). Each sample was analyzed for 45 seconds (time resolution, 0.1 seconds) with a BD Biosciences FACScan, with a forward scatter-side scatter gate to exclude cellular debris. Percent cells with sub-diploid DNA content (apoptotic cells) was calculated using CellQuest software by gating on those cells whose PI fluorescence was less than the diploid peak in control cultures.

Di(OC_6)$_3$ Staining

Di(OC_6)$_3$ (Molecular Probes, Eugene, OR) was added to 200µl of cells at 1nM final concentration. Samples were incubated for 30 minutes at 37°C in a CO_2 incubator. The cells were then washed twice with PBS and resuspended in 200µl PBS for flow cytometric analysis using a forward and side scatter gate.

Histochemical Analysis

Cell cultures were scraped and 200µl samples were removed, washed twice with 200µl of PBS, and centrifuged onto microscope slides (Shandon Cytospin 2, ThermoShandon, Pittsburgh, PA). Slides were stained with Wright Giemsa (WG) (Sigma Aldrich Chemicals) or with acid toluidine blue (0.2 % Toluidine Blue, 0.1 M citric acid, in 50 % EtOH in dH_20). Non-specific esterase staining was performed using a kit and protocol

provided by Sigma (Napthol AS-D Chloroacetate Esterase and α-Naphthyl Acetate Esterase, Procedure No. 91).

Results

IL-10 reduces cell numbers in an IL-3 and SCF-rich environment

Murine bone marrow cells were cultured in the presence of IL-3+SCF with or without IL-10 for 21 days. In these cultures, IL-10 reduced relative cell numbers in a concentration-dependent manner, with a 75% decrease when IL-10 was present at 10 ng/ml (Figure 1). To determine the kinetics of this effect, samples were measured at various time points during the 21 day culture period (Figure 2). The effects of IL-10 were not significant during culture days 0-10, but between days 10 and 21 the relative cell numbers reduced rapidly (slope = 0.5 days 0-10; slope = -3.2 days 10-21). The inhibitory effects of IL-10 required a relatively long exposure period. Not only did cell numbers remain unchanged for the first 10 days of culture, but removal of IL-10 from these cultures during the first 15 days prevented any significant decrease in cell numbers when cells were harvested on day 21 (Figure 3). Likewise, IL-10 added after the first 7 days of culture had no effect on viable cell numbers when cultures were harvested on day 21. Thus IL-10 greatly reduced the survival of developing bone marrow cells, exerting its effects with delayed kinetics that required its presence early in differentiation.

Figure 1.

Effects of IL-10 on bone marrow cell numbers in culture. Concentration-response of IL-10 effects on viable cell numbers. Bone marrow cells were cultured in IL-3+SCF +/- IL-10. Cultures were harvested after 21 days and viable cell numbers were measured by timed flow cytometric counting as described in Materials and Methods. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 5 separate experiments.

Figure 1.

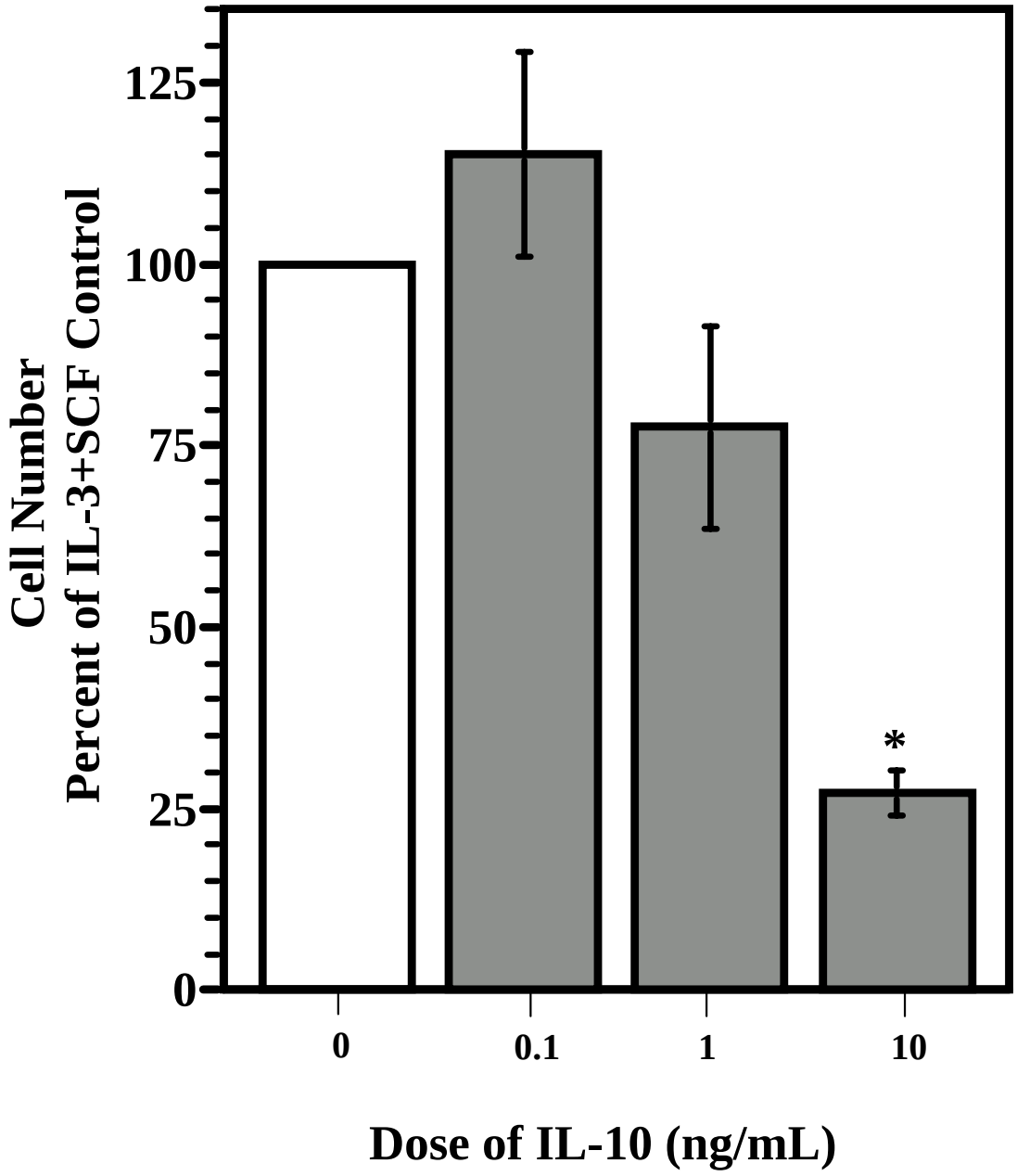

Cell Number
Percent of IL-3+SCF Control
125
100
75
50
25
0
*
0
0.1
1
10
Dose of IL-10 (ng/mL)

Figure 2.

Time course of IL-10 effects. Bone marrow cells were cultured in IL-3+SCF +/- IL-10 at 10 ng/ml. Viable cell numbers were determined on the indicated days by timed flow cytometric counting. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 5 separate experiments.

Figure 2.

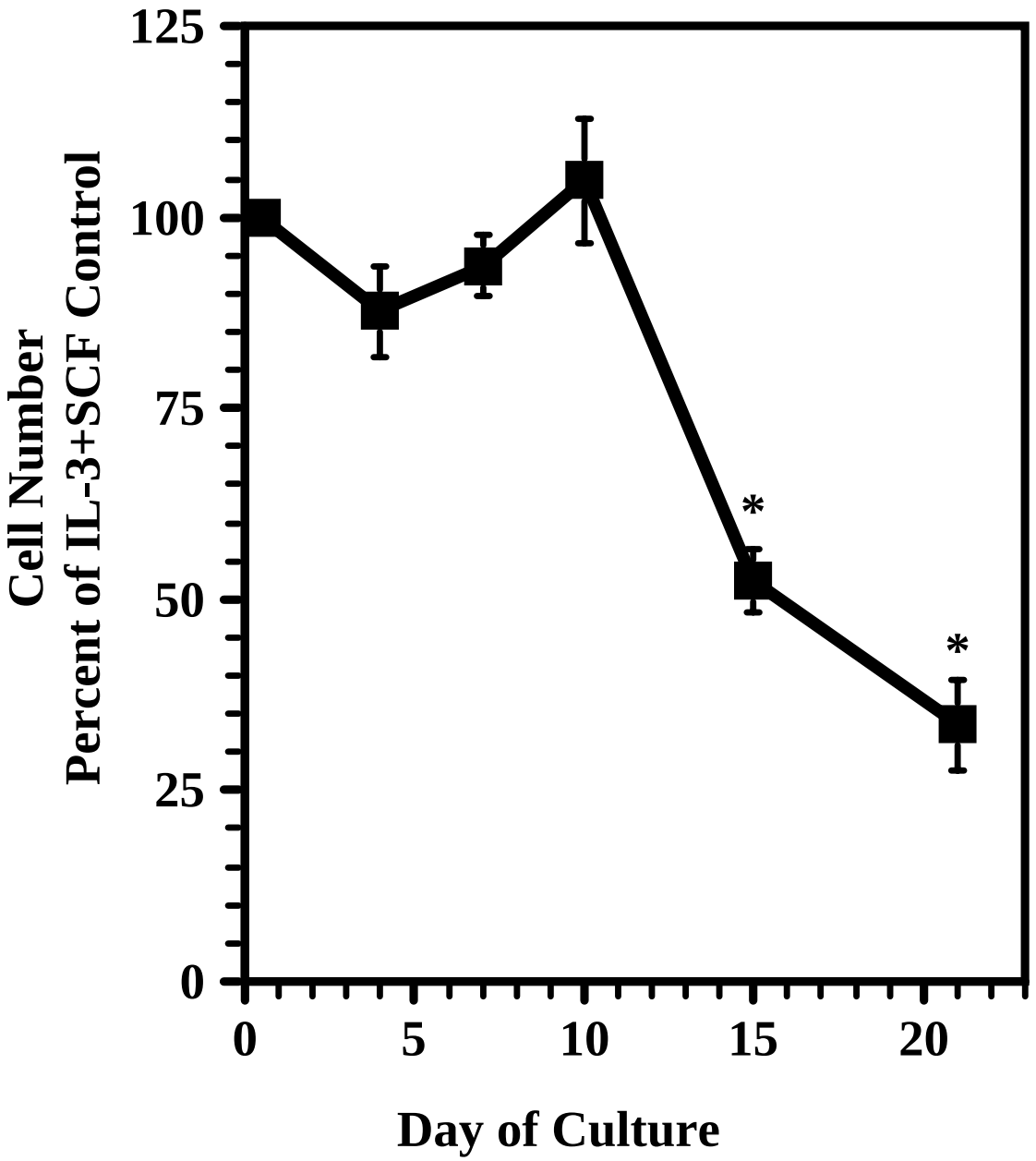
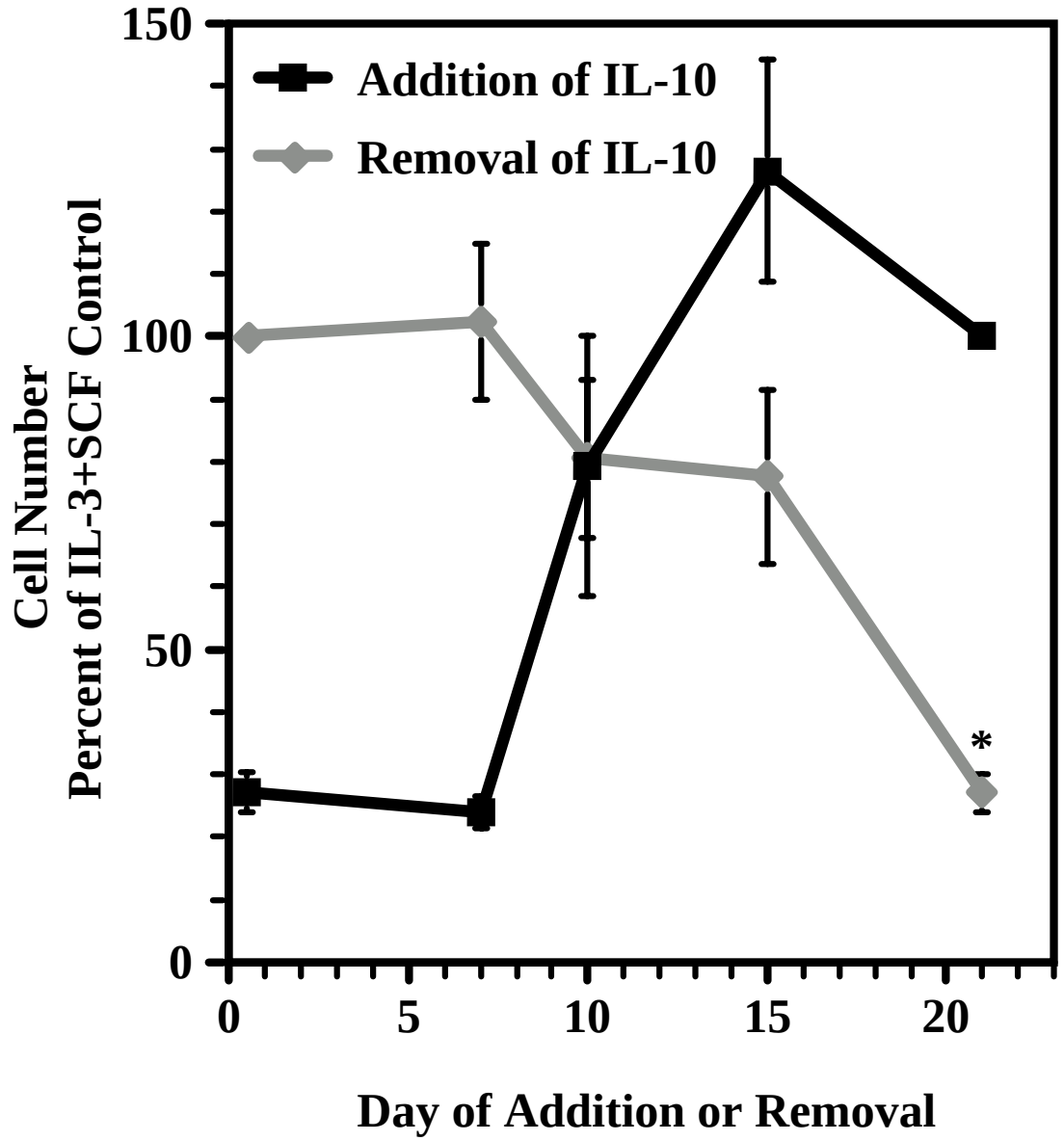

125
100
75
50
25
0
Cell Number
Percent of IL-3+SCF Control
*
*
0
5
10
15
20
Day of Culture

Figure 3.

Addition/removal of IL-10 (10 ng/ml) to bone marrow cultures containing IL-3+SCF. IL-10 was added to or removed from cultures containing IL-3+SCF on the days indicated. All cultures were harvested on day 21. Viable cell numbers were determined by timed flow cytometric counting. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 3.


150
100
50
0
Addition of IL-10
Removal of IL-10
Cell Number
Percent of IL-3+SCF Control
*
0
5
10
15
20
Day of Addition or Removal

Mast cell but not macrophage development is impaired by IL-10

In addition to inhibiting cell survival, IL-10 may be altering the differentiation of developing bone marrow progenitors. Growth in IL-3+SCF with selection against adherent cells gives rise to mast cells; however we wanted to determine the effects of IL-10 without bias for a specific lineage. Therefore, these cultures, like those studied in Figure 1, were performed without selection. On day 21 of culture, samples were removed and assessed by morphological appearance. This initial analysis revealed that the majority of cells had either mast cell or monocyte/macrophage morphology (Figure 4). Hence, we also employed specific histochemical stains for the mast cell and macrophage lineages. The percentage of mast cells was significantly decreased in cultures containing IL-3+SCF+IL-10 as determined by morphology (Wright Giemsa stain) or by acidic toluidine blue staining (Figure 5). By contrast, the fraction of macrophage-lineage cells, indicated by alpha-naphthyl acetate esterase staining, increased significantly (Figure 6).

Figure 4.

IL-10 alters IL-3+SCF-induced differentiation. Bone marrow cells were cultured in IL-3+SCF with or without IL-10 for 21 days. Mast cell or monocyte/macrophage morphology was determined by Wright Giemsa (WG), acidic toluidine blue (TB) or non-specific esterase (NSE) staining. Black bar represents 10μM length. Solid arrowhead indicates mast cell morphology; open arrowhead indicates monocyte/macrophage morphology.

Figure 4.

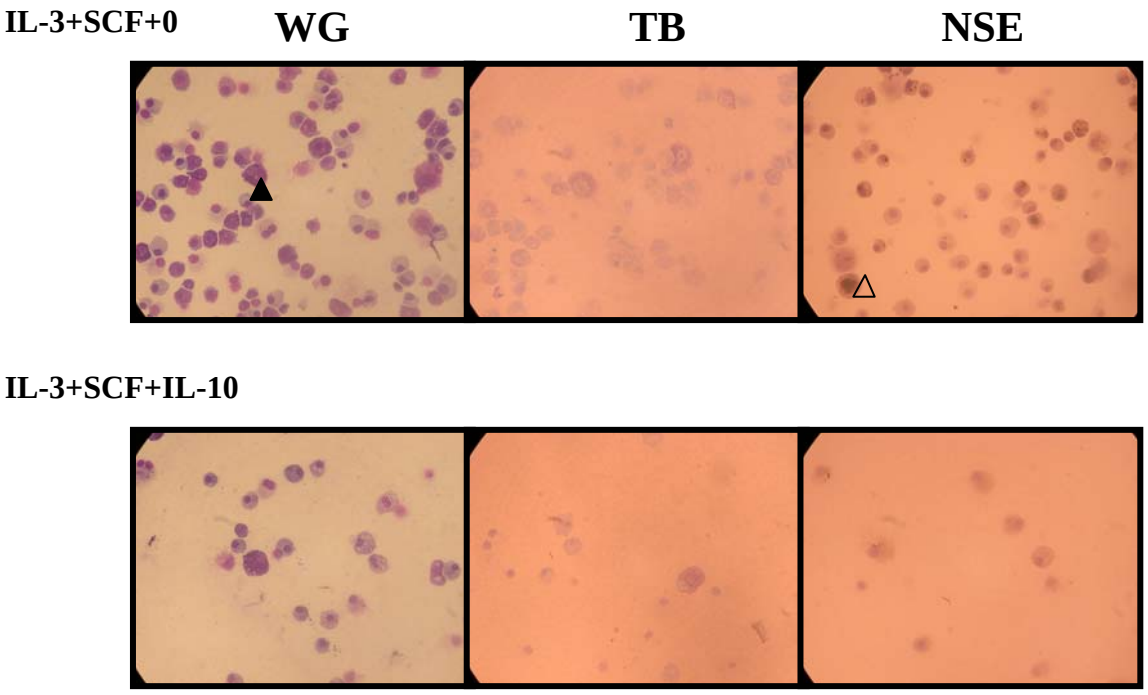


Figure 5.

Histochemical stains were assessed by 400X light microscopy. Slides were quantified by assessing WG stained slides for cells possessing mast cell morphology, which is depicted as percent mast cells. Percentages are based on at least 100 total counts from at least 3 random fields from at least 4 experiments. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$).

Figure 5.

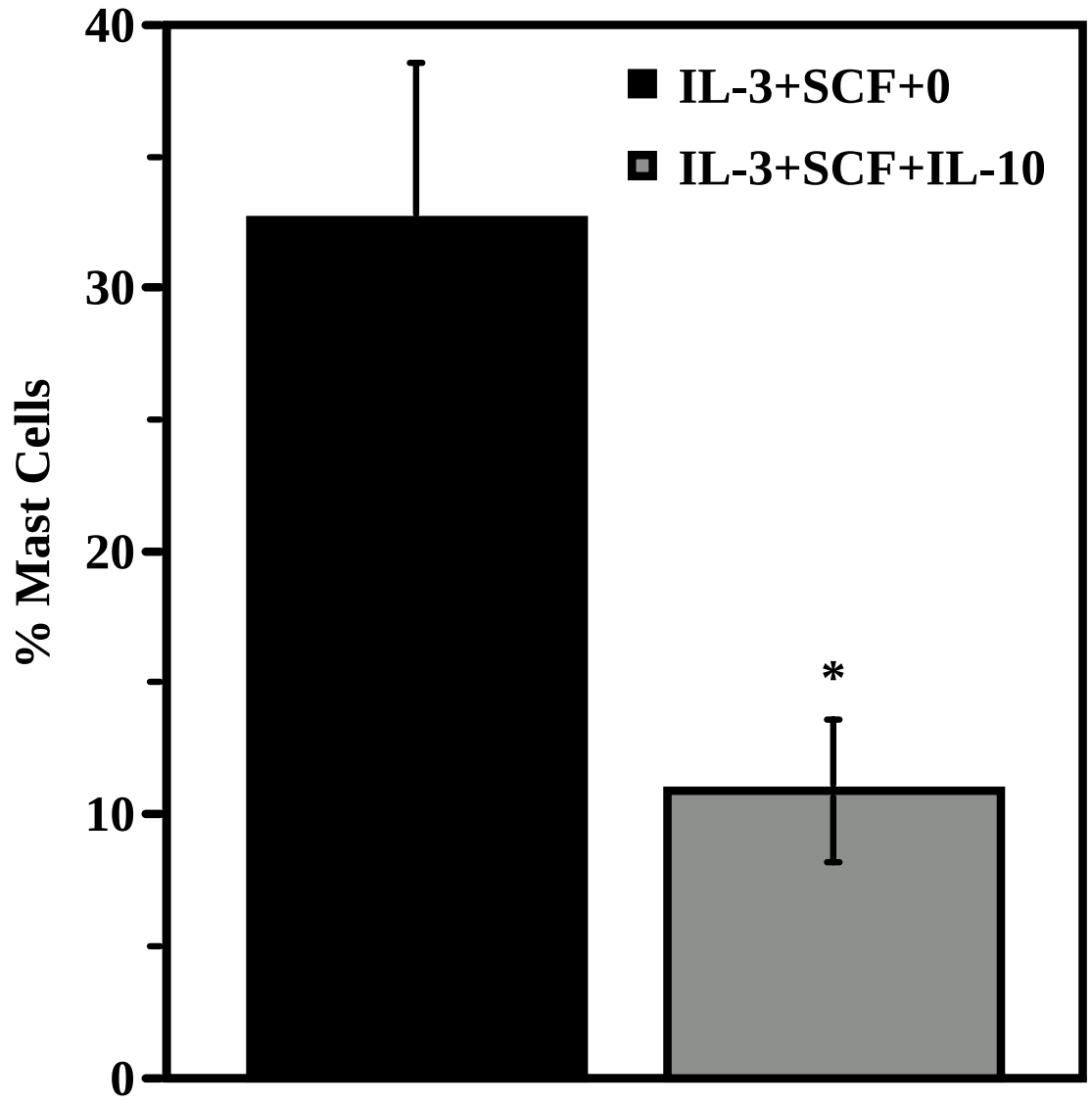
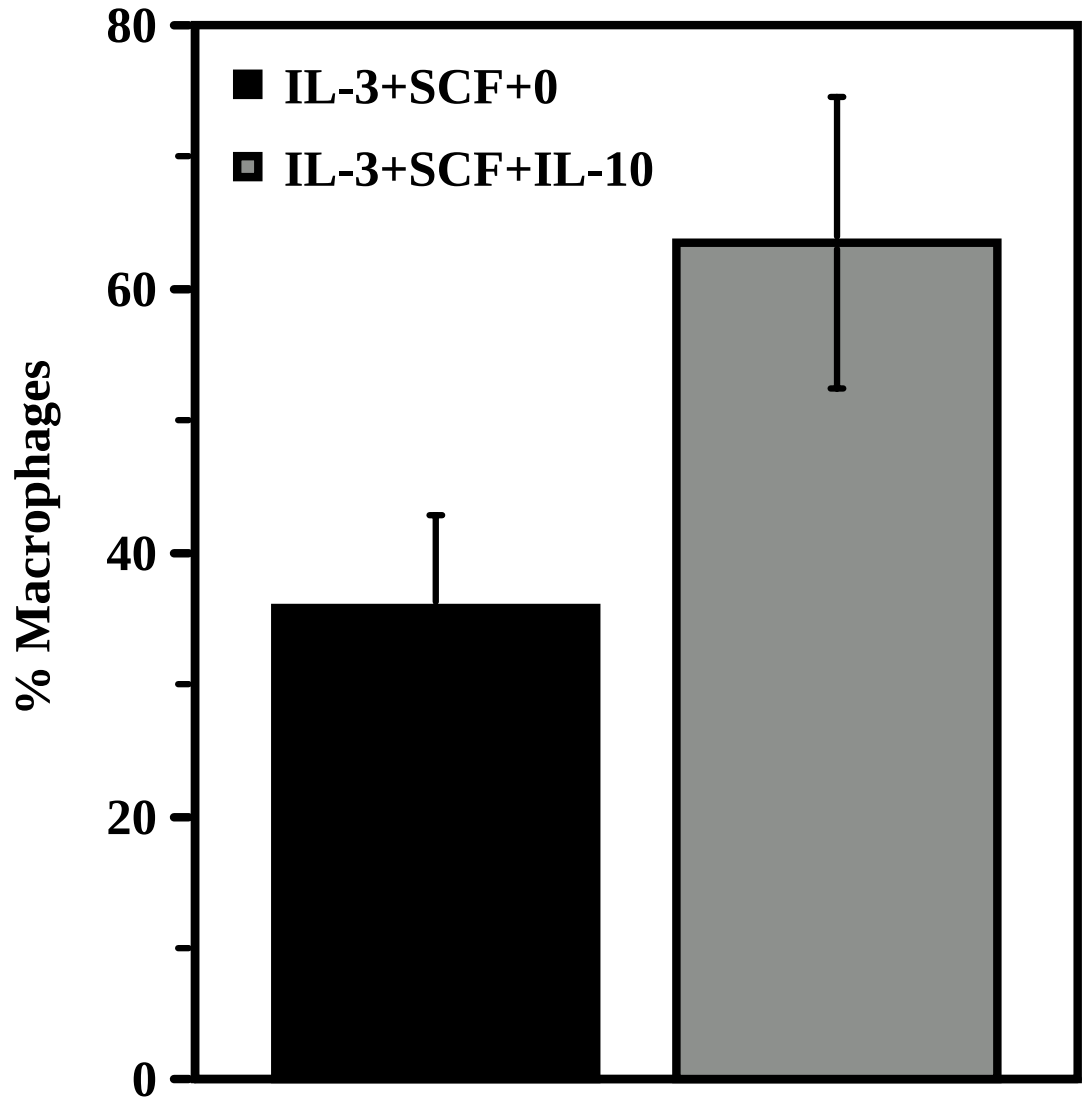


Figure 6.

Histochemial stainss were assessed by 400X light microscopy. Slides were quantified by assessing NSE slides for NSE-positive staining, which is depicted as percent macrophages. Percentages are based on at least 100 total counts from at least 3 random fields from at least 4 experiments. Statistical analysis was conducted using ANOVA pairwise comparison.

Figure 6.



To further characterize IL-10-induced changes in differentiation, the expression of cell surface markers was measured. Mast cells express the receptor tyrosine kinase Kit and the IL-1-related receptor T1/ST2. Early in our cultures (day 10), before a significant decrease in cell numbers occurred, the percentage of cells co-expressing Kit and T1/ST2 was not significantly altered by the presence of IL-10. However, by day 21, a point at which cell numbers were significantly reduced, IL-10 stimulation had greatly reduced the fraction of cells co-expressing Kit and T1/ST2 (Figure 7). Interestingly, IL-10 did not decrease the relative amount of Kit expression on Kit-positive cells (Figure 8), and actually increased T1/ST2 expression (Figure 9). In contrast to the effects on mast cell markers, IL-10 did not alter the percentage of cells expressing the monocyte/macrophage surface antigen CD11b (Mac-1) on either day 10 or 21 (Figure 10), and had no effect on the intensity of its expression (Figure 11).

Based on the combined histochemical and flow cytometric data, it appeared that within the population of surviving cells (approximately 25% of the starting population), IL-10 significantly reduced the proportion of mast cells and had little effect on macrophages derived by culture in IL-3+SCF. Rather, it had opposing effects on the final differentiation patterns of these cells, limiting the expression of toluidine blue-staining proteoglycans and mast cell surface antigens, but increasing macrophage esterase expression. Thus while the most overt effects of IL-10 were a decrease in live cells, this cytokine also affects cell development.

Figure 7.

IL-10 alters IL-3+SCF-induced suface antigen expression. Bone marrow cells were cultured in IL-3+SCF with or without IL-10 for 21 days. The percentage of mast cell surface antigens was determined by flow cytometric analysis, measuring expression of Kit and T1/ST2. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 4 separate experiments.

Figure 7.

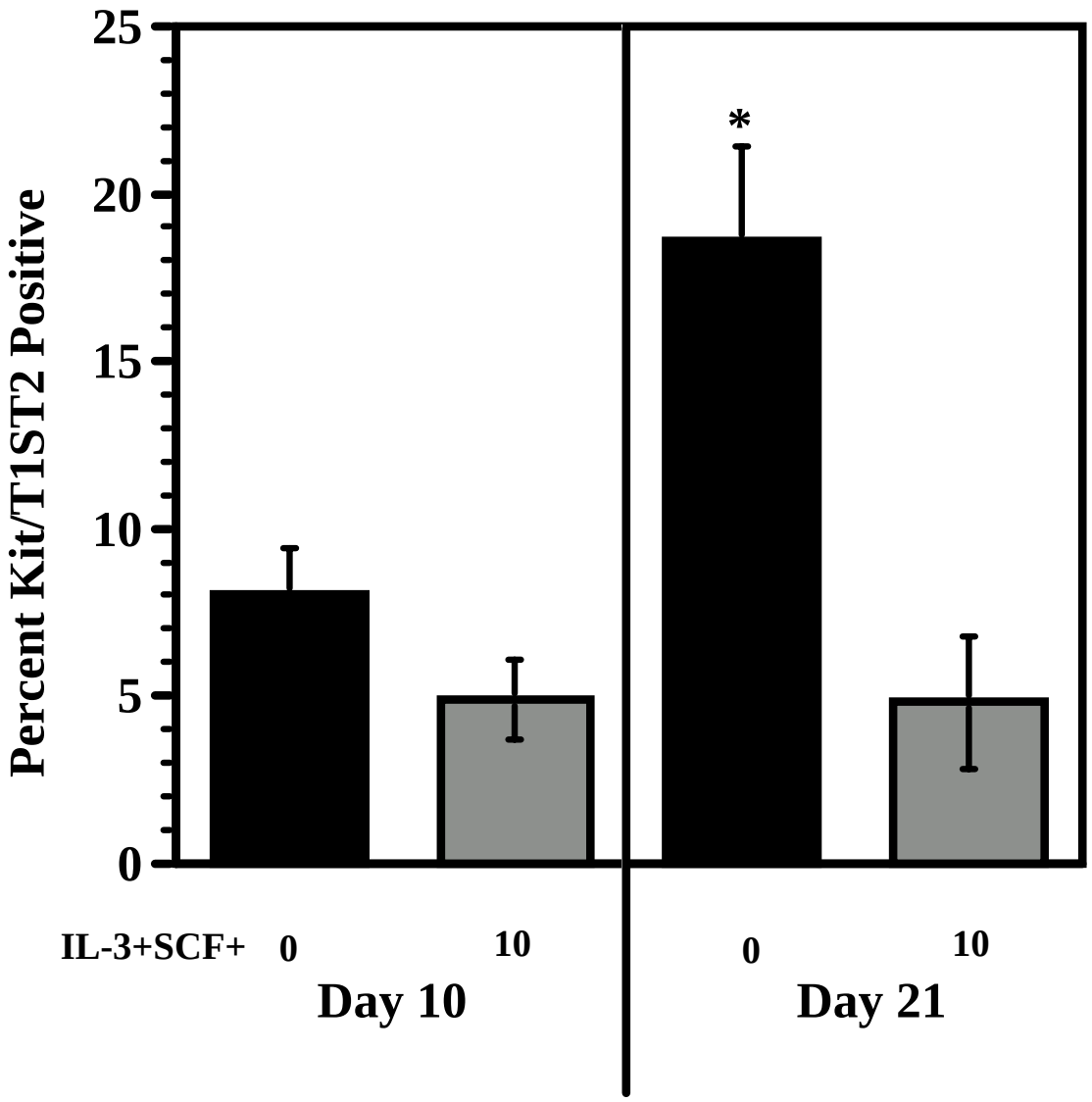

25
20
15
10
5
0
Percent Kit/T1ST2 Positive
*
IL-3+SCF+
0
10
0
10
Day 10
Day 21

Figure 8.

IL-10 alters IL-3+SCF-induced suface antigen expression. Bone marrow cells were cultured in IL-3+SCF with or without IL-10 for 21 days. Mean fluorescence intensity of the mast cell surface antigen Kit was determined by flow cytometric analysis. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 4 separate experiments.

Figure 8.

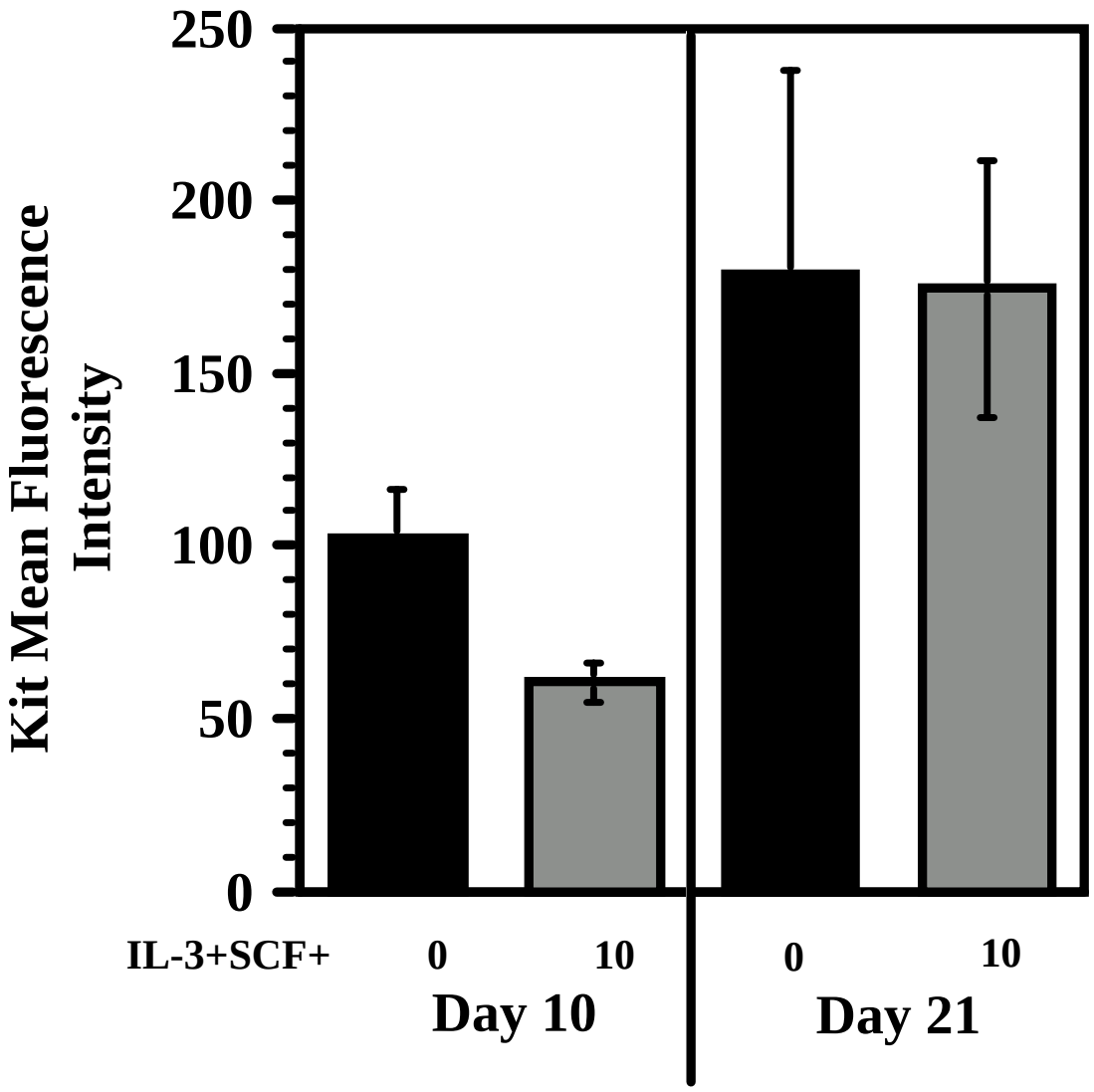

Kit Mean Fluorescence Intensity
250
200
150
100
50
0
IL-3+SCF+
0
10
0
10
Day 10
Day 21

Figure 9.

IL-10 alters IL-3+SCF-induced suface antigen expression. Bone marrow cells were cultured in IL-3+SCF with or without IL-10 for 21 days. Mean fluorescence intensity of the mast cell surface antigen T1ST2 was determined by flow cytometric analysis. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 4 separate experiments.

Figure 9.

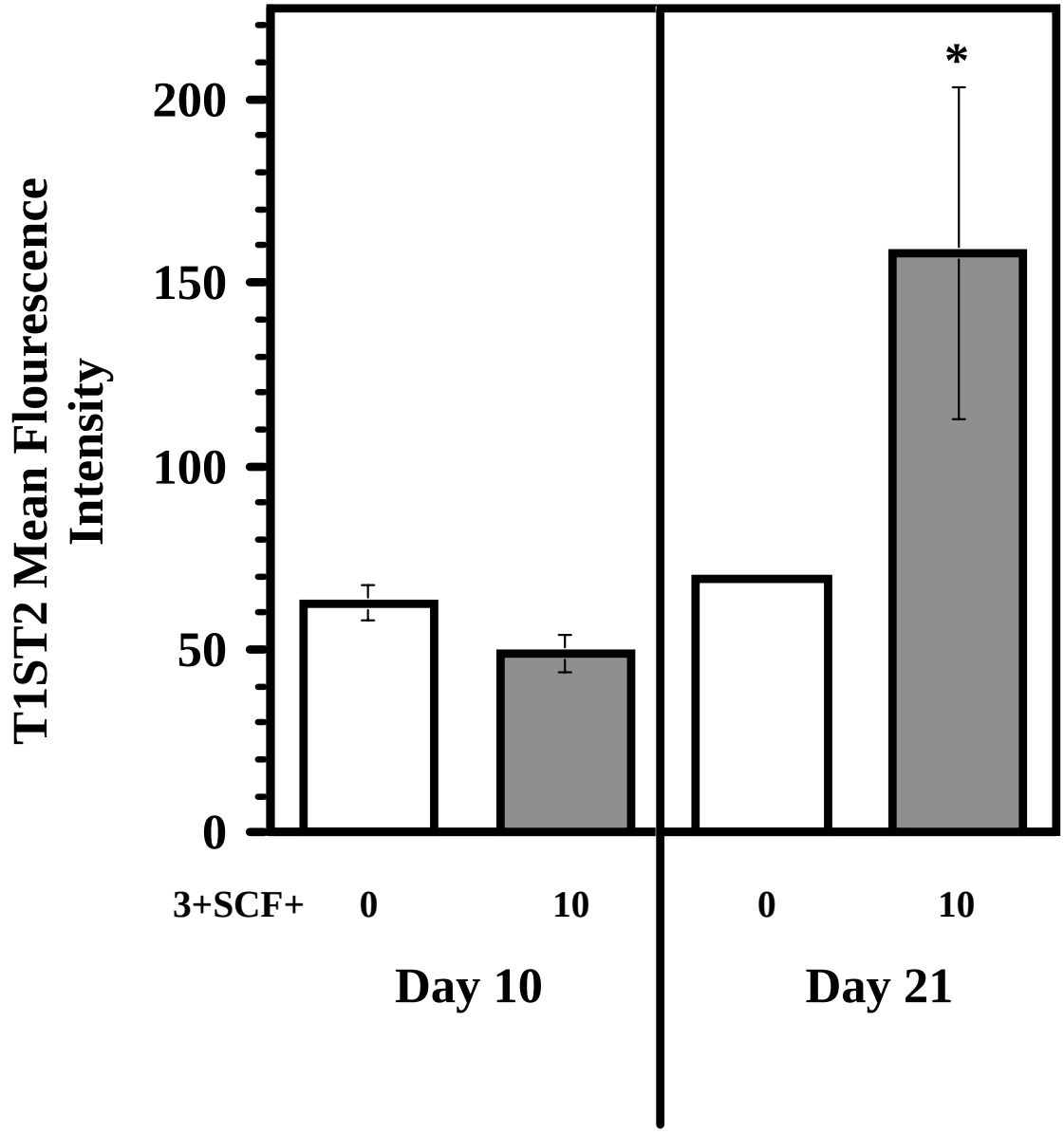

T1ST2 Mean Flourescence Intensity
200
150
100
50
0
*
3+SCF+
0
10
0
10
Day 10
Day 21

Figure 10.

IL-10 alters IL-3+SCF-induced suface antigen expression. Bone marrow cells were cultured in IL-3+SCF with or without IL-10 for 21 days. The percentage of macrophages was determined by flow cytometric analysis, measuring expression of CD11b (MAC-1). Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 4 separate experiments.

Figure 10.

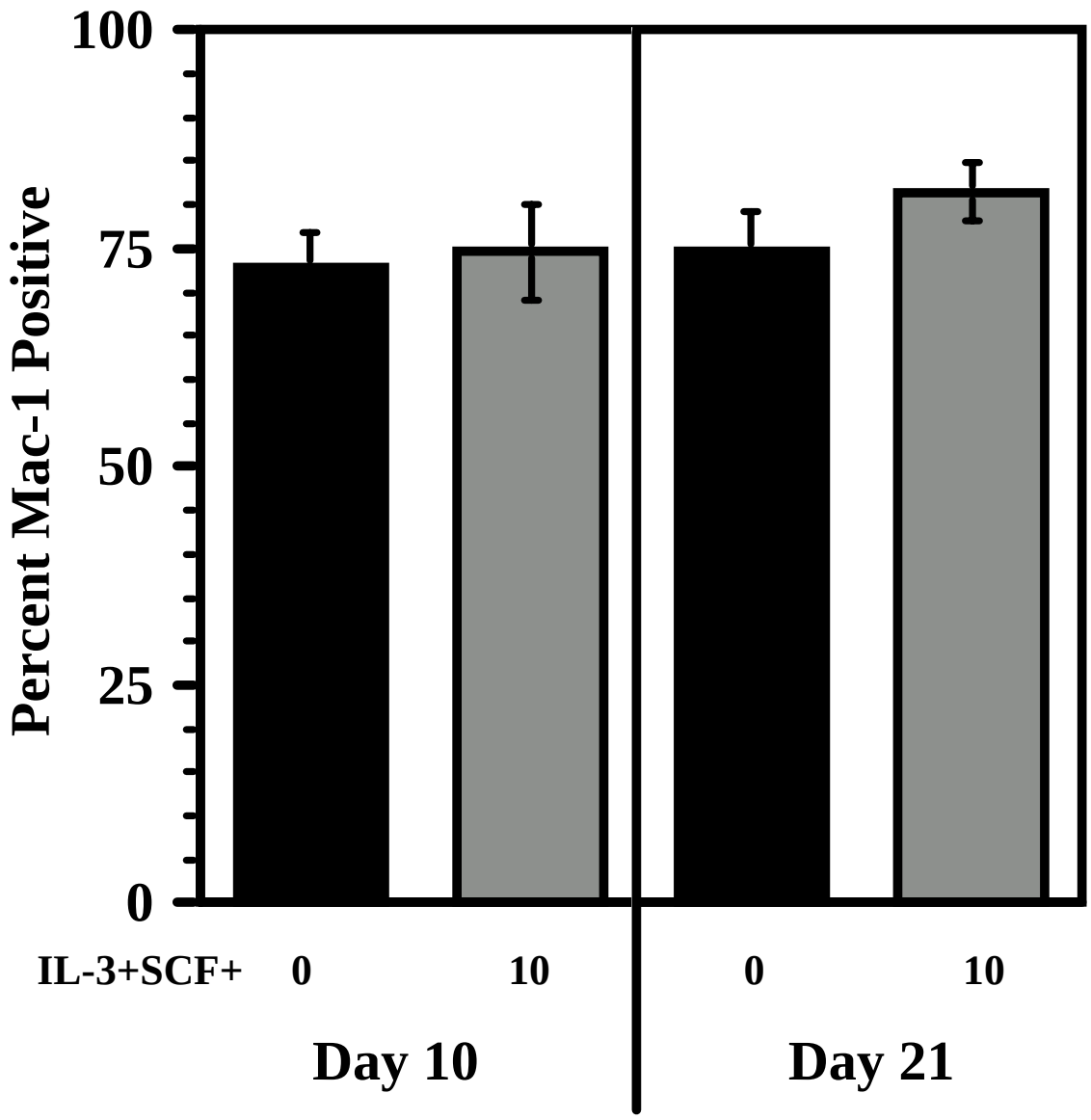
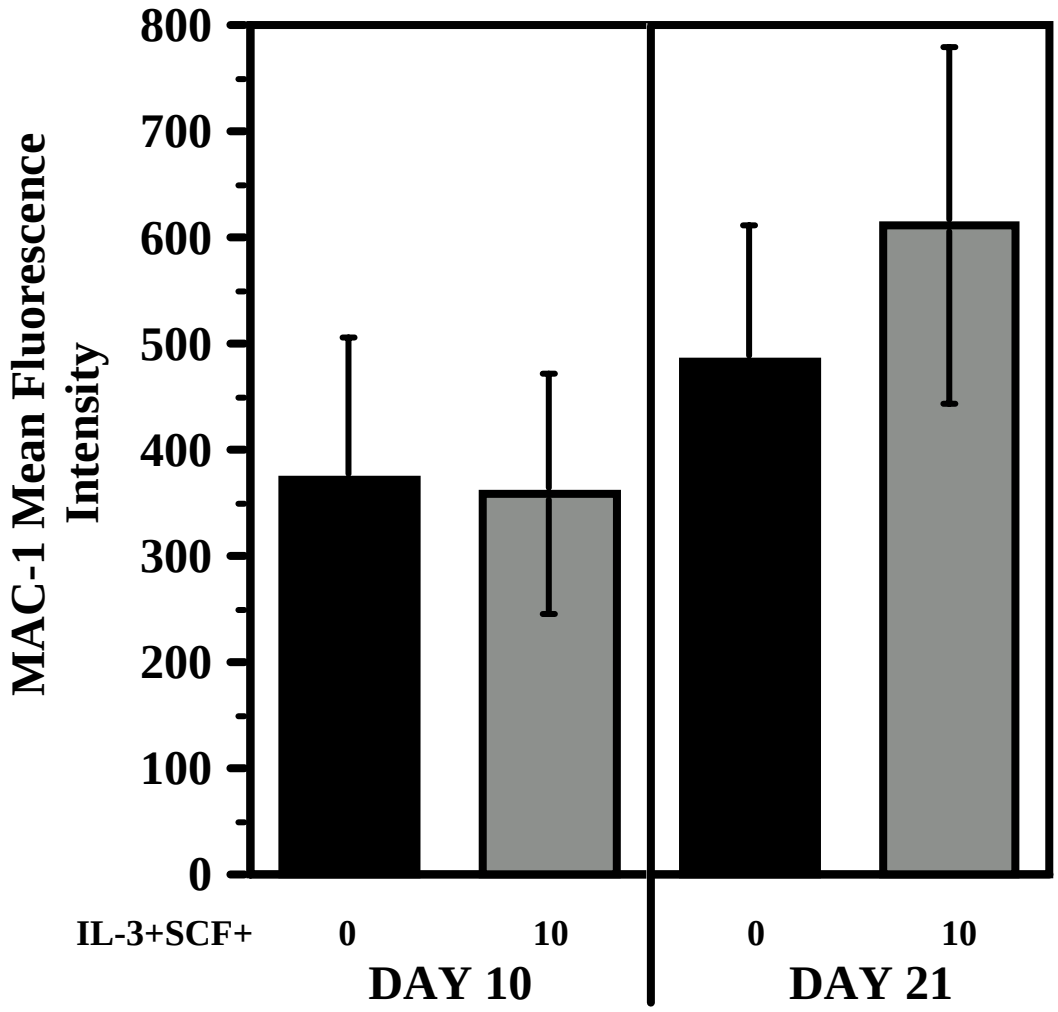

100
75
50
25
0
Percent Mac-1 Positive
IL-3+SCF+
0
10
0
10
Day 10
Day 21

Figure 11.

IL-10 alters IL-3+SCF-induced suface antigen expression. Bone marrow cells were cultured in IL-3+SCF with or without IL-10 for 21 days. Mean fluorescence intensity of the macrophage surface antigen MAC-1 was determined by flow cytometric analysis. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 4 separate experiments.

Figure 11.


MAC-1 Mean Fluorescence Intensity
800
700
600
500
400
300
200
100
0
IL-3+SCF+
0
10
0
10
DAY 10
DAY 21

Apoptosis is enhanced by the presence of IL-10

The decrease in cell numbers observed after IL-10 addition could have been the result of diminished proliferation or enhanced cell death. Propidium iodide (PI) DNA staining was performed to ascertain if an increase in sub-diploid DNA content correlated with the reduction in live cell numbers noted during days 10-21 (Figure 2). Sub-diploid DNA is representative of the DNA fragmentation associated with apoptosis (Figure 12). As seen in (Figure 13), IL-10 had no effect on apoptosis on days 0-10, but conveyed a sharp and significant increase to include 60% of the population between days 10 and 21. Activation of the death-effector enzyme caspase-3 immediately precedes the onset of apoptosis (Figure 14). We measured caspase-3 activation on day 15 and found significantly higher activity in cells that had been treated with IL-10 (Figure 15). These data show that the reduction in live cells numbers caused by IL-10 correlated with an increase in apoptosis.

Figure 12.

IL-10 increases bone marrow cell apoptosis. Example histogram of PI-DNA staining from samples harvested on day 21 of culture in IL-3+SCF (black line) or IL-3+SCF+IL-10 (gray line) are shown. Horizontal marker indicates sub-diploid (apoptotic) cells.

Figure 12.

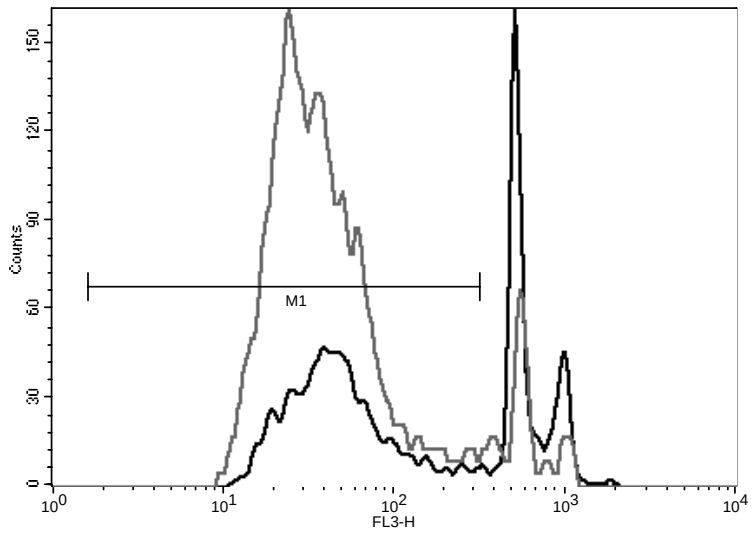


Figure 13.

IL-10 increases bone marrow cell apoptosis. PI-DNA staining Flowcytometric quantification of samples harvested on day 21 of culture in IL-3+SCF (black line) or IL-3+SCF+IL-10 (gray line) are shown. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 4 separate experiments.

Figure 13.

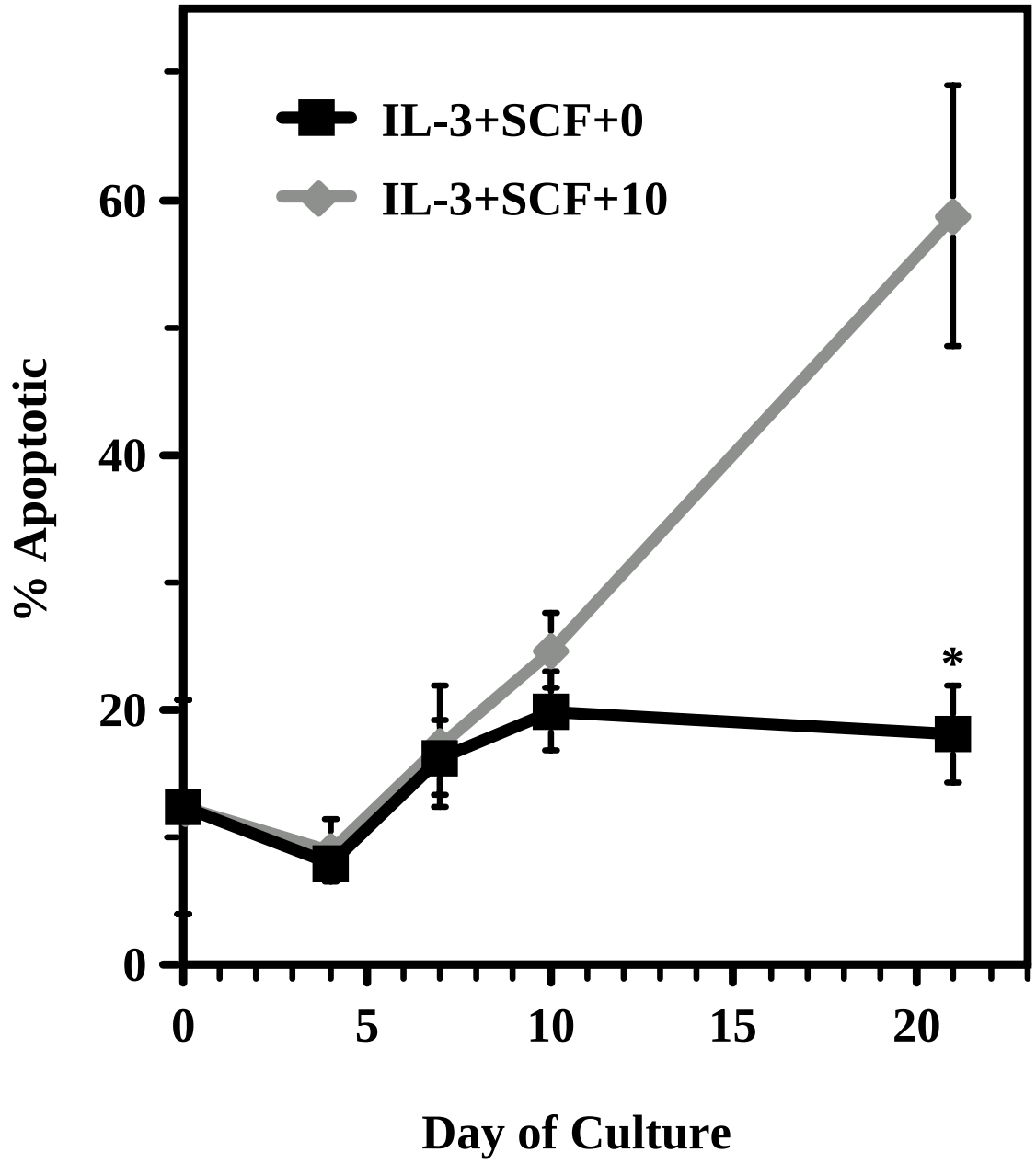

IL-3+SCF+0
IL-3+SCF+10
% Apoptotic
0
20
40
60
0
5
10
15
20
Day of Culture
*

Figure 14.

IL-10 enhances caspase-3 activation. Example histogram of caspase-3 staining from samples harvested on day 21 of culture in IL-3+SCF (black line) or IL-3+SCF+IL-10 (gray line) are shown. Horizontal marker indicates fraction of population with active caspase-3.

Figure 14.

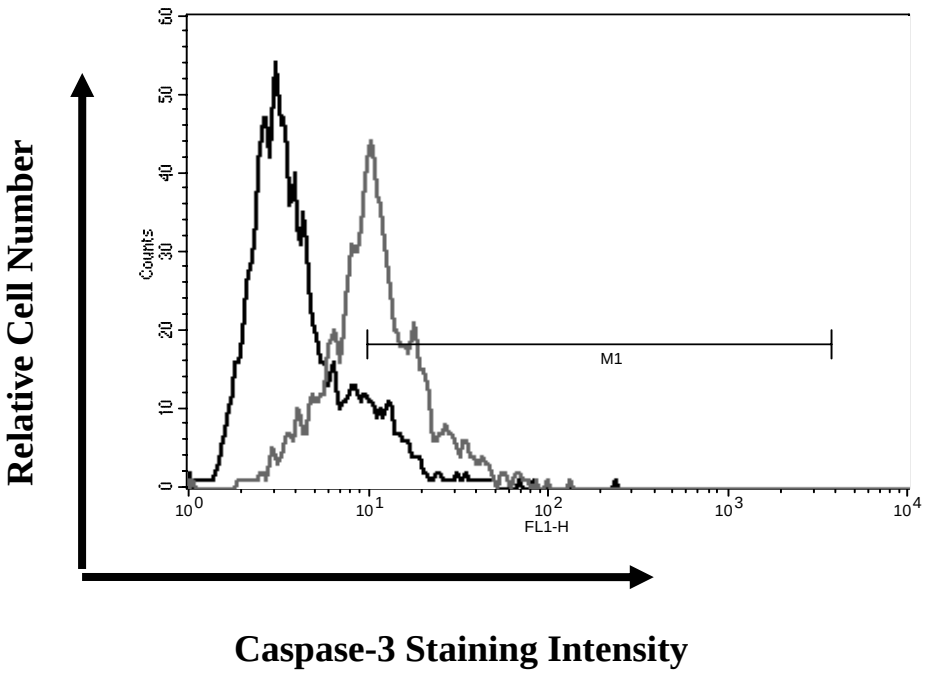
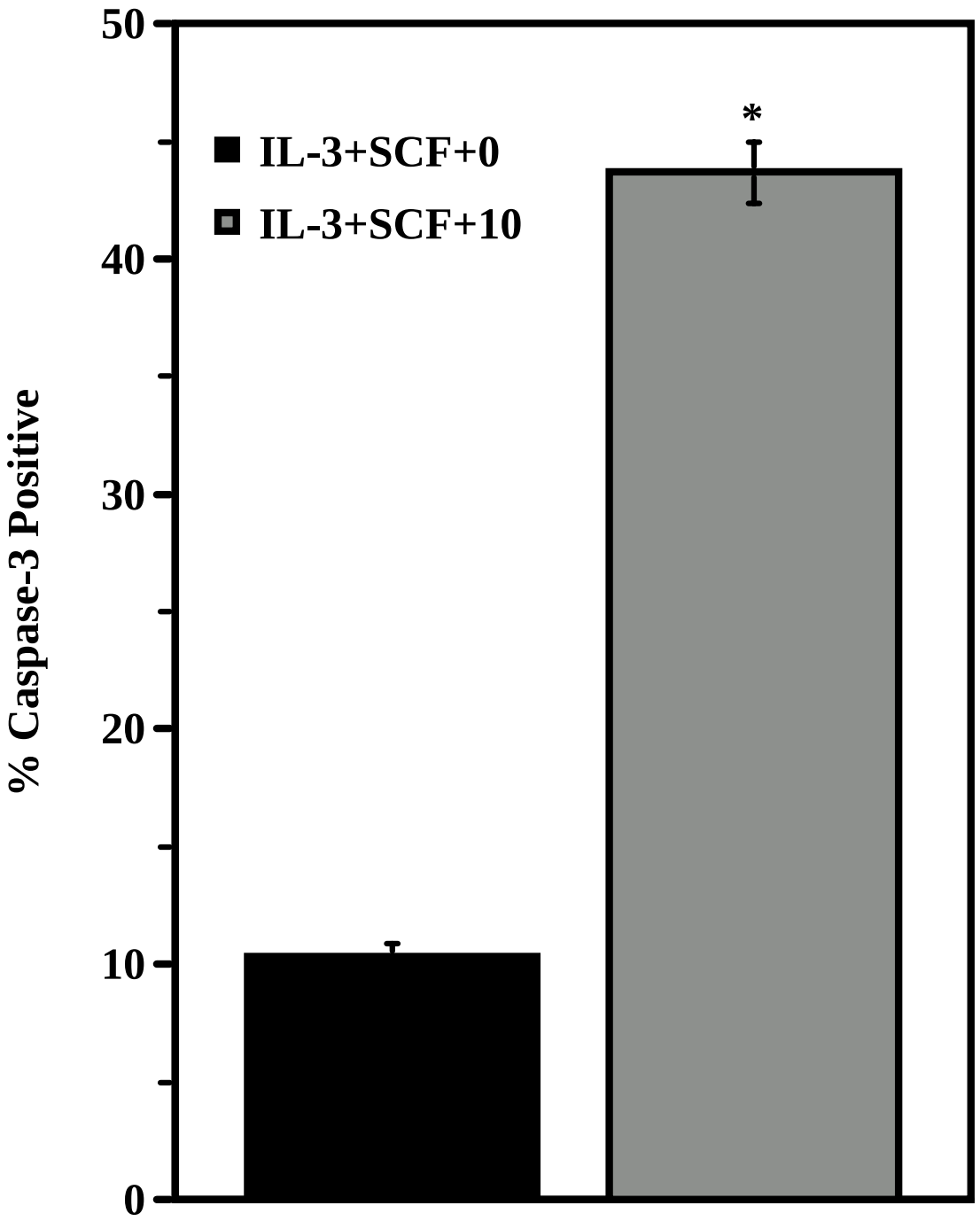

Relative Cell Number
Counts
0
10
20
30
40
50
60
M1
10^0
10^1
10^2
10^3
10^4
FL1-H
Caspase-3 Staining Intensity

Figure 15.

IL-10 enhances caspase-3 activation. Caspase-3 flowcytometric quantification of samples harvested on day 21 of culture in IL-3+SCF (black bar) or IL-3+SCF+IL-10 (gray bar) are shown. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 4 separate experiments.

Figure 15.



IL-10 induces changes in mitochondrial membrane stability

Changes in mitochondrial membrane potential ($\Delta\psi M$) can occur during apoptosis (Figure 16), often resulting from alterations in the Bcl-2 gene family expression or function [21]. IL-10-mediated changes in $\Delta\psi M$ were measured via $Di(OC_3)_6$ staining. As shown in (Figure 17), IL-10 altered the pattern of $Di(OC_3)_6$ staining, with evidence of both hypo- and hyper-polarization. Thus the presence of IL-10 resulted in disruption of $\Delta\psi M$.

We next employed bone marrow cells from transgenic and gene-deficient mice to determine the importance Bcl-2 family proteins and the p53 transcription factor that can alter their expression. Bone marrow cells from mice deficient in pro-apoptotic Bax or p53 expression, or from mice overexpressing anti-apoptotic Bcl-2 were cultured in IL-3+SCF +/- IL-10. Loss of Bax expression did not alter the IL-10-mediated decrease in cell numbers. By contrast, p53 deficiency or overexpression of Bcl-2 increased survival approximately 2-fold; however neither completely reversed the effects of IL-10 (Figure 18). These observations indicate that the inhibitory effects of IL-10 correlate with loss of normal mitochondrial membrane potential and can be partially corrected by increasing Bcl-2 expression or deleting p53.

Figure 16.

IL-10-mediated apoptosis involves a mitochondrial pathway. Representative histogram of changes in mitochondrial membrane potential were assessed by $Di(OC6)_3$ staining. The area marked M1 indicates hypopolarization and the area marked M2 indicates hyperpolarization.

Figure 16.

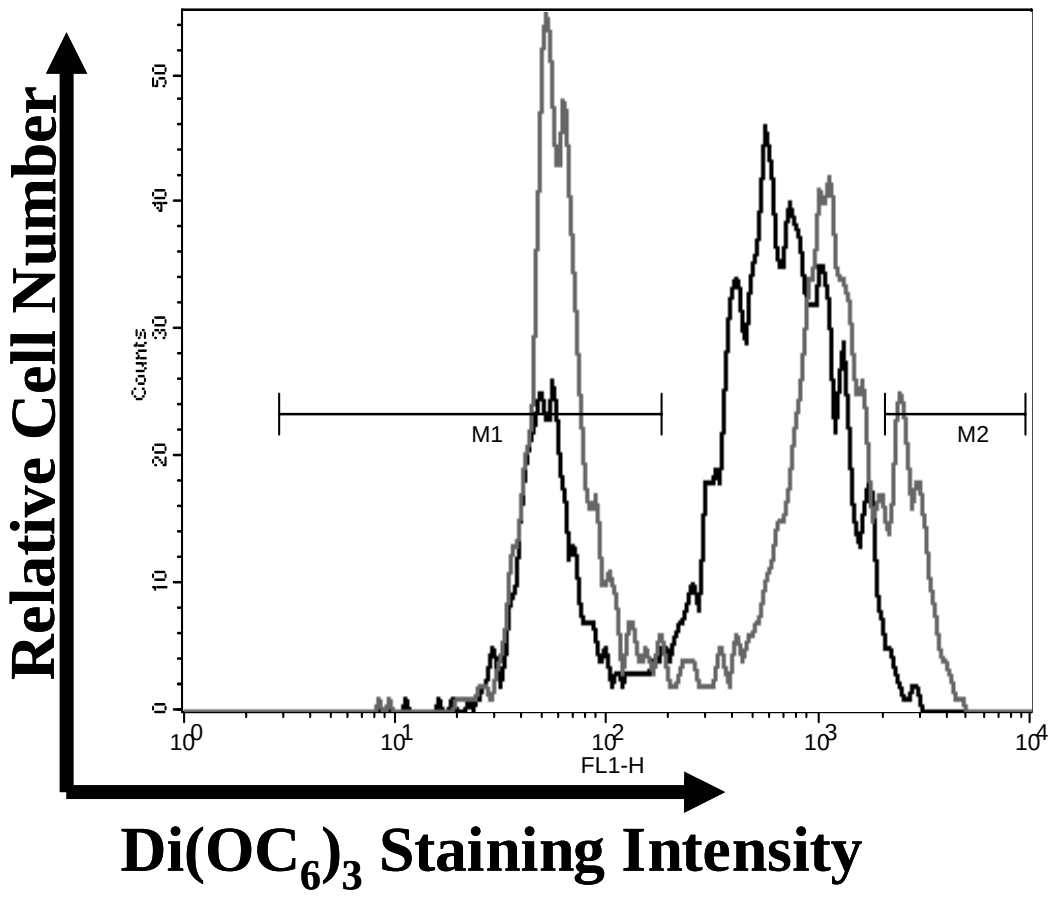

Relative Cell Number
Counts
0
10
20
30
40
50
M1
M2
10^0
10^1
10^2
10^3
10^4
FL1-H
Di(OC6)3 Staining Intensity

Figure 17.

IL-10-mediated apoptosis involves a mitochondrial pathway. Quantification of changes in mitochondrial membrane potential were assessed by $Di(OC6)_3$ staining. Quantification Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 17.

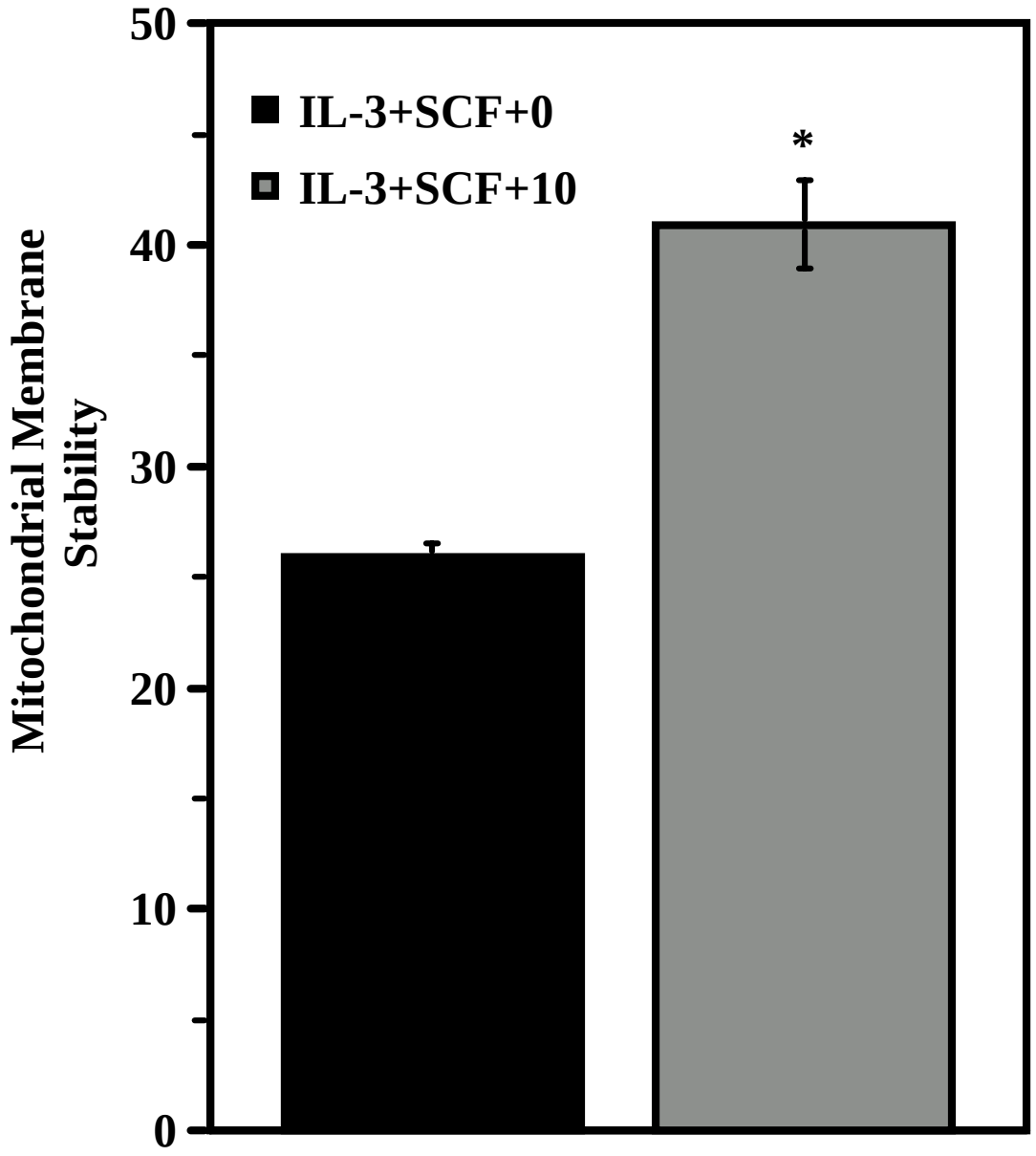
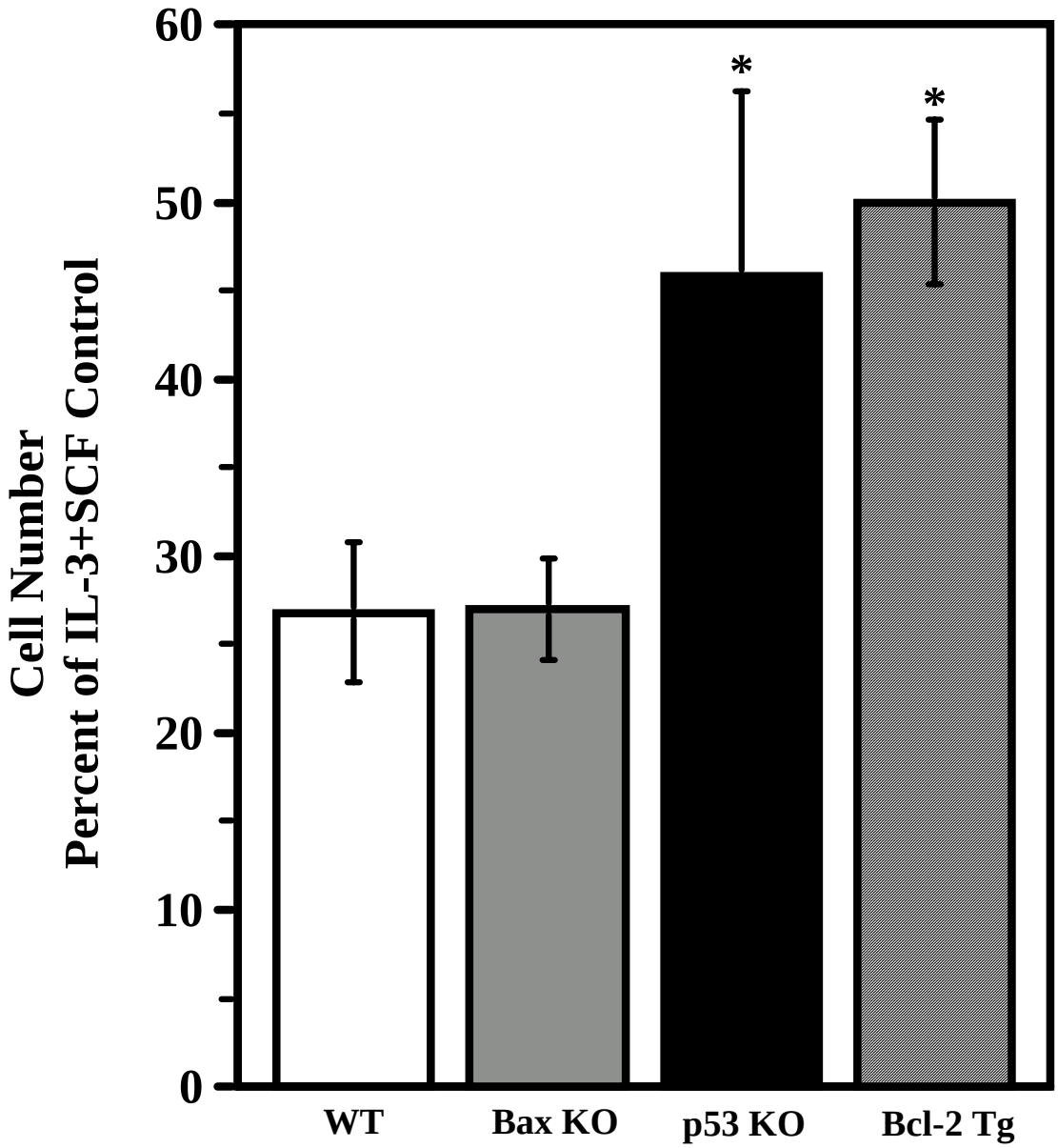

50
40
30
20
10
0
Mitochondrial Membrane Stability
IL-3+SCF+0
IL-3+SCF+10
*

Figure 18.

IL-10 induces apoptosis via a p53 and Bcl-2 dependent pathway. WT, Bax-deficient (Bax KO), p53-deficient (p53 KO) and H2K-Bcl-2 transgenic (Bcl-2 Tg) bone marrow cells were cultured for 21 days in IL-3+SCF +/- IL-10. Viable cell numbers were assessed on day 21 by timed flow cytometric counting, and calculated as a percentage of IL-3+SCF cultures. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 18.


Cell Number
Percent of IL-3+SCF Control
60
50
40
30
20
10
0
*
*
WT
Bax KO
p53 KO
Bcl-2 Tg

IL-10 inhibits IL-3 Receptor Expression

The growth factors IL-3 and SCF promote cell development and survival. Inhibition of growth factor signaling has been shown to result in p53-dependent apoptosis that proceeds through a mitochondrial pathway, also referred to as the "intrinsic" death pathway [21, 22]. Since IL-10 signaling resulted in an apoptotic response coincident with changes in $\Delta\psi M$, and was partially corrected by p53 deletion or Bcl-2 overexpression, we measured changes in IL-3 and Kit receptor expression during the onset of apoptosis. We chose day 15 of culture for these assays, as it was the earliest point at which changes in $\Delta\psi M$ were detected. Importantly, in these assays we cultured bone marrow with selection against adherent cells, resulting in purified mast cell cultures. This enabled us to measure changes in Kit and IL-3 receptor expression on a purified population expressing these receptors.

As we had found on day 21 using non-selected cells, IL-10 had no effect on either the total percentage of Kit-only expressing cells or Kit expression levels (Figure 19, 20). In contrast, IL-10 diminished the expression intensity of the IL-3R beta subunit by more than 60%, without altering the percentage of cells expressing this protein (Figure 21,22). Thus IL-10 appeared to selectively target the IL-3 receptor beta subunit, decreasing expression of this survival receptor in a manner that correlated with apoptosis.

Figure 19.

The effect of IL-10 on growth factor receptor surface expression. Bone marrow cells were cultured in IL-3+SCF +/- IL-10, with selection against adherent cells. Percentage of Kit-positive cells measured by flow cytometric staining with anti-Kit. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 19.

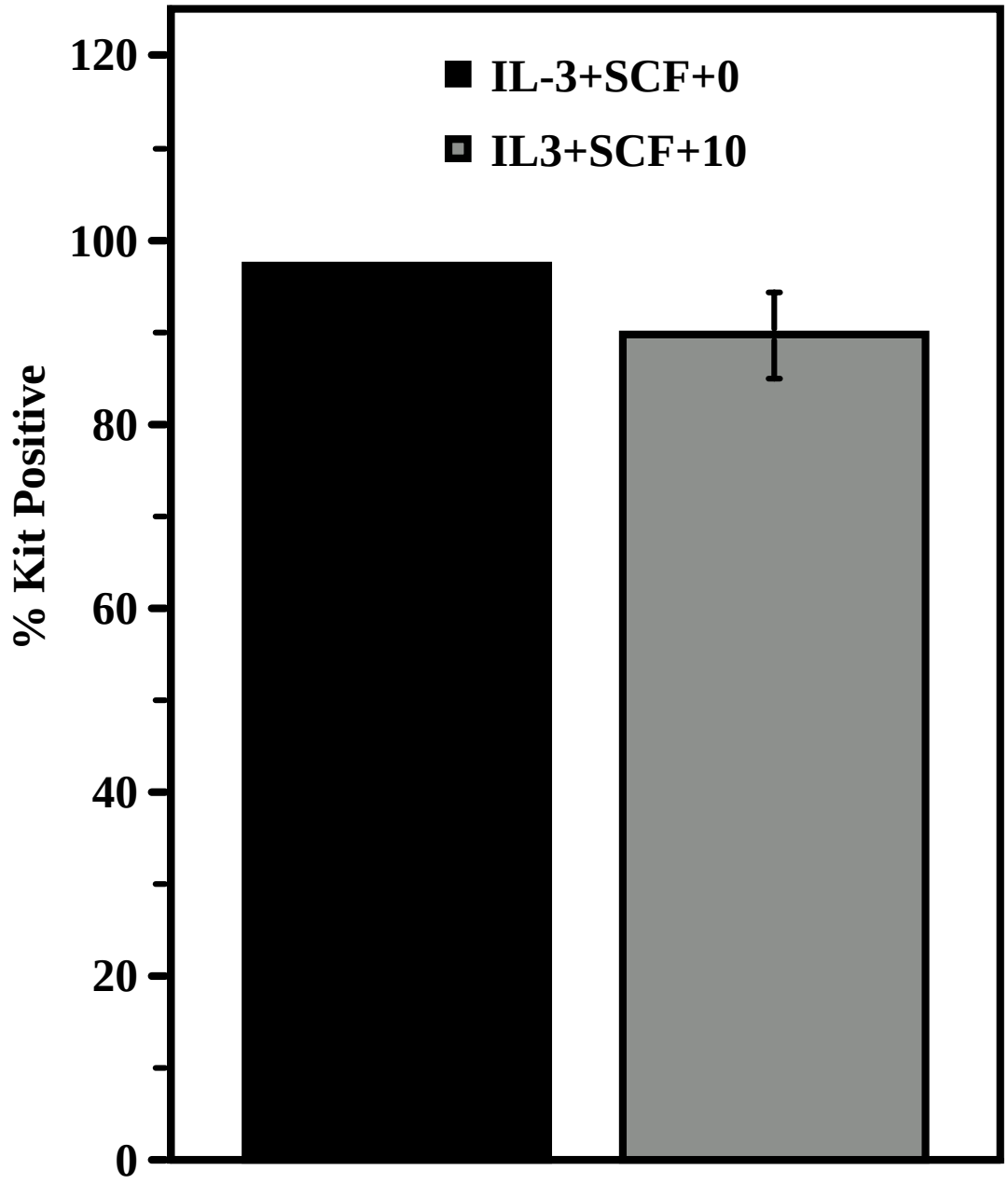


Figure 20.

IL-10 alters surface expression of growth factor receptors. Bone marrow cells were cultured in IL-3+SCF +/- IL-10, with selection against adherent cells. Expression intensity of Kit-positive cells measured by flow cytometric staining with anti-Kit. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 20.

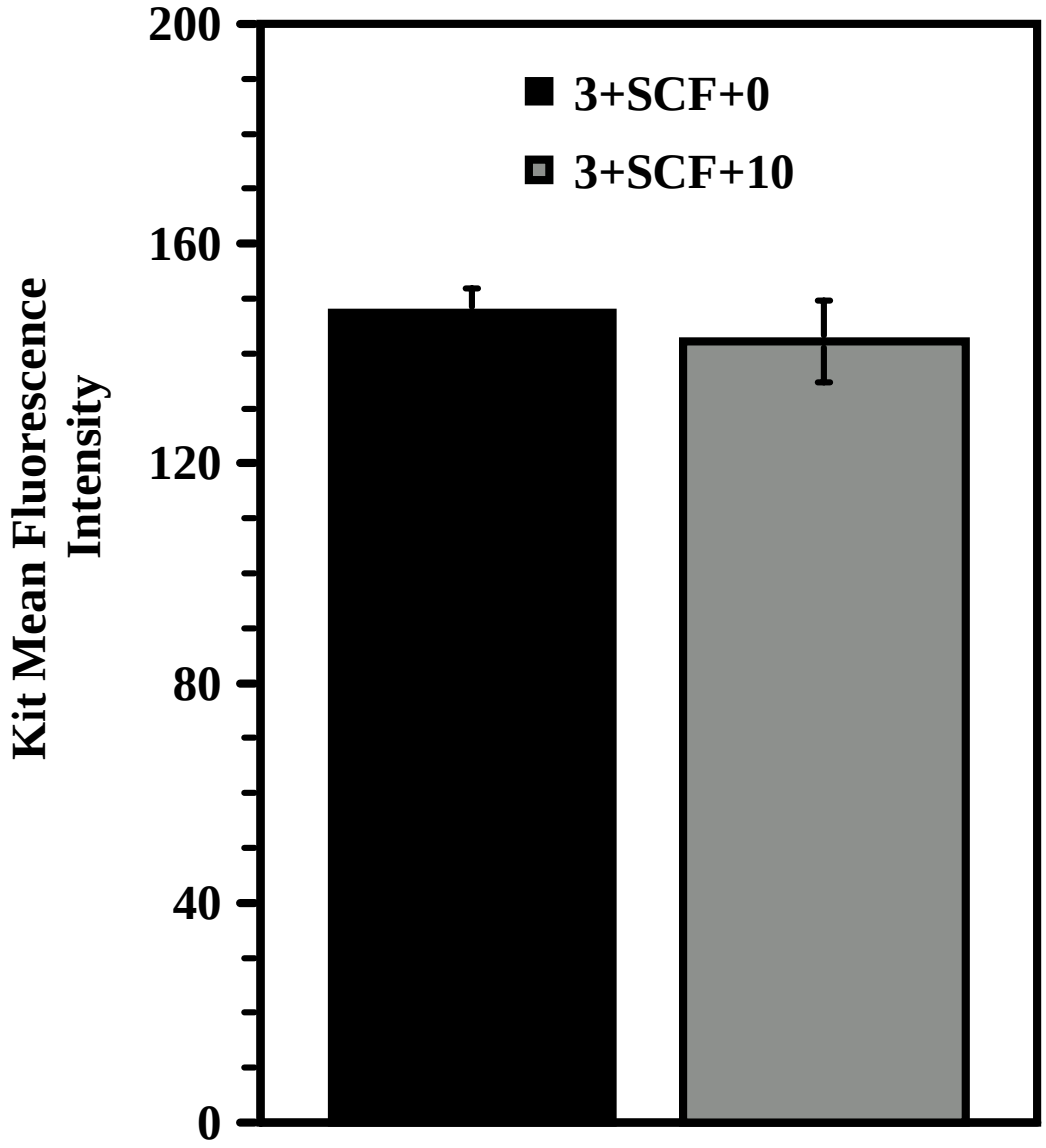

200
160
120
80
40
0
Kit Mean Fluorescence Intensity
3+SCF+0
3+SCF+10

Figure 21.

IL-10 alters surface expression of growth factor receptors. Percentage of IL-3 receptor-beta positive cells measured by flow cytometric staining with anti-IL-3 receptor beta . Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate

experiments.

Figure 21.

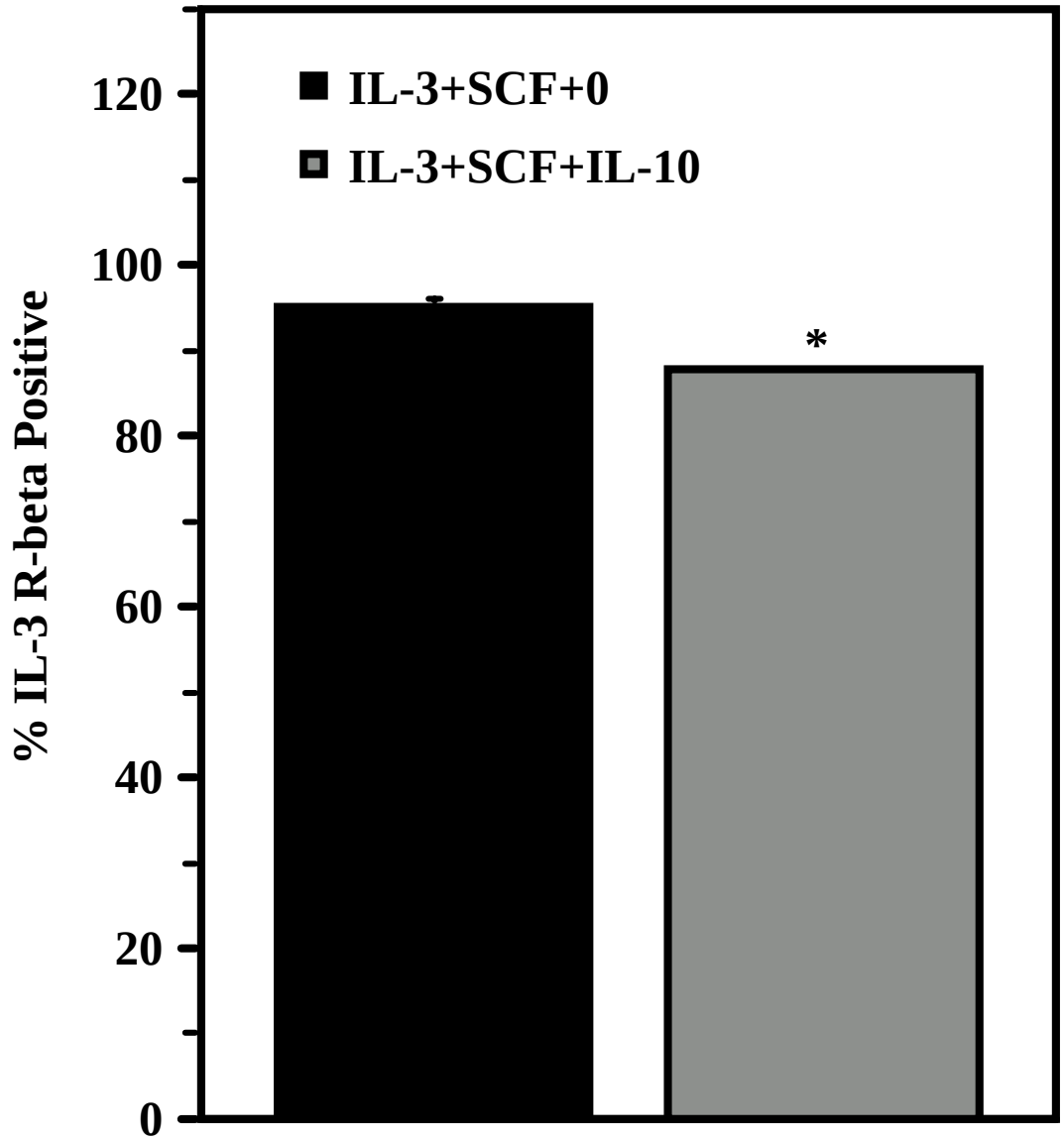
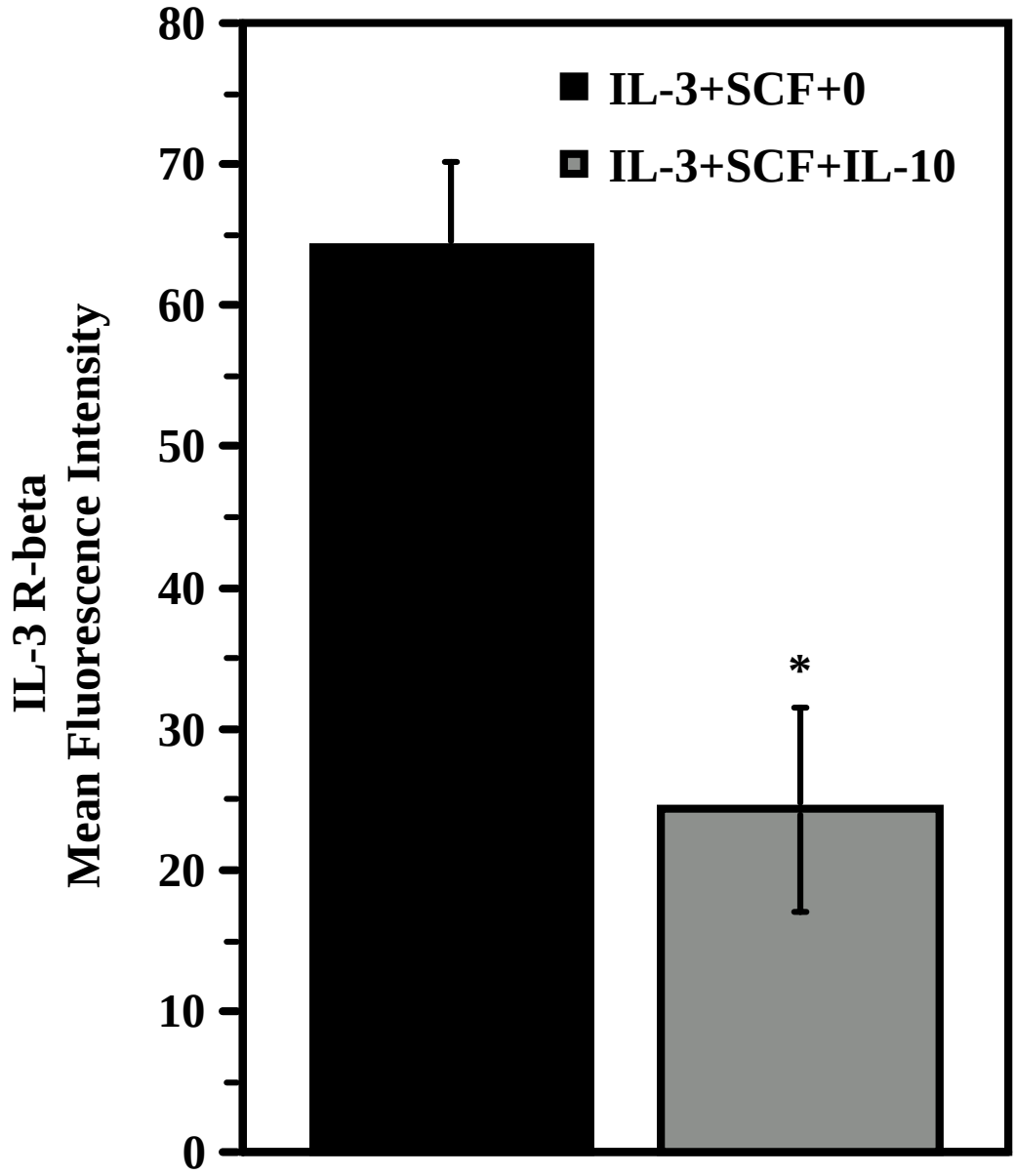


Figure 22.

Expression level of IL-3 receptor-beta measured by flow cytometric staining with anti-IL-3 receptor beta . Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 22.


80
70
60
50
40
30
20
10
0
IL-3 R-beta
Mean Fluorescence Intensity
IL-3+SCF+0
IL-3+SCF+IL-10
*

IL-3 and SCF responsiveness is diminished in cells that have been cultured in IL-10

Activation of the intrinsic, mitochondrial apoptotic pathway can be triggered by loss of growth factor receptor function. Since changes in survival signaling can occur with or without alterations in receptor expression, it was essential that we determine if IL-10 disrupted IL-3 and SCF-mediated survival. To measure these effects, we employed short term survival assays. Bone marrow cells were cultured for 21 days in IL-3+SCF +/- IL-10, after which equal numbers of cells were washed and re-plated in decreasing concentrations of IL-3 or SCF for 4 days. The percentage of surviving and apoptotic cells was judged by the presence of sub-diploid DNA, as detected by PI-DNA staining. As shown in (Figure 23, 24), cells that had previously been cultured in IL-10 were significantly less responsive to IL-3 or SCF in these assays. Under conditions where 55-70% of the cells previously cultured in IL-3+SCF survived in IL-3 or SCF alone, cells previously stimulated with IL-10 showed survival rates of 20%. These data indicated that IL-10 stimulation inhibited survival signaling by both the Kit and IL-3 receptor complexes, resulting in a factor-withdrawal response that is consistent with the intrinsic, mitochondrial apoptotic cascade we have observed.

Figure 23.

Cells cultured in IL-10 show diminished survival in response to IL-3. Equal numbers of cells cultured in IL-3+SCF +/- IL-10 for 21 days were washed and re-plated in decreasing concentrations of IL-3 for 4 days. After PI-DNA staining, viable cell numbers were measured by timed flow cytometric counting. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 23.

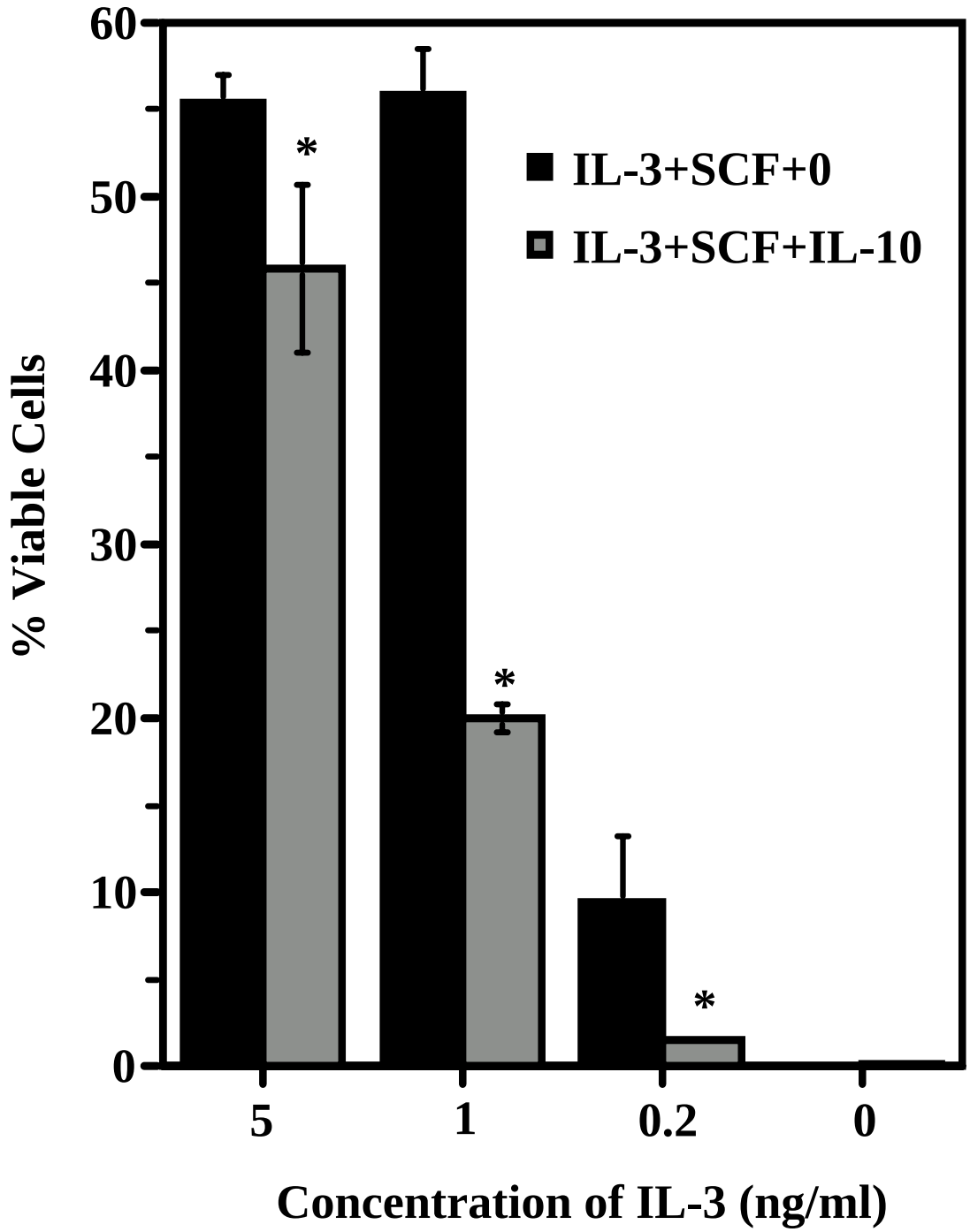
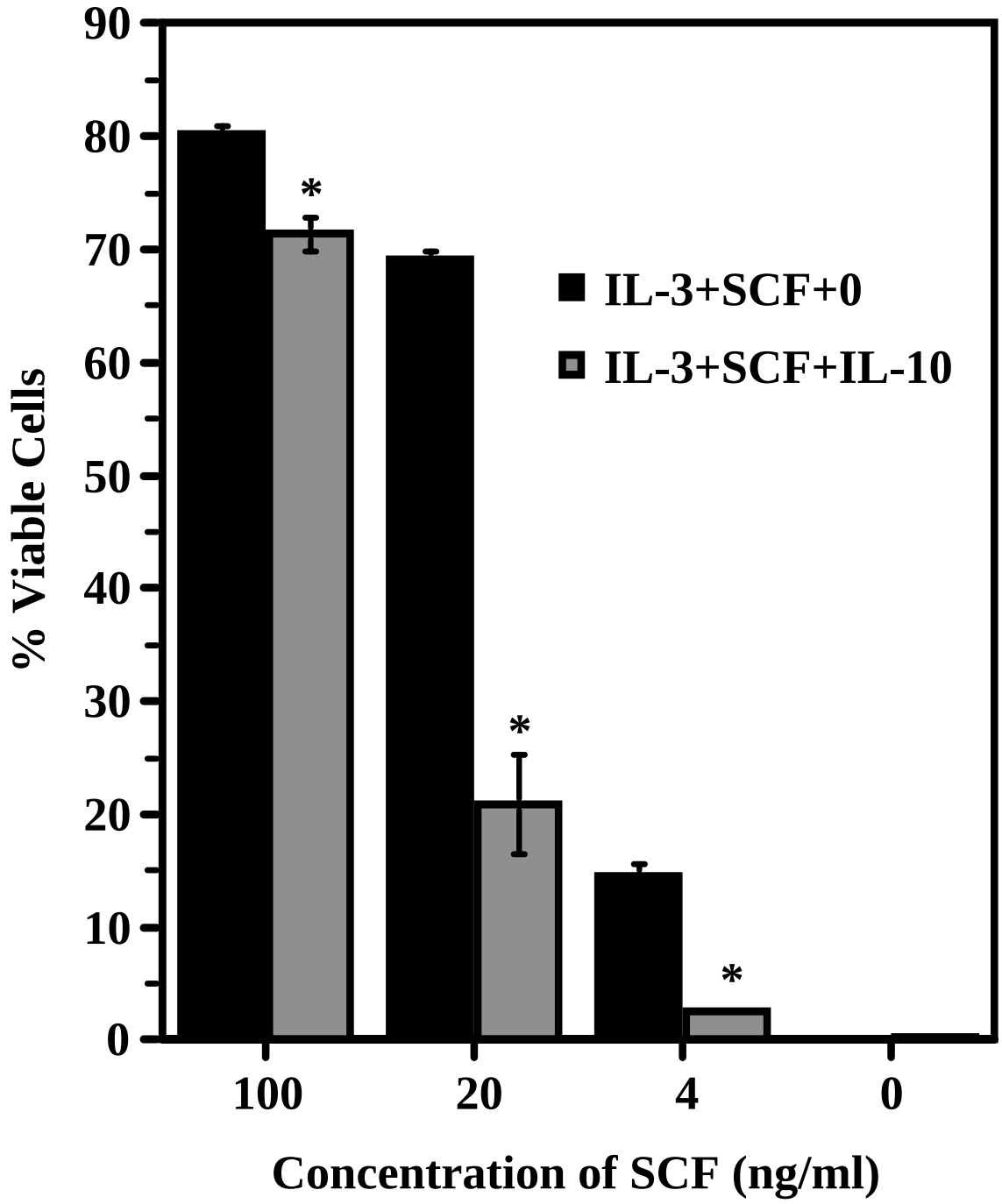

60
50
40
30
20
10
0
% Viable Cells
IL-3+SCF+0
IL-3+SCF+IL-10
*
*
*
5
1
0.2
0
Concentration of IL-3 (ng/ml)

Figure 24.

Cells cultured in IL-10 show diminished survival in response to SCF. Equal numbers of cells cultured in IL-3+SCF +/- IL-10 for 21 days were washed and re-plated in decreasing concentrations of SCF for 4 days. After PI-DNA staining, viable cell numbers were measured by timed flow cytometric counting. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 24.


90
80
70
60
50
40
30
20
10
0
% Viable Cells
*
*
*
IL-3+SCF+0
IL-3+SCF+IL-10
100
20
4
0
Concentration of SCF (ng/ml)

STAT3 is necessary for IL-10 mediated apoptosis

STAT3 is essential for IL-10 receptor signaling [18, 19] STAT3-deficient mice die during embryogenesis [18], precluding their use in our assays of mast cell and macrophage survival. To study the importance of STAT3 in IL-10-mediated apoptosis we employed a lineage-restricted cre/lox system in which STAT3 is deleted in the macrophage lineage by lysozyme M-driven expression of the cre recombinase [19) Bone marrow from these mice and their littermate controls were cultured in M-CSF +/- IL-10, and viable cells numbers were measured by PI-DNA staining and timed flow cytometric counting on day 7 of culture. Like its effects on IL-3+SCF-mediated survival, IL-10 significantly reduced the number of wild type cells cultured in M-CSF. However, STAT3 deletion completely reversed this effect. In STAT3-deficient cultures, IL-10 induced a 2-fold increase in viable cell numbers (Figure 25). We confirmed the loss of STAT3 expression in these cultured cells by western blotting (Figure26). These data indicate that STAT3 expression is required for the death-inducing effects of IL-10 on developing bone marrow cells.

Figure 25.

STAT3 deletion increases bone marrow cell survival. Utilizing a Cre/Lox System, STAT3 was deleted in cells of the monocyte/macrophage lineage. Bone marrow was cultured for 7 days in M-CSF +/- IL-10 and cell numbers were measured by timed flow cytometric analysis. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 3 separate experiments.

Figure 25.

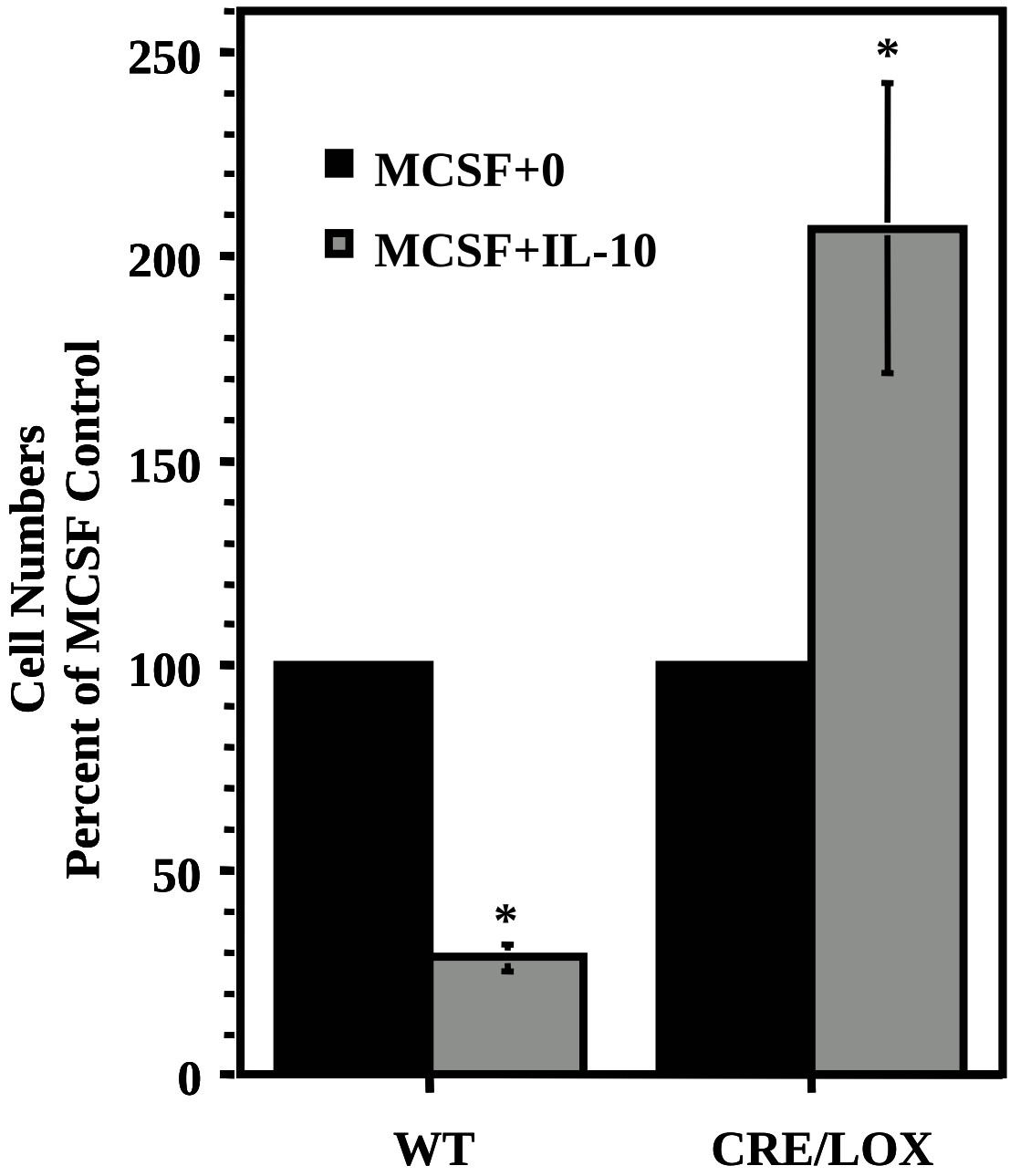
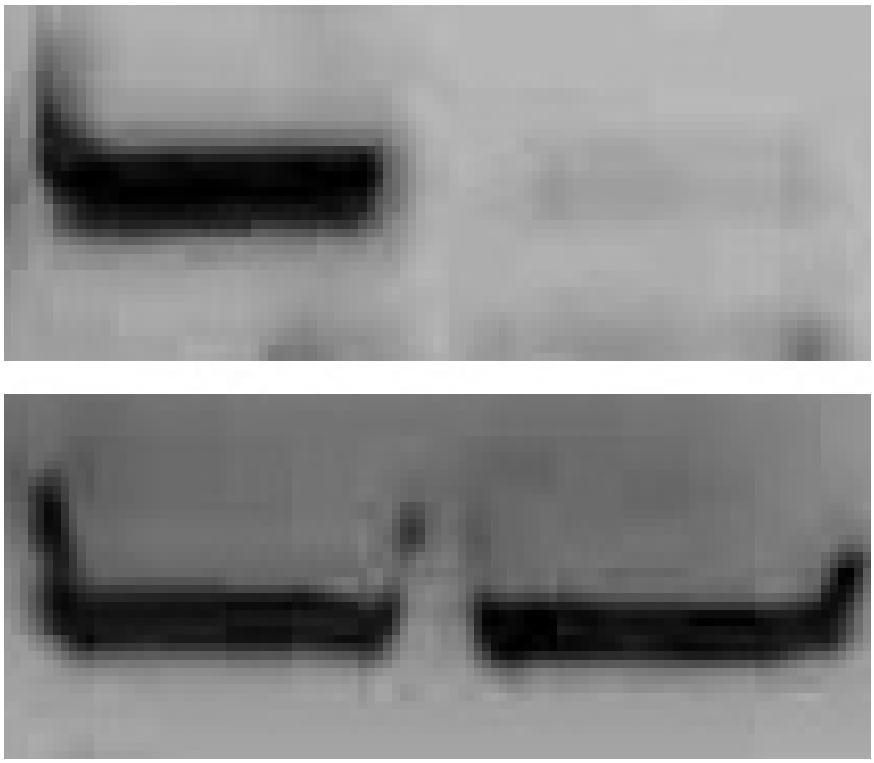

250
200
150
100
50
0
Cell Numbers
Percent of MCSF Control
MCSF+0
MCSF+IL-10
*
*
WT
CRE/LOX

Figure 26.

Western blot showing deletion of STAT3 protein in STAT3 flox/-;

lysMcre cells.

Figure 26.



IL-4 augments IL-10 mediated apoptosis

Previous findings by our laboratory have shown that the Th2 cytokine IL-4 can also inhibit mast cell development from hematopoietic stem cells (Bailey et al). We sought to determine if the addition of IL-4 to cultures of IL-10 caused any biologically significant effect on IL-10 mediated apoptosis. When whole bone marrow was cultured in the presence of IL-10+IL-4 we observed a faster and more significant decrease in viable cell numbers as compared to when cultured with only IL-10 (Figure 27). Apoptosis was also induced earlier when IL-4 was added to these cultures (Figure 28). These findings indicate that IL-4 augments the inhibitory effects of IL-10. In our previous study of the effects of IL-4 on mast cell development we found that STAT6 was both necessary and sufficient in mediating IL-4 induced apoptosis. The combined signaling of both STAT3 and STAT6 may enhance the induction of apoptosis.

Figure 27.

Time course of IL-4+IL-10 effects. Bone marrow cells were cultured in IL-3+SCF +/- IL-4, IL-10, or IL-4+IL-10 at 10 ng/ml. Viable cell numbers were determined on the indicated days by timed flow cytometric counting. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 5 separate experiments

Figure 27.

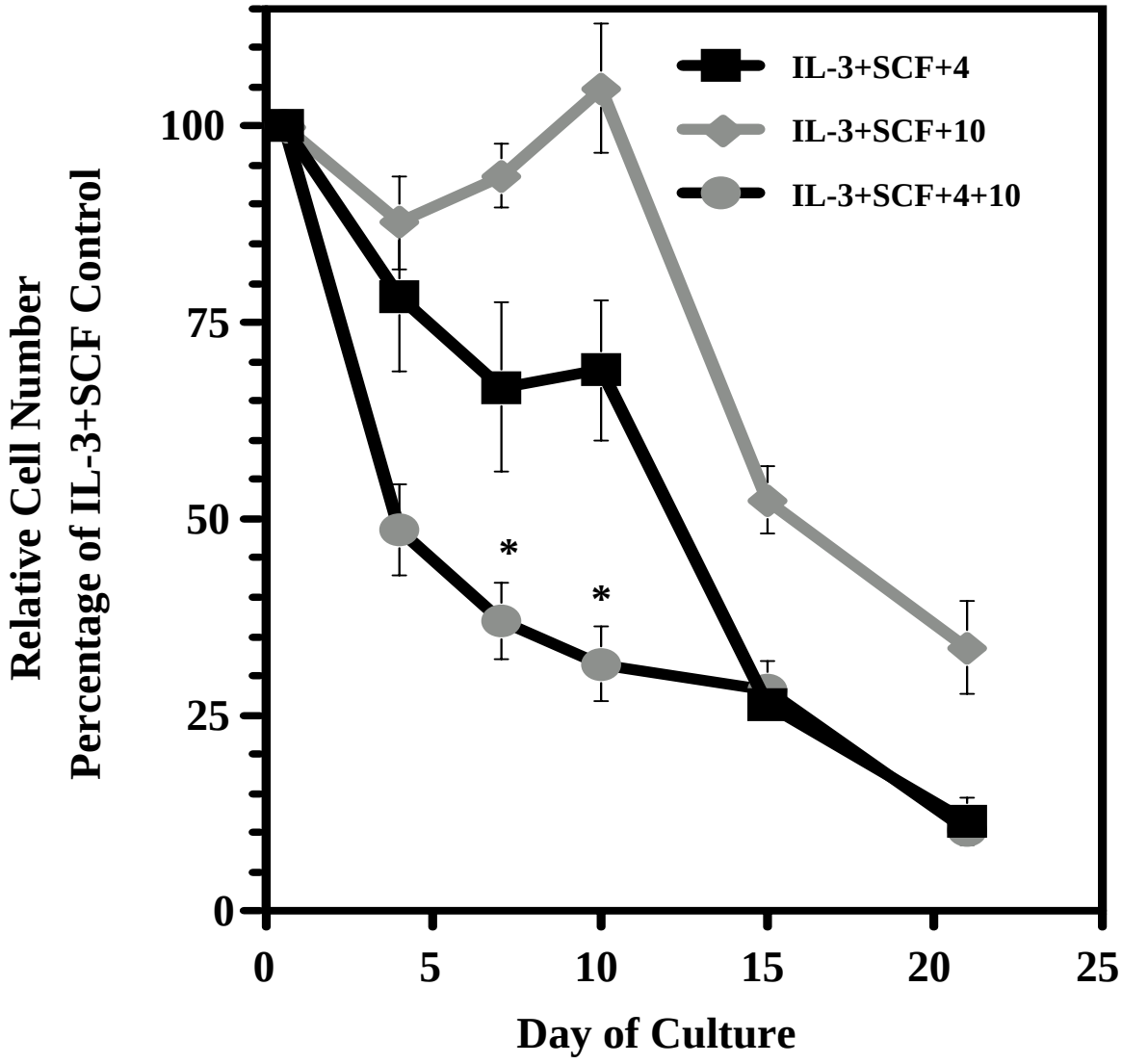
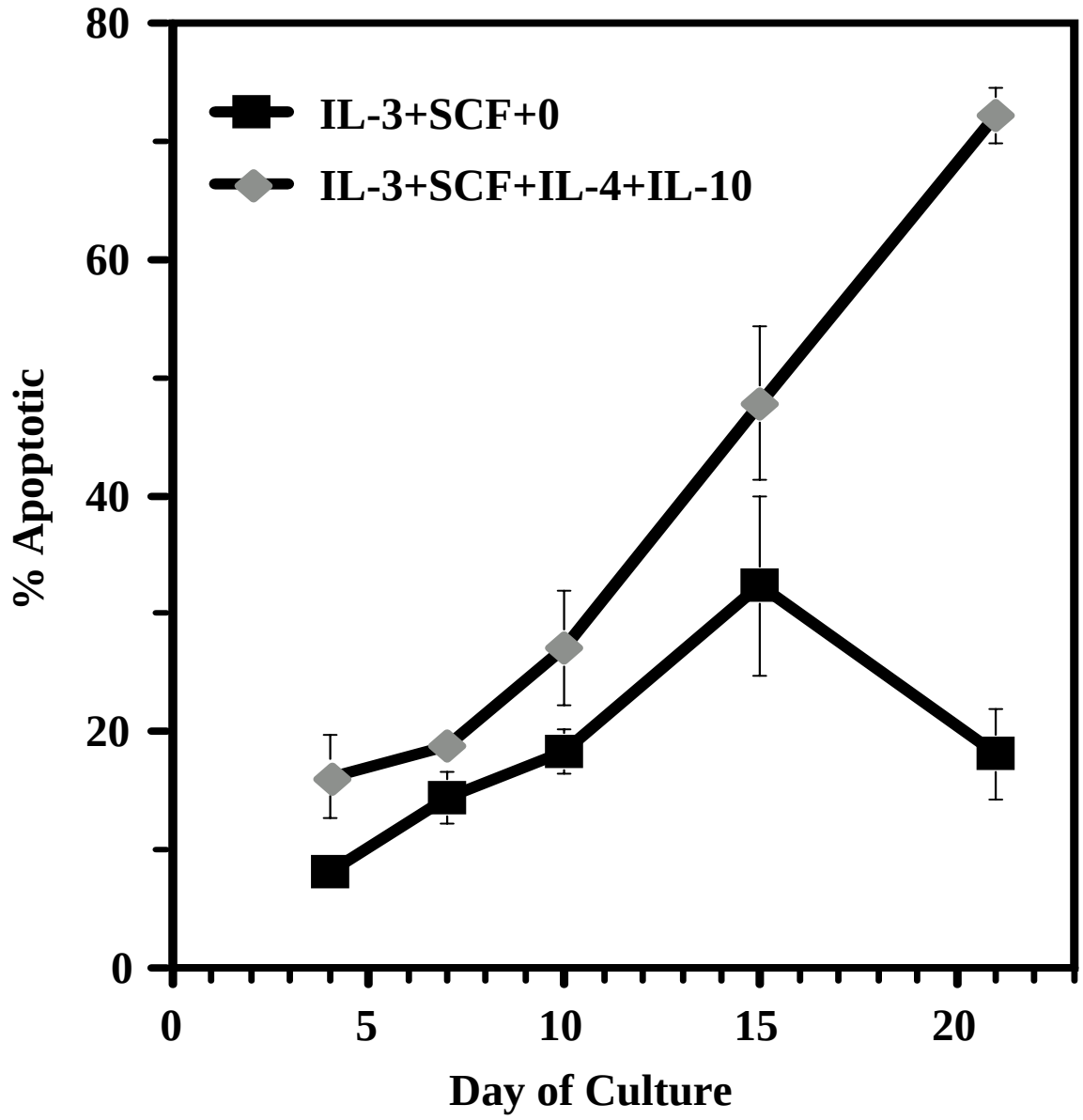

IL-3+SCF+4
IL-3+SCF+10
IL-3+SCF+4+10
Relative Cell Number
Percentage of IL-3+SCF Control
100
75
50
25
0
0
5
10
15
20
25
Day of Culture
*
*

Figure 28.

IL-4+IL-10 increases bone marrow cell apoptosis. Bone marrow cells were cultured in IL-3+SCF +/- IL-4, IL-10, or IL-4+IL-10 at 10 ng/ml and assessed by PI-DNA staining on day 21 culture. Statistical analysis was conducted using ANOVA pairwise comparison (*$p<0.05$). Each data point represents at least 5 separate experiments

Figure 28.


80
60
40
20
0
% Apoptotic
IL-3+SCF+0
IL-3+SCF+IL-4+IL-10
0
5
10
15
20
Day of Culture

Discussion

Maintenance of cellular homeostasis is critical for prevention of pathological changes observed in cancer, autoimmune disease, and immunodeficiency. To this end we have employed a mast cell model system to investigate immune homeostasis, focusing on cytokine mediated regulation of survival and function. Mast cells participate in a wide range of inflammatory responses that are orchestrated by T lymphocytes. Cytokines such as IL-4 and IL-10 produced by T cells and mast cells during an inflammatory response regulate mast cell homeostasis at multiple levels. Our theory is that cytokines such as IL-4 and IL-10 provide feedback regulation for mast cells in a bi-phasic manner. During the first several days of stimulation IL-4 and IL-10 support mast cell survival, proliferation and critical receptors necessary for mast cell activation. After this inflammatory period, these same cytokines inhibit survival, proliferation, maturation, and expression of the critical proteins FcERI and c-Kit on mature mast cells. This regulatory circuit could allow mast cells to perform critical protective functions and prevent the establishment of infection.

The IL-10 receptor is expressed on a variety of hematopoietic cells including B cells, T cells, macrophages, and mast cells, where it regulates many aspects of the inflammatory response. We reasoned that changes in systemic IL-10 concentrations during inflammatory responses could affect not only mature mast cells and macrophages, but their precursors as well. Importantly, IL-10 has been shown to promote murine mast cell proliferation. For example, Rennick et. al. showed IL-10 to be a cofactor for SCF-dependent mast cell progenitor growth and maturation (136, 137). IL-10 was also reported to promote the growth of IL-3-dependent mast cell progenitors (138, 139). In

contrast to our study, these experiments employed committed mast cell precursors. It appears likely that the effects of IL-10 may be dependent upon the stage of differentiation. In support of this, we found that IL-10-mediated apoptosis of developing mast cells required that IL-10 be added to bone marrow progenitors during the first 7 days of culture, the period during which commitment to the mast cell lineage occurs (140).

Our focus is how Th2 cytokines like IL-10 and IL-4 alter the mast cell response. We have previously shown that both cytokines inhibit mast cell IgE receptor and Kit expression, and can combine to induce apoptosis in mature mast cells (112, 119, 120, 141). We also found that IL-4 could induce the apoptotic death of developing mast cells (115). Importantly, apoptosis induced by IL-4 in developing mast cells, and by IL-4+IL-10 in mature mast cells proceeded through a mitochondrial pathway that was blocked by Bcl-2 overexpression (115, 141), consistent with a factor withdrawal response characterized by the intrinsic pathway for apoptosis. Given these results and the close association with elevated serum IL-10 in a wide range of inflammatory conditions, we assessed the role of IL-10 in mast cell progenitor development.

IL-10 Exposure induces apoptosis and changes in cell ontogeny

By studying unseparated bone marrow cells we sought to mimic the microenvironment in which mast cell precursors would be exposed to IL-10 during an inflammatory response. Adherent cells, predominantly macrophages, were retained in these assays to examine changes in differentiation patterns in a non-discriminatory fashion. The differentiation of cells surviving the 21-day assay period was altered by IL-10. Macrophage maturation was not altered, as judged by the insignificant change in

esterase expression (Figure 6), while the percentage of granulated mast cells decreased. Thus it appeared that IL-10 decreased macrophage survival while inhibiting mast cell development.

During the 21-day assay period viable cell numbers remained stable for up to 10 days of culture at which point cell numbers declined significantly. To determine if the duration of exposure to IL-10 related to the observed decrease in cell numbers we performed an addition and removal assay. The addition of IL-10 to cells cultured in IL-3+SCF on various days during the assay showed that IL-10 could induce its effects when added as late as culture day 5. When IL-10 was added post day 10, we found that it could even promote higher cell numbers. We also observed that cells could be exposed to IL-10 for up to 15 days before IL-10 had an effect on cell numbers. This data promotes the hypothesis that there is a commitment phase of cells to either continued lineage development or abortion of development leading to possible loss of cell proliferation or cell death. These data are congruent with observation by Rennick et al (136, 137) in which mast cell committed precursors flourished in the presence of IL-10. Therefore we believe that by day 10 of culture the cells have committed to the mast cell lineage as long as favorable conditions exist.

This hypothesis is supported by the presence of phenotypical mast cells as assessed by histochemical staining and cell surface protein expression. Our observation of relatively low numbers of mast cells on day 21 of culture shows that mast cells are and were present in these conditions. It appears that because of the unfavorable environment, mast cells could differentiate but prolonged survival was not attainable.

Reduced Cell numbers results from induction of apoptosis and activation of intrinsic death pathway

Apoptosis or programmed cell death is a highly regulated process in which cells undergo self-destruction (142-148, 149). This process is characterized by membrane blebbing, cell shrinkage, nuclear condensation, and DNA fragmentation (149). Apoptosis can be activated by the presence of a stimulus or the removal of a stimulus. Cells undergo apoptosis through two major pathways, the extrinsic pathway (death receptor pathway) or the intrinsic pathway (the mitochondrial pathway). The extrinsic pathway is initiated via activation of specific receptors on the cell surface, namely TNFα and FasL. These receptors are associated with a death domain complex that associate with and cleave caspase-8 thereby activating it (150-151). The active form of caspase-8 then activates executioner/effector caspases, such as caspase-3 leading to cell death (152).

The intrinsic pathway can be initiated via a variety of stress stimuli including UV radiation, DNA damage, and loss of survival signals (153). In general, the mitochondria are engaged in intrinsic cell death through loss of mitochondrial membrane stability and subsequent activation of caspase-3. Mitochondrial hyperpolarization (MHP) or increased $\Delta\Psi M$ has been shown to preceed the activation of caspases, phophatidylserine (PS) externalization in apoptotic cells. This initial hyperpolarization is followed by mitochondrial membrane hypopolarization and complete disruption of the mitochondrial membrane potential. Maintenance of the mitochondria outer membrane is critical for cell survival. Anti-apoptotic members of the Bcl-2 protein family are responsible for ensuring stability of the mitochondria. Disruption of the outer membrane results in the release of cytochrome-c from the mitochondria (154). The discharge of

cytochrome-c activates caspase-9 by aggregation of the adaptor Apaf-1, leading to activation of the effector caspase 3 (Reviewed in 142, 155). Disruption of the mitochondrial outer membrane can be initiated via pro-apoptotic proteins such as Bax, Bad and Bid (156). In this study we evaluated the mechanism by which progenitor cells undergo apoptosis by focusing on proteins specific to the intrinsic death pathway.

The most overt effect of IL-10 was a 75% reduction in viable cells, caused by an apoptotic cascade that was consistent with the intrinsic pathway mediated by mitochondrial damage. This theory is supported by findings of caspase activation and DNA fragmentation occurring coincidental to mitochondrial damage. Moreover, Bcl-2 overexpression or p53 deletion partially blocked cell death. Since IL-3 withdrawal induces apoptosis via a mitochondrial pathway accompanied by p53 activation (157), we suspected that IL-10 acts to interfere with survival signals conveyed by IL-3 and/or SCF. Consistent with this idea, exposure to IL-10 greatly reduced IL-3- or SCF-mediated survival. While IL-10 decreased IL-3 receptor beta chain expression, the loss of Kit signaling was not matched by changes in expression. We suspect that IL-10 induces the expression of inhibitory molecules such as members of the SOCS family to dampen IL-3 and SCF signaling.

While IL-10 can signal via several pathways, STAT3 may be the most critical of these. For example, STAT3 deletion greatly impairs IL-10 signaling in macrophages (37, 40, 41). Accordingly, we employed a lineage-restricted gene targeting system to examine the effects of IL-10 on macrophage survival in the absence of STAT3 expression. The effects of IL-10 were completely reversed in the absence of STAT3, highlighting its importance in IL-10-mediated cell death. Given these findings, we hypothesize that IL-

10 acts via STAT3 to promote apoptosis in developing mast cells and macrophages, killing cells by blocking survival signals.

The delayed kinetics of the IL-10 response are consistent with an immune homeostasis controlled by Th2 cytokines. We have found that IL-4 and IL-10 inhibit the function of mature mast cells only after 3 days of signaling (114, 112, 120), allowing sufficient time for a protective inflammatory response, but preventing chronic inflammation. Within 6 days of signaling, mature mast cells are removed by IL-4+IL-10 through mitochondrially-mediated apoptosis (141). Finally, mast cell precursors, as well as developing macrophages, are halted in their development, pushed into an intrinsic apoptosis pathway after 14 days of increased IL-10 levels. As stated, such prolonged increases in circulating IL-10 are noted in a wide range of pathological conditions. It is interesting to note that polymorphisms in the IL-4 and IL-10 promoters, as well as the IL-4 receptor are linked to the establishment of chronic allergic disease (58, 102, 128, 129). Perhaps this pathological immune response results in part from loss of homeostatic controls over mast cell function and development. Our data demonstrate that IL-10 has potent STAT3-mediated apoptotic effects on developing mast cells and macrophages that may assist in preventing the establishment of chronic inflammation. Loss of these protective effects may underlie chronic inflammatory disease.

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