EXPRESSION OF THE COSTIMULATORY MOLECULE CD28 AND CYTOKINES BY MAST CELLS

by

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This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.

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FINAL READING APPROVAL

To the Graduate Council of the University of Utah:

I have read the dissertation of Eric Vincent Marietta in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

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ABSTRACT

Cytokine expression by murine bone marrow-derived mast cells in response to interleukin-3 (IL-3) treatment and CD28 ligation was investigated. Cytokine uptake and cell-to-cell interactions via cell surface molecule ligations are two factors that may play an important role in how the microenvironment can affect the function and development of resident cells. IL-3, a mast cell growth factor, was observed to affect the cytokine profile of mast cells, and CD28, when crosslinked at the surface of mast cells along with a costimulus, was also observed to affect the cytokine profile of mast cells.

Transcript analysis via Reverse Transcriptase-Rapid Polymerase Chain Reaction (RT-RPCR) demonstrated that IL-3 treatment of Stem Cell Factor (SCF)-derived bone marrow mast cells causes an increase in the level of IL-13 transcripts within 30 min and IL-10 transcripts within 1.5 hr. FACscan analysis using intracellular staining indicated that IL-3 derived bone marrow mast cells constitutively express IL-10. FACscan analysis also revealed

that SCF-derived bone marrow cells express IL-10 4 days after IL-3 treatment.

FACscan analysis also demonstrated that CD28 is expressed constitutively at low levels by IL-3-derived bone marrow mast cells and also expressed at higher levels by SCF-derived bone marrow mast cells at the cell surface after treatment with phorbol myristate acetate (PMA) or either of the bacterial products, lipopolysaccharide (LPS) or the *Borrelia burgdorferi*-derived outer surface lipoprotein, OspA. Transcript analysis via RT-RPCR determined that crosslinking of CD28 alone at the surface of the IL-3-derived bone marrow mast cells led to an increased level of c-jun transcripts but not c-fos transcripts. Transcript analysis via RT-RPCR also revealed that, with SCF-derived bone marrow mast cells, crosslinking of CD28 simultaneously with treatment of PMA led to increased levels of IL-13 transcripts. These results indicate that mast cells are capable of expressing CD28 and that some portions of the mast cell CD28 signaling pathway are similar to the T cell CD28 signaling pathway.

Together, these results demonstrate that mast cells are capable of changing their cytokine profile in response to at least two stimuli within their microenvironment, cytokine uptake (IL-3) and cell surface molecule ligation (CD28).

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CHAPTER I

INTRODUCTION

Mast Cell Associated Diseases

Mast cells are best known for their association with allergic reactions (hypersensitive immune responses). With these diseases, an individual responds in an atypical fashion to an antigen (allergen). The type of symptoms exhibited in a hypersensitive immune response depend upon the type of allergen and the site of introduction. For example, air-borne allergens are inhaled and enter mucosal areas of the respiratory tracts. For those individuals with an atypical response to an air-borne allergen such as ragweed or pollen, increased levels of histamine, an inflammatory mediator that causes vasodilation, will be released systemically, followed by bronchoconstriction (1). If the allergen is introduced into the skin however, an individual will experience the delayed-type hypersensitive reaction (late phase) with the hallmark "wheal and flare" response (2). The wheal and flare response is first generated with a localized release of histamine and other proinflammatory agents like TNF-α and IL-1 followed by an influx of lymphocytes including neutrophils and eosinophils, resulting in further inflammation of the tissue (3, 4). In a severely atypical individual, the release of histamine and other inflammatory agents is not controlled, resulting in abnormal swelling. Depending on the severity of the reaction, the

symptoms may become systemic, resulting in the systemic release of histamine and bronchoconstriction.

It was first observed that mast cells were associated with the production of histamine with a study in 1953 by Riley et al., in which they noted that there was a distinct correlation between the presence of mast cells and the quantity of extractable histamine or heparin from various tissues (5). Most striking was the fact that mastocytomas yielded the most histamine and heparin relative to tissue mass. This was a pivotal find, since for the previous 70 years, mast cells had been studied at a histological level.

It was from these histological studies, in fact, that these cells were designated "mast" cells. The term "mast" in mast cells was derived from the mast cell's first designation, "mastzellen," made by Paul Ehrlich around 1880 (5, 6). This is a literal description of these cells, in that mastzellen means "fattened" cell in German. This "fattened" nature, or increased size, of the mast cell is due, in part, to the number of large granules within the cell. Between 1880 and 1950 then, these cells were considered an oddity, "fat" cells without a function. However, once the association with histamine and mast cells was made, the mast cell generated greater interest, with more studies based on the production of histamine by mast cells (5).

Later studies focused then on how the mast cell can release such large quantities of histamine in such a short time period. The observation that histamine was released immediately into the circulatory system indicated that the histamine was present in the mast cell in a preformed state and released upon activation of the mast cell. Studies by Bach et al. determined that mast cells could immediately exocytose histamine and other granule contents in response to the crosslinking of the high affinity receptors for IgE or IgG at the surface of the cell, via the binding of specific antigens to the IgE or IgG present in the receptors (7). Because of these results, it was later determined that the mast cell is not actually the dysfunctional element of the atypical individual as previously thought. The dysfunctional element is actually the overproduction of IgE specific against an allergen. Normally, the IgE level in a normal or typical individual is less than 1 μg/ml in total circulating serum and is polyclonal. Individuals with severe atopy, though, have an abnormally high level of IgE in their serum (1000 μg/ml), due to an abnormal production of IgE that is specific for one antigen (the allergen) (8). This results in mast cells becoming abnormally sensitive to one antigen. An abnormal response to an allergen will occur then, when the allergen is bound by the overabundant type of

IgE bound to the surface of the mast cell. Recently, it has also been observed that mast cells, in addition to contributing to allergies, may also be associated with the development of other diseases and pathologies, such as the development of rheumatoid arthritis (9), asthma (10), allergic rhinitis (11), scleroderma (12), and interstitial cystis (13).

Of course, some diseases are due to mast cells acting "inappropriately," with increased degranulation occurring. This increased degranulation occurs primarily due to an abnormally high turnover of mast cells and can result in urticaria, syncope, anaphylaxis, and diarrhea (6). Also, various types of mastocytomas can lead to similar pathologies. This typically results in sites of inflammation and tissue damage developing at sites of mast cell proliferation (6).

Mast Cell Mediators

Other inflammatory mediators besides histamine are produced by mast cells and include leukotrienes, prostaglandins, cytokines, and chemokines. These inflammatory agents, once released into the tissue, can cause vasodilation and the recruitment of leukocytes into the local tissue (14). The vasodilation and bronchoconstriction are

due to the immediate release of preformed granule components such as histamine, Tumor Necrosis Factor-alpha (TNF-α), leukotrienes, and prostaglandins (1). Leukocyte recruitment and further inflammation are due to the production of cytokines and chemokines (15).

Mast cell granule contents can be released via exocytosis to the surrounding areas as a response to various stimuli. One stimulus that induces mast cell degranulation is the above mentioned antigen specific stimulation through the crosslinking of the IgE or IgG receptors at the surface of the mast cell due to cell surface bound IgE or IgG binding to antigen. Other stimuli that induce degranulation include high concentrations of stem cell factor (SCF) (16), the complement proteins C5a and C3a (17), and calcium ionophores, such as A23187 (18). After degranulation, the mast cell has a greatly reduced number of granules and, as such, looks dramatically different than a fully granulated mast cell. The degranulated mast cell is called a "phantom" mast cell, so designated for its newly acquired transparency (19).

Mast Cells and the Innate Immune Response

The innate immune response, as opposed to the humoral, or antigen specific response, is characterized by the initiation of an

immune response in the absence of T cell help to foreign substances, such as bacteria and bacterial components. One way to initiate an innate immune response is through the escalation of the complement cascade. The complement cascade can be initiated through the classical pathway or the alternative pathway. The alternative pathway can be initiated by the cleavage of C3 into C3a and C3b (20, 21). This cleavage can take place on activating surfaces such as bacteria and viruses. Cleavage of C5 into C5a and C5b will occur later in the cascade. C5a promotes chemotaxis of neutrophils and monocytes/macrophages (22). With leukocyte recruitment, an initial influx of neutrophils occurs within 8 hours followed by an influx of macrophages into the site 8 hours later (23).

The above mentioned capability of mast cells to degranulate in response to the complement proteins C5a and C3a indicates an ability of the mast cell to participate in innate immune responses. Indeed, injections of C5a or C3a into humans results in wheal and flare responses with the typical neutrophil recruitment (24, 25). The development of the wheal and flare response is due not only to the ability of C5a and C3a to cause mast cells to degranulate but also their ability to recruit mast cells in a chemotactic fashion (17, 26). Specific receptors for these proteins are expressed by human mast cells as observed by Nilsson et al. (26).

Responding to C5a and C3a is not the only example of mast cells being able to participate in the innate immune response. Other examples include the capability of mast cells to bind to enterobacteria. In one study by Malaviya et al. (27), it was observed, that mast cells can adhere to enterobacteria that express the FimH fimbrial protein, phagocytose the enterobacteria and process and present bacterial antigens through MHC Class I. Other studies have observed that mast cells can also respond to the bacterial product lipopolysaccharide (LPS). LPS is produced by gram negative bacteria and is present in the outer membrane of their cell wall (28). Rat peritoneal mast cells treated with LPS will secrete IL-6 and will release very little histamine (29). Another study observed that rat peritoneal mast cells treated with cholera toxin will increase their IL-6 synthesis and decrease their TNF-α production, without any measurable release of histamine. These data indicate that the mast cells are not degranulating in response to LPS or cholera toxin. However, type 1 fimbriated *Escherichia coli* can induce mouse mast cell degranulation, resulting in the release of histamine (31). Thus, mast cells are capable of responding to bacterial products, with some products inducing degranulation, and others inducing different cytokine expression patterns.

Another response to LPS by mast cells was observed to be an increased expression of MHC Class II molecules. In that study, bone marrow-derived mast cells, when treated with LPS, upregulated their expression of MHC Class II and were then capable of presenting antigen to MHC Class II restricted T cells. Of interest, was that this upregulation of MHC Class II molecules was observed to be inhibited by treatment with IL-3 or IFN-γ. These data suggest that mast cells may be capable of acting as antigen presenting cells in bacterial infections.

Cytokine Production

The types of cytokines that mast cells express is diverse. These include both inflammatory and anti-inflammatory cytokines, growth factors, and chemokines. For example, the current list includes, but is not limited to, Lymphotactin (Ltn), TNF-α, Transforming Growth Factor-beta (TGF-β), Granulocyte/Monocyte-Colony Stimulating Factor (GM-CSF), Interferon-gamma (IFN-γ), Macrophage Inflammatory Protein-1 alpha (MIP-1α), Macrophage Inflammatory Protein-1 beta (MIP-1β), IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, and IL-13 (33-40). Those cytokines that contribute to a cellular type of immune response are TNF-α, IFN-γ, IL-2, and IL-12.

This classification is based on the Th1/Th2 cytokine profile (for review see 41). Those cytokines that contribute to a humoral type of immune response, again based on the Th1/Th2 cytokine profile, are IL-4, IL-10, and IL-13. GM-CSF, IL-5, and IL-3 have been characterized as hemopoietic growth factors (for review see 42). The chemokines produced by mast cells include MIP-1α, MIP-1β, and IL-8 for the human. Cells that respond to these chemokines are macrophages (MIP-1α, MIP-1β) and neutrophils (IL-8) (for review see 43). IL-5 also serves as a potent chemoattractant for eosinophils (44).

Clearly, this is a wide diversity of cytokines. Either all the cytokines are expressed simultaneously after degranulation, or alternatively, some levels of regulation might exist within the mast cell to control both the timing and the type of cytokines that are released by the mast cell. This latter model, with regulated timing and qualitative release, is supported by observations that the expression or release of cytokines is temporally different for many cytokines. For example, immediately upon degranulation via FcεRI crosslinking, preformed cytokines, such as TNF-α, are released (45). An hour or two after the degranulation event, other cytokines are transcribed, such as GM-CSF and IL-6 (34). At still later time points,

4-8 hours, other cytokines are transcribed or their transcript levels increased, such as TNF-α (34). It appears then, that the expression of cytokines and chemokines by mast cells is under some regulation. For the mast cell to produce such a wide diversity of reagents at different time points would also suggest that the mast cell is targeting a large number of different cell types at different times. The receptors for these cytokines are found on T cells, B cells, macrophages, neutrophils, and eosinophils, among other cells. Receptors for IL-2 are found on T cells. Receptors for IL-4, IL-13, and IL-10 are found on B cells and monocytes (for reviews see 47-48). IL-5 receptors are found on eosinophils, and IL-8 receptors are found on human neutrophils (49). This would suggest that either a very chaotic and unregulated explosive release of cytokines occurs with mast cell degranulation or that a highly regulated temporal series of events occurs, which may be dependent upon signals derived from the local microenvironment in which the mast cell is localized.

Mast Cell Lineage

The wide diversity of cytokine expression by mast cells suggests a similarity with many cell types, including monocytes,

T cells, and B cells. Are mast cells, then, of a monocytic or lymphoid lineage? Because mast cells contain granules, mast cells have been considered to be a typical granulocyte. Therefore, many studies were devoted towards demonstrating that mast cells are of a myeloid lineage, since granulocytes are of the myeloid lineage. Some studies have demonstrated that human monocytes cultured in the presence of fibroblast supernatant gave rise to a cell type that is a granulocyte, expresses IgE receptors, and contains histamine, all mast cell characteristics (50, 51).

However, more recent studies have demonstrated that the basophil, which is more similar to a monocyte than a mast cell, also is a granulocyte, contains histamine, expresses FcεRI, and can release histamine via FcεRI crosslinking (52). More recent data cataloging the expression of a wide variety of cell surface molecules of the CD classification (CD11b, CD18 among many others) on human mast cells, basophils, and monocytes indicates that human monocytes, basophils, and mast cells are actually three distinct cell types (53). One later study even determined that by isolating mature peripheral monocytes using a monocyte marker, CD14, that mast cells do not develop from these cell types (54). This reopens the question of

where in the hematopoietic lineage, myeloid or lymphoid, the mast cell falls.

Similarities do exist between mast cells and lymphoid cells. Some similarities between mast cells and T cells include the diverse cytokine/interleukin production and the production of common integrins, such as αM290. αM290 is a molecule expressed by both intraepithelial T cells localized in the mucosa and mucosal-like mast cells (55). There are even studies from the 60s and early 70s where mast cells were cultured from the thymus, peripheral and mesenteric lymph nodes, and thoracic duct lymph by incubating these cells on top of fibroblast monolayers (56-58). Thus, although the mast cell has been considered to be of a myeloid lineage, recent data and older data suggest that the mast cell is a unique cell type, possibly with its own hematopoietic lineage and possibly derived from the precursor to both the myeloid and the lymphoid lineages (54).

Murine and Human Mast Cell Subclasses

Murine mast cells are categorized into two distinct groups, connective tissue-like mast cells and mucosal-like mast cells. The mast cells that are found in the mucosa are designated as mucosal-like mast cells and those mast cells that are found in connective

tissue, such as the skin, peritoneum, and blood and lymphatic vessels are designated as connective tissue-like mast cells (3). The classification of human mast cells is different from the classification of murine mast cells. With human mast cells, the difference between mucosal-like mast cells and connective tissue-like mast cells is less distinct and is based upon the production of two proteases, tryptase and chymase. In the human, mast cells localized in the skin predominantly express chymase with some tryptase (MC_{TC}), whereas mast cells localized in the mucosa predominantly express tryptase and no chymase (MC_T) (for review see 59).

More distinct differences exist between the murine mucosal-like mast cell and the murine connective tissue-like mast cell. One of these differences, among others, is proteoglycan production. Chondroitan sulfate proteoglycan is produced by mucosal-like mast cells but not connective tissue-like mast cells. Heparin sulfate however, is produced by murine connective tissue-like mast cells but not mucosal-like mast cells (59). Another difference between the two types of cells include histological staining. Mucosal-like mast cells are alcian blue positive, safranin red negative, whereas connective tissue-like mast cells are safranin red positive, alcian blue negative (59). Another difference is the level of histamine within each cell, where the connective tissue-like mast cell contains high

levels of histamine and the mucosal-like mast cell contains low levels of histamine (59).

Other important differences between the two types of murine mast cells exist in the production of cytokines. One study by Smith et al. (39) observed constitutive expression of IL-4 by IL-3-derived bone marrow mast cells, which resemble mucosal-like mast cells, whereas the SCF-derived bone marrow mast cells which resemble connective tissue-like mast cells had a constitutive expression of IL-12. Therefore, at least in the murine system, distinct differences do exist between the connective tissue-like mast cell and the mucosal-like mast cell.

Mast Cell Growth Factors

These two types of murine mast cells exist in two different microenvironments as mentioned before. One factor that contributes to the differences in these microenvironments is the cytokine makeup of the microenvironment. Connective tissue-like mast cells reside in the connective tissue, such as the skin, where large numbers of fibroblasts also reside (60). Based on the close association of mast cells with fibroblasts, studies were performed to see if fibroblasts alone could support mast cell growth *in vitro*. Many

studies with both mouse and rat mast cells demonstrated that indeed, fibroblast monolayers alone could support mast cell survival (61, 62). Further studies demonstrated that one cytokine expressed by fibroblasts could replace the fibroblast monolayer in supporting the growth of mast cells (63). This cytokine was designated stem cell factor (SCF), which is also called mast cell growth factor (MGF), or *c-kit* ligand. The latter designation, *c-kit* ligand, was given, since the cell surface molecule that SCF binds to and ligates is the *c-kit* receptor. Further studies, based on the observation that mast cells are of a hematopoietic lineage, demonstrated that mast cells with a connective tissue-like phenotype could be generated from bone marrow by culturing mouse bone marrow mast cells in the presence of SCF for 3-4 weeks (64).

The other mast cell subclass, mucosal mast cells, resides in the mucosa, which can have a high level of IL-3 production, due to the presence of activated T cells (for review see 65). In fact, it was observed that nude mice, which lack T cells due to the absence of a thymus, have decreased numbers of mucosal-like mast cells in situations of rapid hyperplasia, but normal levels of connective tissue-like mast cells (66). It was later determined that this lack of mucosal-like mast cells in situations of rapid hyperplasia was a result of the lack of IL-3 with the study by Abe et al. (67). In this

study, it was observed that athymic nude (nu/nu) mice that were infected with the parasitic nematode *Strongyloides ratti* were unable to expel the worms and had low levels of mucosal-like mast cells. However, when these mice were repeatedly injected with IL-3, the number of worms was considerably lower, and the number of mucosal-like mast cells significantly increased (67). Further studies observed that IL-3 could drive the differentiation of mast cells from bone marrow, and purified cultures of mast cells could be obtained after 3-4 weeks of culture (68).

Clearly, SCF and IL-3 are significant to the differentiation and proliferation of mast cells. However, the similarity between these two growth factors is minimal. SCF is expressed by fibroblasts, keratinocytes, endothelial cells, and adipocytes and is a crucial growth factor in the differentiation and migration of embryonic cells (69, 70). Its receptor, the *c-kit* tyrosine kinase receptor, is expressed by developing hematopoietic progenitors, primordial germ cells, melanoblasts, erythrocytes, and mast cells (71). Developing lymphocytes that express the *c-kit* receptor are pro-B, pre-B, and Pro-T cells (71). *C-kit* receptor expression has also been observed in nerve cells and a small subset of natural killer cells (72). The receptor is expressed as a transmembrane protein and when bound by its ligand, SCF, will dimerize, leading to enhanced activity of the

tyrosine kinase (73). For these developing cells, SCF may also serve as a chemoattractant for embryonic nerve cells, primordial germ cells, and hematopoietic progenitors (74, 75). Interestingly, SCF retains many of these functions for the mast cell. For the mast cell, SCF can serve as growth factor, chemoattractant, and degranulating agent (75, 76). SCF has been demonstrated to be a very potent cytokine/chemokine, since mice that are homozygous knockouts for SCF do not survive. The only mice that survive with mutations in the SCF gene are mice that are heterozygous. The best example of a mouse that is SCF deficient is the Steel (SL) mouse, Sl/Sl^{d}, which is macrocytic anemic and sterile and lacks cutaneous melanocytes (77). Similar phenotypes are observed with the white spotted mouse (W), for which at least two alleles, v and s, have been isolated (77). The v allele is a rearrangement of the *c-kit* receptor locus, and the s allele is a small deletion of the *c-kit* gene (77). It was noted that these mice exhibited deficiencies in coat color (piebaldism), mental capabilities and other neural associated functions (retardation), erythrocyte numbers (anemia), and reproduction (sterility) (77). However, these mice are not truly mast cell deficient, with mast cells in the white spotted mouse approximately <1% of the number of mast cells in a wild type mouse, but nevertheless, still present (77).

Similarly, with steel mice, <1% of the numbers of mast cells was present compared with the congenic normal mice (77).

Similarly, IL-3 is a pleiotropic cytokine. Similar to SCF, IL-3 is a growth factor that is important for the proliferation of hematopoietic cells, including pre-B and pro-B cells, and pro-T lymphocytes (78, 79). Its cellular sources include activated T cells, mast cells, and keratinocytes (65, 80, 81) and normally is not produced in the bone marrow (65). Since all of these cells are found in the periphery, IL-3 was thought to contribute to the proliferation of hematopoietic cells in an emergency. However, a recent study by Nishinakamura et al. indicated that IL-3 probably does not play a unique role in this function (82). In their study, which consisted of generating a mouse that lacked the βc subunit of the IL-3R (AIC2B), no striking abnormalities associated with IL-3 function deficiency was observed. However, the βc subunit is also utilized by the IL-5 receptor and the GM-CSF receptor (82). In this family of cytokine receptors (IL-3R, IL-5R, GM-CSFR), the βc subunit serves as the signaling component, and the alpha subunits serve to confer specificity for the cytokine ligand (83, 84). Thus, a βc knockout removes some IL-3 and all IL-5 and GM-CSF function. In this particular study, then, no abnormalities were associated with the

decreased level of IL-3 receptor function, but abnormalities associated with the lack of IL-5 and GM-CSF were observed. This included a decrease in the number of peripheral eosinophils and alveolar proteinosis-like symptoms. These results would indicate that IL-3 serves a redundant role with other cytokines. Due to the possibility that another β signaling subunit can be utilized by the IL-3R in the mouse (β_{IL-3} or AIC2A), a double mutant mouse, βc deficient and an IL-3 deficient, was generated. This effectively removed all IL-3 dependent functions in the mouse. Again, no dramatic abnormalities associated with IL-3 were observed to develop. This would also indicate that IL-3 serves a redundant role and that other cytokines, besides just IL-5 and GM-CSF, can substitute for IL-3 in its absence.

In the mouse system, other cytokines that have been demonstrated to increase further mast cell proliferation are IL-4, IL-9, IL-10, and IL-15 (6, 85). However, although they do contribute, they are not capable of generating increased proliferation by themselves. IL-15 is similar to IL-2, in that it can serve as a growth factor for T cells (86). It has also been demonstrated to induce a Th1 phenotype in $CD4^+$ T cells, in that $CD4^+$ T cells incubated with IL-15 will increase their production of IFN-γ (87). In contrast, IL-4, IL-9,

and IL-10 have a primarily anti-inflammatory effect on other cell types. For example, IL-4 and IL-10 are both expressed by the anti-inflammatory type of T helper cell, the Th2 type (for review see 41). IL-4 can contribute to the class switching of antibody by a B cell to the IgE class, can cause the differentiation of T helper cells into Th2 like cells, and can cause the macrophage to downregulate its expression of IL-6, IL-1, and TNF-α (for review see 48). IL-10 has similar properties in that it, too, can cause macrophages to downregulate their expression of IL-6, IL-1, and TNF-α production (88). IL-9 is produced by activated T helper clones of the Th2 type and, as such, is categorized as a Th2 type cytokine (89). IL-9 functions primarily as a growth factor for T helper cell lines, myeloid cell lines, and mast cells. One report has demonstrated that it may have the potential for activating T cells. It was observed that a murine T cell line treated with IL-9 had increased levels of transcript for the granzymes A and B. So far, the inflammatory or anti-inflammatory inducing nature of these cytokines as seen with other cell types has not correspondingly been addressed with respect to the mast cell. However, some studies have demonstrated that these cytokines alter the granule content of mast cells.

One change in granule content exhibited by mast cells towards these cytokines is a change in protease content. Systematic analyses of the different types of proteases that are expressed by mast cells in different cytokine milieus have been performed. Eklund et al. demonstrated that IL-3-derived bone marrow mast cells express high steady state levels of transcripts for murine mast cell protease 5 (mMCP5) and that KL treatment of these cells can induce the accumulation of mMCP4 transcripts (92). IL-10 or a combination of IL-9 and KL can induce the accumulation of mMCP1 and mMCP2 transcripts. IL-4 suppresses the effects of IL-9 and IL-10 in that accumulation of mMCP1 and 2 transcripts is inhibited (92). These studies indicate that mast cells have a variety of phenotypes that may not be limited to just a mucosal-like phenotype or connective tissue-like phenotype. Mast cell phenotypes may be a complex set of phenotypes dependent upon the combination of extracellular cytokines present in the microenvironment of the mast cell.

Costimulatory Molecules

Costimulatory molecules are a group of molecules that were originally identified on the surface of T cells and B cells. These molecules participate in the stimulation process that occurs when B cells present antigen to T cells. They serve primarily to enhance or

increase the stimulation level that is achieved by both the T cell and B cell during antigen presentation. However, there are exceptions. For example, some costimulatory molecules serve to downregulate the stimulatory process. Costimulatory molecules that are expressed by the T cell include CD28, Cytotoxic T Lymphocyte-Associated Molecule-4 Receptor (CTLA-4), 4-1BB, and CD40 Ligand (CD40L) (93-97). Costimulatory molecules expressed by the B cell are B7-1 (CD80), B7-2 (CD86), a putative B7-3, 4-1BB Ligand (4-1BBL), and CD40 (98, 99). Costimulatory molecules are also expressed by cells other than T cells and B cells. Those costimulatory molecules that are expressed by T cells and are expressed by other cell types are CD28 [natural killer cells (100)] and CD40L [human vascular endothelial cells, macrophages, and smooth muscle cells (101)]. Those costimulatory molecules that are expressed by B cells and are expressed by other cell types are B7-1 and B7-2 [activated monocytes, dendritic cells, and activated T cells (102)], CD40 [B cells, monocytes, dendritic cells, endothelial cells, and fibroblasts (103)], and 4-1BBL [splenic dendritic cells (104)].

The expression level of these costimulatory molecules varies with cell type and activation status. CD28 is expressed constitutively by T cells and NK-1.1+ cells, and its expression level can be increased at the surface of the T cells with activation (100, 105). As of yet, 4-

1BB and CTLA-4 have only been observed to be expressed by activated T cells, with CTLA-4 expressed at higher levels on $CD8^+$ cells than $CD4^+$ cells (106). With CD40L, human endothelial cells, smooth muscle cells, and macrophages have a low constitutive expression level, but with IL-1β, TNF-α, or IFN-γ treatment the cell surface expression of CD40L can be increased (101). 4-1BBL is expressed on activated B cells and activated macrophages (104, 107). B7-1 and B7-2 are expressed by antigen presenting cells and can be induced with the ligation of CD40 at the surface of the antigen presenting cells (108). B7-1 and B7-2 have also been observed to be expressed by human endothelial cells, with B7-2 expressed 24 hr after stimulation, and B7-1 expressed 72 hr after stimulation (108). This pattern of expression, where B7-2 expression is increased first and B7-1 expression increased later, was also seen with human B cells that were activated. Again, B7-2 expression was observed to be greatly increased within 24 hr after activation, and B7-1 was observed to be expressed 2-3 days later (99, 102). Observations such as these have led to the theory that the principle ligand for CD28 is B7-2 and the principal ligand for CTLA-4 is B7-1 (102). Both B7-1 and B7-2 have also been observed to be expressed by activated T cells (109, 110). With CD40, constitutive expression has been

observed with B cells, monocytes, dendritic cells, and fibroblasts (103).

Costimulation via costimulatory molecules is based on the interaction of these molecules expressed by T cells and B cells. CD28 on the T cell, when ligated by B7-1 or B7-2, can lead to, among other consequences, increased IL-2, IFN-γ, GM-CSF, Lymphotoxin, IL-3, IL-4, IL-5, or TNF-α production by T cells, due to either or both mRNA stabilization or increased transcription (111-114). These cytokines, especially IL-2, will lead to the further growth and proliferation of the T cells in both a paracrine and autocrine fashion. CTLA-4 also binds to B7-1 and B7-2, and binds with 10- to 20-fold greater affinity than CD28 for either of these molecules (98). However, CTLA-4 is an exception in the costimulatory molecule group, in that the signal derived from CTLA-4 does not result in the increase of T cell proliferation, due to the absence of IL-2 production. Ligation of CTLA-4 may actually lead to the inhibition of IL-2 accumulation. CD40L is similar to CTLA-4 in that ligation of CD40L also does not appear to lead to the increased proliferation of the T cell (116). For the B cell, however, ligation of CD40 at the surface of the B cell by CD40L at the surface of an activated T cell will result in the class switching of antibody to IgE by the B cell, increased levels of B7-1

and B7-2 expression, increased proliferation of the B cell, and increased expression of 4-1BBL by the B cell (104). Interestingly, 4-1BB on the activated T cell can serve as a costimulatory molecule in the absence of CD28, resulting in a strong proliferative response and cytokine production by the T cells when 4-1BB is ligated (104, 117).

More insight into the functions of these costimulatory molecules was achieved with the production of mice where the gene for the costimulatory molecule was knocked out. So far, knockout mice have been generated for CD28, CTLA-4, B7-1, CD40, and CD40L. The knockout mouse for B7-1 did not display any critical reduction in the immune response. No reduction in the number of T cells or B cells was observed, nor any reduction in the concentration of serum immunoglobulins (118). These B7-1 deficient mice also respond normally to mitogens (118). These data indicated a second ligand for CD28 or CTLA-4 and, in fact, led to the isolation and cloning of B7-2 by Freeman et al. (118). As of yet, a double knockout for B7-1 and B7-2 has not been generated to test the hypothesis that a putative B7-3 exists (99). For the CD40 knockout, the phenotype was more dramatic. B cells from CD40 deficient mice were unable to function as antigen presenting cells. However, B cells from CD40 deficient mice, once treated with LPS, which upregulates the expression of B7 molecules on B cells, were able to present antigen and stimulate an

alloresponse. Similarly, mice that are CD40L deficient do not form germinal centers in response to thymus dependent humoral immune responses, nor do they produce antigen specific IgG1 or elevate IgM levels in response to thymus dependent antigens. Thus, one critical CD40 function, which was illuminated with the CD40 deficient mice, is to stimulate B cells and increase the expression of both B7 molecules on B cells (119). For the CD28 deficient mice, very little was observed in the way of a dramatic altered phenotype. The development of T and B cells was normal (120). Immunoglobulin production was 20% of normal, and the antibody class that was most reduced was IgG2a (120). As was expected, B7-dependent functions, such as antigen presentation to T helper cells were inhibited (121). However, most other T cell functions were unaffected, such as cytolytic functions. This absence of a dramatic deficiency in T cell function led to the development of a CTLA-4 knockout mouse. This, surprisingly, of all the costimulatory molecules addressed so far, had the most dramatic phenotype. CTLA-4 deficient mice had an increased level of lymphoproliferation, resulting in fatal multiorgan failure (122). These mice exhibited severe myocarditis and pancreatitis and died within 3-4 weeks of age. Most interesting was the observation that the CTLA-4 deficient mice had a fivefold increase in the number of $CD4^-CD8^-$ T cells in the thymus along with a

10-fold decrease in $CD4^+CD8^+$ T cells in the thymus. This indicated a critical negative role for CTLA-4 in the costimulatory process, and may indicate a role in thymocyte maturation for CTLA-4. Also observed was an increase in the number of total $CD3^+$ T cells in the spleen and the lymph nodes, possibly indicating a role for CTLA-4 in the regulation of peripheral T cells (122).

CD28 Signaling Pathway

Of the costimulatory molecules expressed by T cells, CD28 is the best characterized with respect to cell signaling. The CD28 cell signaling pathway has been analyzed primarily in human T cell lines, such as Jurkat T cells. CD28 is expressed constitutively at the cell surface on $CD4^+$ T helper cells and approximately 50% of $CD8^+$ T cells (123). The expression level of cell surface CD28 on T cells can be increased with activation with PMA or anti-CD3 and will peak at about 48 hours after the activation event (105). Once crosslinked, CD28 then activates various phosphotyrosine kinases at the cell membrane, including phosphatidylinositol 3'-kinase (PI3 kinase) (124). This results in the phosphorylation of CD28 and various downstream kinases, such as MAPK and SAPK. These downstream kinases phosphorylate transcription factors such as *c-jun*. The activated *c-jun* binds to *c-fos*, forming the transcription factor AP-1.

AP-1 binds to the nuclear factor, NF-AT, that is activated through the ligation of the T cell receptor. The NF-AT/AP-1 complex can then bind to the CD28 response element (CD28RE) in various genes. NF-κB can also bind to the CD28RE. Some genes that have the CD28RE in their promoter region are IL-2, GM-CSF, IFN-γ, IL-3, and human IL-8 (125). These increases in cytokine expression are a result of both TCR ligation and CD28 ligation, since NF-AT is provided by the ligation of the TCR, and AP-1 ligation is provided by the ligation of CD28 (124). Thus, in the absence of TCR ligation, CD28 crosslinking alone will lead to processes that do not have NF-AT involved. An example of some events that occur with CD28 ligation in the absence of TCR ligation is the activation of *c-jun*, but not *c-fos*. Thus, in this situation CD28 crosslinking does not lead to increased IL-2 production or other cytokine production, so no proliferation of the T cell is observed. Other cytokines that are increased after CD28 crosslinking but may or may not have CD28RE in their promoters are IL-13, IL-4, and IL-5 (127, 128). The end result is that CD28 crosslinking simultaneously with TCR crosslinking leads to the increased production of these cytokines.

IL-13

IL-13 was originally isolated in a screen looking for novel cytokines that are expressed by activated human T cells (127). IL-13 is very similar in function to IL-4, in that it can cause B cell proliferation, increased CD23 expression, and antibody class switching to IgE (129, 130). Also similar to IL-4 and other Th2 type cytokines, IL-13 can downregulate the production of IL-6 by peripheral blood mononuclear cells and the accumulation of mRNA for IL-1β and TNF-α (127). However, in contrast to IL-4, T cells that are cultured in the presence of IL-13 do not develop into Th2 like cells (131).

Introduction to Work in This Thesis

Material presented in this thesis addresses two means by which the mast cell can "sense" its microenvironments, cytokine uptake, and costimulatory molecule ligation. Specifically, the effect of IL-3 on the expression of cytokines by mast cells was analyzed, and the expression of CD28 and its effect on cytokine expression by the mast cell was analyzed. These studies were based on the premise that the composition of mast cell granule components can be changed in different situations or microenvironments.

An indication of IL-3 altering the cytokine expression of mast cells comes from the observation that bone marrow cells grown in the presence of IL-3 for 3-4 weeks are phenotypically similar to mucosal-like mast cells. Direct support for IL-3 affecting cytokine expression by mast cells came from the observation by Smith et al. that IL-3-derived bone marrow mast cells express IL-4 transcripts constitutively, but SCF-derived bone marrow mast cells do not (39). Extending this observation, the expression of other anti-inflammatory cytokines by IL-3-derived mast cells was addressed, as well as the effect of IL-3 treatment upon SCF-derived bone marrow-derived mast cells. Transcript analysis via RT-RPCR determined that IL-3-derived bone marrow mast cells do have constitutive levels of transcripts for two anti-inflammatory cytokines, IL-10 and IL-13. FACscan analysis using intracellular staining demonstrated that IL-3-derived bone marrow mast cells express IL-10 protein constitutively, presumably storing it in the granules. Also observed was the production of IL-10 by SCF-derived mast cells 4 days after IL-3 treatment.

The expression of costimulatory molecules was proposed when it was observed that mast cells can present antigen via MHC Class II to T cells (32). Although the expression of B7-1 and B7-2 was not addressed specifically, their expression by mast cells is possible,

since successful antigen presentation via MHC Class II requires the presence of a costimulatory molecule, such as B7-1 or B7-2. FACscan analysis determined that IL-3-derived bone marrow mast cells express CD28 constitutively at low levels, and SCF-derived bone marrow cells can be made to increase their expression of CD28 by treatment with PMA, LPS, or the bacterial lipoprotein OspA. Also, mast cell CD28 was found to share similar signaling components with T cell CD28, since with both cell types, CD28 crosslinking led to increases in the *c-jun* transcript levels and IL-13 transcript levels as determined by transcript analysis via RT-RPCR.

These results would indicate, then, that mast cells in response to extracellular signals such as cytokines and costimulatory molecule ligations can potentially alter their granule contents and cytokine secretion. These results also further emphasize the differences between the two types of mast cells, mucosal-like and connective tissue-like, and also provides similarities between mast cells and cells of the lymphoid lineages.

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CHAPTER II

BONE MARROW-DERIVED MAST CELLS

Abstract

In order to conduct the studies that are presented later in this thesis, it was necessary to generate a large number of mast cells. Because the yield of purified, homogeneous mast cells from the peritoneum, or mucosal regions, including the intestine, is very low, bone marrow-derived mast cells were used. These mast cells have been previously demonstrated to provide larger numbers of cells that are phenotypically similar to *in vivo* derived mature mast cells (1). Previous studies on bone marrow-derived mast cells used fibroblast monolayers and recombinant rat SCF as sources of SCF, and WEHI-3 cell supernatant as a source of IL-3. The similarities between bone marrow-derived mast cells and *in vivo* derived mature mast cells include the production of histamine, the production of various proteases, the production of proteoglycans, and the ability to degranulate in response to antigen bound IgE (1). Bone marrow mast cells generated in this work were generated with recombinant mouse SCF and recombinant mouse IL-3. Via FACscan analysis, these SCF-derived bone marrow mast cells were determined to express *c-kit* and IgE receptors, both of which are mast cell markers. Via degranulation assays that analyzed the release of β-glucuronidase, it was determined that both the SCF and IL-3-derived

bone marrow mast cells were capable of degranulating in response to treatment with IgE and antigen. These results indicate, then, that using supernatant from mIL-3 producing 653 myeloma cells and supernatant from mSCF producing CHO cells with murine bone marrow cultures yields mast cells.

Introduction

Generating mast cells from mouse bone marrow requires culturing mouse bone marrow cells in the presence of either SCF or IL-3 for at least 3 weeks. These two models were originally based on the observations that the nude mouse has a decreased level of mucosal-like mast cells and that fibroblasts contribute to the growth and proliferation of mast cells (2, 3). Other cytokines/growth factors besides IL-3 and kit ligand have been observed to contribute to increased proliferation of bone marrow-derived mast cells, including IL-4, IL-9, and IL-10 (4). Our specific system used IL-3 and SCF that was derived from transformed cell lines that had been transfected with cDNA for mouse IL-3 or mouse SCF. This is in contrast to culturing the bone marrow cells in the presence of cells that normally express SCF (fibroblast monolayers) or IL-3 (activated T cells or keratinocytes) (5, 6). Specifically, we used supernatant from a 653 myeloma cell line that had been transfected with mouse IL-3

cDNA as the source of IL-3, and supernatant from a Chinese Hamster Ovary cell line that had been transfected with the mouse SCF cDNA as a source of SCF (7). Supernatant from these cells were used for the bone marrow culture in a concentration of 10% for both the SCF and IL-3-derived mast cells. Again, this is in contrast to some other studies that use Concanavalin A (ConA)-stimulated splenocytes (6) as a source of IL-3, WEHI-3 cell lines as their source of IL-3 (8), or fibroblast monolayers as their source of SCF. By using the 653 and CHO cell lines, the number of cytokines in the culture was restricted, and a large number of phenotypically similar mast cells could be generated.

In contrast, the WEHI-3 cell line is a myelomonocytic leukemia cell line that had been obtained by injecting paraffin oil and testosterone into a Balb/c mouse. The mouse developed a tumor in the first 6 months of age, from which the WEHI-3 (Walter and Eliza Hall Institute of Medical Research) cell lines are derived (9). Later, Lee et al. demonstrated that the WEHI-3 cell lines constitutively express, among other cytokines, IL-3 (10). This is in contrast to the 653 myeloma cell line, which as a result of a transfection with murine IL-3 cDNA, produces predominantly IL-3 (1 μg/ml per 10^6 cells, 24 hr) and some IL-10 (100 ng/ml per 10^6 cells, 24 hr).

Similarly, a fibroblast monolayer would express other cytokines and growth factors other than SCF and so would also not provide a well-defined means of generating mast cells. Because it is known that other cytokines can have an effect on the growth and proliferation of mast cells, our reasoning from the outset was to restrict the number of variables (cytokines) and analyze the specific effects that one or two cytokines had on the function of mast cells.

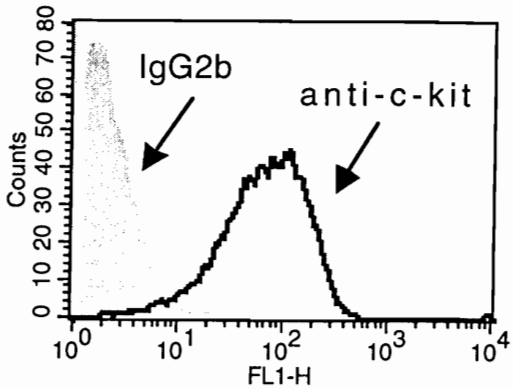
Other groups have generated data that mast cells derived from bone marrow using the WEHI-3b cell line, or fibroblast monolayers, were phenotypically similar to either mucosal-like mast cells or connective tissue-like mast cells (11, 12). However, as we were using different sources of recombinant SCF and IL-3, we needed to demonstrate that our specific models were also generating mast cells. In order to do this, we used various mast cell markers, such as the *c-kit* receptor and IgE receptor to phenotype our bone marrow-derived cells and demonstrate that they were mast cells. Two enzymatic studies analyzing the extent of degranulation in response to high affinity FcεRI crosslinking were used to indicate that the cells generated were functioning mast cells.

Results

As no antibody has been generated that recognizes the mouse high affinity IgE receptor, a different method for detecting IgE receptors has been used. This method was first used by Rottem et al. (13). This consists of detecting IgE bound to the surface of the cell. Rottem's approach was to incubate mast cells with FITC-labeled IgE. A modification of this was the approach of Lantz et al. This consisted of incubating mast cells with IgE first and then staining for the presence of IgE bound to the surface of the cell by using anti-mouse IgE antisera, followed by FACS analysis (14). This does not differentiate between the high affinity IgE receptor versus the low affinity IgE receptor (CD23) but does at least demonstrate that IgE is being bound by the mast cell.

As demonstrated in Figure 2.1, bone marrow cells that were cultured for 7 weeks in the presence of supernatant from the IL-3 producing 653 myeloma cell line express *c-kit* and IgE receptors. As demonstrated in Figure 2.2, bone marrow cells cultured for 14 days in the presence of supernatant from the IL-3 producing 653 cell line also express IgE receptors and *c-kit* receptors. However, at this stage of development, there appears to be a small subpopulation of *c-kit*$^-$ cells still present. Figures 2.3 and 2.4 demonstrate that bone

Figure 2.1 FACscan analysis of *c-kit* and IgE receptor expression by 7-week IL-3-derived mouse bone marrow cells. Mouse bone marrow cells that had been cultured for 7 weeks in the presence of supernatant from mIL-3 transfected 653 myeloma cells were analyzed via FACS analysis for *c-kit* and IgE receptors. FITC conjugated rat IgG2b is the isotype control for the FITC conjugated Rat anti-Mouse *c-kit* antisera (clone 2B8). Biotinylated rat IgG1 is the isotype control for biotinylated Rat anti-Mouse IgE antisera.


IgG2b
anti-c-kit
Counts
0 10 20 30 40 50 60 70 80
10^0 10^1 10^2 10^3 10^4
FL1-H

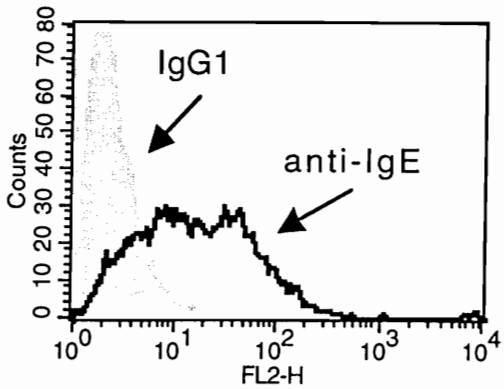
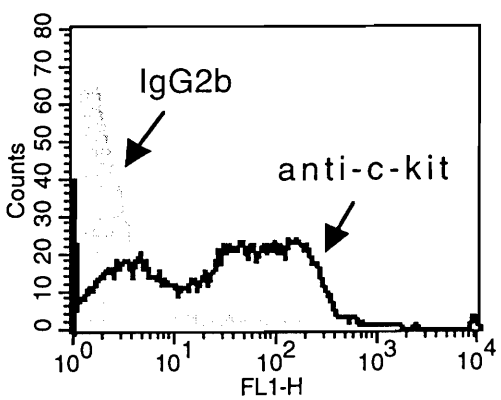

IgG1
anti-IgE
Counts
0 10 20 30 40 50 60 70 80
10^0 10^1 10^2 10^3 10^4
FL2-H

Figure 2.2 FACscan analysis of *c-kit* and IgE receptor expression by 2-week IL-3-derived mouse bone marrow cells. Mouse bone marrow cells that had been cultured for 14 days in the presence of supernatant from mIL-3 transfected 653 myeloma cells were analyzed via FACS analysis for *c-kit* and IgE receptors. FITC conjugated rat IgG2b is the isotype control for the FITC conjugated Rat anti-Mouse *c-kit* antisera (clone 2B8). Biotinylated rat IgG1 is the isotype control for biotinylated Rat anti-Mouse IgE antisera.


IgG2b
anti-c-kit
Counts
0 10 20 30 40 50 60 70 80
10^0 10^1 10^2 10^3 10^4
FL1-H

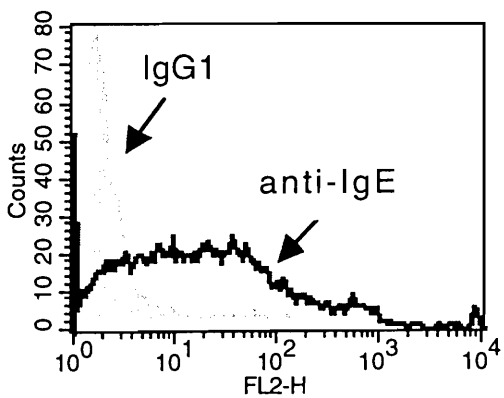

IgG1
anti-IgE
Counts
0 10 20 30 40 50 60 70 80
10^0 10^1 10^2 10^3 10^4
FL2-H

Figure 2.3 FACscan analysis of *c-kit* and IgE receptor expression by 7-week SCF-derived mouse bone marrow cells. Mouse bone marrow cells that had been cultured for 7 weeks in the presence of supernatant from mSCF transfected CHO cells were analyzed via FACS analysis for *c-kit* and IgE receptors. FITC conjugated rat IgG2a is the isotype control for FITC conjugated Rat anti-Mouse *c-kit* antisera (clone ACK-4). Biotinylated rat IgG1 is the isotype control for biotinylated Rat anti-Mouse IgE antisera.

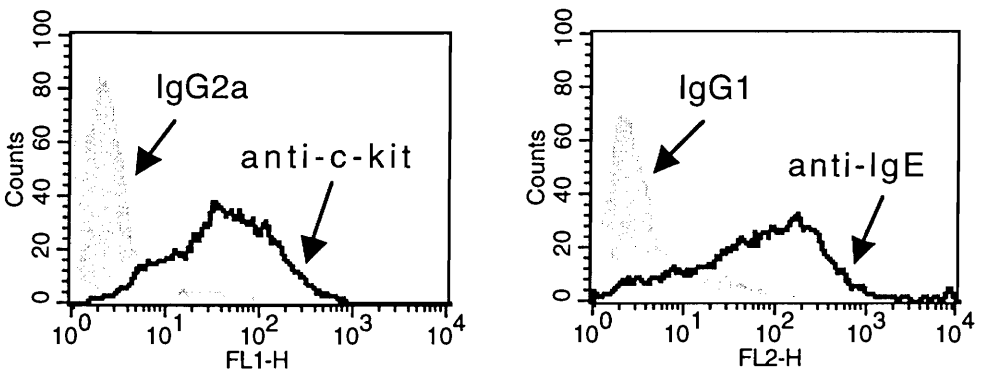
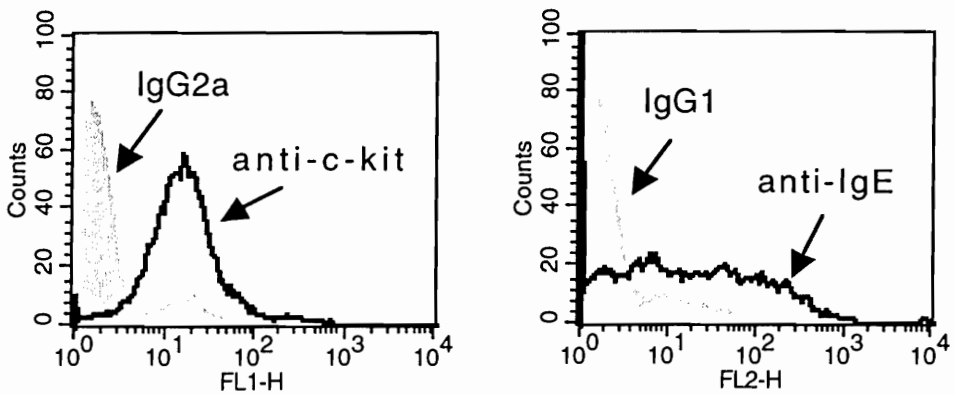

IgG2a
anti-c-kit
Counts
FL1-H
IgG1
anti-IgE
Counts
FL2-H

Figure 2.4 FACscan analysis of *c-kit* and IgE receptor expression by 3-week SCF-derived mouse bone marrow cells. Mouse bone marrow cells that had been cultured for 19 days in the presence of supernatant from mSCF transfected CHO cells were analyzed via FACS analysis for *c-kit* and IgE receptors. FITC conjugated rat IgG2a is the isotype control for FITC conjugated Rat anti-Mouse *c-kit* antisera (clone ACK-4). Biotinylated rat IgG1 is the isotype control for biotinylated Rat anti-Mouse IgE antisera.


IgG2a
anti-c-kit
Counts
FL1-H
IgG1
anti-IgE
Counts
FL2-H

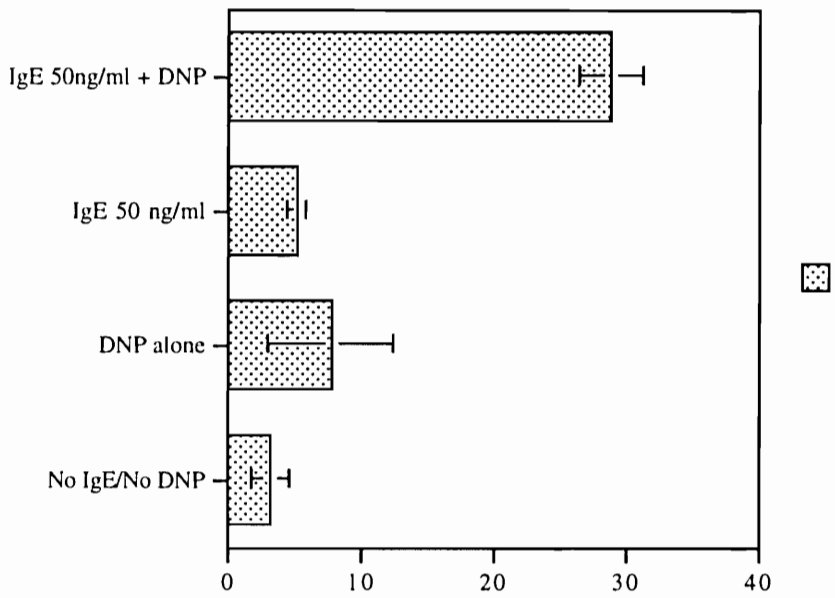
marrow mast cells cultured for 7 weeks or 19 days in the presence of supernatant from the SCF producing CHO cell line also express the *c-kit* receptor and an IgE receptor.

In order to address the functionality of the IgE receptor that was identified as being expressed at the surface of these bone marrow mast cells, we conducted various analyses to examine the degranulation properties of these cells. Briefly, these cells were incubated with IgE specific against dinitrophenyl and then treated with 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) for 45 min. Degranulation was analyzed by examining the quantity of the release of two preformed enzymes into the supernatant. These two enzymes are β-hexosaminidase and β-glucuronidase, both of which are lysosomal enzymes. By measuring the release of these lysosomal enzymes, the amount of degranulation by the mast cell can be measured. Enzyme release can be measured by adding chromogenic substrates for these enzymes to the supernatant of the degranulated mast cell and measuring the activity of the enzyme. For the β-hexosaminidase, the substrate used was p-Nitrophenyl N-Acetyl-β-Glucosaminide, and for the β-glucuronidase, the substrate used was phenolphthalein glucuronic acid (15).

In Figure 2.5, bone marrow-derived cells that had been cultured for 8-9 weeks in the presence of supernatant from IL-3 producing 653 myeloma cells were assayed for degranulation in response to IgE and antigen. Thirty percent release of total β-Glucuronidase activity occurred with the addition of IgE^+ antigen, with background levels of about 5% for β-Glucuronidase release from cells treated with only DNP or only IgE. Similar results were obtained with the β-Hexosaminidase assay. In Figure 2.6, bone marrow-derived mast cells that had been cultured in SCF for 7 weeks degranulated with the addition of IgE and antigen. The percentage of β-glucuronidase release was determined by measuring the fluorescence at 540 nm. Again, similar to the MMC, the CTMC provided about a 30% release of β-Glucuronidase activity. However, background levels of release are twice as high as the MMC. This may be due to the degranulating ability of SCF, since decreasing the concentration of IgE below 1 μg/ml did not reduce the background level of degranulation. Similar results were also obtained with the β-hexosaminidase assay.

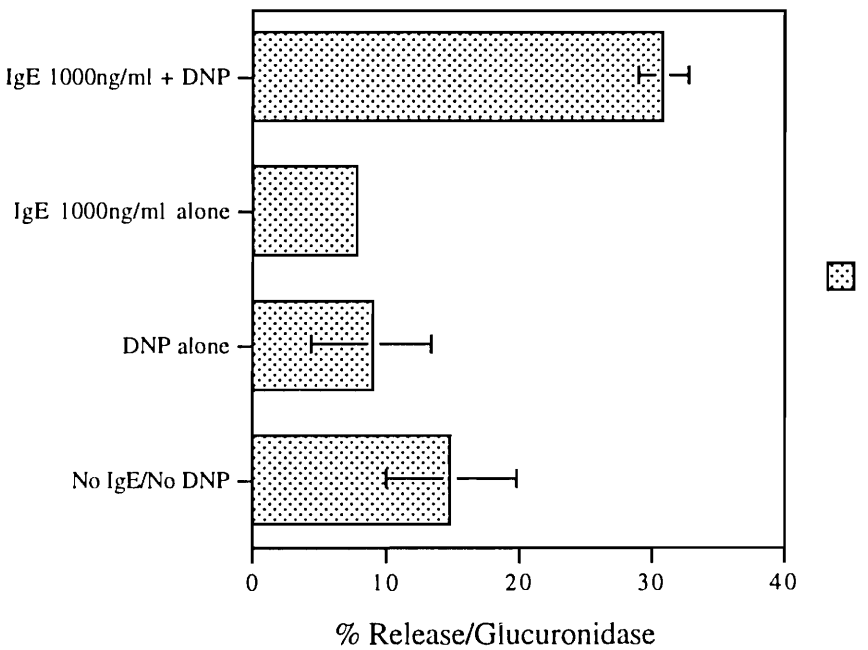
As demonstrated in Figures 2.1-2.6, both types of bone marrow-derived cells do express *c-kit* and IgE receptors and degranulate in response to IgE and antigen. However, the SCF-derived bone marrow mast cells require a higher concentration of

Figure 2.5 Degranulation by IL-3-derived mouse bone marrow cells in response to IgE + antigen. Mouse bone marrow cells that had been cultured for 8-9 weeks in the presence of supernatant from the IL-3 producing 653 cell line were treated with IgE + antigen. Degranulation was measured via the release of β-Glucuronidase. Fluorescence was measured at 540 nm.



% Release/Glucuronidase

Figure 2.6 Degranulation by SCF-derived mouse bone marrow cells in response to IgE + antigen. Mouse bone marrow cells that had been cultured for 6 weeks in the presence of supernatant from SCF producing CHO cells were treated with IgE + antigen. Degranulation was measured via the release of β-Glucuronidase. Fluorescence was measured at 540 nm.


IgE 1000ng/ml + DNP
IgE 1000ng/ml alone
DNP alone
No IgE/No DNP
0
10
20
30
40
% Release/Glucuronidase

IgE for degranulation with antigen than the IL-3-derived bone marrow mast cells. Both concentrations though, 50 ng/ml for the IL-3-derived bone marrow cells and 1000 ng/ml for the SCF-derived bone marrow cells are concentrations that are either the same concentration or lower than concentrations used by other researchers for mast cells.

Discussion

These data indicate, then, that these two cell types, in fact, express IgE receptors. These data also indicate that since the release of the two enzymes by both cell types is dependent upon the crosslinking of the IgE against specific antigen, a portion of these IgE receptors are the high affinity IgE receptors (FcεRI). Also, since these cells express the mast cell marker, *c-kit*, these cells are not basophils but are mast cells. Further characterization of these cells will be presented in Chapters III and IV, such as the lack of IL-2R transcripts. Together these data indicate that these two cell types are indeed mast cells and not basophils, natural killer cells, T cells, B cells, or macrophages.

Therefore, using these two different types of bone marrow-derived mast cells, important questions that will be addressed are

the following: Are these mast cell types capable of responding to cytokines by changing their cytokine profile? Do these cell types express costimulatory molecules, and what signals might the ligation of these molecules provide for the mast cell? Last, do these two types of mast cells respond differently to the same stimuli?

Experimental Procedures

Cells and Tissue Culture

The 653 cell line transfected with mouse IL-3 cDNA was obtained from Ray Daynes. The CHO cell line that was transfected with mouse SCF cDNA sequence was obtained from Genetics Institute, Cambridge, Massachusetts. Bone marrow was flushed from the tibia and femurs of female outbred mice. The bone marrow was then cultured in 90% RPMI and 10% supernatant from either the 653 cells or the CHO cells. Culture times were for 19 days, 6 weeks, or 7 weeks for the SCF-derived bone marrow mast cells and for 14 days, 7 weeks, or 8-9 weeks for the IL-3-derived bone marrow mast cells. Mouse serum was prepared from cardiac punctures.

FACS Analysis

For the analysis of IgE receptors, mast cells were incubated with mouse IgE (monoclonal IgE against DNP-BSA) for 30 min in

RPMI at 37°C. Cells were then washed and stained with Rat anti-Mouse IgE antisera in the presence of mouse serum followed by streptavidin-phycoerythrin.

Antisera used were FITC conjugated anti-*c-kit* (clone 2B8 from Pharmingen cat# 01904D) and FITC conjugated anti-*c-kit* (clone ACK-4 from University of Utah Stem Cell Core Facility). Isotype controls used were FITC conjugated IgG2b (from Pharmingen cat# 11034C) and FITC conjugated Rat IgG2a (from Pharmingen cat# 11024C). Biotinylated Rat anti-Mouse IgE antisera was from Pharmingen (cat# 02132D). Isotype control was biotinylated rat IgG1, obtained from Pharmingen cat# 11012C. Streptavidin-phycoerythrin was obtained from Pharmingen (cat# 13025D).

Degranulation Assay

Mast cells were incubated with 50 ng/ml IgE (IL-3-derived bone marrow mast cells) or 1 μg/ml IgE (SCF-derived bone marrow mast cells) for at least 30 min in RPMI at 37°C. Cells were then washed and resuspended in 1 ml 1X Pipes buffer with 100 ng/ml DNP-BSA. Cells were then plated onto BSA coated 6 well dishes for at least 45 min. BSA coated 6 well dishes were prepared by adding 1-5 ml of 1X Pipes, 1% BSA for at least 30 min with consequent removal

before the addition of cells. Supernatant was then collected from the mast cells incubated in the BSA coated 6 well dishes. Cell pellets were resuspended in 1 ml of 1X Pipes and sonicated. Two hundred microliters of supernatant or sonicate was added to 50 ul of 0.01M phenolphthalein glucuronic acid and 50 ul 0.1M Sodium Acetate buffer pH 4.5 and 200 ul of 1X Pipes. Samples were incubated overnight at 37°C, after which 500 ul of 0.4M Glycine Stop Solution pH 10 was added. The percentage of release was determined by the following equation:

$$\% \text{ Release} = \frac{\text{O.D. supernatant}}{(\text{O.D. supernatant} + \text{O.D. cell pellet})}$$

Fluorescence was measured at 540 nm on a spectrophotometer (15). Phenolphthalein glucuronic acid was obtained from Sigma (cat# P-0501). Mouse IgE specific against DNP was obtained from Sigma (cat# D-8406). DNP-BSA was obtained from Molecular Probes (cat# A843).

β-Hexosaminidase assay was conducted similarly. The substrate used was 0.01M p-Nitrophenyl N-Acetyl-β-Glucosaminide (Sigma cat# N-9376), with 50 ul added to the enzymatic assay in

place of the phenolphthalein glucuronic acid. Fluorescence was measured at 400 nm on a spectrophotometer.

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CHAPTER III

MODULATION OF EXPRESSION OF THE ANTI-INFLAMMATORY CYTOKINES INTERLEUKIN-13 AND INTERLEUKIN-10 BY INTERLEUKIN-3

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Modulation of expression of the anti-inflammatory cytokines interleukin-13 and interleukin-10 by interleukin-3

Interleukin (IL)-4, IL-10 and IL-13 are cytokines with potent anti-inflammatory activities. Prevention of pathological inflammation at mucosal surfaces appears to be due, in part, to the presence of these cytokines. One potential source for these cytokines is the mast cell which resides at mucosal surfaces. Demonstrated in this report are the findings that bone marrow-derived mucosal-like mast cells constitutively expressed IL-13 whereas bone marrow-derived connective tissue-like mast cells demonstrated IL-13 transcription only after FcεRI-mediated activation or the addition of exogenous IL-3. A similar pattern of expression of IL-10 by these mast cell types was also evident and matches that of IL-4 previously reported. Intracellular cytokine staining indicated that IL-10 protein is constitutively expressed by the bone marrow-derived mucosal-like mast cells but is only evident in the bone marrow-derived connective tissue-like mast cells after induction with IL-3. The increase of IL-13 and IL-10 transcripts in the connective tissue-like mast cells following IL-3 treatment is not mast cell specific, in that splenic and bone marrow cells also demonstrated the same phenomenon. These data suggest that mucosal mast cells may have a constitutive repertoire of Th2 cytokines with potential anti-inflammatory activity, while connective tissue mast cells may not. However, production of such cytokines can be induced in the connective tissue mast cell and other cell types of the immune response by the addition of IL-3.

1 Introduction

The interleukins are a class of cytokines that are produced by a variety of cell types. The production of most interleukins is dependent upon the activation of specific cells via a variety of induction pathways. These pathways include receptor mediated activation via specific ligand binding which can lead to increased proliferation and differentiation, and the production of other cytokines [1]. The response of a specific cell to defined stimuli can either be a pre-programmed response or a response influenced by the specific micro-environment in which the cell is found. The latter case has been invoked to explain the development of the Th1 (IFN-γ and IL-2 producing) T cells and the Th2 (IL-4 and IL-5 producing) T cells from the uncommitted precursor Th0 cell during T cell activation (for review see [2]).

A subset of cytokines known for their anti-inflammatory effects are IL-4, IL-10 and IL-13. Collectively, they are known to enhance the humoral immune response and suppress the cell-mediated response. Il-4 can affect proliferation and differentiation of lymphoid cells, influence class switching of activated B lymphocytes to the IgG1 and IgE isotypes, and is instrumental in the generation of the Th2 phenotype of $CD4^+$ helper T cells. In addition, IL-4 acts to suppress the cytotoxic functions of monocytes and macrophages by down-regulating the production of the pro-inflammatory cytokines and inhibiting IFN-γ-mediated activation (for reviews see [3, 4]).

IL-10 is a product of activated Th2 cells, Ly-1 B cells, macrophages, thymocytes and keratinocytes (for review see [5]). Similar to IL-4, IL-10 can promote the proliferation and differentiation of cells of the immune response. IL-10 plus anti-CD40 antibodies stimulate human B cells to differentiate into plasma cells but, unlike IL-4, IL-10 has no effect upon isotype switching. Similar to IL-4, IL-10 has potent immunosuppressive activity upon macrophages. This suppression includes the prevention of nitric oxide synthesis following IFN-γ stimulation and the inhibition of the production of the inflammatory cytokines IL-1, IL-6 and TNF-α. Additionally, IL-10 can help down-regulate the Th1 response. Interestingly, while mice deficient in IL-4 appear for the most part to be normal, aside from differences in immunoglobulin isotypes [6], mice deficient in IL-10 demonstrate a chronic enterocolitis [7].

IL-13 is structurally and functionally similar to IL-4 (for review see [8]). It was first identified as a product of human T cells after activation of peripheral blood mononuclear cells via CD28 and TCR cross-linking [9]. The homologous sequence in the mouse was similarly defined as a Th2 cell product requiring T cell activation (Con A) for expression [10]. Both IL-13 and IL-4 are found on human chromosome 5 and murine chromosome 11 in a cluster of genes encoding cytokines that have similar functions (IL-5 and granulocyte/macrophage (GM)-CSF). The

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Abbreviations: **BM-MMC:** Bone marrow-derived mucosal mast cell **BM-CTMC:** Bone marrow connective tissue mast cell **KL:** c-kit ligand (also called stem cell factor and mast cell growth factor) **RT-RPCR:** Reverse transcriptase-rapid polymerase chain reaction

Key words: Interleukin-13 / Interleukin-3 / Interleukin-10 / Mast cell / Inflammation

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primary functions of IL-13 have been described as anti-inflammatory, in that IL-13, similar to IL-4 and IL-10, reduces the production of IL-1, IL-6, TNF-α and other cytokines by activated macrophages [11]. Like IL-4, IL-13 has been implicated in B cell isotype switching to the IgE isotype [12]. IL-13 has been described as a product of activated T cells and activated mast cells [9, 13], while IL-4 has been described as a product of T cells, mast cells and basophils [14].

The mast cell is capable of producing a variety of cytokines, including IL-1, IL-4, IL-5, IL-12, GM-CSF, TGF-β, and TNF-α (for reviews see [14, 15]). In a fashion analogous to that of Th0 cells developing Th1 or Th2 cytokine profiles, the cytokine potential of the mast cell is influenced by its environment. For example, connective tissue-like mast cells derived from bone marrow (BM-CTMC) in the presence of c-kit ligand (KL, also called stem cell factor and mast cell growth factor) constitutively transcribe the genes encoding the IL-12 subunits, but not IL-4, whereas mucosal-like mast cells (MMC) derived from bone marrow in the presence of IL-3 (BM-MMC) transcribe IL-4, but not IL-12 [16]. Cytokine release by these two populations of mast cells would be expected to contribute differentially to the differentiation of $CD4^+$ T lymphocytes, since IL-4 has been shown to promote a Th2 phenotype and IL-12 to promote a Th1 phenotype. Since BM-MMC do transcribe the Th2 cytokine IL-4 constitutively, we analyzed different mast cell populations for their ability to produce two other Th2 type cytokines, IL-13 and IL-10.

2 Materials and methods

2.1 Cells and tissue culture

BM-MMC were obtained by culturing BM cells in IL-3-conditioned medium (100–300 ng/ml) derived from the supernatant of a 653 B cell line transfected with the mouse IL-3 cDNA sequence, as described [16]. The cells were analyzed after 2.5–3 weeks of culture. BM-CTMC were obtained by culturing BM cells with KL derived from the supernatant of CHO cells transfected with the mouse KL cDNA sequence. The KL-transfected CHO cell line was provided by Genetics Institute, Cambridge, MA. The BM-CTMC cells were analyzed after 2.5–3 weeks of culture.

Freshly isolated splenocytes and BM cells to be analyzed for IL-13 and IL-10 expression were obtained from outbred NIH mice. Freshly isolated splenocytes were obtained by mincing the spleens in RPMI 1640 with 5% FCS, resuspending and passaging through a nylon mesh to obtain a single-cell suspension.

Activated thymocytes for the positive IL-13 control were obtained from the thymus of an outbred NIH mouse 24 h after intraperitoneal injection of *Escherichia coli*.

2.2 FACS analysis

Biotinylated mAb used for FACS analysis were purchased from Pharmingen (San Diego, CA): IgG2c (Cat. no. 11042C), anti-c-Kit (Cat. no. 01822D), and anti-CD3 (Cat. no. 01082D). Cell-surface staining was performed in the presence of mouse serum as described [16], utilizing a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

2.3 Mast cell activation and IL-3 treatment

Activation of BM-CTMC by IgE cross-linking was done as described by Tertian et al. [17], using 20 μg/ml monoclonal anti-dinitrophenyl mouse IgE (Sigma, D-8406, St. Louis, MO) for 30 min and subsequent addition of 100 ng/ml DNP-BSA for the time indicated in the figure legends.

BM-derived mast cells, splenocytes, and freshly isolated BM cells were stimulated with IL-3 by incubating the cells with IL-3 (100–300 ng/ml) derived from the supernatant of 653 cells for the various times indicated in the figure legends. Cell concentrations for IL-3 treatment assays were 1×10^6–3×10^6 cells/ml for BM-derived mast cells, and 5×10^6–10×10^6 cells/ml for freshly isolated BM cells and splenocytes.

2.4 Reverse transcriptase-rapid PCR (RT-R)PCR analysis

RNA from tissue samples was prepared by using the CsCl/guanidine method [18]. Gene-specific transcripts were quantified by the RT-RPCR protocol as described [19, 20]. Annealing temperatures for each oligonucleotide set are listed below. For all reactions, samples were denatured at 94 °C for 1 s, annealed for 1 s and extended for 10 s at 72 °C. The gene-specific oligonucleotides used in this assay, the annealing temperatures used during RT-RPCR, the size of the resultant products, sequence, and gene location are listed in Table 1.

2.5 Intracellular staining

Intracellular staining was performed by fixing the cells with 4% paraformaldehyde for 10 min, permeabilizing the cells with the concentrations of saponin indicated in the figure legends, blocking for 10 min with mouse serum, incubating for 30–40 min with the antibodies indicated in the figure legends, and then analyzing with a FACScan flow cytometer. Cells were stained at densities of 1×10^6–10×10^6 cells/ml with an antibody concentration of 5–10 μg/ml based on the method developed by Sander et al. [21]. The mAb used were rat anti-mouse from Pharmingen (San Diego, CA): anti-IFN-γ (IgG1, clone XMG1.2), anti-TNF-α (IgG1, clone MP6-XT22), anti-IL-10 (IgG1, clone JES5-2A5), and anti-IL-2 (IgG2a, clone JES6-1A12).

2.6 Isolation of $B220^+$ splenocytes

Separation of splenocytes into $B220^+$ and $B220^-$ populations was performed using magnetic polystyrene beads (Dynal, Oslo, Norway; Dynabeads M-280: cat. no. 112.05) covalently bound to streptavidin. Biotinylated rat anti-mouse antibody against B220 was bound to the Dynabeads according to standard instructions provided by Dynal. The antibody against B220 was purchased from Pharmingen (cat. no. 01122A). Freshly isolated splenocytes were then

Table 1. RT-RPCR oligonucleotides[a], annealing temperature and expected product size

Cytokine	Annealing temperature	Expected product size	Sequence of oligonucleotide primers
IL-1β	60 °C	209 bp	5′ CAT GAG ACT TGC ACA GAT CAG 3′ 5′ GGG TTG GAT GGT CTC TTC CAG 3′
TGF-β	55 °C	150 bp	5′ GAT ACC AAC TAT TGC TT 3′ 5′ CCA AAT ATA GGG GCA GG 3′
TNF-α	55 °C	150 bp	5′ CTC AGA TCA TCT TCT CA 3′ 5′ CAC CAC TAG TTG GTT GT 3′
IL-13	60 °C	240 bp	5′ GGG TGA CTG CAG TCC TGG CT 3′ 5′ TGC AAT ATC CTC TGG GTC CT 3′
β-actin	60 °C	135 bp	5′ GTA ACA ATG CCA TGT TCA AT 3′ 5′ CTC CAT CGT GGG CCG CTC TAG 3′
IL-10	60 °C	246 bp	5′ TCC TTA ATG CAG GAC TTT AAG GGT TAC TTG 3′ 5′ GAC ACC TTG GTC TTG GAG CTT ATT AAA ATC-3′
IL-5	60 °C	166 bp	5′ ATG AGA AGG ATG CTT CTG CAC 3′ 5′ TGA GTA GGG ACA GGA AGC CTC 3′

a) All gene sequences were obtained from GenBank submissions. The PCR products are designed to span one or more introns.

incubated with the Dynabeads two times, each for 30 min at a concentration of 1×10^6 Dynabeads/1×10^6 splenocytes, and the $B220^+$ cells extracted with a magnet. RNA was then extracted from the two resulting pools of cells using the CsCl/guanidine method [18].

3 Results

3.1 FACS analysis of BM-derived mast cells

We and others have demonstrated that cells possessing a mucosal mast cell phenotype can be derived from BM by long-term culture (>2 weeks) in the presence of IL-3 [22–26], while a similar culture in KL produces mast cells possessing a connective tissue phenotype [16, 27–29]. For the experiments described in this report, it was important to assess the homogeneity of these two cell types. The c-kit tyrosine kinase receptor is known to be expressed on mast cells and their precursors. We tested the homogeneity of the BM-CTMC (differentiated in KL) and the BM-MMC (differentiated in IL-3) by staining the cells with antisera against c-kit or CD3 (Fig. 1). These analyses indicated that more than 95 % of the cells possess c-kit, while less than 5 % possess CD3. The cells analyzed in these experiments were cultured for 18 days in their respective cytokines. Identical profiles were obtained with cells maintained in culture for up to 45 days (data not shown), however, freshly isolated BM was heterogeneous in the expression of these two proteins (data not shown).

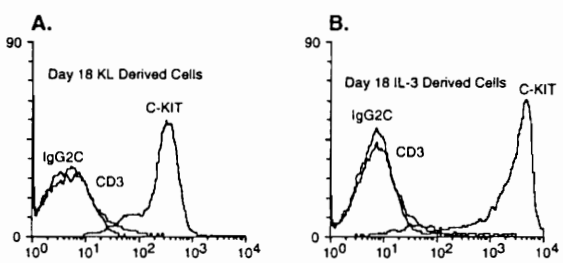

Figure 1. Bone marrow-derived mast cells are c-kit$^+$/CD3$^-$. (A) Bone marrow CTMC-like cells were cultured in c-kit ligand (KL) for 18 days and then analyzed by flow cytometry using rat control IgG2c antibody, hamster anti-mouse antibody against CD3, or rat anti-mouse antibody against c-kit. (B) BM-MMC-like cells cultured in IL-3 for 18 days were analyzed by a FACScan flow cytometer with the antibodies described above.

3.2 IL-13 expression by mouse mucosal mast cells

IL-4 is an anti-inflammatory cytokine produced by Th2 cells, MMC and basophils. Since IL-13 is similar to IL-4 in structure and function, we examined the expression of IL-13 in BM-MMC and BM-CTMC (Fig. 2). We chose to analyze these and other cells for cytokine transcripts using the RT-RPCR protocol developed in our lab [19]. This protocol allows for the analysis of many different gene products from a single mRNA/cDNA source. Additionally, by regulating the numbers of cycles used for product identification, we can ensure that the signal produced during ampli-

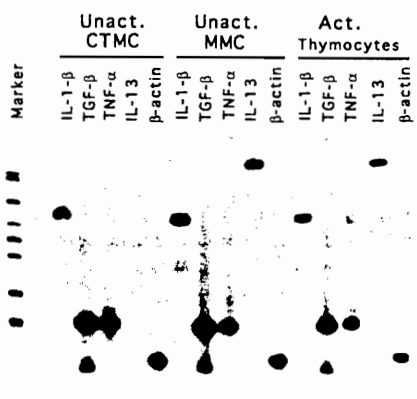

Figure 2. Constitutive expression of IL-13 transcripts by BM-MMC. Transcripts were analyzed by RT-RPCR from total RNA isolated from BM-CTMC-like cells cultured in KL for 20 days (Unact. CTMC), BM-MMC-like cells cultured in IL-3 for 20 days (Unact. MMC), and activated thymocytes (Act. Thymocytes). For the cytokines IL-1β, TGF-β, TNF-α, and IL-13, the number of RPCR cycles was 28, and for β-actin 16 cycles.

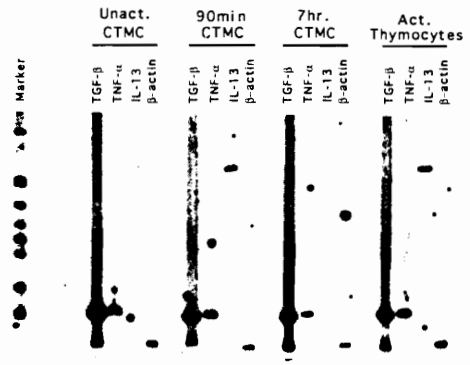

Figure 3. Activated BM-CTMC express IL-13 transcripts. RT-RPCR was used to analyze transcripts from the following RNA samples: BM-CTMC-like cells that were cultured in medium alone for 4 h (Unact. CTMC), BM-CTMC-like cells activated by FcεRI cross-linking and maintained in culture for an additional 90 min (90-min CTMC) or 7 h (7-h CTMC) and activated thymocytes (Act. Thymocytes). All CTMC-like cells used were cultured in KL for 20 days. RPCR cycle numbers were 28 for the cytokines TGF-β, TNF-α, and IL-13, and 16 for β-actin.

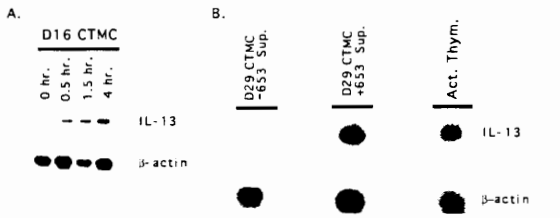

Figure 4. IL-3 treatment increases IL-13 transcript levels in BM-CTMC. (A) RT-RPCR was used to analyze transcripts present in the following RNA samples: BM-CTMC-like cells cultured in IL-3 for 0, 0.5, 1.5, or 4 h. The BM-CTMC-like cells had been cultured in KL for 16 days prior to IL-3 treatment. (B) RT-RPCR was used to analyze transcripts from the following RNA samples: BM-CTMC-like cells cultured for 29 days in KL alone (D29 CTMC – 653 Sup.), BM-CTMC-like cells cultured for 29 days in KL with IL-3 derived from 653 supernatant (D29 CTMC + 653 Sup.) for 48 h, and activated thymocytes (Act. Thym.). RPCR cycle numbers were 28 for IL-13 and 16 for β-actin.

fication is directly proportional to the quantity of transcripts specific for that product in any given cDNA sample [19].

Transcript analysis of these two subsets of cells revealed constitutive expression of IL-13 transcripts in BM-MMC, but not in BM-CTMC. The quantity of IL-13 transcripts in the BM-MMC sample was similar to the quantity of IL-13 transcripts expressed by activated thymocytes, indicating a physiologically relevant number of transcripts in BM-MMC. β-Actin transcripts were used as normalization controls, while IL-1β, TGF-β, and TNF-α were used to compare IL-13 transcript levels with those of other known mast cell cytokines.

3.3 BM-CTMC expression of IL-13 transcripts via FcεRI activation

As demonstrated above, BM-CTMC did not constitutively express IL-13. Since cytokine expression in mast cells can be induced via FcεRI activation [30, 31], we examined IL-13 transcript expression in activated BM-CTMC. This analysis showed a time-dependent course of transcript expression with IL-13 (Fig. 3). That is, IL-13 transcripts appeared within 1.5 h after activation and disappeared 7 h post-activation.

3.4 BM-CTMC expression of IL-13 transcripts via IL-3 treatment

The previous experiment demonstrated that BM-CTMC could be induced to express IL-13 transcripts. However, BM-MMC grown in IL-3 possess IL-13 transcripts in the absence of degranulation, suggesting that another means of increasing IL-13 transcripts exists. To test whether IL-3 could directly induce IL-13 expression, BM-CTMC were treated with IL-3 and analyzed for IL-13 transcripts (Fig. 4A and B). IL-13 transcripts were observable within the first hour after IL-3 addition and were still present 48 h later. The same result was obtained when IL-3 from a variety of different sources was utilized (data not shown), demonstrating that IL-3 was the specific agent resulting in the increase of IL-13 transcripts.

3.5 BM-CTMC expression of IL-10 transcripts via IL-3 treatment

IL-10 is a cytokine which shares a number of functions with that of IL-4 and IL-13. As such, the expression of IL-10 might be expected to be correlated with that of IL-4 and IL-13, especially by cells of the mucosal environment. Accordingly, we examined our mast cell populations for the expression of IL-10. BM-MMC derived in IL-3 constitutively expressed IL-10 transcripts at low levels (Fig. 5 A), as did the BM-CTMC. However, when the BM-CTMC were shifted from a KL culture to one containing IL-3, a marked elevation of IL-10 transcripts was evident. Compared to β-actin, the levels of IL-10 transcripts in these cells were very similar to these of the CH12.LX cell line [32], known to produce high levels of IL-10. The kinetics of the IL-3-dependent increase of IL-10 transcripts in BM-CTMC was determined. The expression of IL-10 transcripts by these cells was readily observable within 4 h and was maintained through 48 h. As a control, IL-5 did not show a similar induction.

3.6 IL-10 protein detected within BM-MMC and BM-CTMC

The presence of IL-13 and IL-10 transcripts in a population of mast cells does not guarantee homogeneous expression of these genes (or their protein products) in all cells of the population. One method designed to analyze single cells for their production of specific proteins, such as cytokines, is intracellular staining. Such an approach was feasible for the analysis of IL-10 but not IL-13, for which specific mAb applicable for intracellular staining have not been described. This procedure was first used to analyze BM-MMC, which constitutively express the IL-10 gene. As

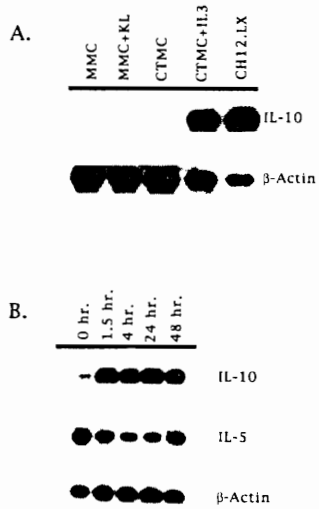

Figure 5. IL-3 treatment increases IL-10 transcript levels in BM-CTMC. RNA isolated from BM-derived mast cells was analyzed for gene-specific transcripts. (A) BM-MMC-like cells maintained in IL-3 alone for 16 days (MMC), BM-MMC-like cells given an addition of KL for 48 h (MMC + KL), BM-CTMC-like cells maintained in KL alone for 16 days (CTMC), BM-CTMC-like cells given an addition of IL-3 derived from 653 supernatant for 48 h (CTMC + IL-3), and CH12.LX cells. RPCR cycle numbers were 30 for IL-10 and 16 for β-actin. (B) BM-CTMC-like cells maintained in KL alone (0 h), or given IL-3 derived from 653 supernatant for 1.5 h, 4 h, 24 h or 48 h. Transcripts analyzed were IL-10 (32 cycles), IL-5 (30 cycles) and β-actin (16 cycles).

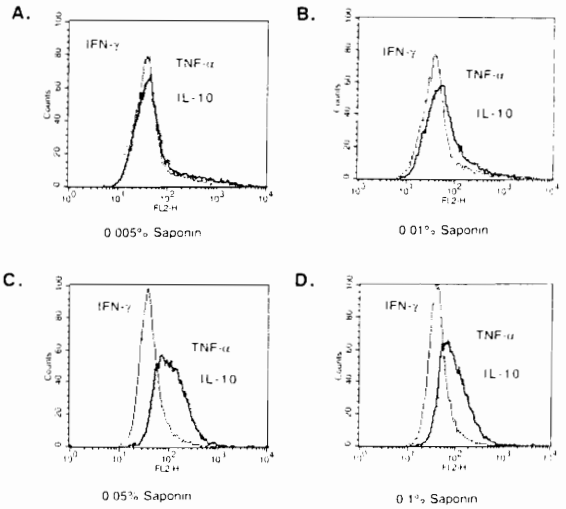

Figure 6. IL-10 is expressed by BM-derived mast cells. Intracellular staining was used to assay IL-10 expression in individual cells. (A–D) BM-MMC-like cells were cultured in IL-3 for 3 weeks and then analyzed by flow cytometry using rat anti-mouse IgG1 antibodies specific for IFN-γ, TNF-α, or IL-10. The different saponin concentrations utilized are indicated [0.005% (A), 0.01% (B), 0.05% (C), and 0.1% (D)].

shown in Fig. 6, various concentrations of the permeabilizing agent saponin were used to optimize staining. Plots A–D demonstrate that increasing concentrations of saponin increase the permeability of the cell and granule membranes, allowing for a non-quantitative demonstration of the presence of the specific cytokines. The optimal response, shown in plot C, was presumably due to the greatest penetration of antisera and least escape of intracellular IL-10. Antisera against IFN-γ and TNF-α were used as negative and positive controls, respectively. These data indicate that the BM-MMC population demonstrated homogeneous expression of both IL-10 and TNF-α.

A similar set of experiments utilizing BM-CTMC treated with IL-3 confirmed the level of staining of IL-10 anticipated from the transcript analysis (Fig. 7). The staining shift for IL-10 is greater than two orders of magnitude when comparing BM-CTMC treated with IL-3 for 96 h (B) with those BM-CTMC cultured without IL-3 (A). A corresponding negative control, using antiserum against IL-2, was performed for both the negative and positive cell populations. Analysis of cells in plot B revealed two peaks of IL-10 staining suggesting heterogeneity in this population for membrane permeability, cytokine recognition, or both. However, both peaks demonstrate a distinct positive shift compared to uninduced BMMC (A) or the IL-2 negative control in (B).

3.7 Induction of IL-13 and IL-10 transcription by splenocytes and BM cells by IL-3 treatment

As described above, IL-3 was shown directly to increase IL-13 and IL-10 transcript levels in BM-CTMC. Activated T cells and degranulated mast cells are the only sources of IL-13 yet to be described, whereas IL-10 sources include activated Th2 cells, thymocytes, macrophages, and Ly1$^+$ B cells. To test whether IL-3 could increase the number of

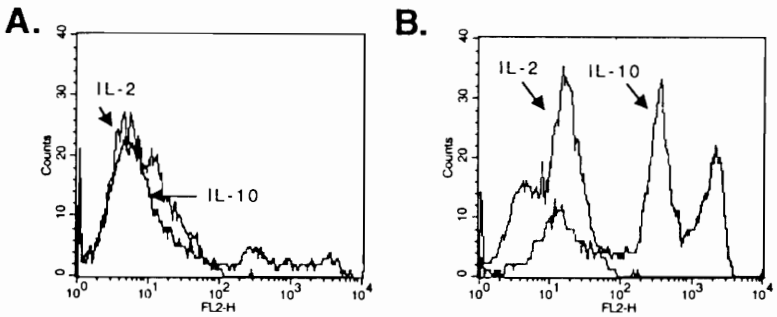

Figure 7. BM-CTMC-like cells were cultured in KL for 3 weeks and then were either cultured with IL-3 for 96 h (B) or without IL-3 (A). The cells were then permeabilized with 0.1% saponin and analyzed by flow cytometry using rat anti-mouse antibodies specific for IL-2 or IL-10.

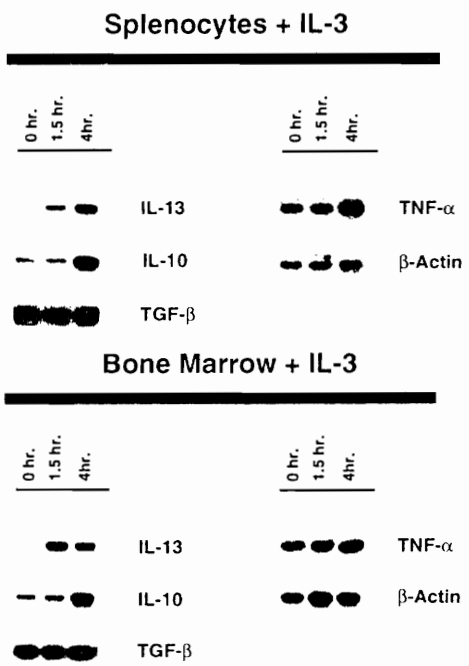

Figure 8. IL-3 increases IL-13 and IL-10 transcription in splenocytes and BM cells. RT-RPCR was used to analyze transcripts from the following RNA samples: (A) freshly isolated splenocytes or (B) BM cells, both of which were treated with IL-3 derived from 653 supernatant for 0, 1.5 or 4 h. RPCR cycle numbers were 28 for IL-13, 28 for IL-10, 24 for TGF-β, 26 for TNF-α, and 16 for β-actin.

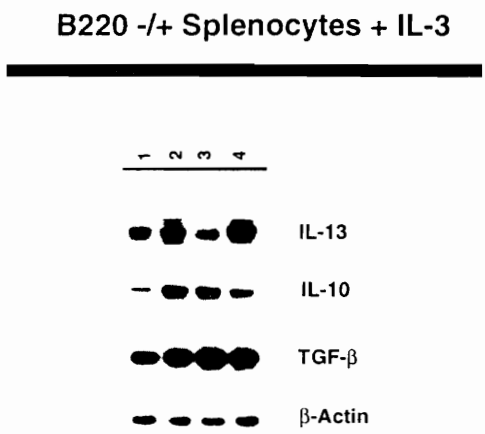

Figure 9. B220$^-$ splenocytes respond differently to IL-3 than B220$^+$. RT-RPCR was used to analyze transcripts from the following RNA samples: (1) Splenocytes cultured for 4 h without IL-3; (2) splenocytes cultured for 4 h with IL-3; (3) B220$^+$ splenocytes cultured in IL-3 and (4) B220$^-$ splenocytes cultured in IL-3. Cells examined for lanes 3 and 4 were treated with IL-3 prior to their separation. RPCR cycle numbers were 28 for IL-13, 28 for IL-10, 24 for TGF-β, and 16 for β-actin.

IL-13, IL-10, or both transcripts in cell types other than mast cells, we incubated cells freshly obtained from the spleen and BM in IL-3 for various lengths of time (thymocytes were also analyzed but possessed a high basal level of IL-13 transcription which was not influenced by the addition of exogenous IL-3; data not shown). Both the splenocyte (Fig. 8A) and bone marrow cultures (Fig. 8B) demonstrated significant increases in IL-13 and IL-10 transcript levels after IL-3 treatment. IL-13 and IL-10 transcripts appeared in the treated splenic and BM cultures between 1.5 h and 4 h, similar to that seen for BM-CTMC, whereas TGF-β and TNF-α were unaffected.

Although mast cells are also present within the spleens of mice, they represent less than 1% of the total cells [33], suggesting they alone cannot be responsible for the increase in IL-13 and IL-10 transcript levels seen in these experiments. Splenic cells were fractionated using magnetic beads conjugated to either anti-B220 or anti-CD3 antibodies. The resultant flow-through cells were analyzed for B220$^+$ or CD3$^+$ staining cells, respectively. This analysis indicated the B220$^+$ selection procedure was more efficient for the removal of cells bearing this marker than the CD3 procedure was for removing CD3-bearing cells. Accordingly, splenic cells were divided into two pools (B220$^+$ and B220$^-$) and transcript quantities analyzed. As shown in Fig. 9, the IL-13 transcript levels were highest in the unbound (B220$^-$) cells (Fig. 9, lane 4), whereas the IL-10 transcript levels were higher in the bound (B220$^+$) population (Fig. 9, lane 3). These data suggest that after IL-3 induction, the B220$^-$ population, which is enriched for T lymphocytes and may contain mast cells, was responsible for most, if not all, of the increased level of IL-13 transcripts, whereas the B220$^+$ population, primarily B cells, was responsible for the increase in IL-10 transcripts.

4 Discussion

4.1 The mouse mast cell as a source of IL-10 and IL-13

Mast cells can make a variety of cytokines and interleukins. While some of these appear to be constitutively expressed during growth and maturation (such as TGF-β), others, such as IL-4 and IL-12, appear only when the mast cells are either activated or cultured under specific cytokine conditions. T cells, mast cells and basophils have been described to produce IL-4. While others have demonstrated the up-regulation of IL-4 gene transcription following degranulation of mast cell lines [30, 31], constitutive expression of IL-4 by BM- or fetal liver-derived mast cells maintained in IL-3 has also been observed [16, 34]. Similarly, when BM-CTMC are treated with IL-3, transcription of the IL-4 gene is induced (unpublished data). Why mast cells (and basophils) produce IL-4 is not entirely clear, although promotion of the Th2 response has been proposed [35].

IL-13 is a recently described cytokine that is very similar to IL-4 [8]. The primary role of IL-13 appears to be as a suppressor of cell-mediated immunity via down-regulating expression of Fcγ receptors and inhibiting the secretion of the inflammatory cytokines TNF-α, IL-1, IL-6 and IL-12 by activated macrophages/monocytes. IL-13 has also been implicated in IgE class switching but, unlike IL-4, appears to have no effect upon T cells. Mature T cells and acti-

vated mast cells have been defined as the only producers of IL-13 [8, 13]. Human IL-13 was first described as a T cell product only after activation with PMA and anti-CD28; PMA alone did not induce IL-13 transcription

IL-10 is a cytokine which shares a number of functions with IL-4 and IL-13. It too suppresses cell-mediated immunity and acts to push an immune response from a Th1 phenotype to a Th2 phenotype [5]. One primary difference between IL-4/IL-13 and IL-10 is that IL-10 appears to have no effect in controlling isotype switching, although it is active in inducing the proliferation and differentiation of B cells. The absence of IL-10 has a profound effect, leading to a highly inflamed intestinal epithelium and chronic enterocolitis [7].

We determined whether BM-derived mast cells could produce IL-13 and IL-10 for a number of reasons. First, mast cells were already known to produce IL-4, IL-5, IL-3 and GM-CSF, all of which are related in chromosomal localization and structure to IL-13. Second, although mast cells have been described to augment the inflammatory process, the presence of IL-4 in MMC suggests that mast cells may be capable of depressing such a response under certain circumstances. Thus, the expression of both IL-13 and IL-10 by these cells might be expected to contribute to the tempering of an inflammatory process.

Mouse mast cells were first analyzed for their expression of IL-13. Those cells derived in IL-3 and possessing a mucosal-like phenotype constitutively expressed IL-13, while those cells derived in KL, representing a connective tissue-like phenotype, did not. These data contrast with those of Burd et al. [13], who demonstrated IL-13 transcription only in activated mast cells.

While BM-MMC did express IL-13, BM-CTMC did not. However, IgE-mediated activation of BM-CTMC did result in a significant increase in IL-13 transcripts. IL-3 was shown to play a role as an IL-13 stimulatory factor by demonstrating that such cells, treated with IL-3, showed elevated levels of IL-13 transcripts. While mast cell degranulation can release IL-3, it is not clear that this cytokine alone was responsible for the IL-13 induction following activation.

Mast cells also express IL-10. While BM-MMC maintained in IL-3 did not show the same level of constitutive expression of IL-10 as seen for IL-4 and IL-13, intracellular staining indicated that the cytokine is stored within the cell. Similar to that found for IL-13, IL-10 transcription was also shown to be responsive to IL-3. Again, these induced cells expressed IL-10 protein intracellularly.

4.2 IL-3 as a Th2-promoting cytokine?

The model derived from the data presented here is that IL-3-responsive cells (mast cells, T cells, B cells, and others) can respond to IL-3 to produce increased levels of three anti-inflammatory cytokines, IL-4, IL-10 and IL-13. IL-3 was first described as a co-factor for granulocyte colony formation *in vitro* from normal BM and was given the name multilineage colony-stimulating factor. It was later defined as a mast cell growth factor in that it supports the differentiation of BM precursors into mucosal-like mast cells. That IL-3 promotes the development of a Th2 anti-inflammatory response would thus be a third function for this cytokine.

The two primary sources described for IL-3 are activated T cells and the mast cells. Mucosal mast cells were first defined as T cell-dependent due to the expansion in numbers of MMC found in the gut after parasitic infection of wild-type but not nude (athymic) mice [36]. This expansion was determined to be due to T cell-derived IL-3. In addition to T cells, mast cells produce IL-3 [37]. We observe IL-3 transcripts in BM-MMC derived in IL-3, but not in BM-CTMC derived in KL (unpublished data). The presence or absence of IL-3 in the culture medium of BM-MMC or BM-CTMC appears directly to influence the cytokine potential of these cells. The CTMC found in the connective tissue and peritoneal cavity of the animal would be expected to promote an inflammatory response to antigen. This has been observed [38, 39]. The MMC found among the intraepithelial lymphocytes (IEL) and lamina propria of the mucosa might, based upon these data, be expected to contribute to an anti-inflammatory response. Clearly, when the anti-inflammatory response in the gut is compromised, as evidenced in the IL-10-deficient animals [7], severe pathology is the result. The role that the mucosal mast cell might play in the maintenance of the anti-inflammatory response of the mucosa is currently under investigation.

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CHAPTER IV

CD28 EXPRESSION BY MOUSE MAST CELLS IS MODULATED BY LPS AND OSPA LIPOPROTEIN FROM *BORRELIA BURGDORFERI*

Abstract

The concept of costimulation has been best defined in T cells and B cells. However, other cells that respond in an antigen specific fashion, such as the mast cell, may also be regulated by similar mechanisms. We have found that murine mast cells express one such costimulatory molecule, CD28, which was previously defined as a T and NK cell specific protein. Although CD28 transcription appears to be constitutive in murine mast cells, its cell surface expression was not. CD28 cell surface expression by mast cells derived from bone marrow with SCF was dependent upon activation with agents such as LPS, the *Borrelia burgdorferi* lipoprotein OspA, and PMA. Peak cell surface expression of CD28 by such cells occurred 24 hr after LPS stimulation, 18 hr after OspA stimulation, and 3 hr after PMA stimulation. In contrast, mast cells derived from bone marrow with IL-3 did not demonstrate induction-specific cell surface expression of CD28. Instead, maturation of such cells *in vitro* allowed for the increased cell surface expression of CD28. Peritoneal mast cells cultured in SCF were also found to express CD28. Mast cell CD28 was functional in that crosslinking of CD28 on the surface of the IL-3-derived cells resulted in an increased level of *c-jun* transcripts. Additionally, crosslinking of CD28 simultaneously with PMA treatment of SCF-derived mast cells resulted in an increased level of

IL-13 transcripts. These data suggest that mast cell CD28 shares functions similar to those of T cell CD28.

Introduction

Mast cells represent a unique link between the innate immune response and the acquired, antigen specific immune response. The presence of antigen-specific IgE held by FcεR1 (1) and antigen specific IgG via Fcγ receptors (2) provides the mast cell with a memory component for specifically recognizing foreign antigen. Alternatively, the presence of receptors for complement by-products (3) and bacterial/parasitic components provides the mast cell with the capacity to respond directly to these products in the absence of memory.

The acquisition of a functional, antigen specific response requires the concerted and coordinated activities of T and B cells. Such cells have developed a two-tiered activation response that requires both an antigen specific stimulus as well as a costimulus provided by specific receptor/ligand interactions. Thus a mature T cell response that is initiated by TCR binding to its cognate peptide/MHC chain requires the costimulation of the T cell CD28 chain binding to its ligand(s), the B7 proteins (CD80 and CD86) which

are expressed by accessory cells (for review see 4). Similarly, the appropriate activation of an antigen specific B cell requires antigen recognition by the surface Ig receptor and ligation of CD40 with the CD40 ligand expressed by T cells (for review see 5). Thus, costimulatory molecules play important roles in the ability of the appropriate T and B cells to proliferate and differentiate.

Since mast cells also possess antigen specificity, might their functions also be modified by the actions of costimulatory molecules? It has been previously reported that human mast and basophil lines express CD40 ligand and can thus be expected to establish cell-to-cell contact with human B cells (6). The same has not been found for murine mast cells because we have not been able to detect CD40 ligand gene products in murine mast cells (unpublished data). Our previous findings that demonstrated that murine mucosal mast cells express homing and retention components previously characterized as the products of IEL T cells (7, 8) led us to investigate whether mast cells also possess the T cell accessory chains CD28 and CTLA-4. No gene products were observed for CTLA-4, but murine mast cell types obtained with a variety of protocols were found to express CD28. In this report, we have focused on characterizing the expression of CD28 by mast cells, and the potential function(s) of this protein in mast cell biology. We have found that although CD28

transcription is constitutive, cell surface expression is not and requires either further maturation of the cell or exogenous activation. Such activation includes bacterial membrane components. Furthermore, mast cell CD28 shares functional activities with that of T cell CD28 in the upregulation of *c-jun* and IL-13 transcription. Thus signaling pathways described for T cell CD28 ligation may also be operative following ligation of mast cell CD28.

Experimental Procedures

Cell Culture

All cells were cultured in RPMI purchased from Gibco BRL, Grand Island, New York (cat# 11875-093), or in DME purchased from Gibco BRL, Grand Island, New York (cat# 11965--092). Both medias contained 5% low endotoxin Fetal Calf Serum purchased from HyClone Laboratories, Logan, Utah (cat# A-1111-L).

IL-3-derived mouse mast cells (9-12) were obtained by culturing femur and tibia bone marrow washes for 18 to 60 days in the presence of the above described RPMI treated with IL-3-derived from the supernatant of a 653 B cell line that had been transfected with the murine IL-3 cDNA sequence (13). The 653 B cell line was cultured in the RPMI described above. The 653 supernatant was used at a 10% vol/vol with fresh cytokine added every second day.

SCF-derived mouse mast cells were obtained by culturing femur and tibia bone marrow washes for 18 to 30 days in the presence of SCF-derived from the supernatant of a CHO cell line that had been transfected with the murine SCF cDNA sequence. The SCF transfected cell line was provided by Genetics Institute, Cambridge, Massachusetts. and cultured in the DME media described above. Bone marrow-derived in the CHO/SCF supernatant was done with a 10% vol/vol with cytokine additions every second day. We have previously shown that IL-3- and SCF-derived bone marrow mast cell cultures are virtually homogenous after 18 days in culture based upon cell surface marker staining to detect the *c-kit* protein or FcεR 1 via the binding of IgE (2, 14, 15) (data not shown).

Peritoneal mast cells were isolated by washing the peritoneal cavity with cold phosphate buffered saline (PBS), removing the adherent cells, and culturing the remaining cells in the presence of SCF-derived from the CHO supernatant (10% vol/vol) for 5-6 weeks. Such cells homogeneously stain for *c-kit*, express all three components of the FcεR1 receptor, the secretory proteoglycan, proteases and integrins characteristic of mast cells, but do not express either CD3 nor components of the IL-2 receptor (Yiyou Chen, Eric Marietta and John Weis, data not shown) which can be found on

c-kit^{+} NK (16). Granule staining also demonstrated a uniform population of cells after 5 weeks in culture. The same long-term population of cells was obtained whether total peritoneal cells were used or if the resident mast cells were first isolated via metrizamide gradient centrifugation (17) prior to tissue culture expansion.

Activated Splenocytes were generated for RNA preparation by injecting $1x10^7$ *E.coli* into one mouse and removing the spleen after 24 hr for RNA extraction.

Stimulation Assays

Reagents used in stimulation assays are as follows. Phorbol 12-Myristate 13-Acetate (PMA) was purchased from Sigma Chemical Co., St. Louis, Missouri (cat# P-8139). Lipopolysaccharide (LPS) from *Salmonella typhosa* was obtained from Sigma Chemical Co., St. Louis, Missouri (cat# L6386). The recombinant lipidated form of the outer surface protein A of *Borrelia burgdorferi* (OspA) was provided by Lorne Erdile (Connaught Laboratories, Swift Water, Pennsylvania) (18) and contained less than 0.3 endotoxin units per 500 mg protein as defined by the Limulus amebocyte lysate assay. Polymyxin B Sulfate (PB) was purchased from Sigma Chemical Co., St. Louis, Missouri (cat# p-1004). Rabbit anti-Hamster antisera was obtained

from Jackson ImmunoResearch Laboratories Inc. West Grove, Pennsylvania (cat# 307-005-003)

To induce CD28 cell surface expression, SCF-derived mast cells (1 X 10^6 cells/ml) were treated with PMA at a concentration of 50 ng/ml for 3 hr.

For the LPS titration curve for CD28 cell surface expression, SCF-derived mast cells (1 X 10^6 cells/ml) were cultured in the presence of LPS at concentrations of 0, 4, 20, or 100 ng/ml for 24 hr.

For the OspA stimulation, titration curve, and polymyxin B treatment, SCF-derived mast cells (1 X 10^6 cells/ml) were cultured in the presence of OspA at a concentration of 500 ng/ml for 0, 3, or 18 hr at 0, 100, or 500 ng/ml for 18 hr and at 0 or 500 ng/ml for 18 hr in the presence or absence of polymyxin B sulfate (10 μg/ml).

For analysis of CD28 transcripts after LPS or OspA treatment, SCF-derived mast cells (1 X 10^6 cells/ml) were cultured in the presence of LPS (500 ng/ml) or OspA (500 ng/ml) for 0, 3, or 18 hr.

For the analysis of *c-fos* and *c-jun* transcript levels following CD28 crosslinking, IL-3-derived mast cells that had been cultured long term (54 days) in the presence of IL-3, were first removed from IL-3 for 12 hr, then treated (1 X 10^6 cells/ml) with Hamster anti-

Mouse CD28 antibody (clone 37.51) (1 μg/ml) for 5 min, followed by treatment with Rabbit anti-Hamster antisera (5 μg/ml) for 30 min. Controls were treatment of IL-3-derived mast cells (1 X 10^6 cells/ml) with Hamster anti-Mouse CD3 (1 μg/ml) for 5 min, followed by treatment with Rabbit anti-Hamster antisera (5 μg/ml) for 30 min, and treatment of IL-3-derived mast cells (1 X 10^6 cells/ml) with Rabbit anti-Hamster antisera alone (5 μg/ml) for 30 min.

For the analysis of IL-13 transcript expression with the combined costimulus of PMA and CD28 crosslinking after CD28 induction with LPS, SCF-derived bone marrow mast cells (3-4 weeks of culture) were treated (1 X 10^6 cells/ml) with LPS (20 ng/ml) for 24 hr or 6 hr, PMA (50 ng/ml) for 6 hr, or with Hamster anti-Mouse CD28 antisera (clone 37.51) (100 ng/ml) for 6 hr. Cells were treated with each reagent in 10 ml of RPMI (5% low endotoxin FCS) in a tissue culture dish and incubated at 37°C for the appropriate time points. Further stimuli were added to the tissue culture dish without washing. For example, the combination of LPS for 24 hr + PMA + anti-CD28 mAb was performed by treating SCF-derived mast cells with LPS for 24 hr; after which both PMA and anti-CD28 antisera

were added. These treated cells were then incubated an additional 6 hr, and RNA extracted.

FACS Analysis

The following antibodies were purchased from Pharmingen, San Diego, California and were provided LPS-free: biotinylated Hamster anti-Mouse TNP (cat# 11092C), biotinylated Hamster anti-Mouse CD3 (cat# 01082D), biotinylated Hamster anti-Mouse CD28 (clone 37.51) (cat# 01672D), FITC-conjugated Rat IgG2b (cat# 11034C), and FITC-conjugated Rat anti-Mouse CD117 (clone 3C1) (cat# 01824D). Hamster anti-Mouse CD117 (clone PV-1) was a generous gift from Dr. Ryo Abe, Naval Medical Research Institute, Bethesda, Maryland. Biotinylated Goat anti-Hamster (cat# 107-065-142) was purchased from Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania. Visualization of biotinylated antibody binding was performed using a streptavidin-phycoerythrin conjugate. Incubations of antibodies with cells were done at 4°C for 25 min in the presence of a 1:50 dilution of mouse serum. Each incubation with antibody was followed by two washes in the staining buffer (0.1% BSA in PBS). Samples were analyzed with a FACScan flow cytometer.

RT-RPCR Analysis

RNA was prepared by the CsCl-guanidine method (19). The RT-RPCR protocol was as previously described (20). RPCR conditions are as indicated in the figure legends. Sequences for PCR analysis were obtained from GenBank submissions. Expected product size and oligonucleotide sets used are as follows: *c-fos* (265bp) 5'-CGT GTC AGG AGG CAG AGC GC-3' and 5'-CGA TTC CGG CAC TTG GCT GC-3'; *c-jun* (240bp) 5'-AGA CAG CCT GGC AGG AGA GC-3' and 5'-GCT CTC GAT GCT GTC AGC AG-3'; *CD28* set #1 (312 bp) 5'-CTC AGG CTG CTG TCC TTG GC-3'and 5'-ATT GAC GTG CAG ATT CCA GAG-3' *CD28* set #2 (321bp) 5'-CTC AGG CTG CTG TCC TTG GC-3' and 5'-ATC TGT GTG ATT GAC GTG CAG-3' *IL-2Rβ* (247*bp*) 5'-CCA ATG TCT CTT GCA TGT GG-3' and 5'-GGT CTT TAC CCT ACG CCA AC-3' *CD14* (231bp) 5'-CGT GGA ACC TGG AAG CCA GAG-3' and 5'-CGC TGG ACC AAT CTG GCT TCG-3' *IL-13* (240bp) 5'-GGG TGA CTG CAG TCC TGG CT-3' and 5'-TGC AAT ATC CTC TGG GTC CT-3' *TNF-α* (150*bp*) 5'-CTC AGA TCA TCT TCT CA-3' and 5'-CAC CAC TAG TTG GTT GT-3' *IL-6* (241bp) 5'-TGT TCT CTG GGA AAT CGT GG-3' and 5'-TGT TGC CTG ACT GGG ACT GT-3' *Crry* (249 bp) 5'-ATG TTC CAA TGG GAA ACA TG-3' and 5'-GCT TTC TAC ACC ATT TGC CG-3' *IgERα* (140bp) 5'-GGA AAA TAC ATA TGT CAG AAG-3' and 5'-CCA GCC ATG GCA TCT GAT GTC-3' *β-actin* (135bp) 5'-

GTA ACA ATG CCA TGT TCA AT-3' and 5'-CTC CAT CGT GGG CCG CTC TAG-3'.

Results

SCF-Bone Marrow-derived Mast Cells Express CD28

There are a number of similarities between mast cells and T cells which include common expression of specific cytokines (14, 21), adhesion molecules (22), signal transduction molecules (23) and similar transcription factors (24). Based upon the previous report of CD40 ligand expression by human mast cells (6), we initiated a series of experiments designed to test for the expression of accessory molecules by murine mast cells, anticipating that such expression would suggest mechanisms with which to modify the function(s) of the antigen specific mast cell. We have not, to date, detected any CTLA-4 or CD40 gene products from murine mast cells, but have detected CD28 gene products.

These studies focused on three sources of primary mast cells: IL-3 bone marrow-derived mast cells, SCF bone marrow-derived mast cells, and peritoneal mast cells. The IL-3-derived cells have been defined as possessing characteristics similar to those of mucosal mast cells due to their expression of cytokines, proteoglycans, and

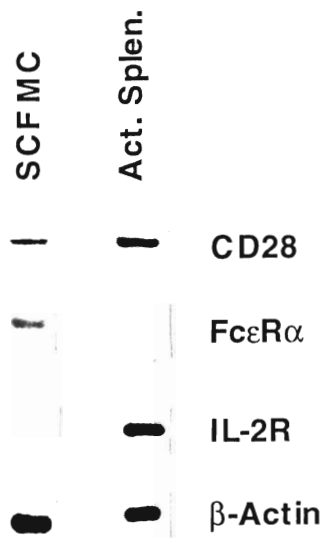
proteases. These cells appear to be a homogenous population of cells within 18-20 days of culture based upon cell surface marker staining (14). These cells can be shifted to a connective tissue phenotype when removed from IL-3 and maintained with SCF (25). Mast cells can also be derived from bone marrow by culture with SCF (also known as mast cell growth factor) (26-28). These cells also appear to be a homogenous population of cells within 18-20 days of culture based upon cell surface marker staining (14). The SCF-derived cells differ from the IL-3-derived cells in their proteoglycan staining, protease and cytokine production. As such these cells appear to possess a connective tissue phenotype which is typified by mast cells within the periphery and peritoneal cavity of the animal, in the absence of T cell derived IL-3. However, based upon cytokine products, the SCF-derived cells can take on a mucosal-like phenotype by shifting such cells into IL-3 (14, 29). Thus both the SCF and IL-3 bone marrow-derived mast cells represent mast cell populations whose phenotypes can be influenced by external stimuli. Peritoneal mast cells can be isolated from peritoneal washes and expanded in culture with SCF. Such cells possess *c-kit* and products of the genes encoding the FcεR1 complex but lack CD3 and IL-2R which would be indicative of NK cell contamination. We did not address the cell

surface expression of CD28 on various transformed mast cell lines since we have previously found that such cells did not express the same range of cell surface proteins (integrins) as did the bone marrow-derived cells (unpublished data).

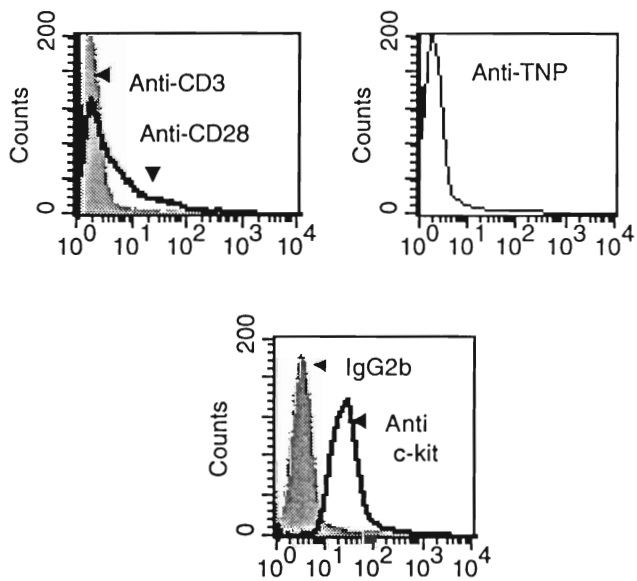
Transcript analysis of RNA samples derived from unstimulated mast cells derived in SCF revealed the constitutive presence of CD28 transcripts in unstimulated cells (Figure 4.1A). An activated splenocyte sample was included as a positive control to detect CD28 and IL-2R transcripts. FcεR1α analysis was included as a mast cell indicator while IL-2Rβ was included as a T cell and NK indicator. Interestingly, whereas transcript analysis indicated CD28 transcripts were apparent, FACS analysis demonstrated only a very low level of constitutive expression of cell surface CD28 (Figure 4.1B). No expression of CD3, utilized as both a T cell marker and the isotype control for the CD28 antisera, was observed, nor did another isotype control, anti-TNP, demonstrate any significant binding to the cell; the high level of expression of *c-kit* was as anticipated. Furthermore, comparison of transcript levels between the SCF-derived mast cells and the murine T cell line TK-1 indicated virtually equivalent levels of transcripts between the two cell lines. However, FACS analysis

Figure 4.1. Analysis of CD28 expression by SCF-derived mast cells via RT-RPCR (A) and FACS (B). (A) RNA samples from unstimulated SCF-derived bone marrow mast cells (SCF MC) and activated splenocytes (Act. Splen.) were analyzed for the expression of transcripts for CD28 (28 cycles), FcεR1α (26 cycles), IL-2Rβ (28 cycles), and β-Actin (16 cycles). (B) Unstimulated bone marrow mast cells that had been derived in SCF for 3-4 weeks were analyzed by FACS for CD28 and *c-kit* expression, using FITC-conjugated anti-*c-kit*, FITC conjugated IgG2b, biotinylated anti-CD28 (clone 37.51), biotinylated anti-TNP, and biotinylated anti-CD3, followed by PE-streptavidin.

A.


SCF MC
Act. Splen.
CD28
FcεRα
IL-2R
β-Actin

B.


Counts
200
0
Anti-CD3
Anti-CD28
Anti-TNP
IgG2b
Anti
c-kit
10^0 10^1 10^2 10^3 10^4

demonstrated CD28 expression only on the surface of the TK-1 cell, not the SCF-derived mast cells (data not shown). These data indicate that CD28 transcripts are present in at least a percentage of the SCF-derived cells but that such cells only express very low levels of the protein on their surface.

PMA Increases CD28 Expression on SCF-derived Mast Cells

Searched for next was a reagent that would increase the level of CD28 at the surface of the mast cell. SCF-derived mast cells were treated with phorbol 12-myristate 13-Acetate (PMA) to test whether such stimulation, presumably through protein kinase C (30), would cause higher levels of cell surface CD28. FACS analysis revealed that CD28 expression levels did increase on such cells with PMA treatment (Figure 4.2). This peak of expression occurred 3 hr after PMA stimulation but was lost within 24 hr. Importantly, the entire population of such cells was shown to express CD28 indicating that all such cells must possess CD28 gene products.

To confirm that the molecule expressed on the cell surface was indeed CD28 and not a related protein, a second monoclonal antibody generated against CD28, clone PV-1 (28), was used (Figure 4.3). Similar PMA-dependent staining of SCF-derived mast cells was

Figure 4.2. Effects of PMA treatment upon CD28 expression by SCF-derived mast cells. Bone marrow-derived cells cultured in SCF and low endotoxin RPMI for 3-4 weeks were treated with PMA (50 ng/ml) for 0, 3, 24, and 48 hr and analyzed for CD28 expression via FACS analysis, using biotinylated anti-CD28 (37.51) followed by PE-streptavidin.

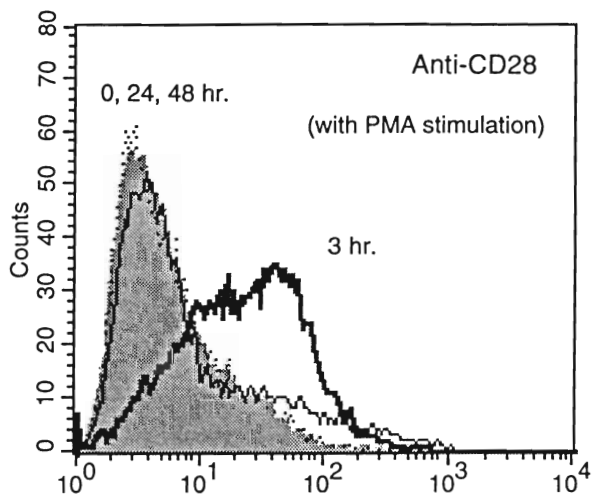
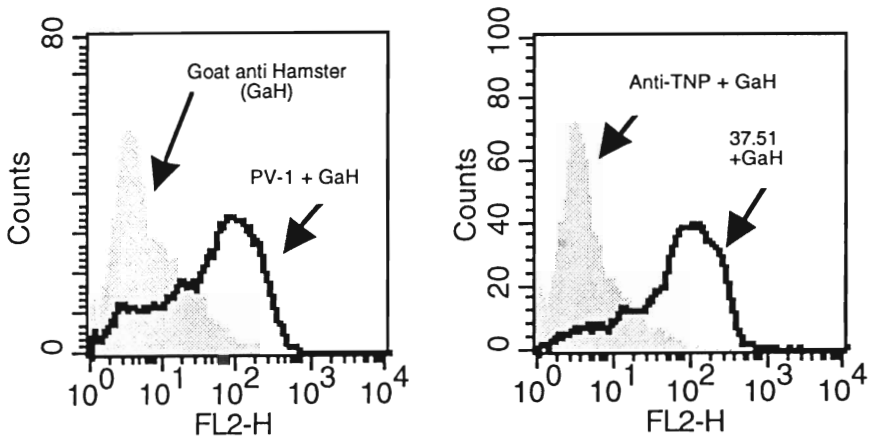

Anti-CD28
0, 24, 48 hr.
(with PMA stimulation)
3 hr.
Counts
0
10
20
30
40
50
60
70
80
10^0
10^1
10^2
10^3
10^4

Figure 4.3. Binding of CD28 by both the 37.51 and PV-1 anti-CD28 antisera. Bone marrow-derived cells cultured in SCF for 3-4 weeks were treated with PMA (50 ng/ml) for 3 hr and analyzed for CD28 expression via FACS analysis using the two anti-CD28 antisera, 37.51 and PV-1. Biotinylated Goat anti-Hamster antibody was used as a secondary antibody, followed by PE streptavidin, in order to detect the 37.51, PV-1, and isotype control anti-TNP antibodies.


Counts
80
0
Goat anti Hamster
(GaH)
PV-1 + GaH
10^0 10^1 10^2 10^3 10^4
FL2-H
Counts
100
80
60
40
20
0
Anti-TNP + GaH
37.51
+GaH
10^0 10^1 10^2 10^3 10^4
FL2-H

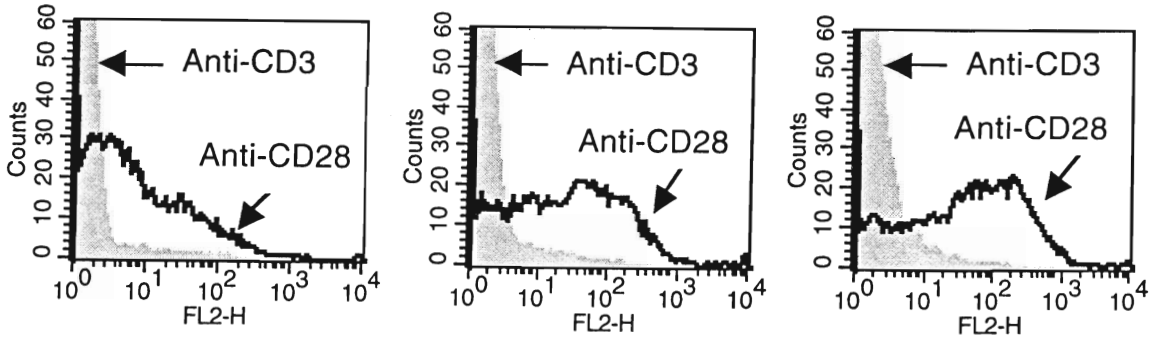
observed with either antisera. These data, plus the RT-RPCR transcript analysis, indicate that SCF-derived mast cells can be induced to express CD28.

LPS Increases CD28 Expression on SCF-derived Mast Cells

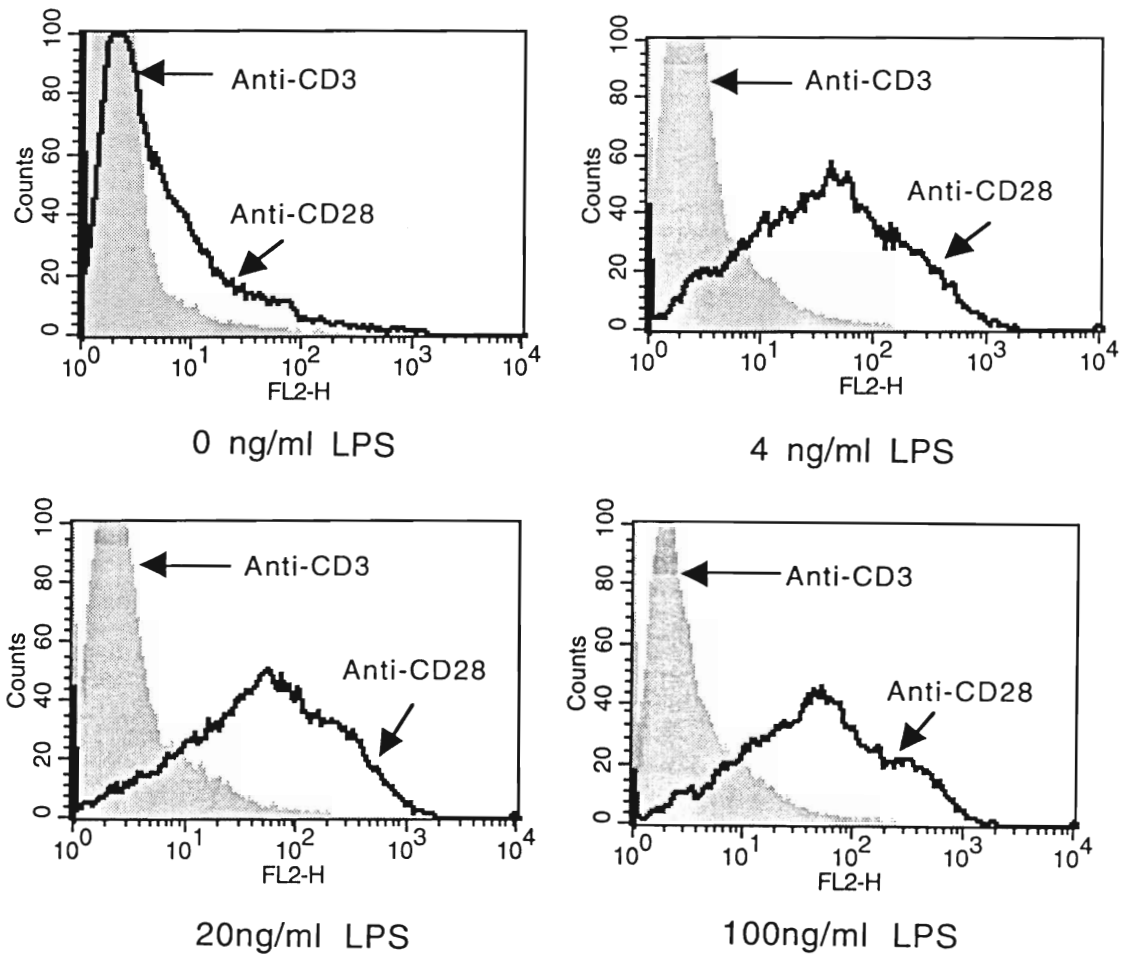
A series of experiments was then initiated to determine if other stimuli would also result in the expression of the CD28 protein. The standard means of activating mast cells, FcεRI-induced degranulation, did not increase the cell surface expression of CD28 (data not shown). LPS has been shown to affect mast cells, resulting in an increase of IL-6 production by rat peritoneal mast cells (32) as well as inducing the differentiation and proliferation of murine mast cells *in vitro* (33). Therefore, the next question addressed was whether LPS treatment would influence the level of expression of CD28 by SCF-derived mast cells. LPS treatment resulted in the maximum expression of cell surface CD28 18 to 24 hr after LPS treatment (Figure 4.4A). Varying concentrations (4 to 100 ng/ml) of LPS for 24 hr demonstrated an increased expression of cell surface CD28 (Figure 4.4B). The level of CD28 cell surface expression returned to control values within 48 to 72 hr (data not shown). The source of LPS was *Salmonella typhosa.* LPS from other sources were not examined.

Figure 4.4. Effects of LPS treatment upon CD28 expression. (A) Bone marrow-derived cells cultured in SCF for 7 weeks were treated with 100 ng/ml LPS for 0, 3, or 18 hr. (B) Bone marrow-derived cells cultured in SCF for 3-4 weeks were treated with 0, 4, 20, or 100 ng/ml LPS for 24 hr. The cells were analyzed for CD28 expression via FACS analysis, using biotinylated anti-CD3 and biotinylated anti-CD28 (37.51), followed with PE streptavidin.

A.


Anti-CD3
Anti-CD28
Counts
FL2-H
0hr. LPS
3hr. LPS
18hr. LPS

B.

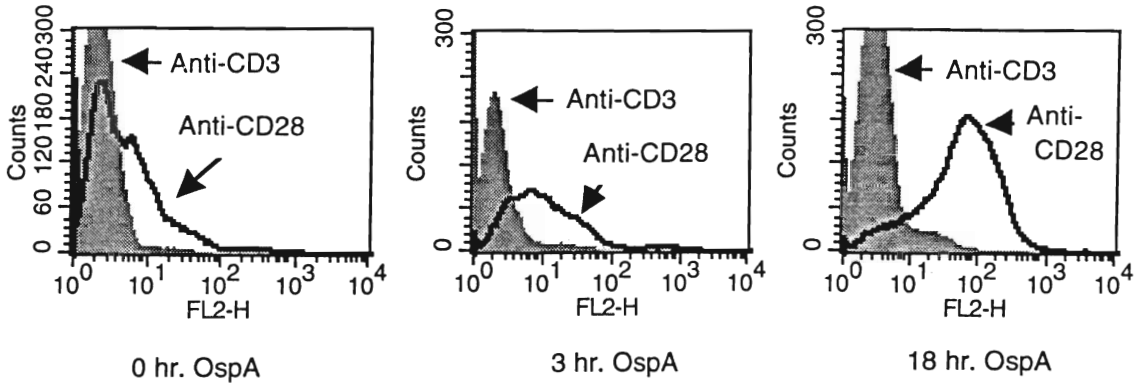

Anti-CD3
Anti-CD28
Counts
FL2-H
0 ng/ml LPS
4 ng/ml LPS
20ng/ml LPS
100ng/ml LPS

OspA Increases CD28 Expression on SCF-derived Mast Cells

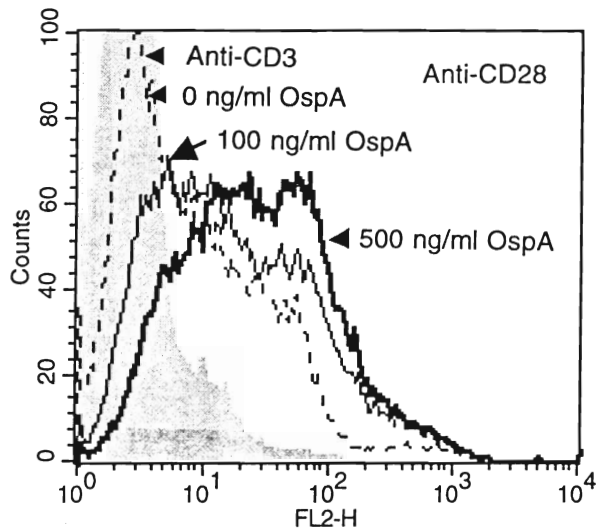
In order to determine whether another bacterial cell product could influence CD28 expression by the SCF-derived mast cells, the lipoprotein OspA from *Borrelia burgdorferi.* was examined. OspA has a lipid modification consistent with the Pam_3Cys structure (34), which is found on a variety of bacterial lipoproteins. It has previously been shown that bacterial lipoproteins including OspA have similar stimulatory effects as LPS on some cell types, such as macrophages (35). FACS analysis, shown in Figure 4.5, demonstrated that OspA treatment resulted in an increase of the level of cell surface CD28 and that the peak increase occurred 18 to 24 hr after OspA treatment (Figure 4.5A). Thus OspA treatment provided similar kinetics for CD28 expression as LPS but again was slower than PMA. A titration curve demonstrated that OspA also stimulated SCF-derived mast cells at low levels (100 ng/ml) but that the peak increase of cell surface expression of CD28 occurred with 500 ng/ml OspA (Figure 4.5B). The possibility of a low level of LPS contamination causing the increase in CD28 expression was examined by treating the OspA preparation with an LPS inhibitor, polymyxin B (PB), for 2 min before adding to the cells (Figure 4.5C). Since polymyxin B treatment did not ablate the increase in CD28

Figure 4.5. Effects of OspA treatment upon CD28 expression. Three- to four-week-old bone marrow-derived cells cultured in SCF were treated with OspA for varying periods of time (A), varying concentrations of OspA (B), or in combination with an LPS inhibitor, polymyxin B (C), and then analyzed via FACS analysis for CD28 expression. (A) The different points of time examined were 0, 3, and 18 hr of OspA treatment (500 ng/ml). (B) The different concentrations of OspA were 0 ng/ml, 100 ng/ml, and 500 ng/ml with an incubation time of 18 hr. (C) Polymyxin B (10 ug/ml) was added either alone (PB) or in combination with OspA (500 ng/ml) (PB + OspA) for 18 hr. Control refers to untreated cells. The two antibodies used were biotinylated anti-CD28 (37.51) and biotinylated anti-CD3, followed by PE-streptavidin.

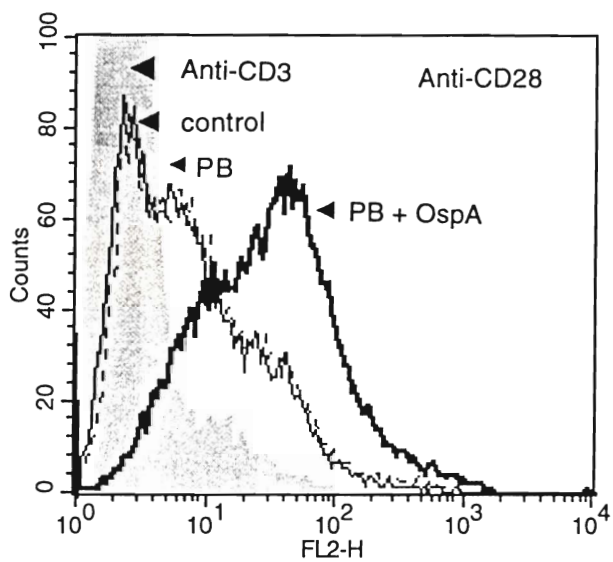
A.


Anti-CD3
Anti-CD28
Counts
FL2-H
0 hr. OspA
3 hr. OspA
18 hr. OspA

B.


Anti-CD3
0 ng/ml OspA
100 ng/ml OspA
500 ng/ml OspA
Anti-CD28
Counts
FL2-H

C.

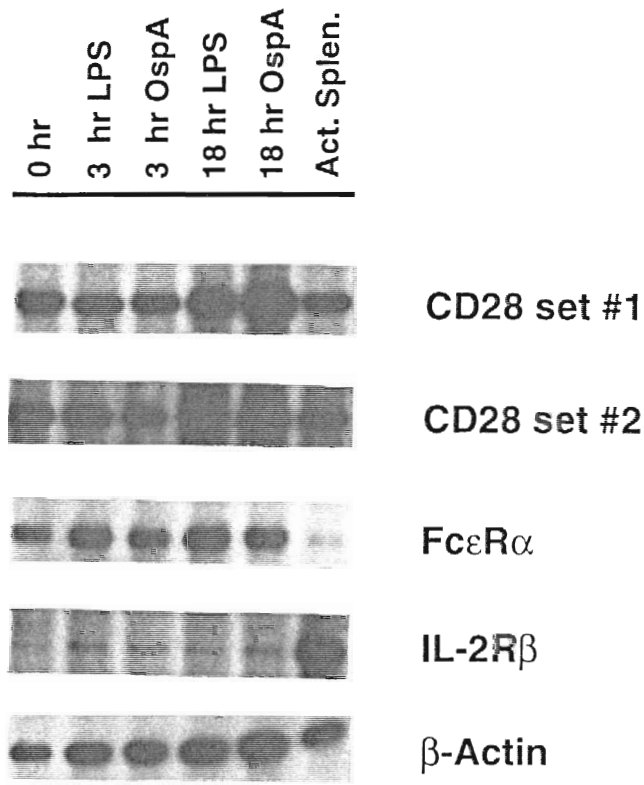

Anti-CD3
control
PB
PB + OspA
Anti-CD28
Counts
FL2-H

expression and polymyxin B alone did not increase the expression of CD28, OspA is capable of stimulating mast cells at low concentrations and increasing CD28 expression within 18 hr.

Both LPS and OspA Elevate CD28 Transcripts

The increase in cell surface CD28 expression on SCF-derived mast cells could be due to increased transcription of the CD28 gene, an increased translocation of preformed CD28 to the cell surface or the extension of the half life of the protein on the cell surface. The immediate cell surface expression of CD28 after PMA treatment of SCF-derived mast cells suggests that either the CD28 protein is translocated from an intracellular site or that the stability of the protein on the cell surface is increased. The longer induction time following LPS and OspA treatment, however, suggested that the level of CD28 transcription might be altered (i.e., increased). Accordingly, SCF-derived mast cells were treated with LPS (500 ng/ml) or OspA (500 ng/ml) for 0, 3, or 18 hr, RNA was extracted, and transcript analysis was performed (Figure 4.6). There did appear to be an increase in CD28 transcripts after 18 hr of LPS or OspA treatment, indicating that an increase in transcripts could account for some of the increased cell surface CD28 expression. It should be noted, however, that the control cells (0 hr) did possess high levels of CD28

Figure 4.6. Analysis via RT-RPCR of CD28 transcript levels after LPS or OspA treatment. Bone marrow mast cells that had been cultured in SCF for 3-4 weeks were treated with either 500 ng/ml LPS or 500 ng/ml OspA for 0, 3, or 18 hr. After RNA extraction, the presence of CD28 (28 cycles), FcεRα (26 cycles), IL-2Rβ (28 cycles), and β-Actin (16 cycles) transcripts was determined via RT-RPCR. Act. Splen. represents RNA isolated from activated splenocytes.


0 hr
3 hr LPS
3 hr OspA
18 hr LPS
18 hr OspA
Act. Splen.
CD28 set #1
CD28 set #2
FcεRα
IL-2Rβ
β-Actin

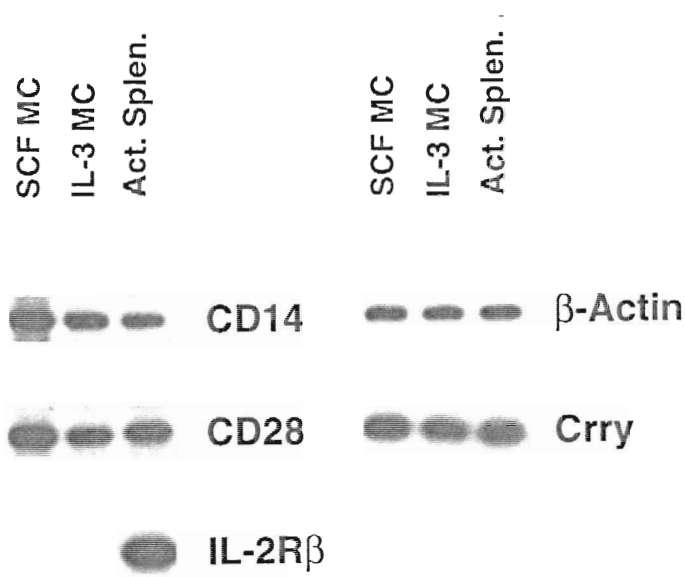
transcripts but no cell surface CD28 protein. Thus the overall level of CD28 on the cell surface may be a combination of transcriptional input and protein stabilization/translocation.

Analysis of CD28 Expression by IL-3-Derived Mast Cells

The data presented thus far have demonstrated the expression of CD28 gene products in SCF-derived bone marrow mast cells. Next examined was the expression of CD28 by IL-3-derived mast cells. As shown in Figure 4.7, the level of CD28 transcripts is largely the same for mast cells derived in either IL-3 or SCF. Again the levels of mast cell CD28 transcripts are comparable to those seen for T cell lines which constitutively express CD28 on the cell surface (data not shown). These RNA samples were also analyzed for transcripts encoding CD14, which they possess (Figure 4.7). CD14 is a receptor for LPS and has been reported to be expressed by macrophages and neutrophils (36). FACS analysis of the IL-3-derived and SCF-derived mast cells also demonstrated CD14 expression on their cell surface (data not shown).

Previous analyses with SCF-derived mast cells were done using cells cultured for a minimum of 21 days. Additional examinations were of CD28 expression by such cells after they had only been in

Figure 4.7. Comparison of constitutive CD28 and CD14 transcript levels between bone marrow mast cells derived in IL-3 (IL-3 MC) and SCF (SCF MC). RNA samples used for transcript analysis via RT-RPCR were extracted from unstimulated bone marrow cells cultured in SCF (SCF MC) for 3-4 weeks, unstimulated bone marrow-derived mast cells cultured in IL-3 (IL-3 MC) for 3-4 weeks, and activated splenocytes (Act. Splen.). Gene specific transcripts analyzed were CD14 (26 cycles), CD28 (28 cycles), IL-2Rβ (28 cycles), β-Actin (16 cycles), and Crry (26 cycles). Crry was included as a constitutive expression control.


SCF MC
IL-3 MC
Act. Splen.
SCF MC
IL-3 MC
Act. Splen.
CD14
β-Actin
CD28
Crry
IL-2Rβ

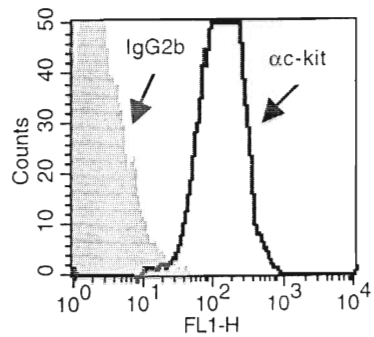
culture for 14 days, and as long as 50 days. In all situations, in the absence of exogenous stimulation, significant levels of cell surface CD28 were not observed, although all such cell populations demonstrated equivalent levels of CD28 transcripts (data not shown). Interestingly the same was not true for IL-3-derived mast cells. As demonstrated in Figure 4.8, after 18 days in culture, maturing IL-3-derived cells stained homogeneously for *c-kit* but lacked CD28. However, after 45 days in culture, the vast majority of the cells now constitutively expressed cell surface CD28 in the absence of any exogenous stimulation. Both populations demonstrated equivalent levels of CD28 transcripts (data not shown). The level of cell surface expression of CD28 by 18 day old IL-3-derived mast cells did not appear to increase after treatment with either PMA or LPS, as was demonstrated with the SCF-derived cells.

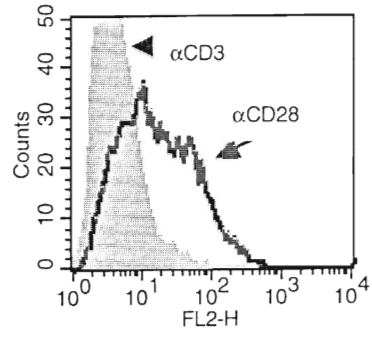
PMC Express CD28

The two types of mast cells examined for the expression of CD28 were those derived from the bone marrow in either IL-3 or SCF. As demonstrated by the maturing IL-3-derived cells, cellular maturation either in culture or within the animal, may play a role in the expression of CD28 on mast cells. *c-kit*$^+$ cells isolated from the peritoneal cavity are mast cells of the connective tissue phenotype

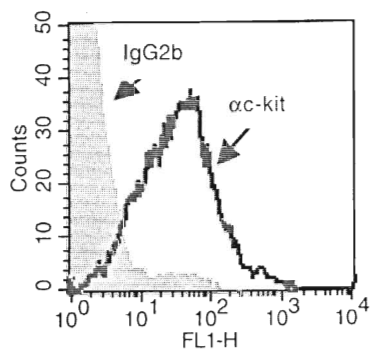
Figure 4.8. Expression of CD28 on unstimulated IL-3-derived mast cells. Bone marrow-derived cells that had been cultured in IL-3 (IL-3 MC) for either (A) 45 days (long term culture) or (B) 18 days (short term culture) were analyzed by FACS analysis for the expression of CD28. Antibodies used were FITC conjugated anti-*c-kit* (α*c-kit*) and IgG2b, and biotinylated anti-CD28 (αCD28) and anti-CD3 (αCD3).

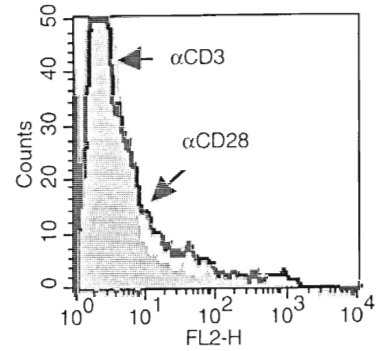
A.


IgG2b
αc-kit
Counts
FL1-H


αCD3
αCD28
Counts
FL2-H

B.


IgG2b
αc-kit
Counts
FL1-H


αCD3
αCD28
Counts
FL2-H

(37). However, they represent only a small percentage of the total peritoneal cell population (1 to 3%) (37). Accordingly *c-kit*+ cells were expanded in culture for 5 to 6 weeks with SCF and tested for their ability to express CD28. As shown in Figure 4.9, such cells clearly express the CD28 and *c-kit* proteins. Peritoneal mast cells freshly obtained from the animals were not analyzed.

Crosslinking of CD28 on IL-3-Derived Mast Cells Increases the Level of *c-jun* Transcripts

It has been previously demonstrated that crosslinking T cell CD28 in the absence of TCR ligation can lead to an upregulation of *c-jun* transcription without a concomitant increase in the accumulation of *c-fos* transcripts (38). This upregulation of *c-jun* is lost hours after induction. We analyzed mature murine IL-3-derived mast cells to determine if crosslinking their CD28 would elicit the same response. As shown in Figure 4.10, when CD28 is specifically crosslinked, an increase in *c-jun*, compared to β-actin, is evident within 30 min. At this time point, however, no alteration in the quantity of *c-fos* transcripts, compared to the ß-actin control, is observed. These data suggest T cells and mast cells possess a similar, if not identical, signal transduction pathway utilized for CD28 signaling.

Figure 4.9. Expression of CD28 on unstimulated PMC. Peritoneal wash cells were cultured for 5-6 weeks in SCF and then analyzed via FACS analysis for the expression of *c-kit* and CD28. Antibodies used were the same as described in Figure 4.8.

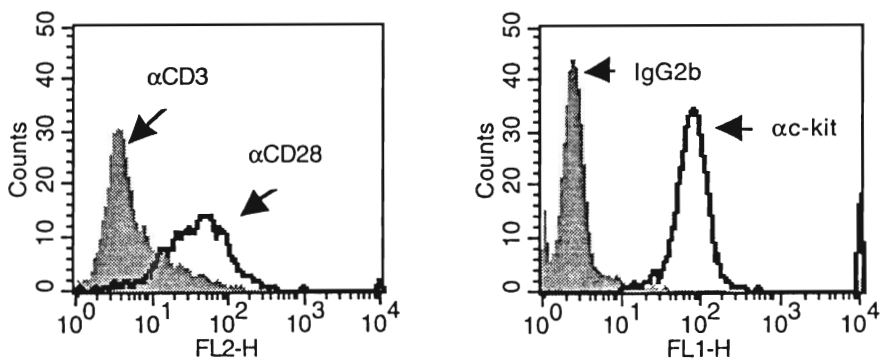
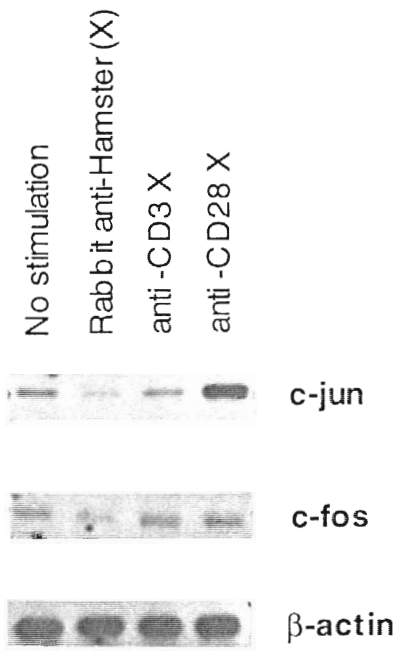

αCD3
αCD28
Counts
FL2-H
IgG2b
αc-kit
Counts
FL1-H

Figure 4.10. Effects of CD28 crosslinking on *c-jun* transcript levels in IL-3-derived mast cells. Bone marrow mast cells derived in IL-3 for 7-8 weeks were treated with Rabbit anti-Hamster (Rabbit anti-Hamster (X)) (5 ug/ml) for 30 min, treated with anti-CD3 (1 ug/ml) and Rabbit anti-Hamster sequentially (anti-CD3 X), or treated with anti-CD28 (37.51) (1 ug/ml) and Rabbit anti-Hamster sequentially (anti-CD28 X) or not treated with antibodies (No stimulation). RNA samples were then extracted and analyzed via RT-RPCR for transcripts for *c-jun* (24 cycles) and *c-fos* (24 cycles) and β-Actin (20 cycles).

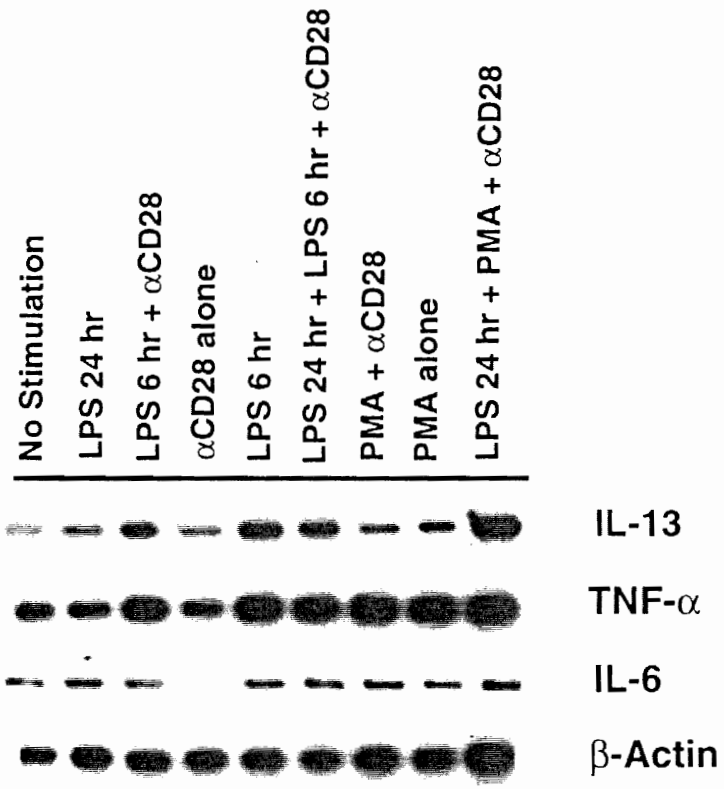

No stimulation
Rabbit anti-Hamster (X)
anti -CD3 X
anti -CD28 X
c-jun
c-fos
β-actin

Crosslinking CD28 on the Surface of SCF-Derived Mast Cells Leads to an Increase in IL-13 Transcripts

In the absence of TCR coligation, CD28 crosslinking on the surface of T cells leads to an increase in *c-jun* transcripts. In the presence of TCR coligation (or PMA stimulation), CD28 crosslinking leads to the increased production by T cells of such cytokines as IL-2 and IL-13 (39). We tested whether similar treatment of mast cells would also increase the level of relevant cytokine transcripts. We chose to examine SCF-derived mast cells because they usually express little, if any, transcripts specific for IL-13 (14).

CD28 cell surface expression on SCF-derived mast cells was induced with a 24 hr LPS treatment, followed by a 6 hr period of secondary stimulation (LPS or PMA) simultaneous with CD28 crosslinking (Figure 4.11). As shown, the greatest induction of IL-13 transcripts required both crosslinking with CD28 as well as a secondary signal (PMA), similar to that reported for T cell induction of IL-13 transcripts (39). Interestingly, the levels of IL-6 and TNF-α transcripts did not show the same pattern of induction as IL-13 suggesting IL-13 transcription is likely to be directly upregulated by the action of the *c-jun* gene product.

Figure 4.11. Effects of CD28 stimulation on the levels of IL-13, TNF-α, and IL-6 transcripts. Bone marrow mast cells derived in SCF for 3-4 weeks were analyzed via RT-RPCR for the expression of IL-13, TNF-α, and IL-6 transcripts after treatment with LPS, PMA, and/or CD28 stimulation. Treatment with LPS (20 ng/ml) was for either 24 hr (LPS 24 hr) or 6 hr (LPS 6 hr). Treatment with PMA (50ng/ml) was for 6 hr. Treatment with anti-CD28 antibody (100 ng/ml) was also for 6 hr. Gene-specific transcripts analyzed were IL-13 (28 cycles), TNF-α (24 cycles), IL-6 (28 cycles), and β-Actin (16 cycles).


No Stimulation
LPS 24 hr
LPS 6 hr + αCD28
αCD28 alone
LPS 6 hr
LPS 24 hr + LPS 6 hr + αCD28
PMA + αCD28
PMA alone
LPS 24 hr + PMA + αCD28
IL-13
TNF-α
IL-6
β-Actin

Discussion

Since its first description, CD28 had been identified as a product of T and NK cells. The realization that mast cells express CD28 introduces some novel possibilities. First, the finding that CD28 is expressed by a cell type other than T cells or NK cells is important because it introduces the concept that costimulation may not be restricted solely for the generation of the antigen specific response, i.e., the differentiation and proliferation of monoclonal T and B cell clones. Second, our finding that mast cell CD28 requires induction (either via maturation or exogenous agents) suggests that other cell types may also express similar costimulatory proteins but only after a similar inductive event. Third, the induction of CD28 cell surface expression via bacterial products suggests the expression of the protein *in vivo* may be a transitory effect of an infectious process. Thus mast cell CD28 may, functionally, have a narrow window of opportunity defined by the presence of an infectious assault. Mast cell CD28 may have no role as a constitutive element of the normal, unstressed immune system. Fourth, the co-expression of CD28 by T cells and mast cells presents yet another example of similarity between these two cells. Both cell types transcribe the gene which suggests they possess similar, if not identical, transcriptional control proteins. Additionally, the effect of CD28 crosslinking in these two

cell types appears to be quite similar in that the transcription of *c-jun* is preferentially upregulated and the increase of cytokine transcripts is evident.

The regulation of the cell surface expression of CD28, however, is not identical between mast cells and T cells. T cell CD28 is expressed constitutively after a set point in differentiation. Although its expression, documented for human T cells, can be increased with TCR activation (40), the protein is still constitutively present on the inactive cell's surface. Alternatively, mast cells which we have developed from bone marrow *in vitro* with SCF do not express the protein (although the mRNA is present) unless specifically stimulated to do so.

One model to explain the absence of constitutive CD28 on the surface of SCF-derived mast cells is that such cells lack a protein (or proteins) that associate with CD28 in the membrane and stabilize its expression. Three proteins that are known to bind to CD28 in T cells are *itk*, GRB-2/SOS, and PI3 kinase (41, 42). Such binding, however, requires the phosphorylation of specific residues in the CD28 cytoplasmic domain. It may be that one or more of these proteins is lacking or that the phosphorylation of such sites is deficient in mast cells. The transient expression of cell surface CD28 by SCF-derived mast cells following activation with PMA (which only requires 3 hr

for maximal induction) suggests an effective short-circuiting of this inhibition, probably through heightened protein kinase activity.

Alternatively, another model could be based on observations made concerning the localization of CD28's counter receptor, CTLA-4. CTLA-4 is expressed only at low levels on the cell surface; the bulk of the protein is stored intracellularly via an intracellular localization motif found within its cytoplasmic tail (43). Similarly, mast cell CD28 might have a functional intracellular localization motif (human T cell CD28 has a nonfunctional intracellular localization motif) (43) that would allow it to be stored intracellularly until needed at the cell surface. We are currently examining mast cells for the presence of intracellular CD28 and the presence or absence of proteins known to bind CD28. We are also determining if these putative proteins are functionally altered following exposure of the mast cells to LPS, OspA, or PMA.

The observation that CD28 expression can be induced by both LPS and bacterial lipoproteins (OspA) suggests that mast cells can respond to both of these compounds in a biologically relevant fashion. OspA possesses a structure similar to that of many other species of lipoproteins (18, 34). That mast cells can respond to it suggests they may be able to respond to similar proteins produced by a wide range of infectious agents. This response capability may

be an important aspect of the innate immune response carried out by the mast cell. This concept is consistent with the finding that mast cells appear to play an important protective role in acute septic peritonitis via the recruitment of neutrophils and the clearance of bacteria (44, 45). It is intriguing that the cell surface expression of CD28 is not constitutive but requires such induction. It may well be that the possible triggering of mast cell CD28 in the absence of an infectious event would be deleterious to the constitutive sentinel role of the mast cell.

This report has described the expression of CD28 by mast cells and some of the responses generated by the mast cell following its ligation. Not defined, yet, is why mast cells express the protein. CD28 has been functionally defined in T cells to augment the signal provided by the binding of the TCR to its appropriate peptide/MHC complex (4). One of the primary proteins of the TCR which helps transduce this signal is the ζ chain. The γ protein of the FcεR 1 complex is extremely homologous to the ζ chain of the TCR. T cells have been described which possess functional TCR containing FcεR1γ instead of, or with, the TCR ζ chain (46). Thus the signal provided to the T cell following TCR ligation would be very similar to that provided to the mast cell following FcεR1 crosslinking. Ligation of

the TCR leads to the functional expression of NF-AT which, in association with *fos/jun*, leads to the upregulation of IL-2 expression (4). A novel member of the NF-AT family has recently been described to be upregulated in mast cells following FcεR1 ligation (24). Therefore, the combined effect of FcεR1 and CD28 ligation on the surface of mast cells should lead to the formation of a transcription complex very similar to that seen following T cell coactivation. The key question that we are currently addressing is whether the coactivation of mast cell CD28 and FcεR1 provides a stimulatory signal different from either alone and whether the expression of gene products is specifically modified by such an event.

One recent publication by Tashiro et al. would indicate that coactivation of mast cell CD28 and FcεRI do provide a stimulatory signal (47). Their results demonstrated that the crosslinking of CD28 simultaneous with FcεRI led to increased levels of TNF-α release. It would appear then that, in certain culture conditions, the crosslinking of mast cell surface CD28 can lead to enhanced cytokine release, such as TNF-α, and enhanced cytokine transcription, such as IL-13. Enhanced cytokine transcription and release are functions exhibited by T cell CD28; thus these two results suggest that mast cell CD28 can function similarly to T cell CD28.

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CHAPTER V

DISCUSSION

Overview

The mast cell is capable of responding to many different stimuli, both innate in nature (complement proteins and the bacterial products LPS, fimH, and OspA) and antigen specific in nature (antigen bound IgG or IgE). The responses that mast cells have to these stimuli are also diverse. In response to many of the stimuli, such as the complement proteins, SCF, and antigen bound IgG and IgE, mast cells will degranulate and release histamine and other inflammatory mediators. However, depending on other signals, the quantity of mediators is different and the composition of mediators is different.

With respect to cytokines, SCF will give rise to a cell type that expresses IL-12 transcripts constitutively. IL-3 will give rise to a mast cell type that expresses more anti-inflammatory cytokines, such as IL-10 and IL-13. These results would indicate that the cytokine profile of mast cells is highly dependent upon the complete cytokine makeup present within the microenvironment of the mast cell. Other examples of cytokines inducing a phenotypic change in mast cells are IL-4, IL-9, and IL-10. These cytokines in combination with SCF or IL-3 will give rise to a number of different combinations of protease expression patterns in the mouse. Mast cells appear then, to be capable of responding to many of the different cytokines present within the microenvironment. These responses include

changes in proliferation, cytokine expression, and granule content. Together, these data indicate that the mast cell is quite plastic and is capable of further phenotypic change within its final microenvironment.

Even though this remarkable ability to respond to such a number of cytokines is demonstrated by the mast cell, it can also respond, via the ligation of cell surface molecules, to the surrounding cells within its microenvironment. These cell surface molecules include various adhesion integrins but now also include co-stimulatory molecules, such as CD28 and probably B7-1 or B7-2. The expression of B7-1 or B7-2 would allow the mast cell to present antigen to MHC Class II restricted T cells. The expression of CD28 appears to contribute to an increased level of IL-13 transcripts, after CD28 is crosslinked simultaneously with PMA treatment. This would indicate, then, that mast cells are capable of "sensing" the immune state not only via the cytokine makeup of the microenvironment but also via the composition of cell types that are present within the microenvironment. With the expression of CD28, the mast cell can potentially interact with any B7 expressing cell type, such as B cells, macrophages, and dendritic cells. Of course, with which cell type the mast cell interacts will depend on the location of the mast cell, the

nature of the immune assault, and the nature of the immune response.

Indeed, the mast cell is present at a diverse number of environments where pathogens are introduced. These environments include the skin, the mucosal areas of the intestine, and the lung. However, an anomaly exists in that mast cells have not been directly associated with the significant elimination of any pathogens in these environments, only indirectly. An exception to this may be the mast cell's ability to phagocytose FimH expressing gram negative bacteria, but whether this plays a significant role in direct bacterial clearance *in vivo* has yet to be addressed. Another exception may be the mast cell's association with nematode clearance. Yet, although the mast cell is implicated in the immune response to nematodes, it has not been demonstrated as being capable of eliminating the nematode. Mast cells have only been observed to be present in the mucosa at higher levels than normal (without nematode infection). Also present in a nematode infection are high levels of eosinophils, which are capable of eliminating the nematode infection. Mast cells, then, do not appear to have a direct effect in the clearance of pathogens, which is similar to T cells.

One well-defined role that the mast cell plays in an immune response, though, is its ability to "sense" in an antigen specific

fashion via its recognition of antigen bound IgE, the introduction of previously recognized pathogens and allergens in the skin (wheal and flare response), or allergens introduced into mucosal sites. In these immune responses, the mast cell acts as a sentinel and recognizes the pathogen or antigen, initiates an inflammatory response, and recruits neutrophils for pathogen clearance. Thus, indirectly, the mast cell has the potential to clear pathogens by being able to affect other cell types of the immune response with the production of vasodilators, chemokines, and cytokines. As mentioned before, mast cells can also respond to injections of C5a or C3a with the exact same response, a wheal and flare response. Thus, whether the immune response is antigen specific or innate, the mast cells can still direct the development of the immune response. Possibly then, the mast cell's "normal" role is not to eliminate pathogens directly as has been proposed with the nematode infection but to direct other cell types and, ultimately, the type of immune response towards pathogen clearance.

By having such a large repertoire of mediators, inflammatory-ctyokines, anti-inflammatory cytokines, vasodilators, and chemokines and being able to respond to a variety of different stimuli, cytokines, bacterial products, complement proteins, antibody complexes, and potentially cell-to-cell interactions via costimulatory

molecule ligations, mast cells certainly have the capability of directing an immune response down a humoral or cellular pathway, similar to T cells. One of the distinct differences between T cells and mast cells, though, is that mast cells are at the periphery and, as such, are one of the first cell types of the immune system to encounter the pathogen. Therefore, mast cells have the potential to initiate and direct the beginning of the immune response in both an antigen specific fashion, similar to T cells, or in an innate fashion, similar to macrophages.

A number of studies have generated data to support the theory that mast cells can direct an immune response. For inflammation, the hallmark study is the contribution of mast cells to the wheal and flare response. Other studies have demonstrated that mast cells can also direct the immune response in other afflictions, including Autoimmune Encephalomyelitis, Ischemia/reperfusion (I/R), and bacterial toxin-induced tissue injury.

Experimental autoimmune encephalomyelitis (EAE) serves as a model for multiple sclerosis (MS) in humans. EAE can be induced in animals by the injection of central nervous system tissue, myelin, myelin proteins, or peptides into the animals. Mast cells are thought to play a role in EAE, since in a study by Orr and Stanley mouse mast cells were observed to be associated with CNS blood vessels at sites

of edema in EAE afflicted SJL/J mice (1). However, the original cause for EAE has been demonstrated to be self-reactive T cells, since encephalitogenic T-cell clones derived from susceptible animals can transfer EAE to naive hosts (2). In another study by Orr and Stanley, histamine levels were found to be significantly increased with the onset of disease (1). With a study by Dietsch et al., mast cell degranulation inhibitors were found to suppress transfer of EAE by sensitized lymphocytes (3). In another study, by Bebo et al., histamine and TNF-α were found to induce leukocyte rolling and adhesion to mouse pial venules. Thus, although not proven to be the sole reason for the recruitment of leukocytes in EAE, the mast cell has been implicated in a number of ways to contribute to the development of the inflammation in EAE and the recruitment of leukocytes by increasing their rolling and adhesion.

Mast cells have also been implicated in the recruitment of leukocytes in I/R. In one study by Kanwar et al. (4), it was observed that increased plasma protease II levels (mast cell degranulation index) occurred following intestinal I/R. Based on these results, another study was performed, looking at the contribution of the mast cell to the development of I/R. In a study done by Kubes et al., sodium cromoglycate, a mast cell stabilizer,

attenuated the rolling of leukocytes in the cat mesentery, reduced neutrophil adhesion by 50%, and inhibited leukocyte emigration out of the postcapillary venules. Again, although not proven to be the only factor responsible for leukocyte recruitment, these data indicate that mast cells do play a role in the recruitment and emigration of leukocytes into the affected tissues (5).

Yet another model of mast cells contributing to the development of an immune response via the recruitment of leukocytes, is that of bacterial toxin-induced tissue injury. In a study by Kubes et al., rat mesentery treated with *Heliobacter pylori* Extract (HPE) was analyzed for mast cell degranulation, leukocyte adherence, and the emigration of leukocytes (5). It was observed that in HPE treated mesentery, 71% of the mast cells degranulated and leukocytes adhered and emigrated through the mesentery. However, in the presence of the mast cell stabilizer, ketotifen, HPE treated mesentery only had 23% mast cells degranulating with a >50% reduction in the number of emigrated leukocytes. Studies done with *Clostridium difficile* toxin A also demonstrated that mast cells contribute to the recruitment of leukocytes (5). Rat mesentery treated with the toxin and lodoxamide, another mast cell stabilizer, decreased the recruitment of leukocytes to the rat mesentery.

Eosinophil recruitment by mast cells has also been addressed. In one study by Hom and Estridge, the contribution of $CD4^+$ T cells and mast cells to antigen-induced eosinophil recruitment was analyzed (6). Although their results revealed that $CD4^+$ T cells play a large role in eosinophil recruitment, studies with mast cell deficient mice, both W/W^v and Sl/Sl^d, indicated that eosinophil recruitment to sites of ragweed injection were reduced by approximately two- to threefold in comparison to congenic control mice.

The above studies demonstrate that mast cells play a role in the recruitment of leukocytes to sites of injury or assault. Mast cells can play a role in the recruitment of neutrophils and eosinophils in a wide variety of tissues. Work presented here indicates that mast cells alter their phenotype in response to cytokines and costimulatory molecule ligations. Possibly then, as changes in the microenvironment occur, such as cytokine milieu and cell influxes, the mast cell alters its cytokine repertoire and granule contents in order to direct the immune response in a specific direction via further degranulation and cytokine secretion. Understanding, then, the contribution of the mast cell to the initiation of this recruitment, to the initiation of the immune response in these situations, and to what degree the tissue localization (cytokine makeup) and the influx of cells (cell-to-cell interactions) has on the further function of the

mast cells in these localized areas will give some insight into whether one of the "normal" roles for the mast cell is to direct an immune response.

Conclusion

Material in this dissertation demonstrates that mast cells can alter their cytokine repertoire in response to stimuli other than IgE and antigen. Mast cells can respond to cytokines alone and, in response, alter their cytokine repertoire. Similar to observations found by Smith et al., where IL-3-derived bone marrow mast cells constitutively express IL-4 transcripts, IL-3-derived bone marrow mast cells also constitutively express transcripts for other anti-inflammatory cytokines, IL-10 and IL-13. Data in this thesis also demonstrate that IL-3 has a similar effect upon SCF-derived bone marrow mast cells. When the SCF-derived bone marrow mast cells are treated with IL-3, the level of transcripts for IL-10 and IL-13 is increased within 90 min. IL-10 protein is expressed by SCF-derived bone marrow mast cells 4 days after IL-3 treatment and is constitutively expressed by IL-3-derived mast cells.

Data in this dissertation also demonstrate that IL-3-derived bone marrow mast cells express CD28 at constitutively low levels at

the cell surface. For SCF-derived bone marrow mast cells, CD28 expression can be increased with the treatment of PMA, LPS, or OspA. FACscan analysis revealed that the CD28 molecule expressed by mast cells is most likely the same CD28 molecule expressed by T cells, and is not a CD28-like molecule. This observation was based on the ability of two monoclonal antibodies, which recognize different epitopes of the T cell CD28 molecule, to both bind to the mast cell CD28 molecule. Further, transcript analysis via RT-RPCR demonstrated that mast cell CD28 shares some components with T cell CD28, since *c-jun* transcripts and IL-13 transcript levels were increased after crosslinking CD28.

Together, these two groups of data demonstrate that mast cells are still potentially capable of further differentiating within different microenvironments, and this can be due to either cytokine uptake or cell-to-cell interactions via costimulatory molecule ligation. These data suggest, then, that depending on the cytokine makeup of the microenvironment and the cells present or that come into the microenvironment, a mast cell will correspondingly change its cytokine repertoire. The observation that mast cells express CD28 also indicates that mast cells have the potential to interact with activated B cells and T cells, macrophages, and dendritic cells. Also, the finding that SCF-derived bone marrow mast cells will respond to

the bacterial products LPS and OspA by increasing cell surface expression of CD28 supports the growing amount of data reinforcing the mast cell's involvement in innate immune response.

Understanding whether mast cells can associate with other cell types that express B7 molecules and under which circumstances (cytokine milieus) they associate will provide insight into just how truly plastic the mast cell is with respect to its granule contents and cytokine secretions.

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