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**Effect of heat-killed *Listeria monocytogenes* and *E. coli* O157:H7 on function of human eosinophil and basophil-like cells**

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# EFFECT OF HEAT-KILLED *LISTERIA MONOCYTOGENES* AND *ESCHERICHIA COLI* O157:H7 ON FUNCTION OF HUMAN EOSINOPHIL-LIKE CELLS AND BASOPHIL-LIKE CELLS

By

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## ABSTRACT

EFFECT OF HEAT-KILLED *LISTERIA MONOCYTOGENES* AND *ESCHERICHIA COLI* O157:H7 ON FUNCTION OF HUMAN EOSINOPHIL-LIKE CELLS AND BASOPHIL-LIKE CELLS

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Using a human cell line model, we tested the hypothesis that exposure to heat-killed *Listeria monocytogenes* (HKLM) and *Escherichia coli* O157:H7 (HKEC) inhibits cell proliferation, IL-4, IL-5, IL-13, eotaxin, ECP and EDN production, expression of CCR3 and FcεRIα by the eosinophils and basophils. Whereas both pathogens inhibited proliferation of eosinophil-like cells, only HKEC inhibited proliferation of basophil-like cells. Both bacteria significantly increased the production of EDN by eosinophil-like cells, and IL-5, IL-13 and eotaxin (CCL11) by basophil-like cells. Notably, both bacteria inhibited IL-4 production by basophil-like cells and CCR3 expression on eosinophil-like cells. Neither pathogens significantly altered the high affinity IgE receptor expression on basophil-like cells. The purpose of this work was not to test what happens when allergic humans are exposed to heat-killed bacteria. Rather our goal was to determine if these bacteria can inhibit specific gene expression (such as IL-4, eotaxin and CCR3), so that the active component of the organism could be isolated and used in future animal model studies to asses protective therapeutic effects. Our findings that both bacteria specifically inhibit IL-4 - a critical type-2 cytokine required for allergic responses, and CCR3 – a major chemokine receptor implicated in allergy pathogenesis suggest the need for future studies aimed to identify the IL-4 and CCR3 inhibiting components of these bacteria.

***Dedicated to my parents, brother and all the people who helped me….***

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| | |
|---|---|
| **HKLM** | Heat-killed *Listeria monocytogenes* |
| **HKEC** | Heat-killed *Escherichia coli* O157:H7 |
| **IL** | Interleukin |
| **MCP** | Monocyte Chemoattractant Protein |
| **MDC** | Macrophage Derived Chemokine |
| **MIG** | Monokine Induced by interferon-$\gamma$ |
| **MIP** | Macrophage Inflammatory Protein |
| **NAP** | Neutrophil Activated Peptide |
| **RANTES** | Regulated on Activation, Normally T-cell Expressed and Secreted |
| **SDF** | Stromal cell Derived Factor |
| **TARC** | Thymus and Activation-Regulated Chemokine |
| **Th** | T helper lymphocyte |
| **OVA** | Ovalbumin |
| **Ig** | Immunoglobulin |
| **ECP** | Eosinophil Cationic Protein |
| **EDN** | Eosinophil Derived Neurotoxin |
| **ELISA** | Enzyme Linked Immunosorbent Assay |

# CHAPTER 1: INTRODUCTION

Allergic disorders such as asthma, allergic rhinitis (hay fever), food allergies, and atopic dermatitis are significant public heath problems. Food allergies in particular often lead to life threatening systemic anaphylaxis. Allergic reactions are immune mediated adverse responses to common environmental antigens such as pollen, animal dander and food. These disorders affect up to 20% of the population in industrialized countries (Trujillo and Erb, 2003) and in the United States nearly 50 million people suffer from allergies (CDC, 2003).

Mast cells, basophils and eosinophils play a key role as effector cells of allergic inflammation. Furthermore, IgE antibody, IL-4, eotaxin, ECP, EDN, Fc$\epsilon$RI and CCR3 are major molecules involved in the pathogenesis.

The prevalence of allergies has been increasing since the early 1980s (Kim and Drake-Lee, 2003; Sheikh and Strachan, 2004). The increase in allergic diseases has been attributed in part to improvements in personal hygiene and reduced exposure to pathogens, bacterial products, infections and increased exposure to allergens during early life (Crane, 2002; Matricardi, 2004). This concept is often termed as 'the hygiene hypothesis'.

According to the hygiene hypothesis suggested by Strachan et al. (1989), infections and allergies are inversely related. In recent years there has been a growing interest in the relationships among infections, pathogens and allergic diseases (Douwes et al., 2004; Hadley et al., 2005; Matricardi et al., 2000; Mojtahedi and Ghaderi, 2005; Platts-Mills et al., 2005). Several animal model studies showed that *L. monocytogenes* and *E. coli* (EC) in particular may prevent allergies (Li et al., 2003a; Li et al., 2003b; Yeung et al., 1998). Thus, two studies have assessed the effect of heat-killed *L. monocytogenes* (HKLM) on food allergies in animal models (Frick et al., 2005; Li et al., 2003b). These studies showed that HKLM inhibited the allergen specific IgE, airways eosinophilia and Th2 cytokine responses (Hansen et al., 2000; Li et al., 2003b). Notably these studies did not examine the effect of *L. monocytogenes* on the function of basophils or eosinophils.

Li et al. (2003) showed that treatment with heat-killed *E. coli* (HKEC) can induce long term downregulation of peanut induced allergenic immune responses in a mouse model. In one other study using oral probiotic *E. coli* and allergic responses such as OVA-specific IgE, total IgE, and IgG1 were suppressed in a mouse model (Kim et al., 2005). Notably these studies did not examine the effect of *E. coli* on the function of basophils or eosinophils.

Since basophils and eosinophils constitute only a small proportion of peripheral blood leukocytes (0-5%), they are difficult to obtain in large numbers (Franklin, 2003). Therefore, several studies have used the cell line KU812 as a model to study basophils and HL-60 clone 15 derived cells as a model to study eosinophils.

**Rationale and statement of the problem:** Studies have been done looking at the effect of HKLM and HKEC on allergic diseases in a mouse model. We are not aware of previous studies examining the effect of HKLM or HKEC on basophil or eosinophil function. Furthermore, there is little evidence for direct interaction between *L. monocytogenes* or non-invasive *E. coli* O157:H7 and basophils and eosinophils.

This study was conducted with following aims and hypothesis

**Aim 1:** To test the hypothesis that HKLM and HKEC inhibit the proliferation of human eosinophil-like cells and basophil-like cells.

**Aim 2:** To test the hypothesis that HKLM and HKEC inhibit the IL-4, IL-5, IL-13 and eotaxin production by human eosinophil-like cells and basophil-like cells.

**Aim 3:** To test the hypothesis that HKLM and HKEC inhibit the ECP and EDN production by human eosinophil-like cells.

**Aim 4:** To test the hypothesis that HKLM and HKEC inhibit the CCR3 receptor expression by human eosinophil-like cells.

**Aim 5:** To test the hypothesis that HKLM and HKEC inhibit the high affinity IgE receptor (Fc$\epsilon$RI$\alpha$) expression by human basophil-like cells.

The purpose of this work was not to test what happens when allergic humans are exposed to heat-killed bacteria. Rather our goal was to determine if these bacteria can inhibit

specific gene expression (such as IL-4, eotaxin and CCR3), so that the active component of the organism could be isolated and for therapeutic effects assessed in future animal model studies.

# CHAPTER 2: REVIEW OF LITERATURE

This review of literature (i) summarizes the hygiene hypothesis for allergies; (ii) assess the role of *L. monocytogenes* and *E. coli* (not O157:H7) in allergic disorders as reported in the literature and (iii) provides an overview on the biology of eosinophils and basophils. It also compares and contrasts the biology of eosinophils, basophils and mast cells with the human eosinophilic cell line (HL-60 clone 15 derived) and human basophilic cell line (KU812) model systems used in this study.

## Hygiene hypothesis:

In 1989, Strachan proposed the hygiene hypothesis to explain the increased prevalence of allergic diseases in industrialized countries. According to this hypothesis, allergic diseases may be prevented by exposure to infectious agents during early childhood. Therefore, the rise in allergic diseases over the last three decades in developed countries has been attributed in part to improved environmental conditions and reduced microbial exposure.

Numerous reports suggested that hygienic environment in modernized societies may be partly responsible for the increased prevalence of atopic diseases. Reductions in allergen sensitization and atopic disease have been found in children of farmers, children with pets and siblings in their homes, children raised in day-care, and children of large families (Matricardi et al., 1998; Ring et al., 1999; Shaheen et al., 1996; Svanes et al., 1999).

Merchant et al (2002) investigated the relationship between asthma, allergies and farm exposure. The aim of this epidemiological study was to estimate asthma prevalence and assess whether farm exposures result in reduced atopy, allergic disease and asthma, while taking into account multiple personal and other environmental risk factors, among this cohort of farm children. In children, farming as the parental occupation was significantly associated with lower rates of sneezing attacks, allergic sensitization, wheezing, itchy skin rash and allergic rhinitis (Riedler et al., 2000, Von Ehrenstein et al., 2000).

Kramer et al. (1999) suggested that early day care attendance (6–11 months) 6% children from small families in Germany (up to three individuals) afforded 5 to 13 years of protection against allergic sensitizations and hay fever. These findings were supported by researchers in Arizona, US (Haby et al, 2000). Attendance at day care during the first six months of life reduced the relative risk of asthma. Children exposed to other children had more wheezing at 2 years of age, but less wheezing from age 6 to 13.

The intestinal microflora established during infancy may be a source for the induction of immune deviation and the flora composition may determine whether allergy disorders develop. Bjorksten et al. (2001) investigated the relationship between allergies and intestinal microflora. Their study found that children who developed allergies during the first two years of life were less often colonized with enterococci, bifidobacteria, and bacteroides, and had higher stool counts of *Staphylococcus aureus* and *Clostridium* compared to healthy infants.

Kalliomaki et al. (2000) investigated the effect of probiotic bacteria on atopic eczema in a double blind placebo control study. Results showed that frequency of atopic eczema in a *Lactobacillus* GG-treated group was half of that in a placebo-treated group, suggesting that *Lactobacillus* GG effectively prevented early atopic disease in high risk children. Another study showed that *Lactobacillus casei* inhibited antigen-induced IgE production by inducing interleukin-12 (IL-12) secretion by macrophages (Shida et al 1998).

Hessel et al. (2000) showed that gram-positive and gram-negative bacteria produced different cytokines in human peripheral blood cells. The difference in OVA- specific fecal IgA and serum IgG1 levels among the experimental bacteria suggest different routes for allergy inhibition.

Gereda et al. (2000) investigated the relationship between endotoxin exposure, type 1 T-cell development, and allergen sensitization in infants at high risk of asthma. This study showed that the homes of allergen-sensitized infants had significantly lower house dust endotoxin levels than those of non-sensitized infants, and levels correlated with IFN-$\gamma$–producing T cells, but not with IL-4, IL-5, or IL-13 producing cell proportions. Exposure to various microbial products early in life may explain the low prevalence of allergy among children of farmers (Leynaert et al, 2001, Riedler et al 2001) and the reported inverse relationship between levels of endotoxin house dust and prevalence of allergy.

In a murine model, oral bacterial extracts (comprised of lyophilized fractions of several common respiratory tract bacterial pathogens) induced an increase in IFN-g and IgG2b, and decrease in IgG1 and IL-4. Their results demonstrated that these bacterial extracts function by enhancing postnatal maturation of Th1 function (Bowman et al 2001).

Matricardi et al. (2000) investigated the relationship between foodborne, oro-fecal exposure and atopy and allergic asthma in Italian cadets. Overall, respiratory allergies and atopy were less frequent in people who were exposed to oro-fecal and foodborne microbes such as *Helicobacter pylori, Taxoplasma gondii* and *hepatitis A* virus, but not to other microbes (such as mumps, measles, chicken pox) transmitted through other routes.

Several studies during the past eight years have provided controversial evidence on the role of infections vs. allergy (Cohet et al., 2004; Hadley et al., 2005; Johnston and Openshaw, 2001; Matricardi et al., 2000; Purvis et al., 2005; Radon et al., 2004). In the following sections evidence on the role of *L. monocytogenes* and *E. coli* in allergy has been reviewed.

***L. monocytogenes exposure* and allergy:** *Listeria monocytogenes*, a Gram positive, non-spore forming, motile, facultative intracellular organism causes listeriosis. It can be isolated from soil, silage, and other environmental sources. Manifestations of listeriosis include septicemia, meningitis, encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion or stillbirth.

It has been shown that *L. monocytogenes* alleviates anaphylactic symptoms and reduces allergen-specific IgE and Th2 cytokine (IL-4, IL-5, and IL-13) responses in murine models. Frick et al. (2005) used a canine model to study the effect of HKL on peanut and milk induced anaphylaxis. Their results suggest that vaccination of dogs allergic to peanut and milk with HKL plus allergen, followed by oral challenge with peanut or milk, HKL, and peanut markedly reduced the peanut induced anaphylactic symptoms and allergen-specific IgE, and increased the oral tolerance for these allergens (Frick et al., 2005).

Another study investigated the effect of HKL on the ovalbumin induced airway hyper-reactivity model of asthma in a murine model. Their results showed that HKL dramatically inhibited airway inflammation, eosinophilia and mucus production, significantly reduced OVA-specific IgE and IL-4 production, and dramatically increased Ag-specific IFN-g synthesis (Hansen et al., 2000). Similar study has done by Mizuki and colleagues using *L. monocytogenes* infection on ovalbumin induced asthma in a murine model. They found that *L. monocytogenes* infection suppresses OVA-induced allergic responses like airway eosinophilia, total serum IgE, OVA-specific IgE and Th2 (IL-4, 5, and 13) responses in spleen cell cultures (Mizuki et al., 2001).

One other study investigated the effect of HKL on primary and secondary Ag-specific immune responses in a murine model. Mice immunized with Ag-keyhole limpet hemocyanin (KLH) mixed with HKL showed increased production of Th1 cytokines and KLH-specific IgG2a antibodies, and decreased IL-4 cytokine production and KLH-

specific IgE antibody. Thus, HKL may be clinically effective in vaccine therapies for allergy and asthma (Yeung et al. 1998).

Heat-killed *Listeria* (HKL) mixed with recombinant peanut proteins as an adjuvant on anaphylactic reactions in a mouse model for peanut allergy. HKL significantly reduced peanut-induced anaphylactic symptoms, and inhibited the peanut specific IgE, plasma histamine, Th2 (IL-4, IL-5 and IL-13) cytokine production in spleen cells. These results show that immunotherapy with modified peanut proteins and HKL is effective for treating peanut allergy in this model (Li et al., 2003). All these studies demonstrated that HKL may mediate immune deviation from a pathological Th2-dominated response toward a protective immune response in asthma and allergic diseases. Notably these works did not study the effect of *L. monocytogenes* on the function of eosinophils, basophils or mast cells.

***E. coli* exposure and Allergies:** Three studies have been looked at effect of *E. coli* on allergic diseases. Oral administration of *E. coli* MC4100 culture to murine model of allergy, before OVA-sensitization. *E. coli* inhibited total IgE production and markedly reduced OVA-specific IgE levels. Additionally, *E. coli* treated mice had significantly fewer of de-granulated mast cells. These results demonstrate that administration of *E. coli* inhibited OVA-specific IgE synthesis and thereby reduced the intensity of allergy symptoms (Kim et al., 2005).

Another study examined the effect of HKE producing engineered Ara h1, 2, and 3 on peanut allergy in a mouse model. Thus, HKE-MP123 (heat-killed *Escherichia coli* producing engineered Ara h1, 2, and 3) suppressed peanut induced anaphylaxis symptoms, peanut-specific IgE, plasma histamine, and IL-4, IL-5, IL-10, IL-13 in spleenocyte culture and increased IFN-γ and TGF-B1 levels. HKE alone also had the same beneficial effects. Treatment with HKE-MP123 can induce long-term "downregulation" of peanut hypersensitivity in this allergy model (Li et al., 2003).

Oral administration of probiotic *E. coli* to children of allergic mothers and colonization after birth influenced the levels of some cytokines (IL-4, IFN-g and TGF-beta) and also reduced the clinical manifestations of allergy (Lodinova et al., 2003, 2004).

Thus, although some studies examined the effect of *E. coli* on T-helper cell function in the mouse model, we are not aware of previous studies examining impact of *E.coli* in general or *E. coli* O157:H7 in particular on the function of basophils or eosinophils.

**Role of eosinophils, basophils and mast cells in allergy:**

Mast cells, eosinophils and basophils are important immune cells in mediating allergic disorders (Galli, 2000; Sampson, 2000). The inflammatory responses associated with atopic disorders are characterized by the recruitment of eosinophils, basophils, and lymphocytes to the site of inflammation (Gangur et al., 2003; Gangur and Oppenheim, 2000). Basophils and eosinophils are circulating granulocytes. They are of myeloid origin and develop from pluripotent stem cells found in bone marrow. These cells are released

from the bone marrow as mature cells and have little capacity for further development and proliferation in peripheral tissues (Murakami et al., 1969). Mediators produced by these cells profoundly influence the orchestration of allergic inflammation.

**Role of eosinophils in allergy:**

Eosinophils, first observed by Wharton Jones in 1846, play an important role in allergic diseases (Franklin, 2003). They vary in size from 12 to 17 μm. Their nucleus is usually bi-lobed and they are characterized by distinctive granules in the cytoplasm. They have a short half-life of 8 to 18 h in circulation and then migrate from circulation to tissues where the epithelial surfaces are exposed to the external environment (Franklin, 2003).

Eosinophils also have been implicated as primary effector cells in some specific eosinophil mediated diseases such as allergic eosinophilic oesophagitis, allergic eosinophilic gastroenteritis, allergic colitis and food protein–induced proctocolitis (Rothenberg, 2004; Rothenberg et al., 2001; Sampson, 2004). Activated eosinophils secrete a variety of proinflammatory or tissue-damaging substances, including granular proteins and several chemokines, and cytokines. Allergic/inflammatory airway diseases such as asthma are characterized by infiltration of eosinophils into lungs (Giembycz and Lindsay, 1999).

Peripheral blood has relatively few eosinophils (0-5%). Therefore it is difficult to obtain them in large numbers (Franklin, 2003). HL-60 clone 15-differentiated eosinophils

present a useful means to perform studies on eosinophil function. HL-60 clone 15, when treated with 0.5 mM butyric acid, can differentiate into cells closely resembling human eosinophil-like cells (Fischkoff, 1988). These eosinophil-like cells express many characteristics of primary eosinophils as summarized in Table 2.1. These cells also express the chemokine receptor (CCR)-3 which is critical in trafficking of eosinophils to sites of allergic reactions (Badewa et al., 2002; Michail and Abernathy, 2004; Tiffany et al., 1998). Recent studies using gene knockout mice showed a key pathogenic role of eosinophils and CCR3-eotaxin chemokine system in a mouse model of ovalbumin-induced allergic gastritis. Thus, they demonstrated that mice genetically deficient in eotaxin (a ligand for CCR3) were protected from eosinophil-mediated allergic gastritis (Mishra and Rothenberg, 2003).

Upon activation, they release granular proteins such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) (Gundel et al., 1991; Harrison et al., 1999; Rosenberg and Domachowske, 2001). These cytotoxic proteins are believed to play a role in the development of subacute and chronic symptoms of allergy (Venge, 2004). In addition, eosinophils are also sources for proinflammatory cytokines such as IL-4, IL-5, IL-6, IL-8 and IL-10 (Bandeira-Melo and Weller, 2005; Nakajima et al., 1996) and chemokines such as CCL5 (RANTES), CCL3 (MIP-1$\alpha$), CCL2 (MCP-1), CCL11 (eotaxin) and CCL24 (eotaxin-2) (Bandeira-Melo and Weller, 2005; Chiba et al., 2005). Eosinophils express various membrane receptors for adhesion, cytokine and chemokines. IL-5 is a

Table 2.1 Comparison of primary human eosinophils and HL-60 clone 15- derived eosinophil-like cells (Franklin et al., 2003)

| **Characteristics** | **Primary human eosinophils** | **HL-60 clone 15 derived eosinophils** |
|---|---|---|
| **Morphological** | | |
| Maturity | Mature | Immature |
| Nucleus | Bi-lobed | Irregular |
| Granules in cytoplasm | + | +/- |
| Staining | Acid dye eosin | Acid dye eosin |
| **Functional** | | |
| Proliferation | - | + |
| Major mediators in cytoplasmic granules (ECP, EDN, EPO, MBP) | + | + |
| Cytokine released upon activation. IL-4, IL-5 and IL-13 | + | NR |
| Chemokines released upon activation, MCP, MIP-1 alpha, RANTES, eotaxin and eotaxin-2 | + | + |
| High affinity IgE receptor expression | - | - |
| Chemokine receptor expression (CCR3) | + | + |

NR : Not reported

Key cytokine in the regulation of eosinophilia and eosinophil activation in humans (Kay et al, 2004). Human eosinophils express several chemokine receptors such as CCR1, CCR3, CXCR3 and CXCR4. CCR3 plays a key role in recruitment of eosinophils to allergic inflammation sites by binding to eotaxins, CCL 7 (MCP-3), CCL13 (MCP-4) and CCL5 (RANTES) (Bandeira-Melo and Weller, 2005; Uguccioni et al., 1997).

In our studies clone 15 HL-60 human eosinophilic cells as the in vitro model along with HL-60 clone 15 cells that expressed CCR3 chemokine receptor and granular proteins such as ECP, EDN, and EPO similar to human primary eosinophils (Tiffany et al., 1998; Tiffany et al., 1995).

**Role of basophils/mast cells in allergy:**

Basophils and mast cells play a key role in the development of immediate hypersensitive reactions, because these are the only cells that bear high affinity receptors for IgE antibody- i.e., the allergy-causing antibody (FcεRIα). Table 2.2 shows the similarities and some differences between mast cells, basophils and the KU812 basophilic cell line.

Upon activation via FcεRIα receptor both mast cells and basophils can produce a similar spectrum of proinflammatory mediators, as well as certain cytokines and chemokines. Both of these cells share some phenotypic and functional properties, but they differ in some important aspects such as natural history, location and mediator content. In contrast to mast cells, basophils are circulating granulocytes that typically mature in the bone

Table 2.2: Comparison of human basophils, mast cells and KU812 basophil-like cells (Franklin et al, 2003, Blom et al 1992, Galli et al., 2000)

| Characteristics | Human basophils | Human mast cells | KU812 basophilic cell line |
|---|---|---|---|
| **Morphological** | | | |
| Location | Circulation | Tissue | NA |
| Granules | + | + | + |
| Maturity | Mature | Mature | Immature |
| Staining | Basic dyes | Cationic dye | Basic dye |
| **Functional** | | | |
| Proliferation | - | + | + |
| Major mediators in cytoplasmic granules (histamine and tryptase) | + | + | + |
| Major lipid mediators released upon activation LTC4 and LTB4. | + | + | + |
| Cytokine released upon activation. IL-4, IL-5, IL-13, TGF-B1, IL-10, IL-3 and IL-6. | + | + | + |
| Chemokines released upon activation, CCL-2,7,8, CCL3, CCL5 | + | + | + |
| High affinity IgE receptor expression | + | + | + |
| Chemokine receptor expression (CCR3) | + | + | NR |

NR-Not reported: NA- Not applicable
IL- Interleukin, LTC4- Leukotriene C4, LTB4- Leukotriene B4

marrow, circulate in the blood as mature cells, and can be recruited into sites of inflammatory responses (Galli et al., 2005). Whereas mast cells can be long-lived and can re-enter the cell cycle to proliferate locally, mature granulocytes such as eosinophils and basophils do not (Galli, 2000; Galli and Hammel, 1994; Wedemeyer et al., 2000).

Basophils were first identified and described in 1879 by Paul Ehrlich (Galli and Hammel, 1994; Franklin, 2003). Mature basophils range in size from 5 to 8 μm, they have a lobed nucleus with the chromatin in the nucleus condensed. Granular contents within the cytoplasm include glycosaminoglycans, which give a metachromatic appearance after staining. Basophils selectively infiltrate into the skin, nose, lung and other tissue sites by expressing specific adhesion molecules and chemokine receptors (CCR3 in particular) that enable their migration from the circulatory system into tissues (Bochner and Sterbinsky, 1991; Wimazal et al., 1999). They express a high affinity IgE receptor (FcεRI) that plays an important role in cellular activation. FcεRI is a tetrameric receptor that consists of one $\alpha$ chain, one $\beta$ chain and two $\gamma$ chains. The most important functional characteristic of basophils is their ability to bind to IgE via the $\alpha$ subunit of high affinity IgE receptor (FcεRI$\alpha$) (Blank et al., 1989; Blank et al., 1991). Allergen cross linking of IgE bound to FcεRI$\alpha$ leads to basophil degranulation and the release of several inflammatory mediators including histamine, proteases, cytokines, chemokines and leukotrienes. Basophils account for all histamine released in circulation (Ishizaka et al., 1972) and are a significant source for Type 2 cytokines such as IL-4 and IL-13, which are now considered critical components in the pathogenesis of allergic diseases (Devouassoux et al., 1999).

IL-4- is a critical Th2 cytokine responsible for important pro-inflammatory functions in asthma and allergic diseases, including T helper cell type 2 lymphocyte differentiation, induction of IgE production, up-regulation of IgE receptors, promotion of eosinophil transmigration into the lungs and mucus hypersecretion. IL-4 is a key therapeutic target in treatment of allergic diseases (Steinke, 2004). Basophils also produce several chemokines such as CCL3 (MIP-1$\alpha$), CCL2 (MCP-1) and CCL5 (RANTES) and also express CCR3 chemokine receptor (Iikura et al., 2004; Li et al., 1996; Uguccioni et al., 1997).

Since basophils constitute only 1% of peripheral blood leukocytes, they are difficult to obtain in large numbers (Franklin, 2003). Basophilic differentiation of KU812 cells occurs by the serum starvation technique. Thus, cells grown in a serum-free medium develop characteristics of human primary basophils and mast cells such as high affinity IgE receptor expression and histamine production (Blom et al., 1992; Franklin, 2003; Galli et al., 2005). Therefore, several studies have used the cell line KU812 model. This model represents an immature basophil precursor and was originally obtained from a patient with chronic myeloid leukemia. While this model is useful for studying basophil biology (Blom et al., 1992). This basophil-like cell line must be viewed with caution given fundamental differences in morphology and function compared with primary human basophils.

## CHAPTER 3: MATERIALS AND METHODS

**HL-60 Clone 15 (Eosinohilic cell line):** The HL-60 clone 15 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in culture media {86% (v/v) RPMI-1640 containing 1.5 g/L sodium bicarbonate and 25Mm HEPES (Sigma, St.Louis, MO), 2 mM L-glutamine (Gibco, Carlsbad, CA), 10% (v/v) fetal bovine serum (Sigma), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin (Gibco); pH 7.6} at 37°C and 5% $CO_2$ (Isotemp $CO_2$ incubators, Fisher scientific, Pittsburgh, PA) Cells were maintained at a starting concentration of 5 x $10^5$ cells/mL. Every 3 days, cells were centrifuged (500 rpm, 37$^O$C, 5 min), and resuspended in fresh culture media at 5 x $10^5$ cells/mL. For experiments, cells were cultured in 0.5 mM sodium butyrate (Sigma) for 3 days and then washed with culture media and re-suspended in fresh culture media containing 0.5 mM sodium butyrate (Sigma) with and without HKLM and HKEC at indicated concentration.

**KU812 Cell line (Basophilic cell line):** The KU812 cell line was obtained from the American Type Culture Collection (ATCC) and grown in culture media {86% (v/v) RPMI-1640 containing 1.5 g/L sodium bicarbonate and 25mM HEPES (Sigma), 2mM L-glutamine (Gibco), 10% (v/v) fetal bovine serum (Sigma), 100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin (Gibco); pH 7.2} at 37°C and 5% $CO_2$ (Isotemp $CO_2$ incubators, Fisher scientific) The cells were maintained at a starting concentration of 5 x $10^5$ cells/mL. Every 3 days cells were centrifuged (500 rpm, 37$^O$C, 5 min), and re-suspended in fresh culture media at 5 x $10^5$ cells/mL. For experiments cells

were first centrifuged and washed with serum-free culture medium. Then the cells were cultured in serum-free culture medium with and without HKLM and HKEC for 4 days.

**Preparation and heat killing of *L. monocytogenes* and *E. coli* O157:H7:**

The following bacterial isolates were kindly provided by Dr. Elliot Ryser (Michigan State University): (i) *Listeria monocytogenes*: ILSI #1 (Scott A, clinical strain, outbreak in Mass,1983), ILSI# 35 (Strain collected from sliced turkey outbreak), ILSI #33 (outbreak isolated from hotdogs); and (ii) *E. coli* O157:H7: AR strain, AD305 strain and AD317 strain.

A loop full of each bacterial isolate from a frozen stock culture (-80°C in TSB-YE and 10% glycerol) was inoculated into 10 ml of trypticase soy broth yeast extract (TSB-YE) medium (30 g of trypticase soy agar (BD biosciences, MD, USA), 6.0 g of yeast extract (Difco laboratories, Detroit, MI), 1000 ml of distilled water and autoclaved at 121°C for 15 min) and incubated at 37°C for 24 h, after which a loop full of culture transferred to 40 mL of TSB-YE medium in sterile 50 mL tubes and incubated at 37°C for 24 h. Cultures were harvested by centrifuged (7000 rpm for 15 minutes) and washed in 1% (w/v) sterile peptone (BD biosciences) and re-suspended in 20 mL of 1% peptone. Bacteria were enumerated after 48 h of inoculation at 37°C. The bacterial cultures were heat-killed by incubating in 50 mL tubes at 72°C for 6 min in water bath and confirmed by inoculating TSA-YE and mix plates. After enumeration, heat-killed bacterial solution was centrifuged (7000 rpm for 15 min) to concentrate the bacteria and supernatant was discarded. Then, bacterial cultures were re-suspended in 2 mL of RPMI-1640 medium

approximately at $1\times10^{11}$/mL and stored at -80°C in 1.0 mL aliquots. Heat-killed bacteria were added to the cell cultures at bacteria: cell ratios of 0:1, 100:1 and 1000:1.

**Experimental approach:** Eosinophil-like cells and basophil-like cells were cultured in 3 mL volumes in duplicates (6 well tissue culture plates, BD biosciences) with or without HKLM and HKEC at bacteria: cell ratios of 0:1, 100:1 and 1000:1 at 37°C, 5% $CO_2$.Cells and cell culture supernatants for controls and treatments were collected at days 1, 3 and 5 for eosinophil-like cells and days 1, 2, 3 and 4 for basophil-like cells.

**Determination of cell proliferation and viability:** After specified incubation periods, cells were mixed thoroughly and 40 µl of cell suspension was transferred to a 0.6 mL eppendorf tube. Forty µl of culture medium and 80 µl of 2% trypan blue (Sigma) were added to the tube and enumerated by manual counting of cells in hemocytometer (Hausser scientific, Horsham, PA) under 40 x magnification of microscope (Fisher scientific). The number of cells in top two and bottom two rows of center big square were counted in field and cell number were calculated.

**Protein Array Analyses:** Protein array kits (RayBio® Human Cytokine Antibody Array VI, Raybiotech Inc, Norcross, GA), were used according to manufacturer instructions except that 2 ml of cell cuture supernatant was used for each membrane. The procedure for protein array analysis and location of cytokines/chemokines/growth factors and positive, negative, blank controls on the protein array membrane are shown in Figures 3.1 respectively. The protocol is described below: Briefly, 2 ml of blocking buffer was added to membrane and incubated for 2h at room temperature. Thereafter membrane was incubated for overnight at 4°C with sample (cell culture supernatants) and followed by

incubation with a cocktail of biotin-labeled antibodies for 90 min at room temperature. Then membrane was incubated with HRP-conjugated streptavidin at room temperature for 2 hours. Finally chemiluminescence signal was detected by exposing to film.

Protein array images were scanned in grayscale at 9600 dpi using HP scanner (Hewlett-Packard, Palo Alto, CA). Quantity One software (version 4.5.1 basic, BioRad, Hercules, CA) was then used to analyze the scanned images. This software converted each circle on the film into a numerical density value relative to the positive and negative controls on the film. These numerical values were entered into an Excel based program (RayBio analysis tool- human VI and 6.1) that performed normalization and background subtraction. These numbers were used to calculate the fold change between each control and treatment group.

**ECP and EDN ELISA:** ECP and EDN ELISA kits were obtained from MBL International and samples were screened according to manufacturer's instructions as described below.

EDN ELISA: The EDN ELISA sandwich kit (MBL International, Woburn, MA) was used to measure human EDN. One hundred μL of each cell culture supernatant and standard were added to wells coated with anti-human EDN monoclonal antibody and then incubated for 60 minutes at room temperature (20-25°C). After washing, peroxidase conjugated anti-human EDN polyclonal antibody was added to the microwells and

Figure 3.2 Human cytokine/chemokine/growth factor protein array membrane. This figure shows the location of various cytokine/chemokine and the positive, negative, blank controls on the membrane spotted with specific antibodies.

| POS | POS | POS | POS | Blank | Angiogenin | BDNF | BLC | BMP-4 | BMP-6 | CK β 8-1 | CNTF | EGF | Eotaxin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NEG | NEG | NEG | NEG | Blank | Angiogenin | BDNF | BLC | BMP-4 | BMP-6 | CK β 8-1 | CNTF | EGF | Eotaxin |
| Eotaxin-2 | Eotaxin-3 | FGF-6 | FGF-7 | Fit-3 Ligand | Fractalkine | GCP-2 | GDNF | GM-CSF | I-309 | IFN-γ | IGFBP-1 | IGFBP-2 | IGFBP-4 |
| Eotaxin-2 | Eotaxin-3 | FGF-6 | FGF-7 | Fit-3 Ligand | Fractalkine | GCP-2 | GDNF | GM-CSF | I-309 | IFN-γ | IGFBP-1 | IGFBP-2 | IGFBP-4 |
| IGF-I | IL-10 | IL-13 | IL-15 | IL-16 | IL-1α | IL-1β | IL-1ra | IL-2 | IL-3 | IL-4 | IL-5 | IL-6 | IL-7 |
| IGF-I | IL-10 | IL-13 | IL-15 | IL-16 | IL-1α | IL-1β | IL-1ra | IL-2 | IL-3 | IL-4 | IL-5 | IL-6 | IL-7 |
| Leptin | LIGHT | MCP-1 | MCP-2 | MCP-3 | MCP-4 | M-CSF | MDC | MIG | MIP-1δ | MIP-3α | NAP-2 | NT-3 | PARC |
| Leptin | LIGHT | MCP-1 | MCP-2 | MCP-3 | MCP-4 | M-CSF | MDC | MIG | MIP-1δ | MIP-3α | NAP-2 | NT-3 | PARC |
| PDGF-BB | RANTES | SCF | SDF-1 | TARC | TGF-β1 | TGF-β 3 | TNF-α | TNF-β | Blank | Blank | Blank | Blank | Blank |
| PDGF-BB | RANTES | SCF | SDF-1 | TARC | TGF-β1 | TGF-β 3 | TNF-α | TNF-β | Blank | Blank | Blank | POS | POS |

incubated for 60 minutes at room temperature. Then plates were washed, after which the peroxidase substrate was added and incubate for 10 min. A stop solution (100 μL of 0.5 M sulfuric acid) added to wells to terminate the enzyme reaction and to stabilize the color development. The optical density (O.D) of each well was measured in dual mode of at 450-620 nm using the KC4 software program (Synergy HT, Multifunction Reader, BioTek). The concentration of human EDN in samples was determined using standard curves generated from recombinant human EDN.

**ECP ELISA:** The procedure for performing the ECP indirect sandwich ELISA (MBL International) was similar to the EDN ELISA except for the coating antibody (anti-human ECP), standard (ECP) and peroxidase conjugated anti-human ECP polyclonal antibody.

**Flow cytometric analysis of FcεRIα receptor expression on basophil-like cells:** For human basophil-like cells, cell surface expression of FcεRIα was assessed by flow cytometry following instructions provided by the antibody supplier (eBioscience, San Diego, CA). In brief, cells were incubated with affinity purified anti- human FcεRIα-chain antibody CRA-1 (1ug/ $10^6$) (eBioscience) for 60 min at 4°C. Then the cells were washed twice in wash buffer (PBS, 0.5% BSA; pH 7.5) and exposed to the FITC-labeled anti-mouse IgG (eBioscience) for 60 min at 4 ° C. Cells were washed twice in PBS and subjected to flow cytometry. As a negative control, mouse subclass-matched polyclonal IgG2b k antibody (eBioscience) was used to stain the cells. Results are represented as mean fluorescence intensity. Flow cytometric analysis was done by Dr. Louis King (Dr. Pam Praker's lab, Department of Biochemistry, MSU).

**Flow cytometric analysis of CCR3 receptor expression on eosinophil-like cells:** For human eosinophil-like cells, cell surface expression of CCR3 was assessed by flow cytometry. In brief, cells were incubated with purified anti-human CCR3 reagent (1$\mu$g/million cells) (Biolegend, San Diego, CA) for 60 min at 4 ° C. Then the cells were washed twice with buffer and incubated with biotin goat anti-mouse IgG (Biolegend) for 60 min at 4 ° C. Following another wash , the cells were incubated with PE labeled strepatavidin (Biolegend) for 30 min at room temperature. To remove unreacted streptavidin-PE, cells were washed twice and then, re-suspended in fixation buffer (PBS, 2% paraformaldehyde). As a negative control, mouse subclass-matched IgG2b antibody was used for staining. Results are presented as mean fluorescence intensity.

**Statistics:** All proliferation, cytokine and chemokine data were analyzed using the unpaired student t-test on duplicate samples at a significance level of $p<0.05$. Statistics were performed using Analyse-It computer software program. All experiments were done in duplicates and each experiment was replicated twice.

**"Images in this thesis/dissertation are presented in color."**

# CHAPTER 4: RESULTS

## Effect of HKLM and HKEC on proliferation of human eosinophil-and basophil-like cells

In order to determine the effect of HKLM and HKEC on proliferation of human eosinophil- and basophil-like cells, cells were cultured with and without HKLM or HKE bacteria: cell ratios of 0:1, 100:1 and 1000:1. Human eosinophil-like cells grown without HKLM continued to proliferate for up to 5 days. HKLM had no effect on proliferation of human eosinophil-like cells at bacteria: cell ratio of 100:1 after 3 days. Although cell numbers decreased at bacteria: cell ratio of 1000:1, this decrease was not statistically significant (Figure 4.1). HKLM significantly inhibited the cell proliferation at 1000:1 bacteria: cell on day 5 (unpaired students *t*-test $p<0.05$).

When compared to the untreated cells, HKEC had no effect on proliferation of human eosinophil-like cells at 100:1 bacteria: cell ratio. HKEC inhibited cell proliferation at 1000:1 bacteria: cell ratio and this inhibition was statistically significant on day 5 (Figure 4.2) (unpaired students *t*-test $p<0.05$).

Culturing human basophil-like cells with HKLM for up to 4 days had no major effect on cell proliferation (Figure 4.3).

We tested the effect of HKEC on proliferation of human basophil-like cells during 4 days. Cells cultured at 1:100 cell: bacteria ratio had no effect on proliferation.

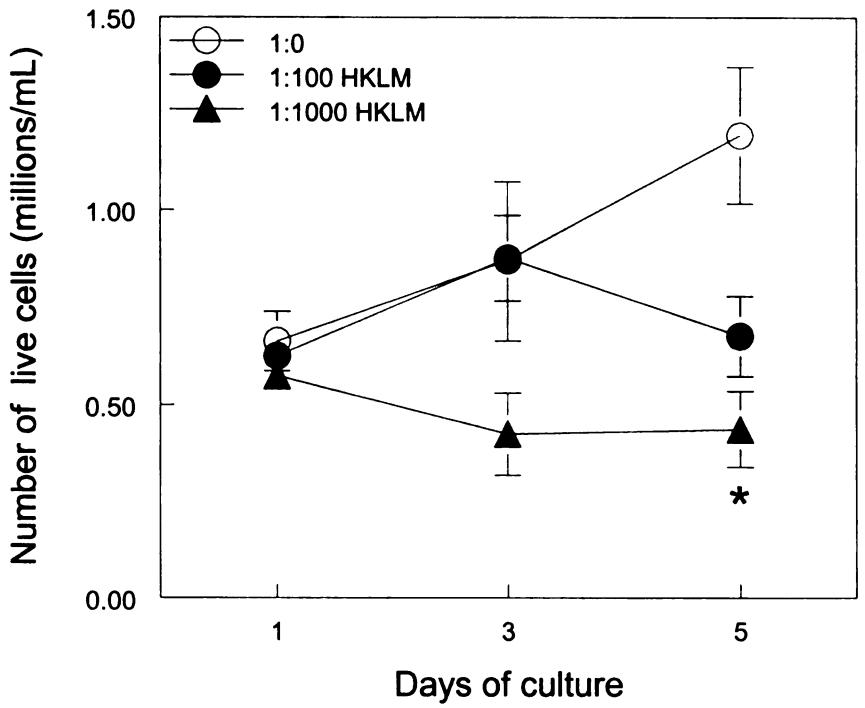



Figure 4.1. Effect of HKLM on proliferation of human eosinophil-like cells. Cells were cultured with or without HKLM at 0:1, 100:1 and 1000:1 bacteria: cell ratio. Data are expressed as mean ± SE of cell densities (* unpaired student t-test $p<0.05$).

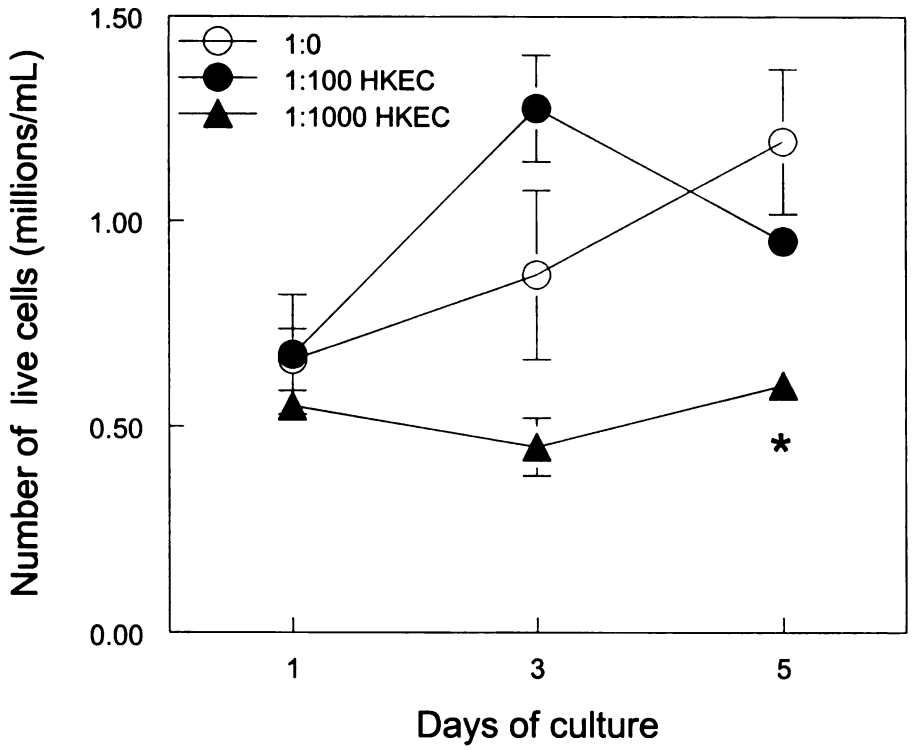



Figure 4.2. Effect of HKEC on proliferation of human eosinophil-like cells. Cells were cultured with or without HKEC at 0:1, 100:1 and 1000:1 bacteria: cell ratio. Data are expressed as mean ± SE of cell densities (* unpaired student t-test $p<0.05$).

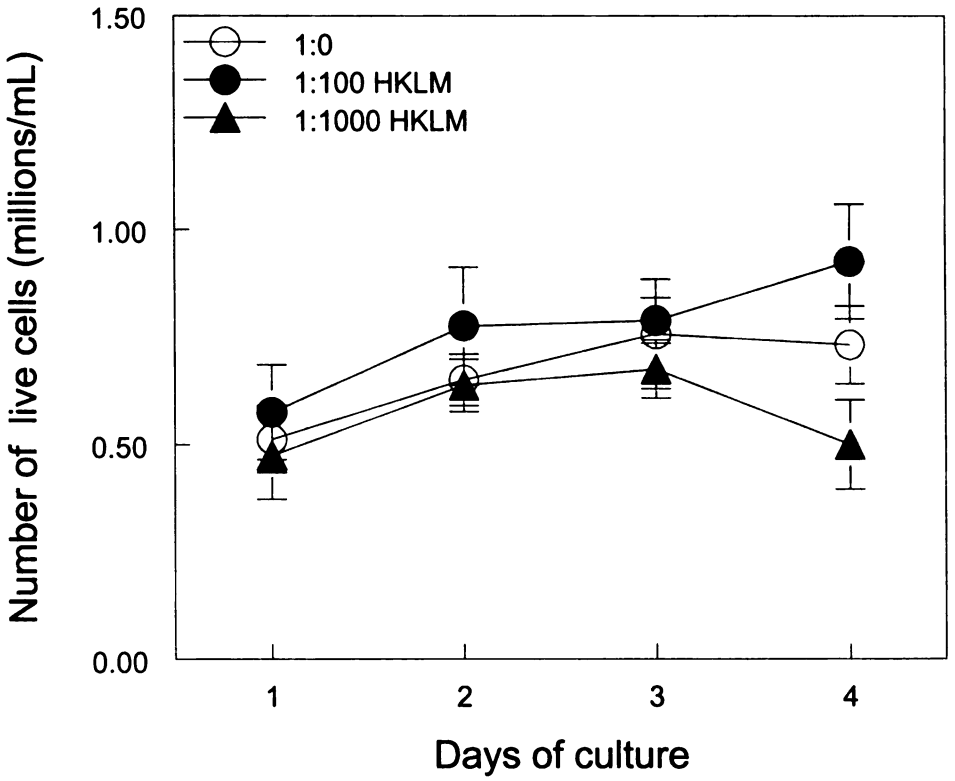



Figure 4.3 Effect of HKLM on proliferation of human basophil-like cells. Cells were cultured with or without HKLM at 0:1, 100:1, 1000:1 bacteria: cell ratio. Data are expressed as mean ± SE of cell densities counted in two different wells.

In contrast, basophil-like cells grown at a 1:1000 cell: bacteria ratio inhibited proliferation on day 4 (Figure 4.4) (unpaired students *t*-test $p<0.05$).

In summary: (i) both HKLM and HKEC inhibited proliferation of human eosinophil-like cells only at bacteria: cell ratio of 1000:1; (ii) HKLM had no major affect on proliferation of human basophil-like cells; and (iii) HKEC inhibited proliferation of human basophil-like cells only at bacteria: cell ratio of 1000:1.

**Effect of HKLM and HKEC on cytokine and chemokine production by human eosinophil-like cells and basophil-like cells.**

Day 3 cell culture supernatants used from eosinophil-like cell experiments were screened for cytokine/chemokine/growth factor production by protein array analysis. Figures 4.5 and 4.6 summarize the effect of HKLM and HKEC on allergy relevant cytokine and chemokine production by eosinophil-like cells respectively. HKLM significantly increased the production of important pro allergic chemokines such as CCL5 (RANTES), CCL1 (I-309), CCL8 (MCP-3) and CCL20 (MIP-3) by human eosinophil-like cells (Figure 4.5). HKEC significantly increased the production of chemokines such as CCL5 (RANTES), CCL24 (Eotaxin-2), CCL22 (MDC), CCL1 (I-309), CCL8 (MCP-3) and CCL20 (MIP-3) by human eosinophil-like cells (Figure 4.6).

Cell culture supernatants of human basophil-like cell experiments (Day 2) were screened the cytokine/ chemokine production by protein array analysis. HKLM clearly increased

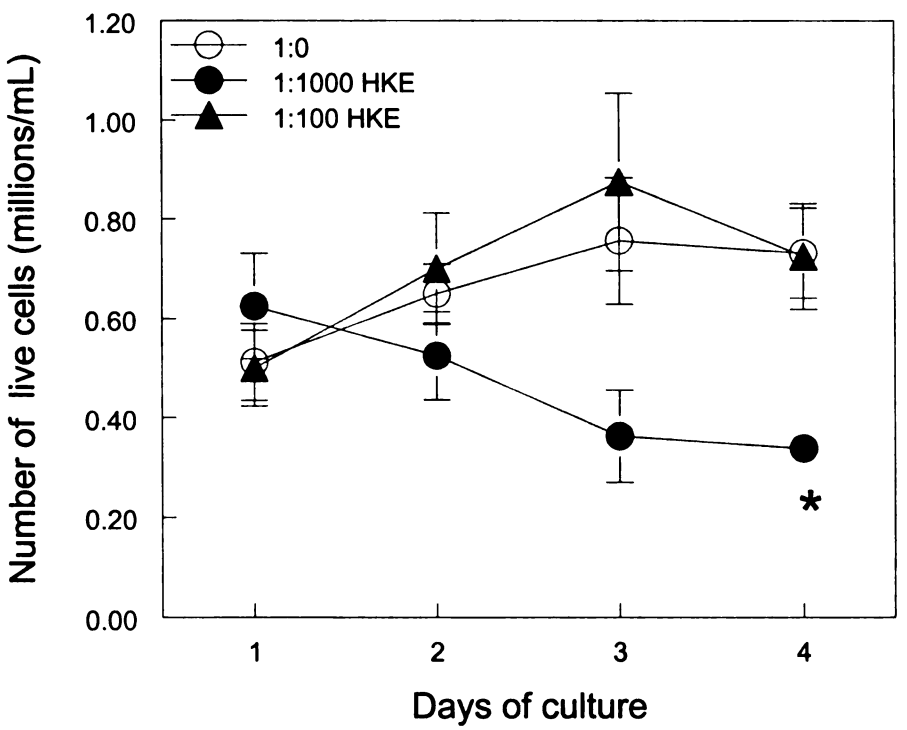



Figure 4.4 Effect of HKEC on proliferation of human basophil-like cells. Cells were cultured with or without HKEC at 0:1, 100:1 and 1000:1 bacteria: cell ratio. Data are expressed as mean ± SE of cell densities (* unpaired student t-test $p<0.05$).

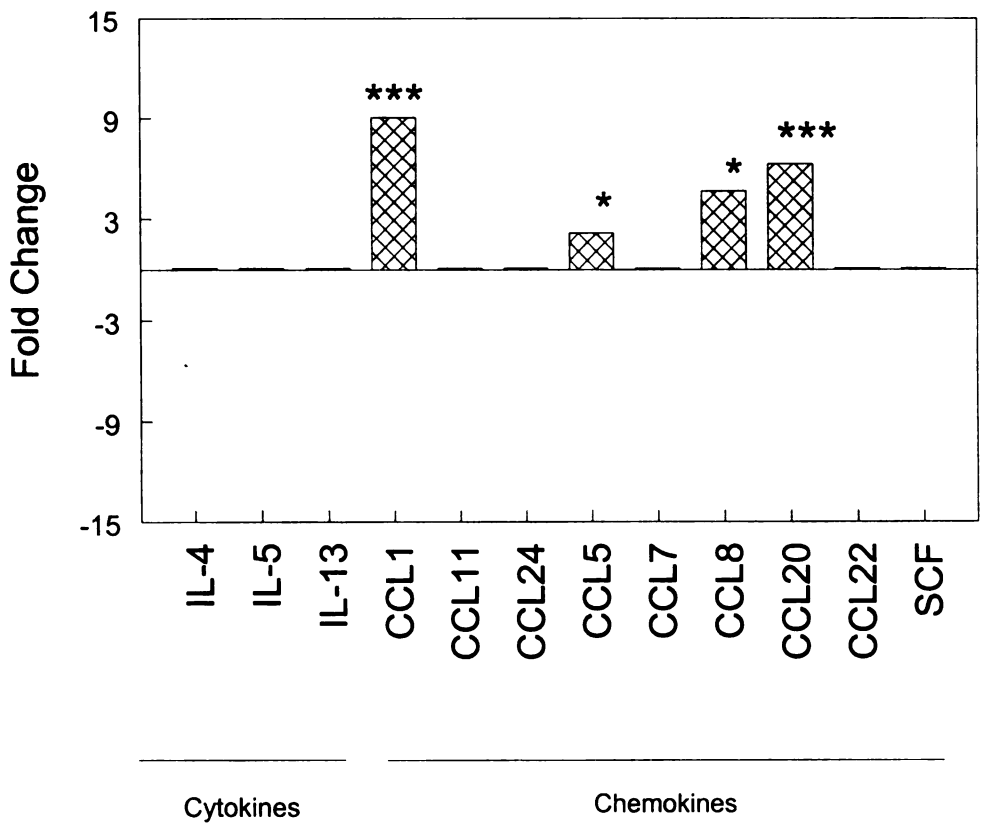



Figure 4.5 Summary of protein array analysis: Effect of HKLM on cytokine and chemokine production by human eosinophil-like cells (* $p< 0.05$, * * *$p<0.001$ student *t*-test).

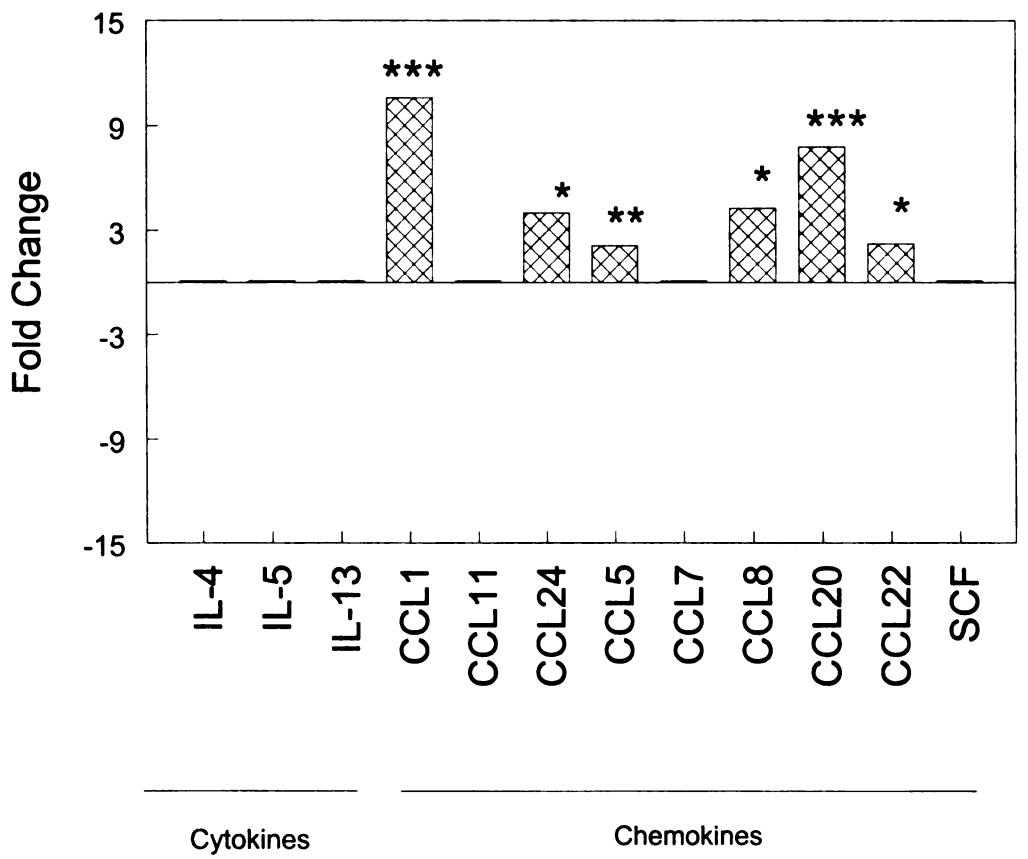



Figure 4.6 Summary of protein array analysis: Effect of HKEC on cytokine /chemokine production by human eosinophil-like cells (* $p< 0.05$, * * $p<0.01$, * * * $p<0.001$ student *t*-test).

The production of cytokines and chemokines such as IL-5, IL-13, CCL5 (RANTES), CCL1 (I-309), CCL7 (MCP-3), CCL8 (MCP-3), CCL11 (Eotaxin) and CCL24 (Eotaxin-2) and decreased IL-4 production (Figure 4.7). HKEC increased important cytokines/chemokines such as IL-5, IL-13, CCL5 (RANTES), CCL1 (I-309), and CCL24 (Eotaxin-2) and decreased IL-4, CCL8 (MCP-2) and CCL7 (MCP-3) (Figure 4.8).

**Effect of HKLM and HKEC on ECP and EDN production by human eosinophil-like cells.**

Culture conditions were optimized for ECP and EDN production. HL-60 clone 15 cells treated with 0.5mM butyric acid produced significantly higher levels of ECP and EDN compared to the control (Figure 4.9 and 4.10). Standard curve were used for calculation ECP and EDN levels. By using these standard curves, we calculated the ECP and EDN concentration in samples.

To assess the effect of HKLM and HKEC on ECP and EDN production eosinophil-like cells, were cultured at bacteria: cell ratio of 0:1, 100:1 and 1000:1 for five days and after which the culture supernatants were screened for ECP and EDN. As evident, ECP production was generally increased by HKLM treatment, but it was not statistically significant (Figure 4.11). In contrast, HKLM significantly increased the production of EDN by human eosinophil-like cells, but not HKEC (Figure 4.12).

In summary, HKLM and HKEC had no significant effect on ECP production by human eosinophil-like cells; and In contrast to HKEC, only HKLM significantly increased EDN

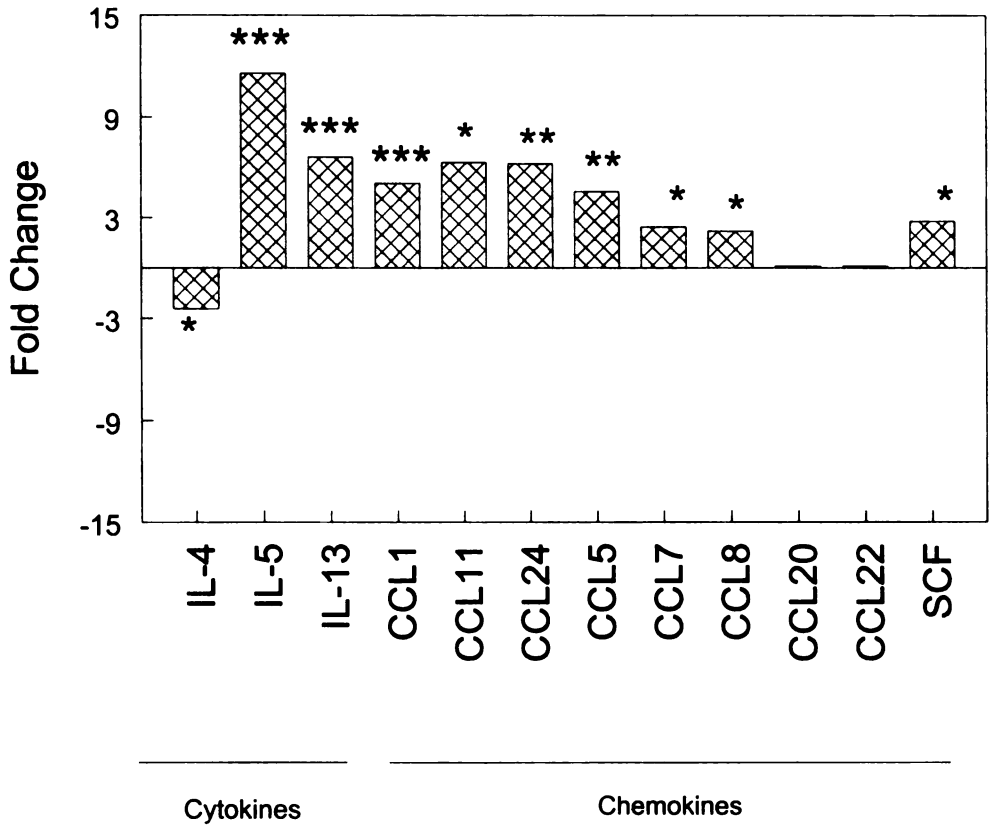



Figure 4.7 Summary of protein array analysis: Effect of heat-killed *L .monocytogenes* on cytokine /chemokine production by human basophil-like cells (* $p<0.05$, * * $p<0.01$, * * *$p<0.001$ student *t*-test)

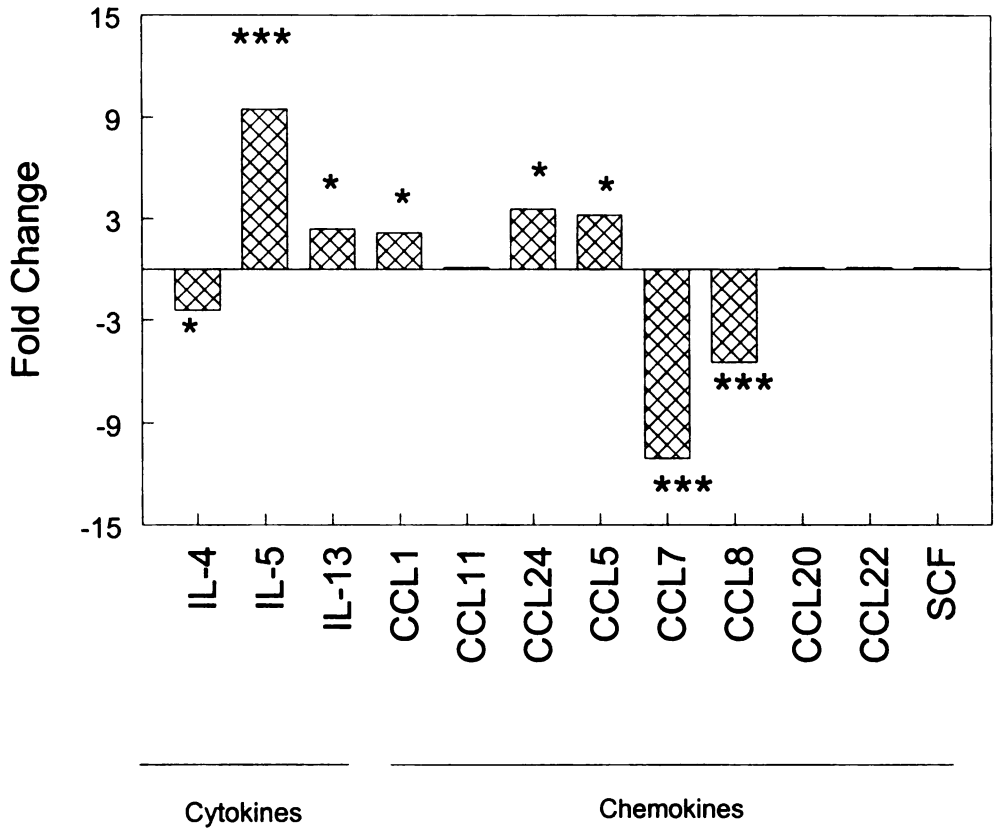



Figure 4.8 Summary of protein array analysis: Effect of heat-killed *E. coli* O157:H7 on cytokine /chemokine production by human basophil-like cells (* $p<0.05$, * * $p<0.01$, * * *$p<0.001$ student *t*-test)

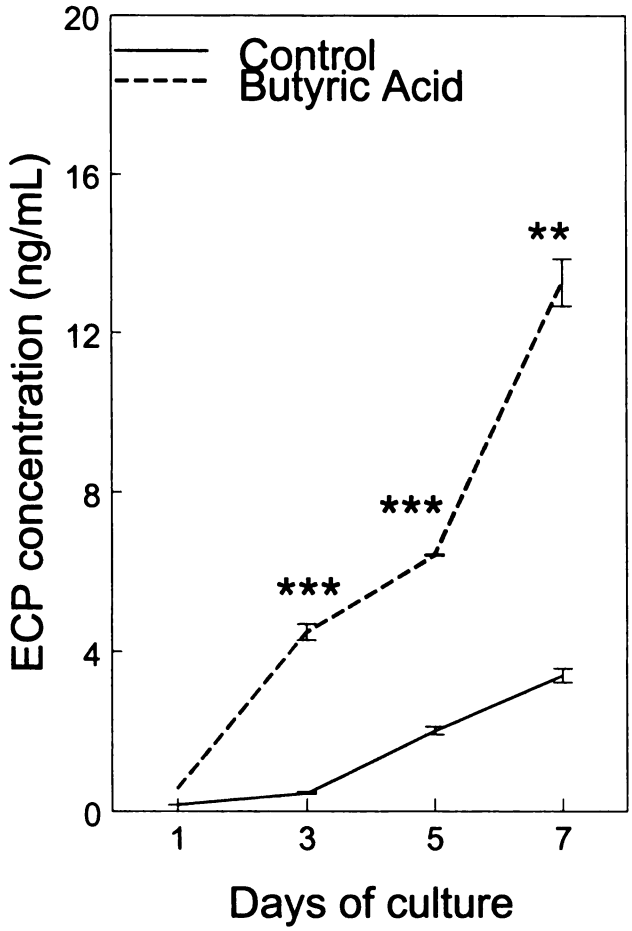



Figure 4.9. ECP concentrations in HL-60 clone 15 cell supernatants cultured with and without 0.5 mM butyric acid. Data are shown as mean +/- SE (unpaired student *t*-test ** $p< 0.01$, *** $p<0.001$).

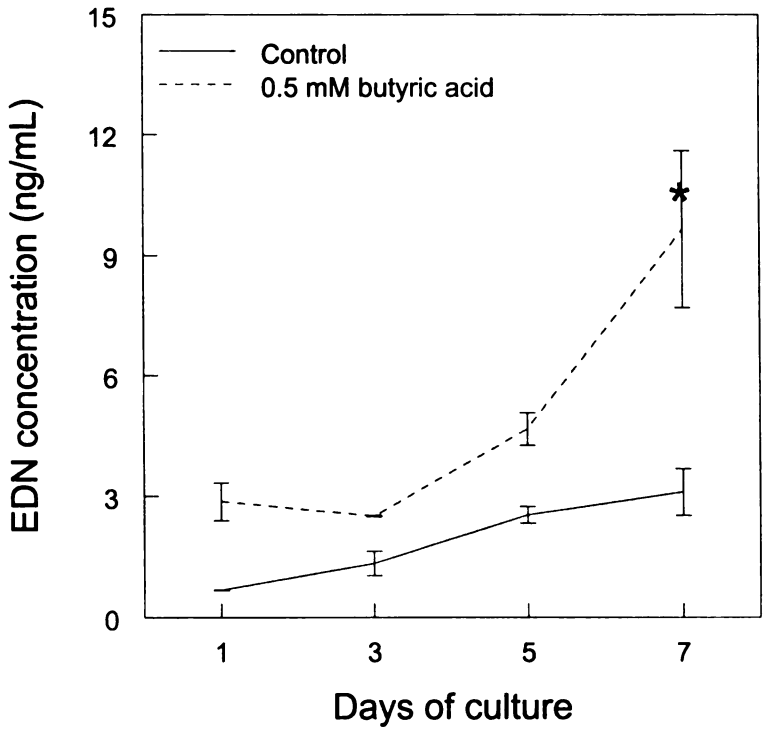



Figure 4.10. EDN concentration in HL-60 clone 15 cell supernatants cultured with and without 0.5 mM butyric acid. Data are shown as mean +/- SE (unpaired student *t*-test $p<0.05$).

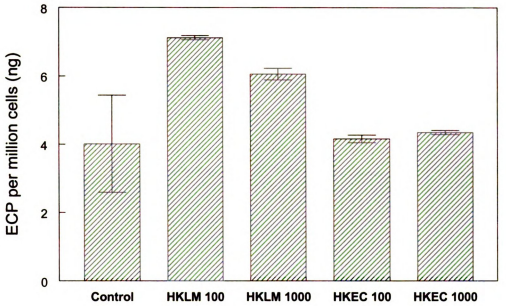



Figure 4.11. ECP production by human eosinophil-like cells cultured with and without HKLM and HKEC. X-axis shows bacteria: cell ratios. Data are shown as mean +/- SE.

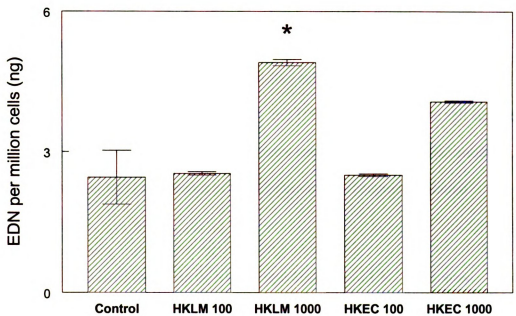



Figure 4.12 EDN production by human eosinophil-like cells cultured with and without HKLM and HKEC. X-axis shows bacteria: cell ratios. Data are shown as mean +/- SE (* unpaired student t-test $p<0.05$).

production by human eosinophil-like cells.

**Effect of HKLM and HKEC on chemokine receptor CCR3 expression by human eosinophil-like cells.**

To determine the effect of HKLM and HKEC on chemokine receptor CCR3 expression by human eosinophil-like cells, human eosinophil-like cells were cultured with heat-killed bacteria at bacteria: cell ratios of 0:1, 100:1 and 1000:1 for 3 days. The cell surface expression of CCR3 was measured by flow cytometry using anti-human CCR3 antibody. Figure 4.13 shows basal level expression of CCR3 chemokine receptor on human eosinophil-like cells.

Using this system, we tested the effect of HKLM and HKEC on CCR3 chemokine receptor expression by human eosinophil-like cells. Both bacteria tested inhibited CCR3 expression on eosinophil-like cells by ~ 50%. This inhibition was clearly seen at bacteria: cell ratios of 100:1 and 1000:1 for HKLM. Whereas HKEC inhibition was only seen at bacteria: cell ratio of 100:1 (Figures 4.14- 4.15).

**Effect of HKLM and HKEC on FcεRI expression by human basophil-like cells.**

To assess the effect of heat-killed bacteria on FcεRI expression on human basophil-like cells were cultured with HKLM and HKEC at bacteria: cell ratios of 0:1, 100:1 and 1000:1 for 48 h under serum-free conditions. Cell surface expression of FcεRI was measured by flow cytometry using the anti-FcεRIα chain antibody CRA-1.

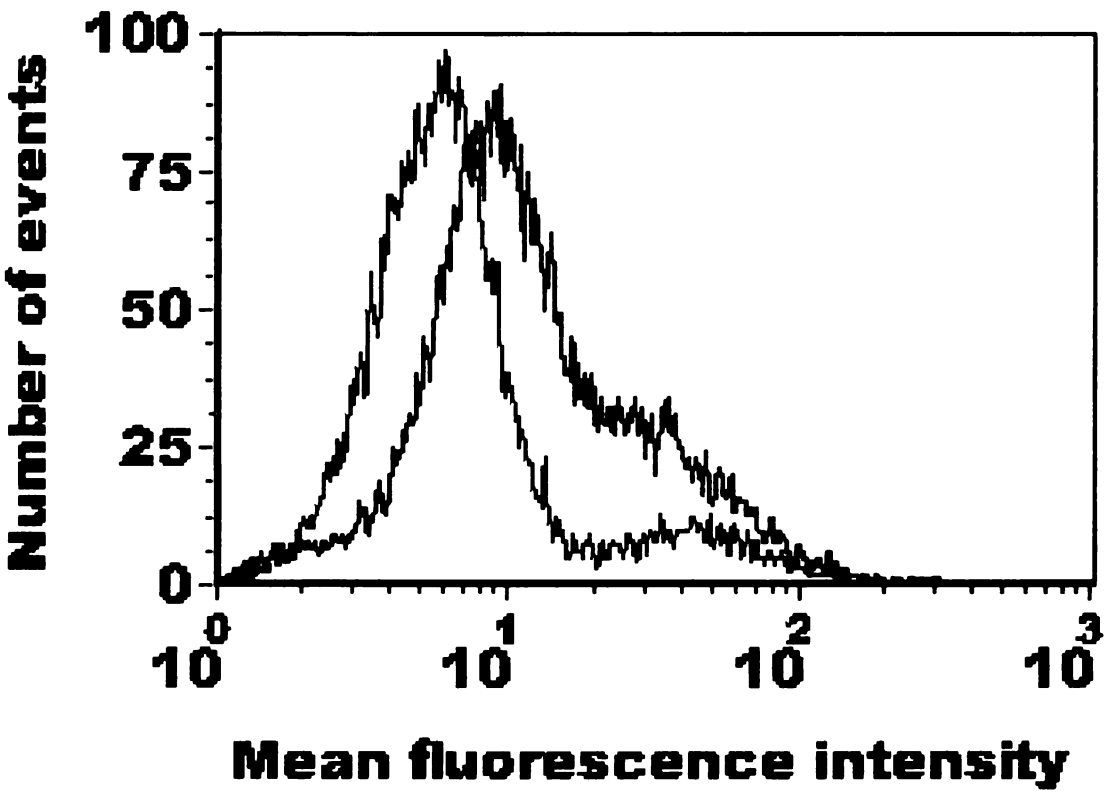
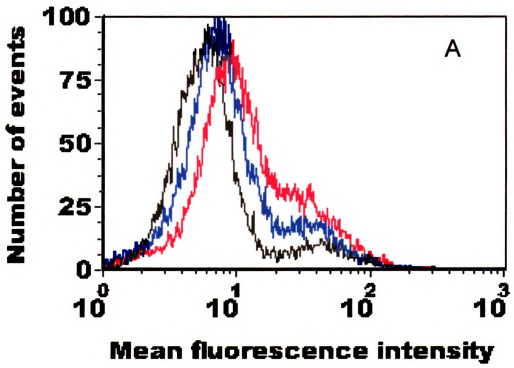



Figure 4.13. CCR3 receptor expression on eosinophil-like cells. Cells stained with control antibody (black line) or anti-CCR3 antibody (redline) were subjected to flow cytometry.

Figure 4.14 (A-B). CCR3 receptor expression by eosinophil-like cells cultured with HKLM at (A) bacteria: cell ratio of 100:1 (B) bacteria: cell ratio of 1000:1 for 3 days. HKLM treated cells stained with anti-CCR3 antibody (blue line) and control cells stained with control antibody (black line) or anti-CCR3 antibody (red line).


A
Number of events
100
75
50
25
0
$10^0$
$10^1$
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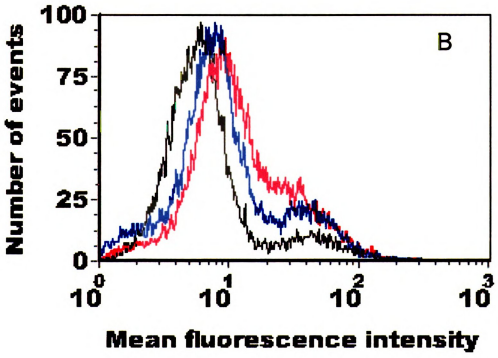
$10^3$
Mean fluorescence intensity


B
Number of events
100
75
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25
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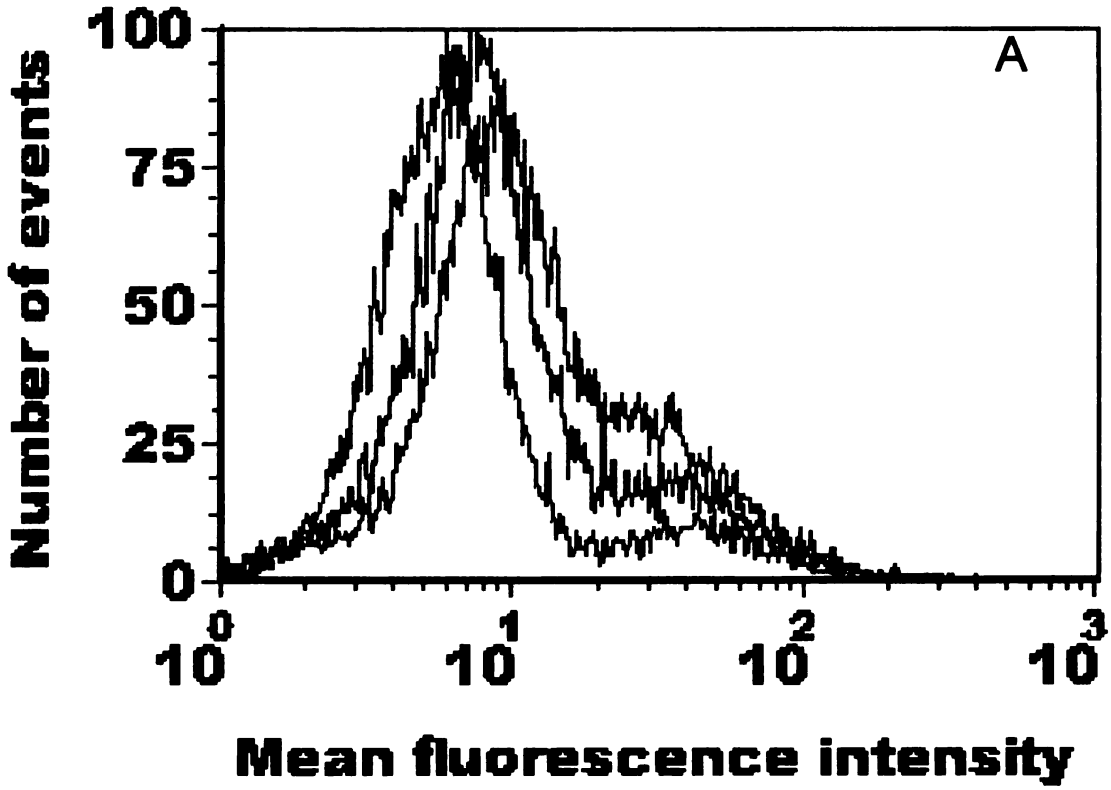
$10^0$
$10^1$
$10^2$
$10^3$
Mean fluorescence intensity

Figure 4.15 (A-B). CCR3 receptor expression by eosinophil-like cells cultured with HKEC at (A) bacteria: cell ratio of 100:1 (B) bacteria: cell ratio of 1000:1 for 3 days. HKEC treated cells stained with anti-CCR3 antibody (blue line) and control cells stained with control antibody (black line) or anti-CCR3 antibody (red line).


A
100
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Number of events
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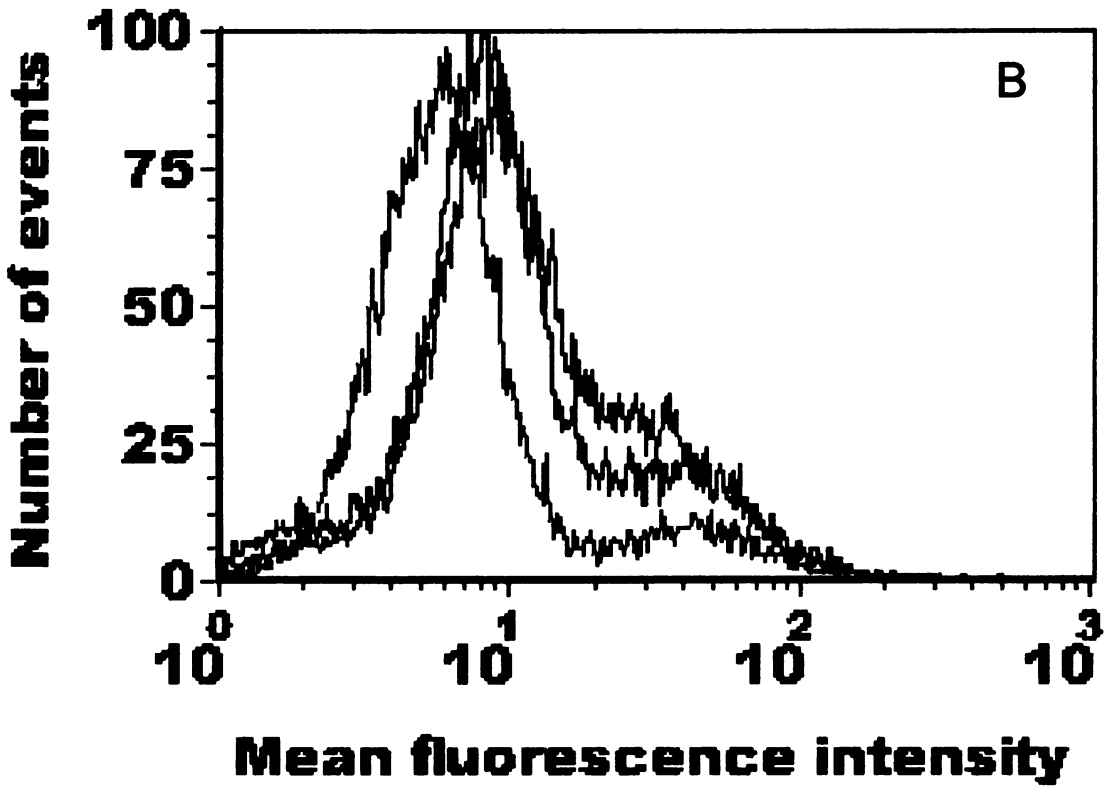
$10^3$
Mean fluorescence intensity


B
100
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Number of events
$10^0$
$10^1$
$10^2$
$10^3$
Mean fluorescence intensity

Figure 4.16 shows the basal level expression of FcεRI on basophil-like cells cultured without any bacteria.

Using this system, we tested the effect of HKLM and HKEC on FcεRI receptor expression by human basophil-like cells. As an overall trend, neither of these bacterial pathogens had significantly affected FcεRIα receptor expression by human basophil-like cells (Figures. 4.17- 4.18).

**"Images in this thesis/dissertation are presented in color."**

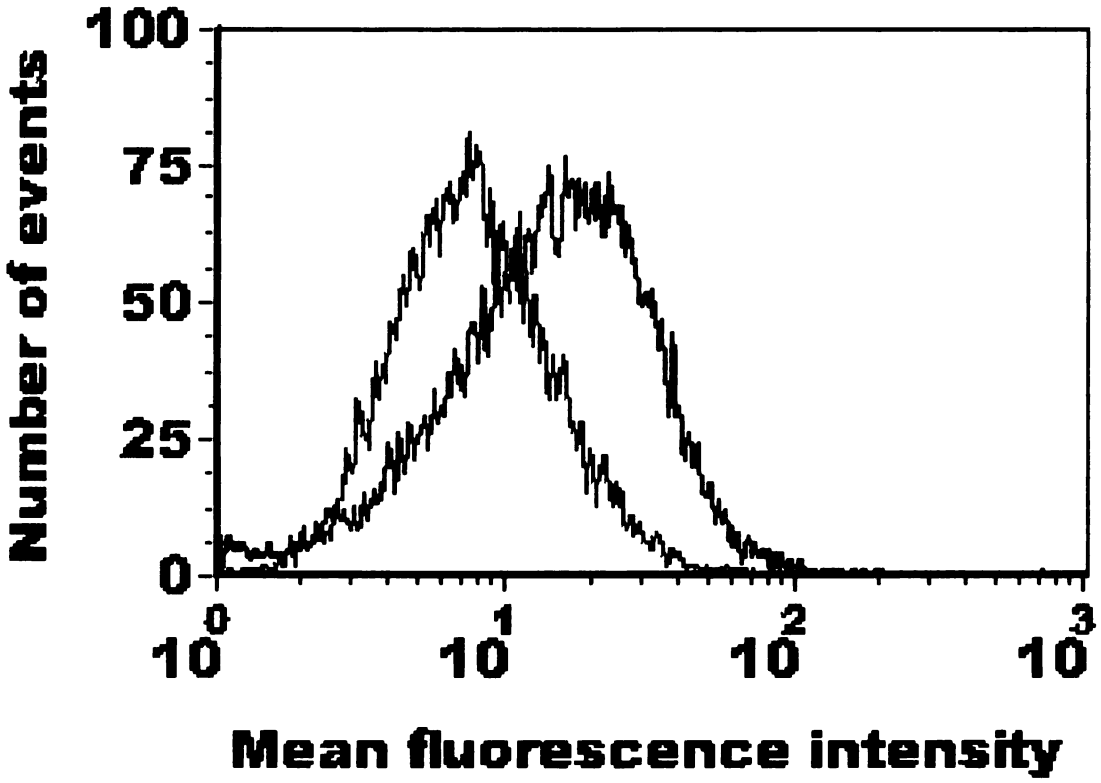
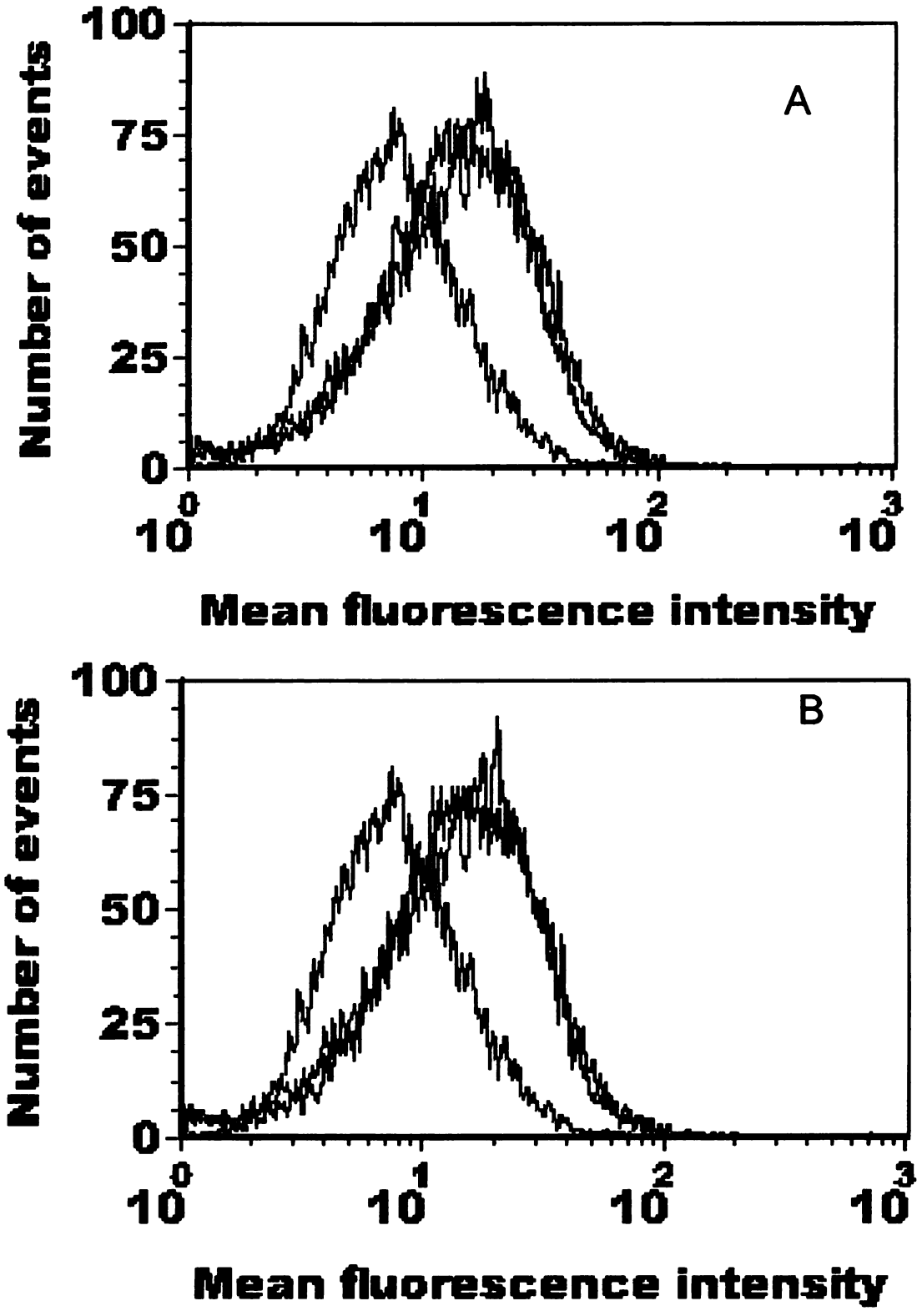



Figure 4.16. FcεRI receptor expression on basophil-like cells. Cells stained with control antibody (black line) or anti-CCR3 antibody (redline) were subjected to flow cytometry.

Figure 4.17 (A-B). FcεRI expression by basophil-like cells cultured with HKLM at (A) bacteria: cell ratio of 100:1 (B) bacteria: cell ratio of 1000:1 for 2 days.. HKLM treated cells stained with anti- FcεRI antibody (blue line) and control cells stained with control antibody (black line) or anti- FcεRI antibody (red line).


A
Number of events
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$10^0$
$10^1$
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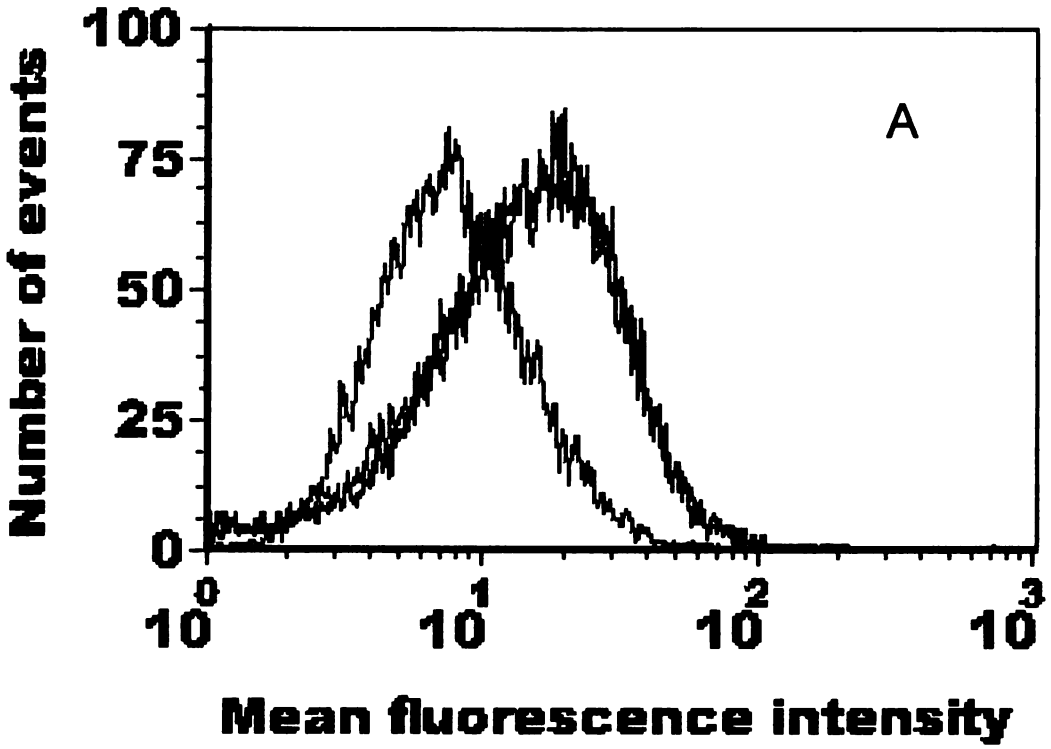
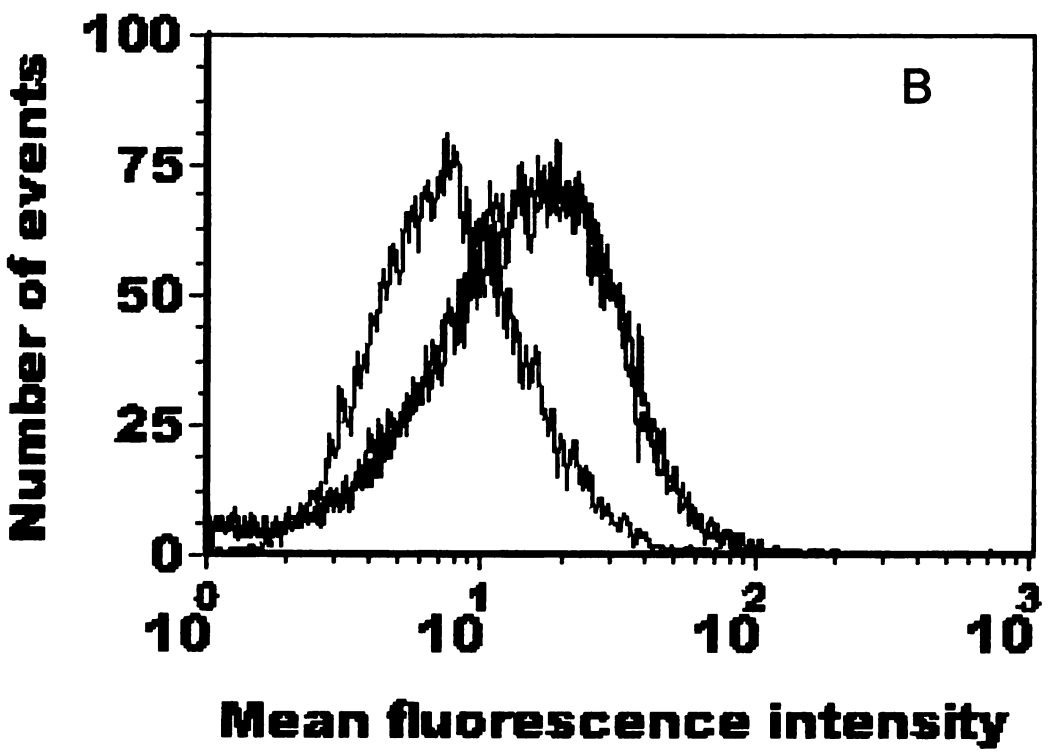
$10^3$
Mean fluorescence intensity
B
Number of events
100
75
50
25
0
$10^0$
$10^1$
$10^2$
$10^3$
Mean fluorescence intensity

Figure 4.18 (A-B). FcεRI expression by basophil-like cells cultured with HKEC at (A) bacteria: cell ratio of 100:1 (B) bacteria: cell ratio of 1000:1 for 2 days. HKEC treated cells stained with anti- FcεRI antibody (blue line) and control cells stained with control antibody (black line) or anti- FcεRI antibody (red line).


A
Number of events
100
75
50
25
0
$10^0$
$10^1$
$10^2$
$10^3$
Mean fluorescence intensity


B
Number of events
100
75
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$10^0$
$10^1$
$10^2$
$10^3$
Mean fluorescence intensity

# CHAPTER 5: DISCUSSION

This study was undertaken to determine whether heat-killed *L. monocytogenes* (HKLM) and *E. coli* O157:H7 (HKEC) contain any components that have the capacity to inhibit the production of allergy-relevant cytokines (such as IL-4, IL-5 and IL-13), chemokines (such as eotaxins), and cell surface expression of chemokine receptor (CCR3) and IgE antibody receptor. The rationale was that, if they were to exhibit such activity then future studies could be designed to isolate the active component of these bacteria and which could be used for potential therapeutic purposes in animal models. Based on our findings, evidence was obtained for the presence of IL-4 and CCR3-inhibiting components in both HKLM and HKEC, but not other targeted genes.

Basophils and mast cells are the primary cell types that express the high affinity receptor for IgE antibody; activation of this receptor is the initiator of an allergic reaction leading to clinical symptoms of allergy (Fujimura et al., 2002; Macglashan, 2005). The mast cell model that we had obtained did not express a functional high affinity receptor for IgE. Basophils can also produce the key allergy relevant cytokines such as IL-4, IL-5, IL-13 and chemokines (such as eotaxins) (Falcone et al., 2000; Galli, 2000; Falcone, 2003). Consequently, we used a human basophilic cell line as a model system to asses the impact of HKLM and HKEC on these gene products of importance in allergy.

Eosinophils express the chemokine receptor CCR3---a major receptor shown to be required for allergic inflammation. CCR3 gene deficient mice exhibit reduced allergic

responses (Ma et al., 2002; Mishra and Rothenberg, 2003) and CCR3 is one of the currently targeted chemokine receptors for novel drug development for allergy and asthma (Gangur et al., 2003). Eosinophils are the primary producers of ECP and EDN---key mediators of nerve damage and inflammation associated with eosinophil mediated allergies including subset of food allergy (Hogan et al., 2001; Romano et al., 1997; Rothenberg et al., 2001a). In view of these facts, we used a eosinophilic cell line model to study the impact of HKLM and HKEC on all these gene products of importance in allergy.

Our goal was not to study the impact of HKLM and HKEC on human allergic diseases, because the cell line model that we used could not have been used for studying disease. Furthermore, others had already studied the effect of these two bacteria on allergic disease in mouse models, although they used *E. coli* strains other than O157:H7 (Kim et al., 2005; Li et al., 2003a). Thus, we used these previous studies as a rationale to focus on the two bacteria that we selected. *Listeria monocytogenes* served as a model Gram-positive bacteria and *E. coli* O157:H7 served as a model Gram negative bacteria.

Our results indicate that both bacterial strains inhibited the expression of IL-4 in basophil-like cells and CCR3 in eosinophil-like cells. Earlier studies using mouse models have shown that HKLM and HKEC inhibit T helper cell production of IL-4 (Hansen et al., 2000; Li et al., 2003a; Li et al., 2003b; Yeung et al., 1998). However, they had not studied IL-4 production by basophils or CCR3 expression. We also found that HKEC inhibited production of MCP-2 and MCP-3 in basophils. Production of these two

chemokines is elevated in allergic diseases (Freyne et al., 2005; Fulkerson et al., 2004; Shang et al., 2002). However, currently there is no evidence implicating an essential role for these two chemokines—as opposed to CCR3 and IL-4, in allergy. It is possible that they may contribute to allergic inflammation as simply secondary participants facilitating additional inflammatory cell recruitment (Gangur and Oppenheim, 2000). Thus, it is unclear if inhibiting MCP-2 and MCP-3 might be of therapeutic value in allergy.

It is notable that inhibition of IL-4 is of special importance, because IL-4 is the prototypic Type-2 cytokine necessary for T helper-2 (Th2) responses. Furthermore, the allergy causing IgE antibody production is Th2 dependent (Lange et al., 1998; Steinke, 2004). Our data show that HKLM and HKEC inhibited IL-4 production by human basophil-like cells. Earlier studies showed that both bacterial strains inhibited IL-4 production by mouse T helper cells (Li et al., 2003a; Yeung et al., 1998). IL-4 can also be produced by certain types of NK cells in mice (Yoshimoto et al., 1995). However, whether these bacteria can also inhibit IL-4 production by NK cells remains to be tested. If HKLM and HKEC inhibit IL-4 production by these three sources of IL-4, then they would be expected to inhibit IL-4 dependent allergic responses and perhaps allergic disease as well. IL-4 gene knockout mice with reduced allergic responses (Lange et al., 1998) and IL-4 have been targeted by biotech industries for developing novel anti-allergy drugs (Steinke, 2004).

We found that co-culture of HKEC and HKLM inhibited the expression of CCR3 on eosinophil-like cells by about ~50%. CCR3 is the chemokine receptor for chemokine

ligands such as eotaxin, eotaxin-2 and eotaxin-3 (De Lucca et al., 2005; Gangur et al., 2003). It was reported that CCR3 gene knockout mice are significantly protected from allergic response (Ma et al., 2002). Inhibition of CCR3 in vivo using specific antibodies or chemical antagonists has shown promising results in blocking allergic reactions in mice and in humans (Gangur et al., 2003; Gangur and Oppenheim, 2000). Consequently, extensive efforts have been undertaken by the biotech and the pharmaceutical industries to develop selective CCR3 antagonists for potential use in allergic disorders (De Lucca et al., 2005; Elsner et al., 2004). Thus, several types of products are being developed as summarized in Table 5.1 (Gangur et al., 2003). Using a novel approach ---i.e., use of human eosinophil-like cells and foodborne pathogenic bacteria, we have found evidence for inhibition of CCR3 expression.

Our results showed that neither HKLM nor HKEC affected on the expression of FceR-I – the high affinity IgE receptor. Although mouse model studies assessed the impact of HKLM and HKEC on allergic response, they did not studied the expression of high affinity IgE receptor (Hansen et al., 2000; Li et al., 2003a; Li et al., 2003b).

We also found that both HKLM and HKEC inhibited proliferation of eosinophil-like cells and basophil-like cells in vitro. The in vivo relevance of these findings is unclear. Previous studies have shown that mature human eosinophils and basophils lack the proliferating capacity (Galli et al., 2005). Our model system represents only the immature form of these leukocytes. We performed the proliferation experiments to determine the dose vs. culture time points that would have minimum impact on cell viability so that we

**Table 5.1 CCR3 Antagonists: Relative specificity and potency (Gangur et al, 2003)**

| CCR3 Antagonists | Specificity | Potency | In vivo Impact |
|---|---|---|---|
| Modified Natural Chemokines | | | |
| Met-Rantes | CCR1, CCR3 | 1 nM (IC50) | Anti-allergic (mice) @ 0.04 mg/Kg |
| Met-Ckβ7 | CCR3 | 10 nM (IC50) | |
| Hybrid Eotaxin/ I-TAC | CCR3 | 4.5 nM (K) | |
| Natural Chemokines: | | | |
| CXCL9 (MIG) | CCR3, CXCR3 | 4065 nM (K CCR3) | |
| CXCL10 (IP-10) | CCR3, CXCR3 | 1583 nM (K CCR3) | Anti-allergic (mice) |
| CXCL11 (I-TAC) | CCR3, CXCR3 | 65 nM (K CCR3) | |
| Peptide/Protein based | | | |
| Phage display derived hexapeptide (CPWYFWPC) | CCR3 | 20 uM (IC50) | |
| Virus Encoded 35 kDa protein vCKBP | Binds CC chemokines | 3M excess vs. eotaxin | Anti-allergic (guinea pigs) |
| vCC1 | Binds CC chemokines | 50 ug/mouse intranasal | Anti-allergic (mice) |
| Non-peptide Small Molecular Antagonists | | | |
| SK&F45323 | CCR3 | 800 nM (IC50) | |
| SB297006 | CCR3 | 60 nM (IC50) | |
| SB328437 | CCR3 | 4.5 nM (IC50) | |

would have sufficient cell numbers for future experiments. Accordingly, experiments described in aims 2 though 4 were performed on day 3 of culture when cell viability had not been seriously impaired.

In this study we tested five hypotheses, among these hypothesis 1 and 4 were tested positive and others were rejected. Our results showed that both bacterial strains significantly increased production of several cytokines/chemokines and EDN production in the model system. Does this mean that heat-killed bacteria, if consumed by humans can aggravate allergic inflammation? Our goal was not to study allergic reaction per se and the model system that we used does not have the power to address this important question. Furthermore, previous animal model studies report inhibition of allergic responses by HKLM and HKEC.

In summary, our data suggest that HKLM and HKEC contain components that inhibit IL-4 production and CCR3 expression in human basophil-like cells and eosinophil-like cells, respectively. Identification of the active components of these bacteria and further testing in other cell types and animal models would reveal whether they may be suitable as novel therapeutic agents in allergic diseases.

# Future directions

(A). Identification of the active components of these bacteria and further testing in other cell types and animal models would reveal whether they may serve as novel therapeutic agents for potential use in allergic diseases.

(B). In this study only CCR3 and FceR-I receptor expression was studied. Future studies aimed at studying the function of these receptors would be valuable. Some studies suggested for future work include: (i) To study the chemotaxis of eosinophili like cells in response to CCR3 agonists such as eotaxins; (ii) To study the calcium signaling events in response to CCR3 agonists; these studies are especially important because inhibition of CCR3 expression was only by ~50% in our study; and (iii) to study the release of histamine by IgE receptor activation for basophil like cells.

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## Appendix

Table 1 Other gene products that were affected by HKLM in human eosinophil-like cells

| Gene product | Significant alteration * | Fold change** | P value*** |
|---|---|---|---|
| **Chemokines** | | | |
| CCL15 (MIP-1 delta) | ↑ | 1.93 | <0.01 |
| **Growth factors** | | | |
| Light | ↑ | 2.81 | <0.05 |
| PDGF-BB2 | ↑ | 8.3 | <0.001 |

*Significant alteration – Significant increase or decrease in cytokine/chemokine/growth factors production by HKLM treated as compared to untreated cells.

** Calculated by dividing specific cytokines/chemokines/growth factors production by the cells that were treated with and without bacteria.

*** Significant differences from controls using the unpaired student *t*-test.

Table 2 Other gene products of eosinophil-like cells that were affected by HKEC

| Gene product | Significant alteration* | Fold change** | P value*** |
|---|---|---|---|
| **Cytokines** | | | |
| TNF-$\alpha$ | ↑ | 3.62 | <0.05 |
| TNF-$\beta$ | ↑ | 2.26 | <0.05 |
| IL-2 | ↑ | 3.49 | <0.05 |
| IL-1 ra | ↑ | 1.42 | <0.05 |
| **Chemokines** | | | |
| CCL3 (MIP-1 $\alpha$) | ↑ | 7.79 | <0.001 |
| CXCL9 (MIG) | ↑ | 2.04 | <0.05 |
| **Growth factors** | | | |
| PDGF-BB2 | ↑ | 6.34 | <0.05 |
| GM-SCF | ↑ | 4.5 | <0.05 |

*Significant alteration – Significant increase or decrease in cytokine/chemokine/growth factor production by HKEC treated as compared to untreated cells.

** Calculated by dividing specific cytokines/chemokines/growth factors production by the cells that were treated with and without bacteria.

*** Significant differences from controls using the unpaired student *t*-test.

Table 3 Other gene products of eosinophil-like cells that were unaffected by HKLM

| Cytokines | Chemokines | Growth factors |
|---|---|---|
| IL-1 ra, IL-1α IL-1β | CCL2(MCP-1) | Angiogenin, BDNF, |
| IL-3, IL-6, IL-7, IL-10 | CCL14(MCP-4) | BLC, BMP-4, BMP-6 |
| IFN-γ, TNF- α, TNF- β | CXCL7(NAP-2) | CK- β 8.1, CNTF |
| IL-15, IL-2, | CCL3(MIP-1α) | EGF, Fractalkine |
| TGF- β 1, TGF- β 3 | CXCL8(MIG) | GCP-2, GDNF |
| | CCL17(TARC) | IGFBP-1, 2, 3, 4 |
| | CCL-26(Eotaxin-3) | IGF-1, PARC |
| | CXCL12(SDF-1) | Leptin, GM-SCF |
| | | M-SCF |
| | | Fit-3 ligand, NT-3 |

Table 4 Other gene products of eosinophil-like cells that were unaffected by HKEC

| Cytokines | Chemokines | Growth factors |
|---|---|---|
| IL-1α IL-1β | CCL2(MCP-1) | Angiogenin, BDNF, |
| IL-3, IL-6, IL-10 | CCL14(MCP-4) | BLC, BMP-4, BMP-6 |
| IFN-γ | CXCL7(NAP-2) | CK- β 8.1, CNTF |
| IL-15, | CCL17(TARC) | EGF, Fractalkine |
| TGF- β 1, TGF- β 3 | CCL-26(Eotaxin-3) | GCP-2, GDNF |
| | CXCL12(SDF-1) | IGFBP-1, 2, 3, 4 |
| | | IGF-1, PARC |
| | | Leptin |
| | | M-SCF, |
| | | Fit-3 ligand, NT-3 |

Table 5 Other gene products that were affected by HKLM in human basophil-like cells

| Gene product | Significant alteration * | Fold change** | P value*** |
|---|---|---|---|
| **Cytokines** | | | |
| TNF-$\beta$ | ↑ | 2.8 | <0.05 |
| IL-10 | ↑ | 3.73 | <0.01 |
| IL-15 | ↑ | 1.92 | <0.01 |
| IL-1 $\alpha$ | ↑ | 1.62 | <0.05 |
| IL-1 $\beta$ | ↑ | 2.41 | <0.05 |
| IL-6 | ↑ | 4.42 | <0.001 |
| IL-3 | ↓ | 6.76 | <0.01 |
| **Chemokines** | | | |
| CXCL9(MIG) | ↑ | 1.58 | <0.05 |
| CCL15 (MIP-1 $\delta$) | ↓ | 2.5 | <0.05 |
| **Growth factors** | | | |
| NT-3 | ↑ | 1.83 | <0.05 |
| PARC | ↑ | 2.74 | <0.05 |
| SDF-1 | ↑ | 2.55 | <0.01 |
| FGF-7 | ↓ | 2.5 | <0.05 |
| BMP-4 | ↓ | 5.61 | <0.05 |
| Fit-3-ligand | ↓ | 1.58 | <0.05 |
| IGF-1 | ↑ | 4.6 | <0.05 |
| Light | ↑ | 2.92 | <0.05 |

*Significant alteration – Significant increase or decrease in cytokine/chemokine/growth factor production by HKLM treated as compared to untreated cells.

** Calculated by dividing specific cytokines/chemokines/growth factors production by the cells that were treated with and without bacteria.

*** Significant differences from controls using the unpaired student *t*-test.

Table 6 Other gene products of basophil-like cells that were affected by HKEC

| Gene product | Significant alteration * | Fold change** | P value*** |
|---|---|---|---|
| **Cytokines** | | | |
| IL-6 | ↑ | 4.22 | <0.001 |
| **Chemokines** | | | |
| CXCL7(NAP-2) | ↓ | 1.64 | <0.05 |
| **Growth factors** | | | |
| BMP-4 | ↓ | 2.7 | <0.05 |
| IGF-1 | ↑ | 4.45 | <0.05 |
| Light | ↑ | 2.04 | <0.05 |
| NT-3 | ↓ | 2.34 | <0.05 |
| FGF-7 | ↓ | 3.48 | <0.05 |
| SDF-1 | ↓ | 2.43 | <0.01 |

*Significant alteration – Significant increase or decrease in cytokine/chemokine/growth factors production by HKEC treated as compared to untreated cells.

** Calculated by dividing specific cytokines/chemokines/growth factors production by the cells that were treated with and without bacteria.

*** Significant differences from controls using the unpaired student *t*-test.

Table 7 Other gene products of basophil-like cells that were unaffected by HKLM

| Cytokines | Chemokines | Growth factors |
|---|---|---|
| IL-1 ra, IL-7 | CCL2(MCP-1) | BDNF |
| IFN-γ, TNF- α, | CCL14(MCP-4) | BLC, BMP-6 |
| TGF- β 1, TGF- β 3 | CXCL7(NAP-2) | CK- β 8.1, CNTF |
| IL-2, IL-16 | CCL17(TARC) | Fractalkine |
| | CCL-26 (Eotaxin-3) | GCP-2, GDNF |
| | | IGFBP-1, 2, 3, 4 |
| | | Leptin, GM-SCF |
| | | M-SCF |

Table 8 Other gene products of basophil-like cells that were unaffected by HKEC

| Cytokines | Chemokines | Growth factors |
|---|---|---|
| IL-1 ra, IL-1α IL-1β | CCL2(MCP-1) | Angiogenin, BDNF, |
| IL-3, IL-7, IL-10 | CCL14(MCP-4) | BLC, BMP-6 |
| IFN-γ, TNF- α, TNF- β | CXCL8(MIG) | CK- β 8.1, CNTF |
| IL-15, IL-16 | CCL17(TARC) | EGF, Fractalkine |
| TGF- β 1, TGF- β 3 | CCL-26(Eotaxin-3) | GCP-2, GDNF |
| IL-2 | CCL3(MIP-1α) | IGFBP-1, 2, 3, 4 |
| | | PARC |
| | | Leptin, GM-SCF |
| | | M-SCF, |
| | | Fit-3 ligand, NT-3 |