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TITOLO TESI

DEVELOPMENT OF SCREENING ASSAYS TO TEST NOVEL INTEGRIN ANTAGONISTS IN ALLERGIC INFLAMMATION

Presentata da:

Dott. Antonino Maria Spartà

Coordinatore Dottorato

Chiarissimo Prof.
Lanfranco Masotti

Relatore

Chiarissimo Prof.
Santi Spampinato

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ABSTRACT

Aim of the research: to develop a prototype of homogeneous high-throughput screening (HTS) for identification of novel integrin antagonists for the treatment of ocular allergy and to better understand the mechanisms of action of integrin-mediated levocabastine anti-allergic action.

Results: This thesis provides evidence that adopting scintillation proximity assay (SPA) levocabastine (IC_{50}=406 μM), but not the first-generation antihistamine chlorpheniramine, displaces [^{125}I]fibronectin (FN) binding to human $\alpha_4\beta_1$ integrin. This result is supported by flow cytometry analysis, where levocabastine antagonizes the binding of a primary antibody to integrin α_4 expressed in Jurkat E6.1 cells. Levocabastine, but not chlorpheniramine, binds to $\alpha_4\beta_1$ integrin and prevents eosinophil adhesion to VCAM-1, FN or human umbilical vein endothelial cells (HUVEC) cultured *in vitro*. Similarly, levocabastine affects $\alpha_L\beta_2$/ICAM-1-mediated adhesion of Jurkat E6.1 cells. Analyzing the supernatant of TNF-α-treated (24h) eosinophilic cells (EoL-1), we report that levocabastine reduces the TNF-α-induced release of the cytokines IL-12p40, IL-8 and VEGF. Finally, in a model of allergic conjunctivitis, levocastine eye drops (0.05%) reduced the clinical aspects of the early and late phase reactions and the conjunctival expression of $\alpha_4\beta_1$ integrin by reducing infiltrated eosinophils.

Conclusions: SPA is a highly efficient, amenable to automation and robust binding assay to screen novel integrin antagonists in a HTS setting. We propose that blockade of integrin-mediated cell adhesion might be a target of the anti-allergic action of levocabastine and may play a role in preventing eosinophil adhesion and infiltration in allergic conjunctivitis.

CHAPTER 1
INFLAMMATION

1.1 Historical background

Inflammation was first described by the Egyptians (3000 BC), but a more extensive and accurate picture was available after Celsus, a Roman writer of the first century AD. He identified the four *cardinal signs of inflammation*: **dolor**, **rubor**, **tumor**, and **calor** (pain, redness, swellness, and heat). This symptoms are more common in the acute form of inflammation, than in the chronic one. Centuries later Virchow added a fifth sign: *functio laesa* (loss of function) (Tracy, 2006; Heidland et al., 2006). Further details of the inflammatory process where added in the course of the centuries thanks to the development of the technology used. For example, Cohneheim first used the microscope to observe inflamed blood vessels (Plytycz et al., 2003) and the subsequent edema caused by increased vascular permeability and the characteristic leukocyte emigration.

During the twentieth century many other discoveries were carried out, starting from Paul Ehlrich and Ilya Metchnikov – who shared the Nobel prize in Physiology or Medicine in 1908 for the humoral theory of immunity and phagocytosis, respectively (Germain, 2004) – to the nowadays break throughs by the means of molecular biology.

Figure 1.1
Aulus Cornelius Celsus (ca 25 BC – ca 50 AD) on the far left (from the internet); Iliya Metchnikov (1845 – 1915) in the middle and Paul Ehrlich (1854 – 1915) on the right (from Schmalstieg et al., 2008)

1.2 Essential meaning of inflammation

Inflammation is a complex reaction to injurious agents such as microbes and damaged, usually necrotic, cells that consists of *vascular responses*, *migration and activation of leukocytes*, and *systemic reactions*: the unique feature of the inflammatory process is the reaction of blood vessels, leading to the accumulation of fluid and leukocytes in extravascular tissues. Inflammation serves to destroy, dilute, or wall off the injurious agent, and sets into motion a series of events that try to *heal* and *reconstitute* the damaged tissue. The injured tissue is replaced through regeneration of native parenchymal cells, by filling of the defect with fibrous tissue (**scarring**) or, most commonly, by a combination of these two processes. Inflammation, in fact, is fundamentally a protective response, the ultimate goal of which is to rid the organism of both the initial cause of cell injury (e.g. microbes or toxins) and the consequences of such injury (e.g. necrotic cells and tissues). However, inflammation and repair may be potentially harmful. Inflammation reactions, for example, underlie common chronic diseases such as rheumatoid arthritis, atherosclerosis, and lung fibrosis, as well as life threatening hypersensitivity reactions to insect bites, drugs, and toxins.

The inflammatory response consists of *two main components*: a **vascular reaction** and a **cellular reaction**. Many tissues and fluids are involved in these reactions, including the *fluid and proteins of plasma*, *circulating cells*, *blood vessels*, and *cellular and extracellular constituents of connective tissue*. The circulating cells include **neutrophils**, **monocytes**, **eosinophils**, **lymphocytes**, **basophils**, and **platelets**. The connective tissue cells are the **mast cells** which intimately surround blood vessels; the connective tissue **fibroblasts**; resident **macrophages**; and **lymphocytes**. The **extracellular matrix** consists of the structural fibrous proteins (collagen and elastin), adhesive glycoproteins (fibronectin, laminin, nonfibrillar collagen, tenascin, and others), and proteoglycans. The **basement membrane** is a specilized component of the extracellular matrix consisting of adhesive glycoproteins and proteoglycans.

Inflammation can be **acute** or **chronic**. The first is rapid in onset (seconds or minutes) and is of relative short duration, lasting for minutes, several hours, or a few days; its main characteristics are the exudation of fluid and plasma proteins (edema) and the **emigration of leukocytes**, predominantly **neutrophils**. Chronic inflammation is of longer duration and is associated histologically with the presence of **leukocytes** and **macrophages**, the **proliferation of blood vessels**, **fibrosis**, and **tissue necrosis**. The

vascular and celluar responses of both acute and chronic inflammation are mediated by chemical factors that are derived from plasma proteins or cells and are produced in response to or activated by the inflammatory stimulus.

Inflammation is a defensive mechanism of the body that occurs after a noxious stimulus of various nature (a toxin, a xenobiotic, a microorganism, a chemical-physical insult). The length of the process depends on many factors and on this basis we can distinguish acute and chronic inflammation. It is also important to underline that the inflammatory process itself includes the healing process, which intervenes concomitantly. However, some forms of inflammation can be dangerous for the organism which cannot resolve them with its own means, causing harmful conditions. It is therefore of no surprise the abundance of anti-inflammatory drugs available in pharmacies, which are one of the most sold class of medicines.

1.3 Acute inflammation

In recent years, inflammation has emerged as a new and major process underlying many prevalent diseases. These include Alzheimer disease, cardiovascular disease (Hansson et al., 2006), and cancer (Erlinger et al., 2004) which now join the well-known inflammatory disorders such as arthritis and periodontal disease (Nathan et al., 2002; Serhan et al., 2007).

Acute inflammation is a rapid response to an injurious agent that serves to deliver mediators of host defense (leukocytes and plasma proteins) to the site of injury. It has three major components: *alterations in vascular caliber and that lead to an increase in blood flow*, *structural changes in the microvasculature that permit plasma proteins and leukocytes to leave the circulation*, and *emigration of the leukocytes from the microcirculation*, *their accumulation in the focus of injury*, and *their activation to eliminate the offending agent*.

Acute inflammation is triggered by a variety of stimuli such as infections, trauma, physical and chemical agents, tissue necrosis, foreign bodies, immune reactions (also called hypersensitivity reactions). During this process blood vessels undergo a series of changes that are designed to maximize the movement of plasma proteins and circulating cells out of the circulation and into the site of injury or infection. **Changes in vascular flow and caliber** begin early after injury and develop at varying rates depending on the severity of the injury: (1) VASODILATION: is one of the earliest manifestations of acute inflammation. It

produces an *increase in blood flow* which is the cause of the heat and the redness. Vasodilation is induced by the action of several mediators, notably **histamine** and **nitric oxide**, on vascular smooth muscle; (2) INCREASED PERMEABILITY OF THE MICROVASCULATURE: with the outpouring of protein-rich fluid into the extravascular tissues; (3) STASIS: the loss of fluids results in concentration of red cells in small vessels and increased viscosity of the blood, reflected by the presence of dilated small vessels packed with red cells and slower blood flow. With mild stimuli, stasis may not become apparent until 15 to 30 minutes have elapsed, whereas with severe injury, stasis may occur in a few minutes; (4) ACCUMULATION OF LEUKOCYTES: leukocytes, principally neutrophils, accumulate along the vascular endothelium. Leukocytes then stick to the endothelium, and soon afterward they **migrate** through the vascular wall into the interstitial tissue.

The loss of protein from the plasma reduces the intravascular osmotic pressure and increases the osmotic pressure of the interstitial fluid that, together with the increased hydrostatic pressure owing to increased blood through the dilated vessels, leads to a *marked outflow* of fluid and its accumulation in the interstitial tissue causing edema. Other suggested mechanisms are:

- *Formation of endothelial gaps in venules*: this is the most common mechanism of vascular leakage and is elicited by **histamine**, **bradykinin**, **leukotrienes**, the neuropeptide **substance P**, and many other classes of chemical mediators. It occurs rapidly after exposure to the mediator and is usually reversible and short lived (15 to 30 minutes); it is thus known as the *immediate transient response*. Classically, this type of leakage affects *venules* 20 to 60 μm in diameter, leaving capillaries and arterioles unaffected. Parenthetically, many of the later leukocyte events in inflammation, such as adhesion and emigration, also occur predominantly in the venules in most organs. Binding of mediators, such as **histamine**, to their receptors on endothelial cells activates intracellular signalling pathways that lead to phosphorylation of contractile and cytoskeletal proteins, such as **myosin**. These proteins contract, leading to contraction of the endothelial cells and separation of intercellular junctions. Thus, the **gaps** in the venular endothelium are largely intercellular or close to the intercellular junctions. **Cytokines** such as

interleukin-1 (IL-1), tumor necrosis factor (TNF), and interferon-γ (IFN-γ) also increase vascular permeability by inducing a structural reorganization of the cytoskeleton, such that the endothelial cells retract from one another.

- *Direct endothelial injury, resulting in endothelial necrosis and detachment*: this effect is usually encountered in **necrotizing injuries** and is due to direct damage to the endothelium by the injurious stimulus as, for example, *severe burns* or *lytic bacterial infections*. **Neutrophils** that adhere to the endothelium may also injure the endothelial cells. In most cases, leakage starts immediately after injury and is sustained at a high level for several hours until the damaged vessels are thrombosed or repaired. The reaction is known as the immediate sustained response. All levels of the circulation are affected, including venules, capillaries, and arterioles.
- *Delayed prolonged leakage*: in this case the increase of permeability begins after a delay of 2 to 12 hours, lasts for several hours or even days, and involves venules as well as capillaries. It may result from the direct effect of the injurious agent, leading to delayed endothelial cell damage (perhaps by apoptosis), or the effect of cytokines causing endothelial retraction.
- *Leukocyte-mediated endothelial injury*: leukocyte adhere to endothelium and become activated releasing toxic oxygen species and proteolytic enzymes, which then cause endothelial injury or detachment, resulting in increased permeability.
- *Increased transcytosis*: certain factors, for example vascular endothelial growth factor (VEGF), appear to cause vascular leakage by increasing the number and the size of the channels between the cells.
- *Leakage from new blood vessels*: during repair, endothelial cells proliferate and form new blood vessels in the process called **angiogenesis**; new vessels sprouts remain leaky until the endothelial cells mature and form intercellular junctions.

Although all these mechanisms are separable, all may play a role in response to one stimulus. The vascular leakage induced by all these mechanisms accounts for the life-threatening loss of fluid in severely burned patients.

1.4 Cellular events in acute inflammation

A critical function of inflammation is to deliver leukocytes to the site of injury and to activate the leukocytes to perform their normal functions in host defense. Leukocytes ingest offending agents, kill bacteria and other microbes, and get rid or necrotic tissue and foreign substances. A price that is paid for the defensive potency of leukocytes is that they may induce **tissue damage** and prolong inflammation, since the leukocyte products that destroy microbes and necrotic tissues can also injure normal host tissues.

The process of **extravasation** can be divided in the following steps:

1. *In the lumen*: **margination**, **rolling**, and **adhesion**. Vascular endothelium normally does not bind circulating cells or impede their passage. In inflammation, the endothelium needs to be activated to permit it to bind leukocytes, as a prelude to their exit from the blood vessels.
2. **Transmigration across the endothelium**.
3. **Migration in interstitial tissues** toward a chemotactic stimulus.

In normal flowing blood in venules, erythrocytes are confined to a central axial column, displacing the leukocytes toward the wall of the vessel. Because blood flow slows early in inflammation (**stasis**), hemodynamic conditions change, and more white cells assume a peripheral position along the endothelial surface, a phenomenon called **margination**. Subsequently, individual and then rows of leukocytes tumble slowly along the endothelium and adhere transiently (a process called **rolling**), finally coming to rest at some point where they adhere firmly. After firm adhesion, leukocytes insert pseudopods into the junctions between the endothelial cell, squeeze through interendothelial junctions, and assume a position between the endothelial cell and the basement membrane and escape into the extravascular space. *Neurophils*, *monocytes*, *lymphocytes*, *eosinophils*, and *basophils* all use the same pathway to migrate from the blood into tissues.

Leukocyte adhesion and transmigration are regulated largely by the binding of complementary adhesion molecules on the leukocyte and endothelial surfaces, and chemical mediators (chemoattractants and certain cytokines) affect these processes by *modulating the surface expression or avidity of such adhesion molecules*. The adhesion receptors involved belong to four molecular families: the **selectins**, the **immunoglobulin superfamily**, the **integrins**, and **mucyn-like glycoproteins** (see table 1.1 and table 1.2).

Table 1.1

Endothelial/Leukocyte Adhesion Molecules

SELECTINS	**E-selectin** (CD62E, ELAM) confined to the endothelium; **P-selectin** (CD62P, GMP140, PADGEM), present in endothelium and platelets; **L-selectin** (CD62L, LAM-1), expressed on most leukocytes. *Selectins bind through their lectin domain to sialylated forms of oligosaccharides (e.g. sialylated Lewis X), which are themselves covalently bound to various mucin-like glycoproteins (GlyCAM-1, PSGL-1, ESL-1, and CD34)*
IMMUNOGLOBULIN FAMILY	**ICAM-1** (**intercellular adhesion molecule 1**) and **VCAM-1** (**vascular cell adhesion molecule 1**). *Both these molecules serve as ligands for integrins found on leukocytes*
INTEGRINS	*Transmembrane heterodimeric glycoproteins made up of* α *and* β *chains that are expressed on many cell types and bind to ligands on endothelial cells, other leukocytes, and the extracellular matrix.* **LFA-1** (leukocyte function-associated 1; $\alpha_L\beta_2$) and **Mac-1** (macrophage antigen 1, $\alpha_M\beta_2$, CD11a/CD18) are β_2 **integrins** and bind to ICAM-1, while β_1 **integrins** (such as **VLA-4**, very late antigen 4) bind to VCAM-1
MUCIN-LIKE GLYCOPROTEINS	**Heparan sulphate**, is an example. *Serve as ligands for the leukocyte adhesion molecule called* **CD44**. *This proteins are found in the extracellular matrix and on cell surfaces*

The recruitment of leukocytes to sites of injury and infection is a multistep process involving attachment of circulating leukocytes to endothelial cells and their migration through the endothelium. The first events are the induction of adhesion molecules on endothelial cells by a number of mechanisms. Mediators such as **histamine**, **thrombin**, and **platelet activating factor** (**PAF**) stimulate the redistribution of P-selectin from its normal intracellular stores in granules (*Wibel-Palade bodies*) to the cell surface (Rondaij et al., 2006).

Table 1.2

Endothelial Molecule	Leukocyte Receptor	Major Role
P-selectin	**Sialyl-Lewis X** **PSGL-1**	Rolling (neutrophils, monocytes, lymphocyes)
E-selectin	**Sialyl-Lewis X**	Rolling, adhesion to activated endothelium (neutrophils, monocytes, T cells)
ICAM-1	**CD11/CD18 (integrins)** **(LFA-1, Mac-1)**	Adhesion, arrest, transmigration (all leukocytes)
VCAM-1	**$\alpha_4\beta_1$ (VLA-4) (integrins)** **$\alpha_4\beta_7$ (LPAM-1)**	Adhesion (eosinophils, monocytes, lymphocytes)
GlyCAM-1	**L-selectin**	Lymphocyte homing to high endothelial venules
CD31 (PECAM)	**CD31**	Leukocyte migration through endothelium

Agonist-induced release of these endothelial cell specific storage granules provides the endothelium with the ability to rapidly respond to changes in its micro-environment. Originally being defined as an intracellular storage pool for von Willebrand factor, it has recently been shown that an increasing number of other components, including **P-selctin**, **interleukin-8** (**IL-8**), **eotaxin-3**, **endothelin-1**, and **angiopoietin-2**, is present within this subcellular organelle, implicating a role for Weibel-Palade bodies exocytosis in inflammation, hemostasis, regulation of vascular tone and angiogenesis. In fact, resident tissue **macrophages**, **mast cells**, and **endothelial cells** respond to injurious agents by secreting the cytokines **TNF**, **IL-1**, and **chemokines** (chemoattractant cytokines). TNF and IL-1 act on the endothelial cells of post-capillary venules adjacent to the infection and induce the expression of several adhesion molecules. Endothelial cells lining the vasculature, provide a tightly regulate barrier that regulate a number of physiological processes including extravasation of leukocytes to the underlying tissues, neovascularisation in response to a vascular injury, vascular tone and hemostasis (Aird, 2006). Endothelial cells phenotypes are differentially regulated in *space* and *time*. Endothelial cells work as an input-output adaptive device that contains an elaborate non-linear array of dynamic signalling pathways that couple extracellular signals to cellular responses or phenotype changes. *As input is coupled to output, variations in the extracellular environment across space and time leads to phenotypic heterogeneity* (see Appendix I). Interestingly, Ary Goldberger et al. (2002) has argued that health is associated with organized (fractal chaotic-like) complexity, and disease with loss of variability and so the endothelium: a quiescent endothelium is associated with pathological conditions. Therefore, an inflammatory stimulus induces phenotipical changes in the endothelium that, within one or two hours, the begin to express E-selectin. The concept of **endothelial cell activation** first arose from *in vitro* studies demonstrating the ability of well defined stimuli (e.g. lectin phytohemagglutinin, endotoxin, tumor necrosis factor α (TNF-α), interleukin-1 (IL-1) and others) to induce the expression of so-called *activation antigens* on their surface (Ia-like antigen, ELAM-1, later designed E-selectin). Leukocytes express at the tips of their microvilli carbohydrate ligands for the selectins. These are low-affinity interactions with a fast-off rate, and they are easily disrupted by the flowing blood. As a result, the bound leukocytes detach and bind again, and thus begin to roll along the endothelial surface. TNF and IL-1 also induce endothelial expression of ligand for integrins, mainly **VCAM-1** (the ligand for the VLA-4 integrin) and **ICAM-1** (the

ligand for the LFA-1 and Mac-1 integrins). Leukocytes normally express these integrins in a low-affinity state. Meanwhile, chemokines that were produced at the site of injury enter the blood vessel, bind to endothelial at the site of injury enter the blood vessel, bind to endothelial cell heparan sulphate glycosamminoglycans, and are displayed at high concentrations on the endothelial surface. These chemokines act on the rolling leukocytes and activate the leukocytes. One of the consequences of activation is the conversion of a VLA-4 and LFA-1 integrins on the leukocytes to **high-affinity state** (a process described more in detail in the following chapters of the manuscript). The combination of induced expression of integrin ligands on the endothelium and activation of integrins on the leukocytes results in **firm integrin-mediated binding of the leukocytes to the endothelium at the site of infection**. The leukocytes stop rolling, their cytoskeleton is reorganized, and they spread out on the endothelial surface (Fig. 1.1).

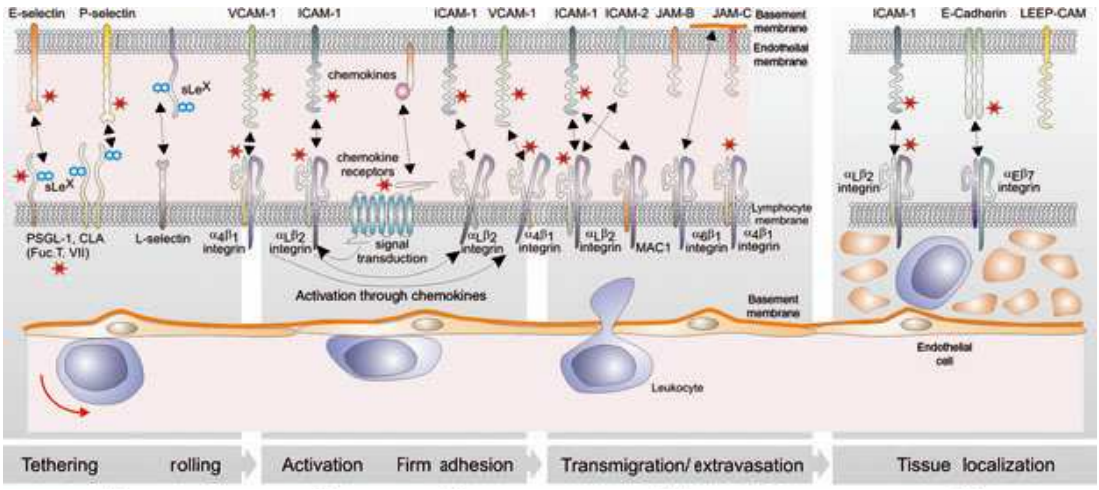

Figure 1.2
Mechanism of leukocyte rolling, adhesion and diapedesis. The initial interactions between the activated leukocyte and endothelium are weak: attachment and detachment alternate causing leukocyte rolling. On the contrary, the integrin-mediated binding is firm, so that the activated leukoccytes are steadily localized at the level of the inflammatory process and can move through the endothelium to the extracellular space, in a process called diapedesis. (from Vicente-Manzanares et al., 2004)

Chemokines act on the adherent leukocytes and stimulate the cells to migrate through interendothelial spaces toward the chemical concentration gradient, that is toward the site of injury or infection. Once the leukocytes enter the extravascular connective tissue, they are able to adhere to the extracellular matrix by virtue of β_1 integrins and CD44 binding to matrix proteins. The most telling proof of the importance of adhesion molecules is the existence of genetic deficiencies in the leukocyte adhesion proteins, which result in impaired leukocyte adhesion and recurrent bacterial infections, as in the *leukocyte adhesion deficiency type 1 (LAD1)*, the *leukocyte adhesion deficiency type 2 (LAD2)*, and the *leukocyte adhesion deficiency type 3* (see Box 1.1).

Box 1.1

Leukocyte adhesion deficiency type 1 (LAD1) is a rare, inherited immunodeciency that affects 1 in 1 million people yearly and usually presents with recurrent, indolent bacterial infections of the skin, mouth and respiratory tract and impaired pus formation and wound healing. Features of this disease result from mutation of the CD18 gene, which is encoded on chromosome 21q22.3. This gene codes for the common subunit of the leukocyte integrins **LFA-1**, **Mac-1**, and **p150,95**. Failure to produce a functional subunit results in the defective expression of all three leukocyte integrins, and the leukocytes of LAD have subnormal adhesion properties.

Leukocyte adhesion deficiency type 2 (LAD2) is caused by the absence of sialyl-Lewis X, the fucose-containing ligand for E-selectin, owing to a defect in fucosyl transferase, the enzyme that attach fucose moieties to protein backbones.

While in LAD1 and 2 the defect in the adhesion cascade is restricted to leukocytes, all four cases of **leukocyte adhesion deficiency type 3 (LAD3)** described to date also had defects in platelet aggregation. These patients suffered from recurrent bacterial infections and a severe bleeding tendency. All cases were reported to have activation defects in all major integrin subfamily members expressed in circulating leukocytes and platelets (Etzioni et al., 2004). In three of the cases, consanguinity was reported suggesting an autosomal recessive type of inheritance. While in LAD1 and LAD2 only a specific molecule was involved in adhesion, CD18 and CD15a respectively, in LAD3 β_1, β_2 and β_3 integrins are affected and thus both leukocytes and platelets are defective in their ability to adhere and hence a tendency for bleeding accompanied the severe recurrent infections (Alon et al., 2003).

The **type of emigrating leukocytes** varies with the age of the inflammatory response and with the type of stimulus. In most form of acute inflammation **neutrophils** predominate in the inflammatory infiltrate during the first 6 to 24 hours, then are replaced by **monocytes** in 24 to 48 hours. There are several reasons that justify this event:

neutrophils are more numerous in the blood, they respond more rapidly to chemokines, and they may attach more firmly to the adhesion molecules that are rapidly induced on endothelial cells, such as P- and E-selectins. In addition, after entering the tissues, neutrophils are short-lived; they undergo apoptosis and disappear after 24 to 48 hours, whereas monocytes survive longer. There are exceptions to this pattern of cellular exudation. In certain infections neutrophils predominate over 2 to 4 days; in viral infections, lymphocytes may be the first cells to arrive; in some hypersensitivity reactions, eosinophilic granulocytes may be the main cell type.

Microbes, products of necrotic cells, antigen-antibody complexes, and cytokines, including chemotactic factors, induce a number of responses in leukocytes that are part of the defensive functions of the leukocytes (neutrophils and monocytes/macrophages) and are referred to under the title of leukocyte activation. Activation results from several signaling pathways that are triggered in leukocytes, resulting in increases in cytosolic Ca^{2+} and activation of enzymes such as protein kinase C (PKC) and phospholipase A_2 (PLA_2). The intracellular elaboration of the stimuli results in:

- Production of arachidonic acid metabolites
- Secretion of cytokines
- Modulation of leukocyte adhesion molecules
- Toll-like receptors (TLRs) and GPCRs expression
- Expression of receptors for opsonins

1.5 Termination of the acute inflammatory response

The inherent capacity of the acute inflammatory response to cause tissue injury need to be tightly controlled to minimize the damage. In part, inflammation declines simply because the mediators of inflammation have short half-lives, are degraded after their release, and are produce in quick bursts, only as long as the stimulus persists. In addition, as inflammation develops, the process also triggers a variety of stop signals that serve to actively terminate the reaction (figure 1.2). These active mechanisms include a switch in the production of pro-inflammatory leukotrienes to anti-inflammatory **lipoxins** from arachidonic acid; the liberation of anti-inflammatory cytokines, transforming growth factor-

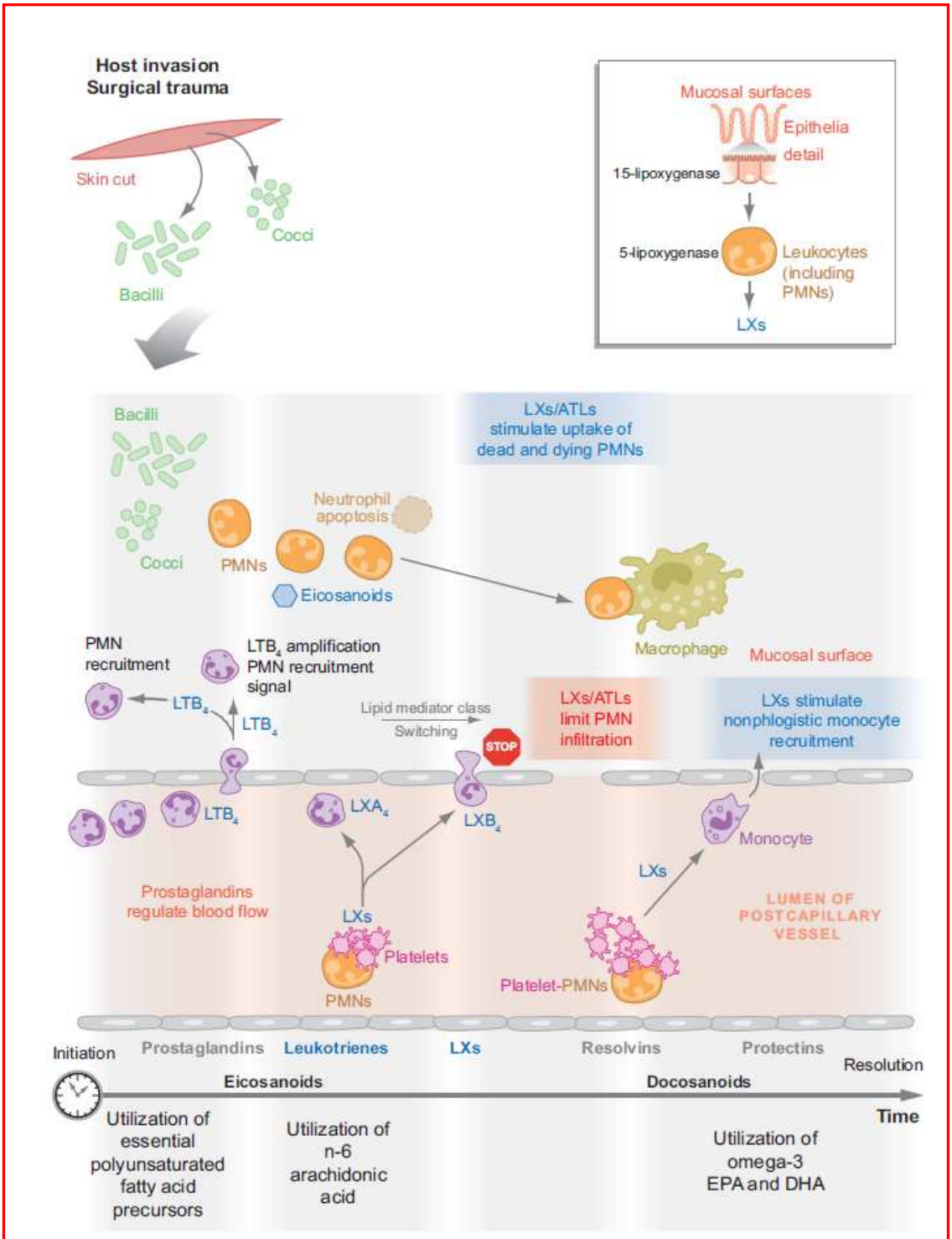

Figure 1.2
Time course of inflammation from the beginning to the resolution. (Serhan et al., 2007)

β (TGF-β), from macrophages and other cells; and neural impulses (*cholinergic discharge*) that inhibit the production of TNF in macrophages. They also reduce vascular permeability, promote nonphlogistic recruitment of monocytes, and stimulate clearance of apoptotic neutrophils via macrophages (Serhan, 2007). Recently, new families of local-acting mediators were discovered that are biosynthesized from the essential fatty acids eicosapetaenoic (EPA) acid and docosahexaenoic acid (DHA) (Ariel et al., 2007). These new chemical mediators are endogenously generated in inflammatory exudates collected during the resolution phase, and were termed **resolvins** and **protectins** because specific members of these families control the magnitude and duration of inflammation in animals. Elucidating the mode of action of **omega-3 PUFAs** (polyunsaturated fatty acids, or simply fish oils), novel enzymatic oxygenated products generated *in vivo* were identified in murine systems and in humans by pathways initiated from the precursors EPA and DHA. These new families of compounds are biosynthesized and contribute functionally to the resolution of inflammatory exudates and neuroprotection (Serhan et al., 2000; Serhan et al., 2002; Hong et al., 2003; Marcheselli et al., 2003; Mukherjee et al., 2004; Lukiw et al., 2005). Resolvins are endogenous local mediators possessing stereospecific and potent anti-inflammatory, in addition to immunoregulatory, actions (Serhan et al., 2002). They reduce neutrophil trafficking, cytokine and oxyne reactive species release, and lowering the magnitude of the inflammatory response. The terms **protectin D1** (**PD1**) and **neuroprotectin D1** (**NPD1**) – when generated in neural tissue – were introduced given the general anti-inflammatory and protective actions of this unique 10R,17S-dihydroxy-docosatriene in neural systems, stroke, animal models of Alzheimer's disease, and peritonitis (Serhan et al., 2006). These new families of anti-inflammatory and pro-resolving mediators contrast with the earlier omega-3 PUFA-derived oxygenated products that possess similar structures to previously known arachidonic acid originated eicosanoids, but were less potent pro-inflammatory mediators or devoid of bioactions (Lee et al., 1984). Members of the resolving and protectin families specifically evoke potent stereoselective bioactions evident in the nanomolar and picomolar ranges *in vitro* and *in vivo*. Because the precursors to both resolvins and protectins are the essential omega-3 PUFAs, their relation to dietary supplementation by omega-3 PUFAs also raises new and interesting questions, given the widely appreciated notion that omega-3 supplementation reduces inflammatory diseases and brings about an anti-inflammatory status. Lipoxins, specifically LXA_4 and LXB_4, as well as

their aspirin triggered forms, stop further polymorphonuclear cells entry into the exudates as well as counter-regulate the main signs of inflammation. As new polymorphonuclear cells (PMN) enter the exudates, older and apoptotic immune cells must be removed from the site in a timely fashion for the inflammation to resolve (figure 1.3). Once PMN enter an exudate, they interact with other cells (such as other leukocytes, platelets, endothelial cells, and fibroblasts) in their immediate vicinity and can perform **transcellular biosynthesis** to produce lipoxin and eventually new mediators: when platelets adhere to PMNs the platelet-PMN aggregates become a major intravascular source of lipoxin that in turn halts further PMN diapedesis and recruitment.

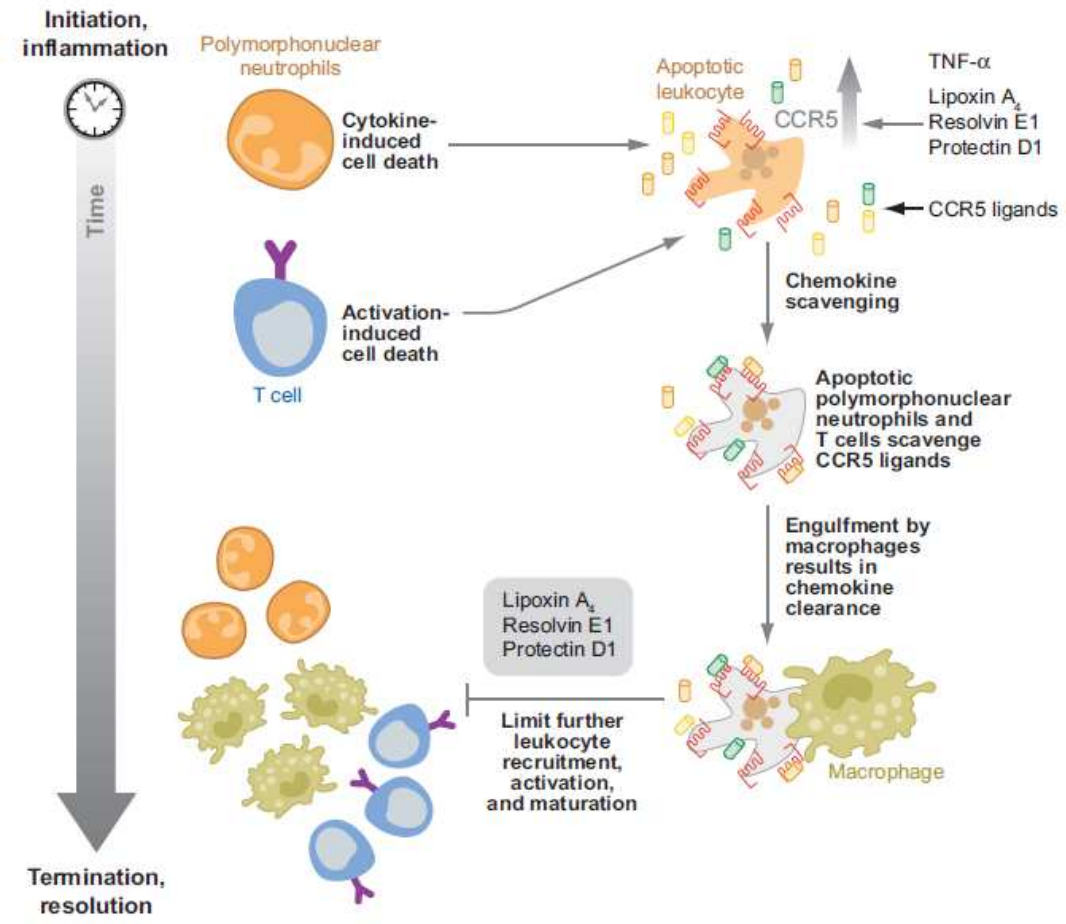

Figure 1.3
Resolution of inflammation. (from Serhan, 2007)

These events clearly demonstrate the ability of PMNs to switch their phenotype changing the profile of lipid mediators produced depending upon their local environment (Levy et al., 2001; Serhan et al., 2000). Therefore, during the course of inflammation and complete resolution, mediator switching also occurs between families of lipid mediators, namely from eicosanoids to resolvins as well as protectins (Serhan et al., 2002; Bannenberg et al., 2005).

1.6 Chemical mediators of inflammation

The chemical mediators of inflammation orchestrate the inflammatory process working in a coordinated manner. They can originate either from plasma or from cells. Plasma-derived mediators (e.g. complement proteins, kinins) are present in plasma in precursor forms that must be activated, usually by a series of proteolytic cleavages, to acquire their biologic properties. Cell-derived mediators are normally sequestered in intracellular granules that need to be secreted (e.g. histamine in mast cell granules) or are synhesized de novo (e.g. prostaglandins, cytokines) in response to a stimulus. The major cellular source are platelets, neutrophils, monocytes/macrophages, and mast cells, but mesenchymal cells (endothelium, smooth muscle, fibroblasts) and most epithelia can also be induced to elaborate some of the mediators. The production of active mediators is triggered by microbial products or by host proteins, such as the proteins of the complement, kinin, and coagulation systems, that are themselves activated by microbes and damaged tissues. Most mediators perform their biologic activity by initially binding to specific receptors on target cells. Some, however, have direct enzymatic activity (e.g. lysosomal proteases) or mediate oxidative damage (e.g. reactive oxygen and nitrogen intermediates). Furthermore, one mediator can stimulate the release of other mediators by target cells themselves. These secondary mediators may be identical or similar to the initial mediators but may also have opposing activities. They provide mechanisms for amplifying or, in certain instances, counteracting the initial mediator action. Once activated and released from the cell, most mediators are short-lived. They quickly decay or are inactivated by enzymes, or they are otherwise scavenged or inhibited. There is thus a system of checks and balances in the regulation of mediator actions.

Among the most important mediators are the vasoactive amines histamine and serotonin. They are especially important because they are present in preformed stores in

cells and are therefore among the first mediators to be released during inflammation. Histamine is widely distributed in tissues, the richest source being the mast cells that are normally present in the connective tissue adjacent to blood vessels. It is also found in blood basophils and platelets. Proformed histamine is present in mast cell granules and is released by mast cell degranulation in response to a variety of stimuli: physical injury, such as trauma, cold or heat; immune reactions involving binding of antibodies to mast cells; fragments of complement called anaphylatoxins (C3a and C5a); histamine-releasing proteins derived from leukocytes; neuropeptides; and cytokines. Histamine causes dilation of the arterioles and increases the permeability of venules (it, however, constrict large arteries). It is considered to be the principal mediator of the immediate transient phase of increased vascular permeability, causing venular gaps. It acts on the microcirculation mainly via binding to H_1 receptors on endothelial cells. Serotonin (5-hydroxytryptamine) is a preformed vasoactive mediator with actions similar to those of histamine. It is present in platelets and enterochromaffin cells, and in mast cells in rodents but not humans. Release of serotonin and histamine from platelets is stimulated when platelets aggregate after contact with collagen, thrombin, adenosine diphosphate (ADP), and antigen-antibody complexes. Platelet aggregation and release are also stimulated by platelet activating factors (PAF) derived from mast cells during IgE-mediated reactions. In this way, the platelet release reaction results in increased permeability during immunologic reactions.

Plasma proteins mediate a variety of phenomena in the inflammatory reponse through an interrelated system of complement, kinin and clotting systems.

When cells are activated by diverse stimuli, their membrane lipids are rapidly remodelled to generate biologically active lipid mediators that serve as intracellular or extracellular signals to affect a variety of biologic processes, including inflammation and hemostasis. These lipid mediators are thought of as autacoids, or short-range hormones that are formed rapidly, exert their effect locally, and then either decay spontaneously or are destroyed enzymatically.

Cytokines are proteins produced by many cell types (principally activated lymphocytes and macrophages, but also endothelium, epithelium, and connective tissue cells) that modulate the functions of other cell types. Long known to be involved in cellular immune responses, these products have additional effects that play important roles in both acute and chronic inflammation. Tumor necrosis factor (TNF) and interleukin-1 (IL-1) are

two of the major cytokines that mediate inflammation. They are produced mainly by activated macrophages, but they can be released from many other cell types as well. Their most important actions in inflammation are their effects on endothelium, leukocytes, and fibroblasts, and induction of systemic acute-phase reactions. In endothelium, they induce a spectrum of changes, mostly regulated at the level of gene transcription, referred to as endothelial activation (Mantovani et al., 1997). In particular, they induce the synthesis of endothelial adhesion molecules (see Appendix 1) and chemical mediators, including other cytokines, chemokines, growth factors, eicosanoids, and nitric oxide (NO); production of enzymes associated with matrix remodelling; and increases in the surface of thrombogenicity of the endothelium. TNF also induces priming of neutrophils, leading to augmented responses of these cells to other mediators. IL-1 and TNF induce the systemic acute-phase responses associated with infection or injury. Features of these systemic responses include fever, loss of appetite, slow-wave sleep, the release of neutrophils into the circulation, the release of corticotrophin and corticosteroids and, particularly with regard to TNF, the hemodynamic effects of septic shock: hypotension, decreased vascular resistance, increased heart rate, and decreased blood pH. TNF also regulates body mass by promoting lipid and protein mobilization and by suppressing appetite. Sustained production of TNF contributed to cachexia, a pathologic state characterized by weight loss and anorexia that accompanies some infections and neoplastic diseases.

Chemokines are a family of small proteins that act primarily as chemoattractants for specific types of leukocytes. About 40 different chemokines and 20 different receptors for chemokines have been identified. They are classified in four major groups, according to the arrangement of the conserved cysteine residues in the mature protein (see table 1.3). Chemokines mediate their activities by binding to seven transmembrane G protein-coupled receptors. These receptors usually exhibit overlapping ligand specificities, and leukocytes generally express more than one receptor type. Chemokines stimulate leukocyte recruitment in inflammation and control the normal migration of cells through various tissues. Some chemokines are produced transiently in response to inflammatory stimuli and promote the recruitment of leukocytes to the sites of inflammation. Other chemokines are produced constitutively in tissues and function in organogenesis to organize different cell types in different anatomic regions of the tissues. In both situations, chemokines may be displayed

at high concentrations attached to proteoglycans on the surface of endothelial cells and in the extracellular matrix.

Table 1.3

CLASS	NAME	ACTIONS
C-X-C	IL-8	Secreted by activated macrophages, endothelial cells, and other cell types and causes activation and chemotaxis of neutrophils, with limited activity on monocytes and eosinophils
C-C	MCP-1; eotaxin; MIP-1α; RANTES	Generally attract monocytes, eosinophils, basophils, and lymphocytes but not neurophils. Eotaxin selectively recruits eosinophils
C	Lymphotactin	Relatively specific for lymphocytes
$C\text{-}X_3\text{-}C$	Fractalkine	Exist in two forms: the cell surface bound protein can be induced on endothelial cells by inflammatory cytokines and promotes strong adhesion of monocytes and T cells; and a soluble form derived by proteolysis of the membrane-bound protein that has a potent chemoattractant activity for the same cells

Nitric oxide (NO), a pleiotropic mediator of inflammation, was discovered as a factor released from endothelial cells that caused vasodilation by relaxing vascular smooth muscle and was therefore called endothelium-derived relaxing factor. NO is a soluble gas that is produced not only by endothelial cells, but also by macrophages and some neurons in the brain. NO acts in a paracrine way on target cells through induction of cyclic guanosine monophosphate (GMP), which, in turn, initiates a series of intracellular events leading to a response, such as the relaxation of vascular smooth muscle cells. Since the *in vivo* half-life of NO is only seconds, the gas acts only on cells in close proximity to where it is produced. NO plays an important role in the vascular and cellular components of inflammatory responses. NO is a potent vasodilator by virtue of its actions on vascular

smooth muscle. In addition, NO reduces platelet aggregation and adhesion, inhibits several features of mast cell-induced inflammation, and serves as an endogenous regulator of leukocyte recruitment. Blocking NO production under normal conditions promotes leukocyte rolling and adhesion in postcapillary venules, and delivery of exogenous NO reduces leukocyte recruitment. Thus, production of NO is an endogenous compensatory mechanism that reduces inflammatory responses. NO and its derivatives are microbicidal, and thus NO is also a mediator of host defence against infection.

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CHAPTER 2
ALLERGY

2.1 Introduction to allergy

Allergic disorders, which affect approximately 30% of the population in developed countries, are genetically determined and/or environmentally affected multifactorial, refractory diseases. These disorders are associated with chronic inflammation characterized by influx of a large number of eosinophils and accumulation of mast cells in the lesions and increased IgE production (Parronchi et al., 2000; Valenta et al., 2004). Leukemic patients who did not have any allergic symptoms, developed allergic diseases after therapeutic transplantation of bone marrow stem cells from donors who had allergic disorders, suggesting that bone marrow-derived immune cells contribute to the development of allergic disorders.

The **allergic inflammatory response** is characterized by IgE synthesis, IgE-dependent mast cell activation and connective tissue infiltration by T lymphocytes and eosinophils. The **immediate-phase response** is mediated by a range of inflammatory mediators such as histamine, leukotrienes, and prostaglandins, resulting in vasodilation, oedema, mucus secretion, itching. Individuals who experience a **late-phase response** have further symptoms 4-24 hours after the initial challenge with allergen. Biopsies indicate that the late-phase allergic response involves T lymphocyte activation, production of Th2-type cytokines and tissue **eosinophilia**. The pattern of the inflammatory responses to allergen is mediated by a number of different cells and a complex sequence of release of different **inflammatory mediators**. The cardinal features of allergic inflammation, which distinguish it from other types of inflammation, include **IgE synthesis**, **IgE-dependent mast cell activation**, and tissue infiltration of **T lymphocytes** and **eosinophils**. Allergen interaction with IgE on the surface of mast cells results in the production of a range of inflammatory mediators that may be granule-derived (e.g. histamine and tryptase) or newly formed membrane-derived mediators such as leukotrienes B_4, C_4, D_4, and E_4 and prostaglandins. Further mediators are bradykinin and platelet activating factor (PAF). There is good evidence that these agents mediate the immediate allergic response. Their biological actions

include vasodilation and increased vascular permeability (givin rise to oedema), stimulation of mucous secretion, and stimulation of afferent nerves promoting itching. Late-phase responses are characterized by the presence in the tissue of T lymphocytes and eosinophils. The mechanism of local tissue eosinophilia is not well understood, but it is hypothesized that the regulation of eosinophil movement and IgE production is under the T lymphocytes and mediated by Th2 cytokines. When biopsy tissue is taken from allergic individuals 24 hours after an allergen challenge ouside the pollen season, a marked increased in the number of $CD4^+$ T lymphocyte is seen (Varney et al., 1992). To identify the mediators released by these cells, *in situ* hybridization techniques – using RNA for selected cytokines – were carried out to monitor the increased gene expression. These experiments demonstrated an increased expression of IL-3, IL-4, IL-5, and GM-CSF at mRNA level after local allergen provocation (Durham et al., 1992). This interleukin profile is consistent with activation and proliferation of Th2 lymphocytes. By contrast, the Th1-type cytokines IL-2 and IFN-γ are not increased. Approximately 70-80% of IL-4 and IL-5-producing cells were T cells (Ying et al., 1992). IL-4, along with IL-13, is the key mediator in promoting B lymphocyte isotype switching in favour of IgE production. During the late-phase response, it was demonstrated that not only was there an increase in IL-4, but also significant elevation of Cε and Iε RNA expressing cells, suggesting that increased IgE synthesis may be occurring locally, although further studies to detect IgE protein production by B cells and/or plasma cells are required. Exaggerated IgE production is part of the phenomenon underlying the development of **atopy**. Just why allergic responses are different in each individual – for example some people develop asthma, some rhinitis, and others eczema – is not fully understood. It may be that the local T-cell response and B-cell IgE production predict target organ response, while the underlying predisposition to IgE hyperresponsiveness is determined by intrauterine, developmental or pollution factors. Another important effect of IL-4 is to promote increased expression of **adhesion molecules** (including VCAM-1) on vascular endothelium, which may selectively recruit eosinophils. Increased IL-5 production is also an important observation, since IL-5 is an eosinophil-specific cytokine (Takatsu et al., 2008) that is not only responsible for the terminal differentiation of eosinophils and their release from the bone marrow, but also during allergic reactions it promotes adhesion of the eosinophils to vascular endothelium and prolongation of their survival at the site of the inflammation, possibly by inhibition of

apoptosis. All these actions will have the effect of increasing the number of eosinophils in the tissue. Thus, it would appear that the late phase response is associated with T lymphocyte and eosinophil recruitment and activation and expression of Th2-type cytokines. Cytokine production occurs predominantly in T cells, with a smaller but significant amount being found in mast cells and eosinophils (Bradding et al., 1994).

Mast cells have long been recognized for their role in the genesis of allergic inflammation; and more recently for their participation in innate and acquired immune responses. These cells were first described by Paul Ehrlich in 1879, who called them mast cells from the German word *mastzellen* meaning well fed, as he observed the granular nature of these cells. He was also the first to demonstrate the metachromatic nature of the mast cells using special stains. Typically, mast cells stain metachromatically because of the interaction of the aniline dyes with acid heparin contained in their cell granules. Mast cells reside within tissues and including the skin and mucosal membranes, which interface with the external environment; as well as being found within vascularised tissues next to nerves, blood vessels and glandular structures. Mast cells have the capability of reacting both within minutes and over hours to specific stimuli, with local and systemic effects. Mast cells arise from pluripotential stem cells, mature in tissues, and have the ability to generate inflammation following exposure to a variety of receptor-mediated signals initiated by both innate and acquired immune response mechanisms. Mast cells are easily indentified by the presence of prominent granules within their cytoplasm. These mast cells are heterogeneous in morphology and staining characteristics (Fig. 2.1). Tissue mast cells can be activated in wound healing, fibrosis, cardiovascular disease and auto-immunity in addition to allergic inflammation. Upon activation of mast cells via cross-linking of the high affinity IgE receptor (FcεRI) or non-IgE-mediated activation through complement receptors or Toll-like receptor (TLR) activation, mast cells can release a variable spectrum of pro-inflammatory mediators. These include pre-formed mediators such as histamine, serotonin and proteases; newly synthesized mediators including leukotrienes and prostaglandins; and cytokines and chemokines (see table 2.1). In addition to IgE-mediated activation, human mast cells exposed to IFN-γ can be activated following IgG-mediated aggregation of FcγRI to release similar mediators (Tkaczyk et al., 2002). Addition IgE-independent mast cell triggers have been described. These include SCF, complement factors (C3a and C5a), neuropeptides (substance P), adenosine, TLR and scavenger receptors (Brown et al., 2007).

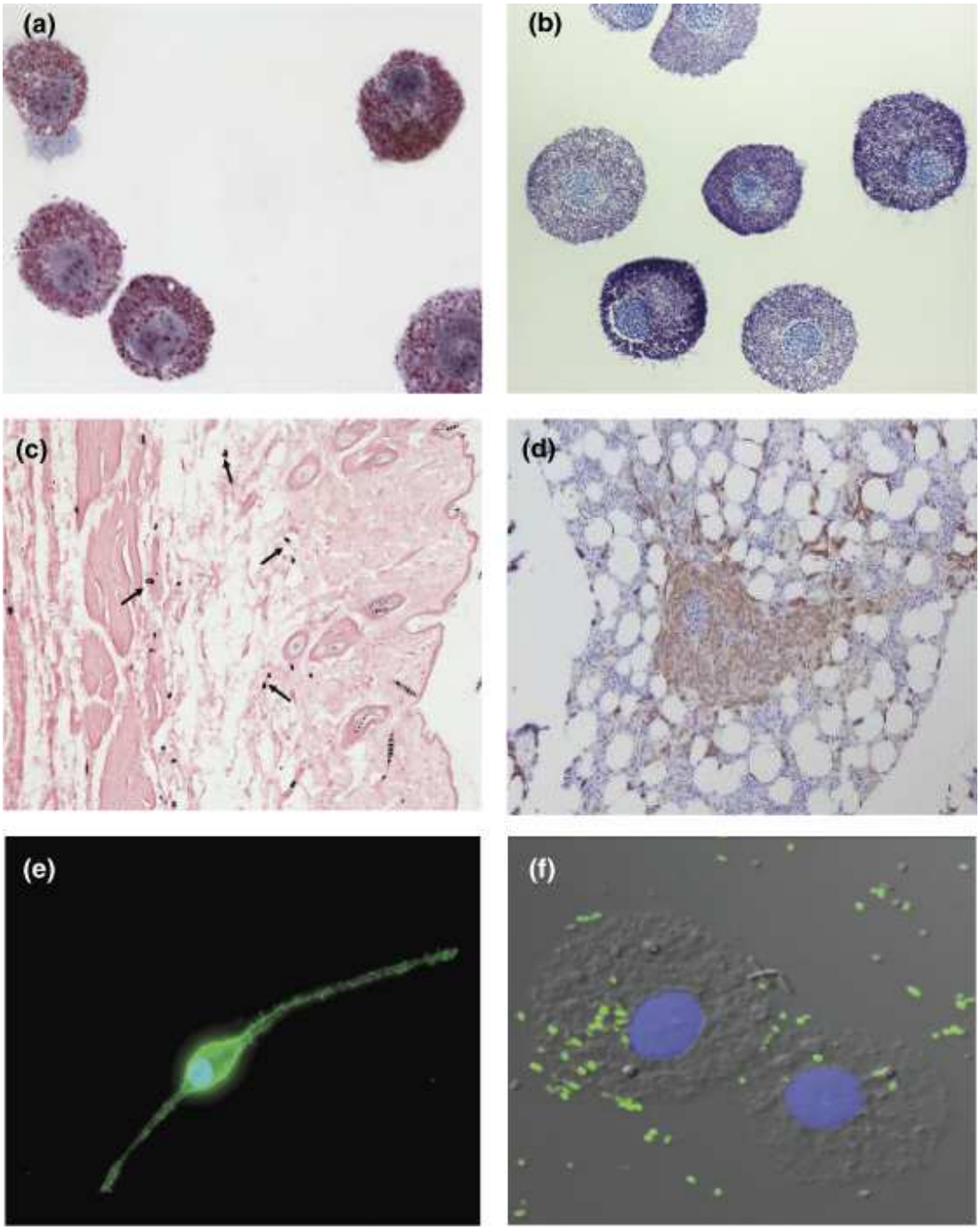

Figure 2.1
Images of mast cells: (a) murine mast cells stained with Wright-Geimsa; (b) human $CD34^+$-derived mast cells stained with toluidine blue; (c) murine skin mast cells stained with toluidine blue; (d) tryptase stained mast cells aggregated in the bone marrow of a mastocytosis patient; (e) HuMC stained with phalloidin for F-actin following treatment with 5-HT; (f) phagocytosed fluorescent *Escherichia coli* particles in BMMC (Brown et al., 2007)

Table 2.1		
Class	**Mediators**	**Physiologica Effects**
Preformed Mediators	Histamine, serotonin, heparin, neutral proteases (tryptase and chymase, carboxypeptidase, cathepsin G), major basic protein, acid hydorlases, peroxidase, phospholipase	Vasodilation/vasoconstriction; angiogenesis; mitogenesis; pain; protein processing/degradation; lipid/proteoglycan hydrolysis; arachidonic acid generation; tissue damage; inflammation
Lipid Mediators	LTB_4, LTC_4, PGE_2, PGD_2, PAF	Leukocyte chemotaxis; vasoconstriction; bronchoconstriction; platelet activation; vasodilation
Cytokines	TNF-α, TGF-β, IFN-α, IFN-β, IFN-γ, IL-1α, IL-1β, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-18, IL-25, SCF, MIF	Inflammation; leukocyte migration/proliferation
Chemokines	CXCL8, CCL2, CCL3, CCL5, CCL7, CCL13, CCL11, CCL19	Chemoattraction and tissue infiltration of leukocytes
Growth Factors	CSF, GM-CSF, bFGF, VEGF, NGF, LIF	Growth of various cell types; vasodilation; neovascularisation; angiogenesis

The level and pattern of mediator release is influenced by cytokines, growth factors and microenvironmental conditions.For example, IL-4 enhances FcεRI-mediated reactions from human mast cells (Bischoff et al., 1999). In addition to enhancing activation of mast cells, several modulatory cytokines produced by regulatory T cells such as IL-10 and TGF-β can decrease FcεRI-mediated reactions (Gebhardt et al., 2005). Mast cell activation and mediator release can independently, as well as in concert with other immune cells, induce much of the pathology observed in allergic inflammatory conditions. Mast cell mediators

such as histamine, leukotrienes, and prostaglandins contribute to eosinophil recruitment, increase vascular permeability, and smooth muscle contraction. Proteases can activate fibroblasts thereby promoting collagen deposition and fibrosis (Levi-Schaffer et al., 2003). Mast cell-derived cytokines have numerous effects on other cells of the immune system as well as endothelial cells. These mediators can cause B cells to class switch to synthesize IgE, induce basophil histamine release, recruit neutrophils and eosinophils, and promote the development of T cells into a T helper 2 (Th2) phenotype (Gauchat et al., 1993). Mast cell products may both induce an immediate reaction and contribute to a late-phase reaction. The immediate phase reaction occurs within minutes of FcεRI cross-linking and its consequences are referred to as an immediate hypersensitivity reaction. Pre-formed granule-associated and newly generated mediators released during this phase include histamine, proteases and lipid-derived mediators. Late phase reactions peak 6-12 h following antigen challenge and are associated with cytokine and chemokine production and release in part form eosinophils, neutrophils and basophils that have entered the inflammatory site following the immediate reaction (Fig. 2.2). Therefore, mast cells have long been recognized for their role in immediate hypersensitivity reactions, by virtue of the presence of high affinity receptors for IgE (FcεRI) on their surface. More recently, mast cells have been postulated to be involved in a variety of chronic inflammatory disorders as numerous mediators released by activated mast cells are characterized. The humoral and cellular inflammatory responses occurring during immediate hypersensitivity reactions are initiated by numerous mediators of mast cells and possibly basophils.

2.2 Mast cells and their mediators

As shown in *Table 2.1* some mediators are stored preformed in the secretory granules of mast cells and are released rapidly upon mast cell activation by a process of regulated secretion or **degranulation**. Other mediators are synthesized *de novo* upon mast cell activation and are released without a prolonged intracellular storaged phase after variable time intervals. In humans, the principal preformed mediators of mast cells include histamine, proteoglycans, neutral proteases, and possibly, basic fibroblast growth factor. Newly generated mediators include the arachidonic acid metabolites prostaglandin D_2, and leukotriene (LT) C_4, and TH2-like cytokines such as interleukins (ILs) 4, 5, 6, and 13, and tumor necrosis factor α (TNF-α):

→ *Preformed granule associated mediators*

BIOGENIC AMINES: **Histamine** is stored in secretory granules of mast cells, is bound to carboxyl and sulphate groups on protein and proteoglycans, and is the sole biogenic amine in human mast cells and basophils. Human mast cells contain 1 to 5 pg of histamine per cell (Schwartz et al., 1987), which is equivalent to a concentration of 0,1 M inside the secretory granules. In constrast, plasma concentration of histamine averages 2nM. Intermediate concentrations in various biologic fluids, such as nasal lavage fluid reflect local rates of production and diffusion or metabolism. After mast cell degranulation, released histamine is metabolized within minutes. Elevated levels of histamine in plasma or urine are found following anaphylactic reactions and indicate mast cell or basophil involvement. Histamine exerts potent biologic effects through its interaction with cell specific receptors designated H1, H2 and H3 receptors (Arrang et al., 1987; Black et al., 1972; Polk et al., 1988). Stimulation of H1 receptors results in vasodilation and increased capillary permeability, contraction of bronchial and grastrointestinal smooth muscle, and increased mucous secretion at mucosal sites. H2 receptor agonists stimulate gastric acid secretion and exerts numerous effects on cells of the immune system, including augmentation of suppression by T lymphocytes, inhibition of mediator secretion by cytotoxic lymphocytes and granulocytes, and suppression of eosinophil chemotaxis (Clark et al., 1975). Additional effects include activation of endothelial cells to release prostacyclin, a potent inhibitor of platelet aggregation. Stimulation of H3 receptors affects neurotransmitter release and histamine production in the central and peripheral nervous system (Arrang et al., 1988). H3 receptors are postulated to mediate interactions between mast cells and peripheral nerves, and they may be involved in histamine-mediated neurogenic hyperexcitability.

PROTEOGLYCANS: the presence of highly sulphated proteoglycans in secretory granules of mast cells confers the metachromatic staining properties of these cells when stained with basic dyes, such as toluidine blue or alcian blue (Fig. 2.2).

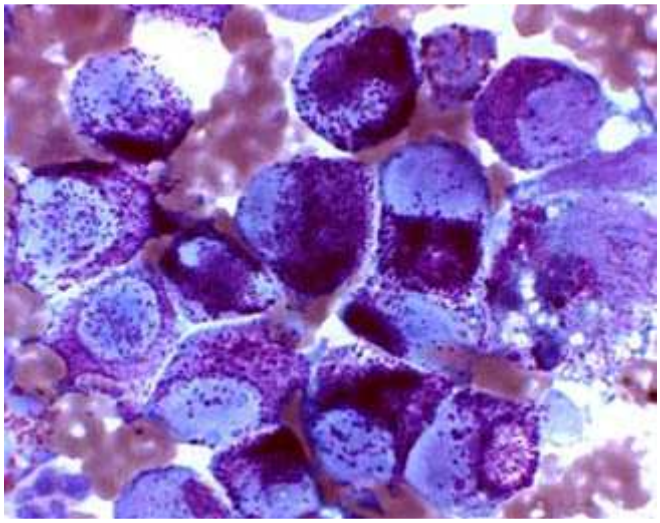

Figure 2.2
Well differentiated canine mast cells. (From the internet)

In human mast cells, heparin and chondroitin sulphate E are the major intracellular proteoglycans (Stevens et al., 1988; Thompson et al., 1988). Heparin is found in all mature mast cells, but not in other cell types. The biologic functions of endogenous mast cell proteoglycans await further clarification. These proteoglycans bind to histamine, neutral proteases, and acid hydrolases at the acidic pH found inside the mast cell secretory granules, and may play an important role in intracellular packaging. Heparin facilitates processing of chymase and tryptase forms to their active forms. The stabilizing effect of heparin and (to a lesser degree) chondroitin sulphate E on tryptase activity may be critical for mast cell physiology. Heparin and chondroitin sulphate E express anticoagulant, anticomplement, and antikallikrein activities. Heparin facilitates fibroblast growth factor activity in cutaneous mast cells and modulates the cell adhesion properties of matrix proteins, such as vitronectin, fibronectin and laminin.

NEUTRAL PROTEASES: cleave peptide bonds near neutral pH and are the dominant protein components of secretory granules in rodent and human mast cells from one another. Tryptase is a serine class protease with tryptic-like activity. It is stored in secretory granules bound to heparin, which

is essential for tryptase to retain its enzymatic activity, and it is not inhibited by biologic inhibitors of serine proteases present in plasma and urine. Tryptase is present in all mast cells at concentrations that vary from 10 pg per mast cell derived from the lung to 35 pg per mast cell derived from the skin. Negligible amounts have been have been detected in human basophils (0,04 pg/cell), but not in other cell types (Xia et al., 1995). Tryptase is released together with histamine during degranulation and serves as a specific marker of human mast cells. Potential biological activities of tryptase include inactivation of fibrinogen, with subsequent anticoagulant effect, which explains the lack of fibrin deposition in reaction involving urticaria and angioedema, and consequently, their rapid resolution. Tryptase augments histamine constriction effects on airway smooth muscles and degrades vasoactive intestinal peptide (an intrinsic neuropeptide with bronchodilating properties), effects that may result in increased bronchospasm. Chymase is a serine protease with chymotryptic-like activity that was purified from human skin. Like tryptase, chymase is bound to heparin, but remains stable after dissociation from heparin. Chymase activity is inhibited by the classical biologic inhibitors of serine proteases. Potential biological activity of chymase include activation of angiotensin I and procollagenase and inactivation of bradykinin. Chymase digests the lamina lucida of the basement membrane at the dermal-epidermal junction of human skin and degrades substance P, vasoactive intestinal peptide, and probably other neuropeptides. Dog chymase stimulates mucous production from glandular cells *in vitro* (Sommerhoff et al., 1989), suggesting a similar role in allergic disorder at various organ sites, such as mucous hypersecretion in asthma and allergic rhinitis and mucous hypersecretion in allergic ocular disorders. Chatepsin G is a serine neutral protease with chymotryptic activity that colocalizes with chymase in human mast cells. It also found in neutrophils and monocytes and therefore cannot be used as a specific marker of mast cells.

→ *Newly generated mediators*

LIPID MEDIATORS: unstimulated mast cells incorporate exogenous arachidonic acid into membrane and cytoplasmic lipid. Upon activation of mast cells, arachidonate released by hydrolysis of these lipids is metabolized along the cycloxygenase pathway, givin rise to PGs and thromboxane, or (along the lipoxygenase pathway) LTs. The production of arachidonic acid metabolites begins within minutes of cell activation and lasts to about 30 minutes. Relative production of these lipid mediators cannot be used to distinguish various types of mast cells. The biologic activity of arachidonic acid metabolites has been underscored by the favourable clinical effects of 5-lipoxygenase inhibitors in atopic and aspirin-induced asthma, where mast cell activation occurs.

CYTOKINES: upon activation human mast cells produce an array of cytokines, including up-regulation of the IL-4 gene cluster on human chromosome 5 (IL-4, IL-5, IL-6, IL-9, IL-13, and granulocyte macrophage-colony stimulating factor), and of TNF-α on human chromosome 6 (Walsh et al., 1991; Bradding et al., 1993; Burd et al., 1995). Mast cells preferentially produce IL-13 over IL-4 as opposed to basophils, which produce more IL-4. Cytokine production occurs minutes to hours following mast cell activation. Cytokines derived from activated **mast cells** serve to activate **endothelial cells** to recruit **eosinophils** and other inflammatory cell types at the site of immediate hypersensitivity reactions, thus giving rise to the late phase of allergic response and sustained inflammation. These cytokines also may be involved in modulating the local distribution of the Th1 and Th2 types of T lymphocytes, thereby expanding the biologic role of mast cells far beyond type I hypersensitivity reactions only.

Another cell type that is involved in the development of the allergic inflammatory response is the Th lymphocyte. Since the discovery of two distinct types of Th cells (IFN-γ-producing Th1 cells and IL-4-producing Th2 cells) in mice by Coffman and collegues (1986), mutual regulation between Th1 and Th2 cells (**Th1/Th2 balance**) has been considered to be important for homeostatic maintenance of the immune system in the whole body. The dysregulation of the Th1/Th2 balance leads to excessive Th1 or Th2 cell

activation, resulting in the development of autoimmune diseases associated with accumulation of Th1 cells or induction of allergic diseases due to accumulation of Th2 cells, respectively (Bach, 2002). Thus, the concept of a Th1/Th2 balance provided the basis for understanding the molecular mechanisms of immune responses and/or disease development and has been widely accepted as a "paradigm" of the immune system for the past two decades. The factors responsible for the Th cell polarization into a predominant Th1 or Th2 profile have been extensively investigated. Current evidence suggest that Th1 and Th2 cells develop from the same Th-cell precursor under the influence of mechanisms associated with antigen presentation. Both environmental and genetic factors influence the Th1 or Th2 differentiation by determining the "leader cytokine" in the microenvironment of the responding Th cell. IL-4 is the most powerful stimulus for Th2 differentiation, whereas IL-12 and IFNs favour Th1 development. A role has been demonstrated for the site of antigen presentation, the physical form of the immunogen, the type of adjuvant, and the dose of antigen. Potent adjuvants and microbial products induce Th1-dominated responses because they stimulate IL-12 production. On the contrary, both the primary source of IL-4 and the mechanisms responsible for its early production involved in the differentiation of naïve Th cells into Th2 effectors are still unclear. Th1- and Th2-dominated responses not only provide different strategies of protection against pathogens, but also play a role in some physiologic and pathophysiologic conditions. Evidence is accumulating in animal models to suggest that Th1 responses are involved in the genesis of organ-specific autoimmune diseases, such as experimental uveo-retinitis, experimental encephalomyelitis, or insulin-dependent diabetes mellitus. Th2 responses are apparently insufficient per se to protect against the majority of infectious agents but can provide some protection against parasites and by limiting potentially harmful Th1-mediated responses. On the other hand, changes in autoimmune diseases that could not be explained by the Th1/Th2 paradigm were also observed in several settings: unexpectedly, IFN-γ/IFN-γ-R-deficiency or neutralization resulted in exacerbation, rather than attenuation, of development of autoimmune diseases such as murine encephalomyelitis (Ferber et al., 1996), arthritis (Vermeire et al., 1997), uveitis (Jones et al., 1997), and nephritis (Ring et al., 1999), which have classically been considered to be Th1-mediated diseases. Therefore, the paradigm has been recently integrated with the Th17 cells (Oboki et al., 2008) (Fig. 2.3). Allergic asthma is considered to be a Th2-dominant chronic inflammatory disease of the

lungs. Th2 are characterized by secretion of IL-4, IL-5, IL-9 and IL-13. The transcription factor GATA-binding protein 3 (GATA3) is crucial for the differentiation of uncommitted T cells into Th2 cells and regulates the secretion of Th2 cytokines (Ho et al., 2007). There is an increase in $GATA3^+$ T cells in the airways of stable asthmatic subjects. Nuclear factor of activated T cells (NFAT) is a T-cell specific transcription factor and enhances the transcriptional activation of the *Il4* promoter by GATA3. Finally, IL-33, a member of the IL-1 family of cytokines, promotes differentiation of Th2 cells by translocating to the nucleus and regulating transcription through an effect on chromatin structure (Carriere et al., 2007), but it also acts as a selective chemoattractant of Th2 cells (Komai-Koma et al., 2007). Increased Th2-cytokine and IgE levels and accumulation/activation of Th2 cells, eosinophils and mast cells are observed in asthmatic lungs (Wills-Karp, 1999; Cohn et al., 2004).

IL-4 plays a critical role in the differentiation of Th2 cells from uncommitted Th0 cells and may be important in initial sensitization to allergens. It is also important for isotype switching of B cells from producers IgG to producers of IgE. IL-13 mimics IL-4 in inducing IgE secretion and causing structural changes in the airways, but does not play a role in promoting Th2 cell differentiation. IL-13 has attracted particular attention as a therapeutic target for the treatment of asthma, as it not only induces airway hyperresponsiveness in animal models of asthma, but also produces several of the structural changes seen in chronic asthma (Wills-Karp et al., 2004).

The transcription factor T-bet is crucial for Th1 cell differentiation and secretion of the Th1-type cytokine IFN-γ. Consistent with the prominent role of Th2 cells in asthma, T-bet expression is reduced in T cells from the airways of asthmatic patients compared with airway T cells from non-asthamtic patients (Finotto et al., 2002). After phosphorylation, T-bet associates with and inhibits the functions of GATA3 by preventing it form binding to its DNA target sequences. In turn, GATA3 inhibits Th1-type cytokines by inhibiting STAT4, the main transcription factor activated by T-bet-inducing IL-12. Th1 cells are the prominent $CD4^+$ T cells, and Tc1 cells the predominant $CD8^+$ T cells expressed in chronic obstructive pulmonary disease lungs. IFN-γ is the predominant cytokine produced by Th1 and Tc1 cells and may play an important role in inflammation by inducing the release of chemokines. In contrast, it is usally found at reduced levels in individual with asthma,

although levels of IFN-γ are increased in patients with more severe disease and acute exacerbations.

Th17 cells are a subset of $CD4^+$ T cells that play an important role in inflammatory diseases and are regulated by the transcription factor retinoic acid orphan receptor-γt (RORγt). IL-6, IL-1β, TGF-β, and IL-23 are all involved in the differentiation of human Th17 cells. Th17 cells also produce IL-21 which is important for the differentiation of these cells and thus acts as a positive autoregulatory factor. It also inhibits expression of the forkhead transcription factor forkhead box P3 (FOXP3) and thereby the development of Treg. IL-22 is also released by Th17 cells and stimulates the production of IL-10 and acute-phase proteins. IL-25 (IL-17E) another member of the IL-17 superfamily, is produced by Th2 cells, mast cells, and epithelial cells. IL-25 induces the expression of IL-4, IL-5, and IL-13, resulting in inflammation mediated by eosinophils, increased IgE production and allergic hyperresponsiveness in mice. A blocking antibody specific for IL-25 inhibits the development of allergic hyperresponsiveness in response to allergens in mice and is in clinical development for asthma (Ballantyne et al., 2007).

Several cytokines implicated in allergic inflammation either promote the differentiation and survival of inflammatory cells or result in proliferation and/or activation of structural cells, contributing to tissue remodelling. Examples are GM-CSF and SCF. Another class of compounds that have growth factor-like characteristics are the **neurotrophins**. Neurotrophins are cytokines that play an important role in the function, proliferation and survival of autonomic nerves. In sensory nerves, neurotrophins increase responsiveness and expression of takychinins. Nerve growth factor (NGF) may be produced by mast cells, lymphocytes, macrophages and eosinophils as well as structural cells, such as epithelial cells, fibroblasts, and smooth muscle cells. Although neurotrophins have predominant effects on neuronal cells, they can also act as growth factors for inflammatory cells, such as mast cells, as well as increasing chemotaxis and survival of eosinophils (Freund-Michel et al., 2008; Nassenstein et al., 2006).

Chemokines play a fundamental role in the recruitment of inflammatory cells from the circulation to the site of antigen exposure. There has been particular interest in the role of cemokines in the allergic inflammation as they signal through GPCRs, for which small molecule antagonists can be relatively easily developed. Chemokines are best discussed in the light of the receptors to which they bind, and these are divided into receptors for CC

chemokines (CCRs), receptors for CXC chemokines (CXCRs), and the receptor for the lone CX3C chemokine.

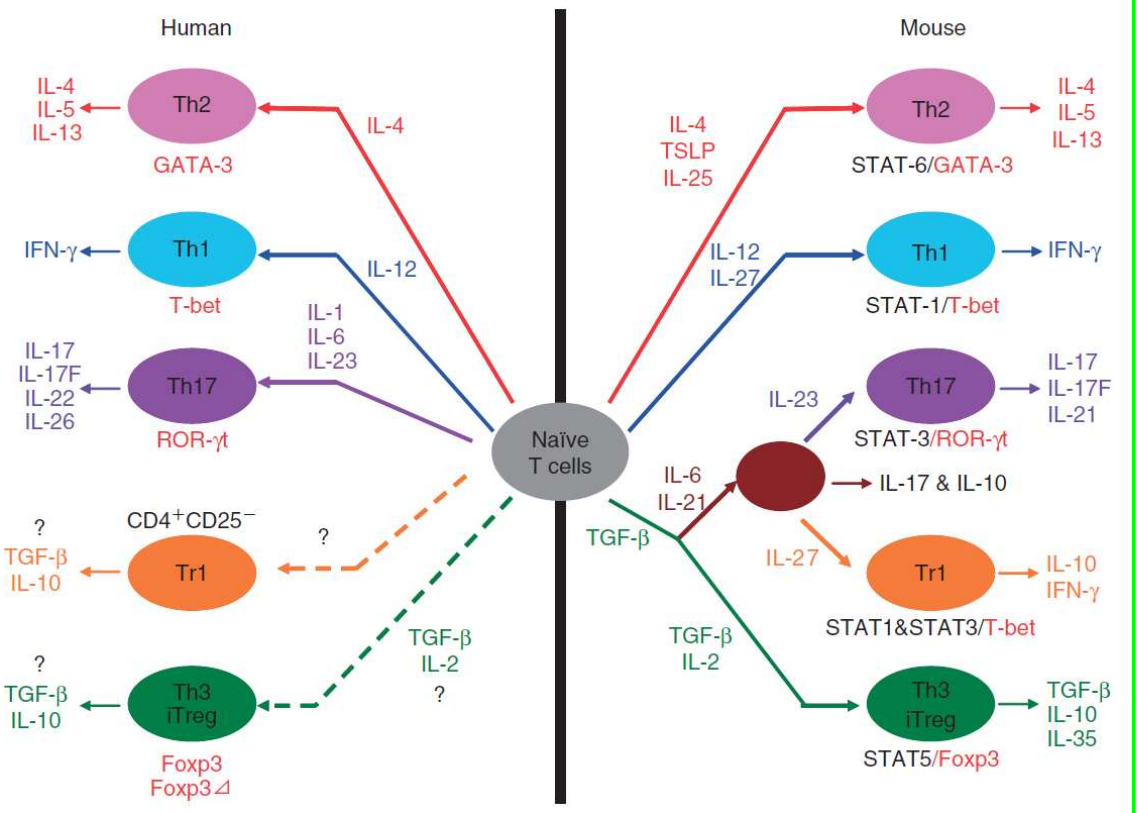

Figure 2.2
The revised scheme of Th cell differentiation in humans and mice. In the mouse, naïve T cells differentiate into Th1 cells in the presence of IL-12 or IL-27, Th2 cells in the presence of IL-4, TSLP or IL-25, Th17 cells in the presence of IL-23 after TGF- β1 plus IL-6- or IL-21-mediated cell differentiation, Tr1 cells in the presence of IL-27 after TGF- β1 plus IL-6- or IL-21-mediated cell differentiation, and Th3/iTreg cells in the presence of TGF-β1 and IL-2. In man, naïve T cells differentiate into Th1 cells in the presence of IL-2, Th2 cells in the presence of IL-4 and Th17 cells in the presence of IL-1β plus IL-6 or IL-1β plus IL-23. The mechanisms of human Tr1 and Th3/iTreg cell differentiation remain unclear.

IL-17 can potentiate bronchial fibroblast, epithelial cell and smooth muscle cell activation enhancing IL-6, IL-8, IL-11 and CXCL1/Groα production by bronchial fibroblast, β-defensin-2, ICAM-1, IL-8, CXCL1, CCL20, G-CSF, MUC5B and MUC5AC expression/production by human bronchial epithelial cells, and IL-6 and IL-8 production by human airway smooth muscle cells. Nevertheless, these IL-17-mediated inflammatory mediators do not seem to contribute to the development of Th2-mediated eosinophil-dominant allergic asthma, since IL-8 and CXCL1/Groα are potent chemoattractant factors

for neutrophils, and IL-6 and G-CSF are important for granulopoiesis, especially neutrophil development. In addition, IL-17 inhalation led to induction of neutrophilia rather than eosinophilia in the airways of rodents (Laan et al., 1999; Hoshino et al., 1999). However, these observations may provide us with new insight into the pathogenesis of "non-Th2-type" asthma. The heterogeneity in the symptoms of asthma has been classified as **atopic** and **non-atopic** and **eosinophilic** and **non-eosinophilic**. Amin et al. (2000) demonstrated that atopic asthmatics showed Th2-type airway inflammation characterized by increased IL-4^+ and IL-5^+ cells and eosinophils in bronchial biopsies and increased serum IgE levels, whereas non-atopic asthmatics exhibited non-Th2-type airway inflammation characterized by increased IL-8^+ cells and neutrophils in bronchial biopsies, and no elevation of serum IgE. On the other hand, increased mast cell accumulation was observed in both atopic and non-atopic patients irrespective of the IgE level.

Allergic and parasitic worm immunity is characterized by infiltration of tissue with IL-4- and IL-13-expressing cells, including Th2 cells, eosinophils and basophils. Tissue macrophages assume a distinct phenotype, designated alternatively activated macrophages. The major mast cell product PGD_2 is released during the allergic response and stimulates the chemotaxis of eosinophils, basophils, and Th2-type T lymphocytes (Schleimer et al., 1985; Lewis et al., 1982). The chemoattractant receptor homologous molecule of Th2 cells (CRTH2) has been shown to mediate the chemotactic effect of PGD_2. PGH_2 is the common precursor of all PGs and is produced by several cells that express cyclooxygenases. Like other prostanoids, PGD_2 rises from the phospholipase A_2/arachidonic acid/cyclooxygenase pathways. PGD_2 is finally isomerized by from the primary cyclooxygenase product, PGH_2, by two specific cytosolic enzymes: lipocalin-type PGD synthase (PGDS) and hematopoietic PGDS. The latter is express in immune cells such as mast cells, antigen-presenting cells, microglia, and Th2-type lymphocytes (Kanaoka et al., 2003; Mohri et al., 2003). In contrast, lipocalin-type PGDS is most abundant in the CNS but can also be found in osteoblasts, male genital organs, the heart, and vascular endothelium (Urade et al., 2000; Grill et al., 2008; Schuligoi et al., 2005; Gallant et al., 2005). Upon damage of these tissues, lipocalin-type PGDS become up-regulated and is also been secreted into the cerebrospinal liquor, seminal fluid, and blood plasma, respectively. In agreement with the expression patterns and localization of PDGS, PGD_2 has been implicated in allergic disease, sleep, nociception, male fertility, and vascular function.

Importantly, Sculigoi et al. (2009) demonstrated that PGH_2 potently induces the chemotaxis of human eosinophils, elicits a shape change, and stimulates intracellular Ca^{2+} flux and that these effects appear to be mediated by the CRTH2 receptor. Also endothelial cells are able to release PGH_2 in response to mechanical stimuli, acetylcholine, endothelin-1, and IL-1β. These studies suggest that activation of endothelial cells results in enhanced metabolization of arachidonic acid to PGH_2, which cannot be metabolized any further by prostacylin synthase or other PG synthases as a result of the limited capacity and, thus, PGH_2 is released untransformed from cells (Folco et al., 2006). The released PGH_2 can promote platelets aggregation and vasoconstriction acting on thromboxane receptor TP (Corey et al., 1975; Saito et al., 2003). Furthermore, PGH_2 can activate two prostanoid receptors CRTH2 and DP that regulate inflammatory cells that are involved in the allergic response and that point to a novel role of PGH_2 in leukocyte-endothelial cell interaction in allergic disease. PGH_2 acts on the CRTH2 receptor of eosinophils causing their shape change and acting rearrangement, almost as potently as eotaxin – which binds the CCR3 receptor and is the most potent eosinophil chemoattractant known – and similarly to basophils. Consistent with its effect to cause eosinophil shape change, PGH_2 also induced the elevation of intracellular free Ca^{2+} in eosinophils to similar levels as PGD_2 and eotaxin. Therefore, the primary role of PGD_2 and CRTH2 in eosinophil function is the regulation of eosinophil trafficking, as activation of CRTH2 results in eosinophil mobilization from bone marrow, direct stimulation of chemotaxis, and priming eosinophil for migration toward other chemoattractants (Hirai et al., 2001; Heinemann et al., 2003; Schratl et al., 2006; Royer et al., 2007). Accumulation of eosinophils at sites of allergic reactions is a hallmark of tissue injury and lung dysfunction. Asthmatic patients that receive treatment based on eosinophil count in sputum have significantly fewer severe asthma exacerbations than patients treated according to standard management therapy (Green et al., 2002). Moreover, genetically modified mice lacking eosinophils are protected against allergen-induced lung injury and asthma. It is now clear that the mast cell-derived and T cell-derived cytokines, such as IL-5 and GM-CSF, are pivotal in eosinophil maturation and chemotaxis (Rothenberg, 1998; Lampinen et al., 1999; Oliveira et al., 1997). Mast cell-derived chemokines and inflammatory cytokines, such as TNF-α and IL-2, also can regulate eosinophil chemotaxis, activation, and function. This mast cell-eosinophil axis needs further study, as it could lead to insights into the pathogenesis of chronic allergic inflammation and may lead to new therapeutic targets.

Mast cells and eosinophils colocalize in diseases such as allergic responses and parasitic diseases. Eosinophil participation in allergic responses is in turn mediated by the release of a plethora of mediators when activated. Studies from several laboratories suggest that ligand-receptor interactions, such as stem cell factor (SCF)-c-*kit* and cytokine-cytokine receptor interaction, as well as tryptase protein-activated receptor 2 (PAR2) binding, may mediated mutual activation and signalling in mast cell-eosinophil interactions. Among the mast cell products that affect eosinophils are cytokines, including IL-1, IL-3, Il-4, IL-5, IL-13, TNF-α, and GM-CSF, and chemokines, including IL-8, eotaxin, regulated upon activation normal T cell expressed and secreted (RANTES), and monocyte chemotactic protein 1 (MCP-1). In human allergies, mast cells may serve as amplifiers of IgE-mediated inflammatory responses and eosinophil influx. Kung et al. (1995) using mast-cell deficient mice demonstrated that these mice develop less eosinophila than their normal counterpart. In another study by Das et al. (1998) mast cells were required for the induction of eosinophilia in response to eotaxin injection in air pouches of sensitized mice. Thus, in all likelihood, based on their prolific cytokine/chemokine synthetic capacity, human mast cells play a pivotal role in allergic inflammation and in eosinophil mediated diseases.

2.3 Cytokine expression in allergic inflammation

Allergic inflammatory responses are driven by cells of the immune system that rely on cytokines to regulate the activity of other immune and structural cells. Cytokines are extracellular signalling proteins produced by different cell types that act on target cells to modulate diverse cellular functions. Some cytokines recruit specific cell types to the site of inflammation; others can act on immune cells to increase activation and survival, whereas some, like INF-γ, suppress cellular activity. The role of cytokines to mount an adequate immune response is further supported by the ability of lymphokines to induce immunoglobulin isotype switch in B cells and to modulate T cell differentiation. Therefore, identifying the pattern of cytokine expression observed during the development of allergic inflammation provides a valuable insight to the underlying immunological mechanisms. Allergen challenge studies have demonstrated that chemotactic cytokines and lymphokines are significantly increased as a result of allergen challenge. Chemokines, such as eotaxin and MIP-1α, are released by immune and structural cells of the injured tissue and are responsible for the late recruitment of eosinophils, neutrophils and basophils to the site of

inflammation. This recruitment is an essential step in the development of the late-phase allergic response and, therefore, it represents a candidate immunological mechanism to be targeted by new therapeutic approaches.

Here follows a list of some important cytokines involved in allergic disease:

- ***SCF (Stem Cell Factor)***: SCF bind to its receptor c-*kit* which is present, in particular, on mast cells and eosinophils. Hence, SCF is also called c-*kit*-ligand and, because it is encoded by the steel locus, it has also been referred to as the steel factor. SCF induces growth of mast cells and also regulates their activation, degranulation, and chemotaxis. Many factors, including mast cell cytokines and chymase, regulate expression of SCF by human eosinophils. Thus, the addition of cytokines or chymase to eosinophils induces SCF secretion. Several studies support the hypothesis that paracrine and autocrine SCF-based loops can regulate bidirectional mast cell-fibroblast-eosinophil interactions, leading to chronic inflammation. SCF is produced by epithelial cells, smooth muscle cells, endothelial cells, fibroblasts, mast cells, and eosinophils. It is a critical growth factor for mast cells and promotes their generation from $CD34^+$ progenitors. SCF induces mast cell survival, adhesion, chemotaxis, degranulation, which releases histamine and tryptase, and synthesis of pro-inflammatory cytokines and chemokines. SCF is upregulated by inflammatory stimuli and shows increased expression in epithelial cells of the inflamed tissue. A bloking antibody specific for SCF inhibits the inflammation mediated by eosinophils after allergen exposure (Berlin et al., 2004).
- ***IL-1β and TNF-α***: important cytokines expressed by human mast cells. They induce the expression of chemokines from adventitial cells, such as fibroblasts, which in turn serve to chemoattract eosinophils. IL-1β increases eosinophil survival through paracrine production of TNF-α, IL-5, and GM-CSF from mast cells in an autocrine manner. TNF-α is stored in mast cells granules and has important effects on eosinophil function inducing the release of GM-CSF that increase their survival and the NF-κB translocation to the nucleus. TNF-α activates eosinophils and stimulate them to release eosinophil peroxidise (EPO). It can act direcly on the smooth muscle increasing the sensitivity to spasmogens and may thus play a role in asthma, or can play a role in the amplification of the

allergic inflammation throught the activation of NF-κB. IL-1β expression is increased is increased in allergic individuals and activates many inflammatory genes that are expressed in allergy. IL-1 receptor antagonist (IL-1ra) administration reduces the allergic response in mice, but human recombinant IL-1ra is not effective.

- ***IL-2***: important eosinophil chemoattractant. Although its dominant source is the T cells, mast cells and eosinophils are able to express this cytokine. IL-2 can enhance eosinophil cationic protein production in platelet activating factor-stimulated eosinophils. IL-2 is an important eosinophilic activator and induces the release of eosinophil peroxidase and IL-6 from these cells. IL-2 can regulate eosinophil chemotaxis, and eosinophilia is a recognized feature of the adverse effect of infusion of recombinant IL-2.
- ***IL-3, IL-5, and GM-CSF***: these cytokines together regulate growth, differentiation and survival of eosinophils, while their absence is cause of apoptosis and death of these cells. This survival is necessary for eosinophils to sufficiently exert their effects. IL-3, IL-5 and GM-CSF delay the apoptosis of eosinophils for a minimum of 12-14 days, whereas in their absence, the life-span of these cells does not exceed 48 hours. They also cause eosinophils to express large numebers of receptors for cytokines, immunoglobulins, and complement. IL-5 induces eosinophil differentiation in the bone marrow and also stimulate eosinophil precursors to synthesize granule proteins. IL-5, like GM-CSF, is also essential for eosinophil activation and may play a role following tissue infiltration by the eosinophil. IL-3 is a hematopoietic cytokine that activates eosinophils and allows them to present superantigens and peptides to T cells. It also downregulates the chemokine receptor CCR3 from the surface of the human eosinophil. IL-3 works in tandem with IL-5 to induce differentiation of eosinophil progenitor cells. GM-CSF induces hypodense, activated eosinophils and prolongs eosinophil survival secondary to its production and release by activated mast cells through TNF-α. GM-CSF can also induce the release of EPO from eosinophils. Thus, induction of autocrine and paracrine GM-CSF production is one of the ways mast cell increase eosinophil survival. The major sources of IL-5 are Th2 cells, Tc2 cells, mast cells, eosinophils, and γδT cells.

IL-5 acts as a co-activator of B cell proliferation in combination with anti-CD38. Eosinophil development is supported by GM-CSF, IL-3 and IL-5. Among these cytokines IL-5 signaling is especially important to develop eosinophilia. In fact, IL-5 plays a key role in inflammation mediated by eosinophils, since it is critically involved in the differentiation of eosinophils from bone marrow precursor cells and also prolongs eosinophil survival. Systemic and local administration of IL-5 to allergic patients results in an increase of in circulating eosinophils and $CD34^+$ eosinophil precursors (Stirling et al., 2001). GM-CSF plays a role in the differentiation and survival of eosinophils, neutrophils, and macrophages. It is secreted predominantly by macrophages, epithelial cells and T cells in response to inflammatory stimuli.

- ***IL-4 and IL-13***: key cytokines responsible for the **late phase** reaction observed in allergic inflammation. Both IL-4 and IL-13 are products of human mast cells. IL-4 regulates IgE synthesis and also allows for eosinophil recruitment. IL-4 also regulates eosinophil apoptosis, thus contributing to the resolution of inflammation. IL-13 can prolong eosinophil survival by inducing autocrine production of IL-3, IL-5, and GM-CSF. IL-4 and IL-13 can also affect eosinophils by also enhancing the activating effect of TNF-α. This effect is also seen with IL-5, suggesting an *in vitro* synergism among all four of these cytokines. IL-4 and IL-13, however, play a pivotal role in transmigration of eosinophils across the endothelium of vasculature by activation of adhesion pathways specific to eosinophils as well as promoting IL-5 and eotaxin production by inflammatory cells. IL-4 induces vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells, allowing eosinophil recruitment into tissue involving VCAM-1- very late antigen 4 (VLA-4) interactions. Eosinophils tether, roll, and accumulate when exposed to endothelial cells activated by IL-4 and expressing VCAM-1. Inhibition of VCAM-1 or VLA-4 integrin leads to the inhibition of eosinohil tethering. When IL-4 is injected into the tissue, leukocyte recruitment occurs, and the majority of these infiltrating cells are eosinophils. IL-4, in conjunction with IL-1 and TNF-α, also induces eotaxin from endothelial cells which, in turn, modulates eosinophil recruitment. IL-13 induces inflammation through stimulating the expression of multiple chemokines,

including CCL11 (also known as eotaxin) from structural cells in the airways, including epithelial cells. IL-13 is produced by several cell types in addition to Th2 cells, (Th1 cells, Tc2 cells, and invariant NKT cells). After antigen challenge there is a transient increase in IL-4, whereas the release of IL-13 is sustained and correlates with the increase in the number of eosinophils (Kroegel et al., 1996).

- ***IL-6***: often works in concert with other cytokines and provides a link between innate and acquired immunity. IL-6 is found in increased amounts in induced sputum of asthmatic patients after mast cell activation. It may play a role in the expansion of Th2 and Th17 cells and therefore have a proinflammatory effect in asthma. IL-6 release C-reactive protein from the liver and can be involved in some systemic features of allergies such as muscle weakness and endothelial dysfunction.
- ***IL-9***: its overexpression in mice induces inflammation mediated by eosinophils, mucous hyperplasia, mastocytosis, airway hyperresponsiveness, and increased expression of other Th2 cytokines and IgE (Zhou et al., 2001). Many of the effects of IL-9 in mice (eosinophilic inflammation and mucous hypersecretion) are mediated via the release of IL-13, whereas its effect on mast cell expansion and B cells seem to be direct. IL-9 plays an important role in differentiation and proliferation of mast cells and interacts synergistically with SCF.
- ***Type I and type III IFNs***: Type 1 INFs (IFN-α and INF-β) and type III INFs (IFN-λ) play an important role in innate immunity against viral infections, but IFN-β and IFN-λ show reduced expression in epithelial cells of asthmatic patients and are associated with increased rhinovirus replication, which may predispose these patients to viral exacerbation of asthma. Low dose IFN-α seems to give marked benefit in patients with severe corticosteroid-resistant asthma but the mechanism is unknown.
- ***IL-12 and related cytokines***: IL-12 plays an important role in differentiating and activating Th1 cells and is produced by activated macrophages, dendrocyte, and airway epithelial cells. IL-12 induces T cells to release IFN-γ, which regulates the expression of IL-12Rβ2 and so maintains the differentiation of Th1 cells, whereas IL-4 suppresses IL-12Rβ2 expression and thus antagonizes Th1 cell differentiation.

- ***IL-18***: Originally described as IFN-γ-releasing factor, IL-18 has a mechanism of action different from that of IL-12 and may enhance Th1 responses independently of IL-12. IL-18 and IL-12 appear to have synergistic effects on the induction of IFN-γ release and the inhibition of IL-4-dependent IgE production and allergic hyperresponsiveness.
- ***IL-27***: is a member of the IL-12 family that promotes Th1 cell differentiation through a STAT1-dependent mechanism independently of IL-12. It is produced by activated antigen-presenting cells and enhances Th1 function by downregulating GATA3 expression and upregulating T-bet expression, thereby favouring the production of Th1-type cytokines, which then act to further inhibit GATA3 expression.
- ***IL-17***: IL-17A and the closely related IL-17F are linked to neutrophil-mediated inflammation by induction of the release of the of the neutrophil chemoattractant CXCL1 and CXCL8 from epithelial cells and smooth muscle cells and thereby may play a role in the neutrophilic inflammation. IL-17 also increases the expression of mucin-encoding genes (*MUC5AC* and *MUC5B*) in human epithelial cells. However, the functional role of IL-17 in asthma is unclear, as it seems to be involved in allergic sensitization of animal models but to inhibit eosinophilic inflammation in sensitized animals (Schmidt-Weber, 2007).
- ***IL-25***: part of the IL-17 superfamily (aka IL-17E) and produced by Th2 cells, mast cells, and epithelial cells. Induces the expression of IL-4, IL-5, and IL-13 leading to an inflammatory condition characterized by the presence of eosinophils, increased IgE production and allergic hyperresponsiveness. It also enhances Th2 cytokine secretion from human Th2 cells.
- ***TSLP***: Thymic stromal lymphopoietin is a cytokine belonging to the IL-7 family that shows a marked increase in expression in the epithelium and mast cells of allergic patients. It is released by epithelial cells, and its synergistic interaction with IL-1β and TNF-α results in the release of Th2 cytokines from mast cells independently of T cells. TSLP also play a key role in programming tissue dendritic cells to release the chemoattractants CCL17 and CCL22 and thus is

important in recruiting Th2 cells to the inflamed tissue. TSLP may also directly stimulate Th2 cytokine release from $CD4^+$ T cells.

- ***TGF-β***: family of pleiotropic cytokines that play several roles in allergy. TGF-β is a multifunctional growth factor that induces the proliferation of fibroblasts and smooth muscle cells, deposition of ECM, and epithelial repair. It also has immunoregulatory effects that are largely mediated by through Tregs through the induction of FOXP3, resulting in suppression of Th1 and Th2 T cells. There is increased expression of TGF-β1, particularly in eosinophils and this has been associated with subepithelial fibrosis. Increased expression of TGF-β2 has also been reported in patients with severe asthma. Many of the fibrotic effects of TGF-β are mediated via increased secretion of connective tissue growth factor (CTGF).
- ***EGF***: EGF activated EGFR tyrosine kinases that are also activated by TGF-α and may play a critical role in regulating mucous secretion in individuals with asthma.
- ***VEGF***: plays an important role in regulating the growth of new vessels and vascular leakage in asthmatic airways. Increased expression of VEGF and VEGF receptors is correlated with increased vascularity in inflamed tissues. Blockage of the VEGF receptors induces apoptosis of endothelial cells and development of emphysema.

2.4 Chemokine expression in allergic inflammation

Chemokines are cellular products that mediate the recruitment of eosinophils, among other inflammatory cells. Chemokines constitute a family of chemotactic cytokines that regulate leukocyte chemotaxis. Most chemokines exert their effect through binding to specific receptors, such as the chemokine receptor CCR3. In fact, **CC chemokines** are chemotactic cytokines that can induce mediator secretion and chemotaxis of leukocytes (Taub, 1997). Leukocyte recruitment from the circulation into inflammatory tissues requires a series of soluble and cell-bound signals between the responding leukocyte and vascular endothelial barrier. Chemotactic factors are believed to be responsible for this selective adhesion and transmigration. A superfamily of small, solube, structurally-related molecules called "chemokines" have been identified and shown to selectively promote the rapid

adhesion and chemotaxis of a variety of leukocyte subtypes both *in vitro* and *in vivo*. Chemokines are produced by almost every cell type in the body in response to a number of inflammatory signals, in particular those which activate leukocyte-endothelial cell interactions. Through a coordinated series of signals generated within the tissue lesion, the local vascular endothelial barrier becomes primed and quite sticky, quickly altering the circulating leukocytes that can intervene in the inflammatory process. Responding leukocytes become rolling along this primed endothelium slowing their transit through the circulation. Upon interaction with a pro-adhesive molecule, leukocyte adhere tightly to and spread out along this endothelial cell layer. These primary adhesion events are prerequisite for the successful trafficking of circulating leukocytes into extravascular tissues. **Chemotaxis** is the process by which leukocytes are directed to site of inflammation under the influence of a concentration gradient of the soluble chemotactic molecules.

Box 2.1

Chemokine receptors are **G protein-coupled receptors**. Classically, one of the initial events in chemokine-receptor signalling is the physical association of heterotrimeric $\mathbf{G_i}$ proteins to the receptor, resulting in the inhibition of adenyl cyclase activity and intracellular calcium mobilization. Consistent with G_i association, the majority of chemokine responses are inhibited by treatment with the pertussis toxin.

Nervertheless, in some circumstances, the pertussis toxin cannot block completely chemokine-induced responses, owing to chemokine-receptor association to G proteins other than G_i, for example, $\mathbf{G_{q/11}}$ or $\mathbf{G_{16}}$ (Viola et al., 2006).

Thus, depending on their **coupling** to different G proteins, chemokine receptors can initiate distinct signal-transduction pathways and exert several biological functions. Therefore, one key question is what specifies G protein-coupling to a chemokine receptor or, in general, to any GPCR.

An intriguing possibility is that GPCRs can adopt **various conformations** at the cell membrane, exposing distinct residues responsible for ligand recognition and G protein-coupling. According to this model, by binding to different receptor sub-states, different agonists might direct trafficking of the GPCR stimulus to different G proteins.

Another factor potentially influencing chemokine receptor conformations and signalling is their **homo**- and/or **hetero-dimerization** an event that changes the receptor pharmacology modifying not only its coupling and signalling but also the GPCR regulation and trafficking.

G protein-coupling might also be influenced by the lipid environment surrounding the receptor. Different G proteins might be targeted differently to distinct membrane microdomains by acylation of the α subunit and, thus, localization in **lipid rafts** could modulate the specificity of GPCRs and G_α coupling.

Upon encountering a chemotactic molecule, responding leukocytes begin to migrate directionally from regions of low ligand concentration toward the sites of chemoattractant production which tipically possess more substantial levels of soluble chemotactic factors. Many chemoattractans have been shown to bind to endothelial cells and various adhesive substrates within the tissue lesion facilitating the haptotactic migration of leukocytes while still maintaining a bound concentration gradient. Many of these chemoattractants not only induce leukocyte migration, but also facilitate leukocyte adhesion to endothelial cells and purified adhesive ligands. This enhanced adhesiveness occurs within seconds of stimulation and, as with migration, is highly concentration-dependent. The subtype of leukocyte that appear in inflammatory infiltrates can differ markedly depending on the identity of the inflammatory irritant as well as the duration of irritation. The chemotactic factors responsible for these differences are likely to be cell type-specific (selective) chemoattractants and/or chemoattractant receptor.

Chemokines appear also to play important roles in cellular activation and leukocyte effector functions. They have been found in the tissues of a variety of disease states characterized by distinct leukocytic infiltrates including rheumatoid arthritis, sepsis, atherosclerosis, asthma, psoriasis, ischemia/reperfusion injury, and a variety of pulmonary disease states (Table 2.2).

The role of chemokines in eosinophil migration and adhesion has currently received a great deal of attention due to their apparent role in asthma and allergy development. RANTES and MCP-3 have been shown to be potent chemoattractants for human eosinophils *in vitro*, as well as MIP-1α although with less efficacy. Interestingly, IL-5-primed eosinophils exhibited an even greater responsiveness to RANTES than non-primed cells. This RANTES effect on eosinophils was further supported by *in vivo* studies demonstrating the profound infiltration of eosinophils into the RANTES injection site in dog skin. Furthermore, within a murine airway inflammation model, MIP-1α and RANTES but not MCP-1 have been shown to play a role on eosinophil accumulation in pulmonary tissues post antigen challenge. Moreover, eotaxin is a C-C chemokine selective for eosinophils. Strangely, eotaxin mRNA also appears to be constitutively expressed in both guinea pig, but not human, intestines and lung and is upregulated in pulmonary allergic inflammation. Subsequently, the intradermal injection of human eotaxin, as a potent chemoattractant for

human eosinophils with no significant effect on human neutrophils or lymphocytes, into rhesus monkeys induced the selective accumulation of eosinophils in the skin of these animals (Gonzalo et al., 1996). These studies, along with the findings of substantial human RANTES and eotaxin in human eosinophil-containing nasal polyp tissue and murine eotaxin in the sensitized pulmonary tissue of murine allergic inflammation model, strongly support a role for these chemokines in allergic inflammation. Interestingly, there is evidence that some mediators as SCF, histamine and TNF-α, are able to potentiate the IgE-mediated chemokine release. Therefore, it is not surprising that treatment of mice with eotaxin (CCL11) produced a time-dependent accumulation of eosinophils that was significantly reduced if the animals were pre-treated with histamine H1 antagonists (Jose et al., 1994). Thus, the effect of mediators from mast cells on other cell populations has not been fully appreciated. One aspect of chemokine biology is the ability of several of them to cause mast cell activation. In fact, recent studies indicate that activation of mast cells with MCP-1 (via CCR2) can induce mediator release, including histamine and leukotriene release, further exacerbating airway hyperreactivity (Campbell et al., 1999). It is, therefore, to take in account that chemokines not only act as chemoattractants, but they elicit a more profound role potentiating the activation of the cells under their influence. Chemokine receptors are, in fact, highly versatile players fine-tuning immune responses.

Table 2.2

Pathological Disease State	Chemokines
Acute Glomerulonephritis	IL-8
Acute Respiratory Distress Syndrome	IL-8; MCP-1
Alcoholic Epatitis	IL-8
Angiogenesis	IL-8; GROα; GROβ; ENA-78; GCP-2; NAP-2
Angiostatic	PF-4; IP-10, mig, GROβ
Asthma	IL-8; ENA-78; MCP-1; MIP-α
Atherosclerosis	MIP-1α; MIP-1β; MCP-1; RANTES; IL-8
Bacteremia	IL-8
Bladder Inflammation	IL-8
Bronchial Carcinoma	IL-8
Cardiac Allograft	MIP-1α; MIP-1β; RANTES

(continued)

Contact Dermatitis	IL-8; MCP-1; IP-10
Cryptococcal Infections	MIP-1α; MCP-1; MIP-2
Cutaneous T Cell Lymphoma	IL-8; IP-10
Cystic Fibrosis Lavage fluids/tissues	IL-8; ENA-78; MCP-1
Endotoxemia and Sepsis	IL-8; MIP-1α; RANTES; MIP-2
Gastritis	IL-8
Gingivitis	IL-8
Gouty Arthritis	IL-8
HIV-infected Monocytes	MIP-1α; MIP-1β
Idiopathyc Pulmonary Fibrosis	IL-8; MCP-1
Inflammatory Bowel Syndrome	IL-8
Kawasaki Disease	IL-8
Lepromatous/Tuberculoid Leprosy Lesions	IP-10
Meningococcal Infections	IL-8
Mycobacterial Infections	IL-8
Nasal Polyps	IL-8; RANTES; eotaxin
Ocular Inflammation	IL-8
Osteoarthritic Synovial Fluids	MIP-1β
Palmoplantar Pustulosis	IL-8
Pancreatitis	IL-8
Peritonitis	Elevated plasma IL-8
Pertussis	IL-8
Pleural Emphysema	IL-8
Post-major Surgery	Elevated plasma IL-8
Pregnancy, Parturition	IL-8
Psoriatic Scale Extracts	IL-8; GROα; MCP-1; IP-10
Pulmonary Reperfusion Injury	IL-8; MCP-1
Pulmonary Fibrosis	IL-8
Red Cell Incompatibility	IL-8
Relapsing Fever	IL-8
Renal Ischemia	GROα; MCP-1
Rejection of Lung Transplant	IL-8; MCP-1
Rheumatoid Synovial Fluids	IL-8; ENA-78; MCP-1; MIP-1α; RANTES
Sepsis, Endotoxemia	IL-8; MCP-1
S. Mansoni-induced Granulomas	MIP-1α; MCP-1; RANTES
Sarcoidosis	IL-8; MCP-1
β-thalassemia	IL-8

(continued)

Transplantation	IL-8; MCP-1
Ulcerative Colitis	IL-8
Uremia	IL-8
Uveoretinitis	IL-8; IP-10; MIP-1α; MIP-1β; RANTES; MCP-1
Wound Healing	IL-8; GROα; PF-4; MCP-1; MIP-1α

- **CCRs**

CCL2 (MCP-1) activates CCR2 on monocytes and T cells. CCL2 is a potent chemoattractant or monocytes and may therefore be involved in the accumulation of macrophages in the inflamed tissue.

CCR3 is expressed predominantly on eosinophils and mediates the chemotactic response to several chemokines, including CCL11, CCL24 (aka eotaxin-2), CCL26 (aka eotaxin-3), CCL13 (aka MCP-4), and CCL5 (aka RANTES), all of which show increased expression in allergic individuals. Small molecule antagonists of CCR3 are effective in inhibiting pulmonary eosinophilic inflammation in mice exposed to CCR3 and may also be important in the differentiation of eosinophils and their release from bone marrow (Ravensberg et al., 2005). Further, CCR3 is also expressed on Th2 cells and mast cells in humans, although most attention has focused on CCL11, CCL24 and CCL26 may play a role in prolonged eosinophilia after allergen exposure (Bocchino et al., 2002).

CCR4 is selectively expressed on Th2 cells and activated by CCL22 and CCL17, which are released from airway epithelial cells and dendritic cells in allergic tissues and are further increased after antigen challenge in association with increased Th2 cytokines. CCL8 is also expressed on Th2 cells but neither it nor its ligand CCL1 (aka I-309) are increased in asthmatic airways.

CCR5 is activated by CCL3 (aka MIP-1α), CCL4 (aka MIP-1β), and CCL5, all of which are elevated in the tissues of allergic individuals. CCR5 is selectively expressed on Th1 and Tc1 cells and contributes to the recruitment of these cells into the tissues of patients with allergy. CCL5 also shows increased increased expression in epithelial and smooth muscle cells.

- **CXCRs**

  CXCL8 activates CXCR1, which is almost specific for this chemokine, and CXCR2, which is also activated by several related CXC chemokines, including CXCL1 (aka GRO-α) and CXCL5 (aka ENA-78). CXCL1 and CXCL8 levels are markedly increased in allergic patients and correlate with the increased proportion of neutrophils. CXCL8 is further increased during exacerbations and CXCL5 shows a markedly increased expression during this process. Small molecule inhibitors of CXCR2 block the chemotactic response of neutrophils and monocytes to CXCL8 and CXCL1 in animals *in vivo*.

  CXCR3 is expressed by T cells and is activated by CXCL9, CXCL10 and CXCL11, all of which are induced by IFN-γ. Since Th1 and Tc1 cells produce IFN-γ, this provides a potential amplification loop to perpetuate T cell accumulation in tissues. CXCR3 may also play a role in the recruitment of T cells after antigen challenge. On the other hand, CXCR3 ligands antagonize CCR3, suggesting that they might suppress eosinophilic inflammation.

  CXCR4 is preferentially expressed on Th2 cells and is activated by CXCL12 (aka SDF-1α). A small molecule inhibitor of CXCR4 (AMD3100) reduces inflammation in sensitized mice, and these effects are associated with a reduction in Th2 cytokines. CCL12 may also be involved in mobilization of eosinophil progenitor cells from the bone marrow as eosinophils express CXCR4.

- **CX3CR1**

  CX3CL1 (aka fractalkine), the sole member of a third family of CX3C chemokines, is produced by epithelial cells after stimulation with TNF-α, IL-1β, and IFN-γ. CX3CL1 is chemotactic for monocytes, T cells and mast cells and also functions as an adhesion molecule in its membrane-bound form (El Shazly et al., 2006).

2.5 Antinflammatory cytokines

Although most cytokines increase or orchestrate the inflammatory process, some cytokines have inhibitory or anti-inflammatory effects. For example, IL-12 through the release of IFN-γ from Th1 cells, can suppress Th2 cytokine release and allergic inflammation. TGF-β has potentially immunomodulatory effects through inhibition of $CD4^+$ T cells, but does not have therapeutic potential in view of its profibrotic actions. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of many inflammatory proteins, including several cytokines (such as TNF-α, GM-CSF, IL-5, and several chemokines) that are overexpressed in allergies, and also inhibits antigen presentation. There is a reduction in *IL10* transcription and secretion from macrophages in individuals who suffer from allergy (John et al., 1998). IL-10 is produced by a subset of Tregs and by macrophages. Specific allergen immunotherapy results in increased production of IL-10-producing Tregs. IL-10 might also be beneficial in the chronic obstructive pulmonary disease as it not only inhibits the production of TNF-α and chemokines, but also MMP-9, which may be involved in the distruction of lung elastin (Takanashi et al., 1999).

2.6 Eosinophilia

A marked accumulation of eosinophils occurs in several important disorders, such as **allergic diseases**, **parasitic infections**, and **cancer**. The level of eosinophils in the body in normally tightly regulated. In normal subjects, eosinophils account for only a small minority of peripheral blood leukocytes, and their presence in tissues is primarily limited to the gastrointestinal mucosa. In certain disease states, however, eosinophils can selectively accumulate in the peripheral blood or any tissue in the body. Any perturbation that results in **eosinophilia**, defined here as abnormal accumulation of eosinophils in blood or tissue, can have profound clinical effects. Eosinophilia can be harmful because of the proinflammatory effects of eosinophils, or it may be helpful, because of the antiparasitic effects of these cells. Eosinophilia occurs in a variety of disorders (Table 2.3) and is arbitrarily classified as mild, moderate or severe depending on the numer of eosinophils present per blood cubic millimiter. The most common cause of eosinophilia worldwide is helminthic infections, and the most common cause in industrialized nations is **atopic disease**.

Table 2.3

Disease associated with eosinophilia

Type of Disease	*Eosinophilia*		*Possible Causes*
	Peripheral Blood	*Tissue*	
Infectious	Present	Present or Absent	Infections with especially invasive helminths
Respiratory	Present or Absent	Present	Eosinophilic pneumonitis, asthma
Gastrointestinal	Present or Absent	Present	Inflammatory bowel disease, eosinophilic gastroenteritis, allergic colitis
Allergic	Present or Absent	Present	Allergic rhinoconjunctivitis, asthma, eczema
Systemic	Present	Present	Idiopathic hypereosinophilic syndrome, vasculitis
Iatrogenic	Present	Present or Absent	Drug reaction, cytokine infusion
Malignant	Present or Absent	Present or Absent	Lymphona, colonic carcinoma

Eosinophils are produced in the bone marrow from pluripotential stem cells. The latter cells differentiate into hybrid precursors with properties of basophils and eosinophils and then into a separate eosinophil lineage. Three cytokines – **IL-3**, **IL-5** and **GM-CSF** – are particularly important in regulating the development of eosinophils. Of the three cytokines, IL-5 (also known as eosinophil differentiating factor) is the most specific for the eosinophil lineage and is responsible for selective differentiation of eosinophils (Sanderson, 1992). IL-5 also stimulates the release of eosinophils from bone marrow into the peripheral circulation. The migration of eosinophils from the circulation into tissues involves a stepwise interaction between eosinophils and endothelial cells. The steps are mediated by **adhesion molecules** on endothelial cells and counter-ligands on eosinophils and are followed by the passage of eosinophils through endothelial cells.

Tissue eosinophilia is a hallmark of atopic diseases and eosinophils are a major effector cell in these disorders. After IgE-triggered activation, mast cells may promote inflammation with eosinophils by producing pro-inflammatory mediators (**TNF-α**, **IL-1**) and eosinophil directed cytokines (e.g. **IL-4**, **IL-5**). These substance, in turn, induce

chemokines that attract eosinophils. Moreover, helper T lymphocytes are essential for the late-phase response, because they produce three cytokines that promote allergic responses: **IL-4** and **IL-13**, both of which regulate **IgE** and **VCAM-1** production, and **IL-5**. The helper cells that orchestrate this type of response are Th2 cells. Antigen presenting cells not only activate Th2 cells, but also secrete pro-inflammatory mediators that induce resident cells (e.g. epithelial cells) to produce the chemokines that attract eosinophils.

Once eosinophils are localized at the inflammatory site, they produce unique toxic inflammatory mediators, which are stored in granules and synthesized after cellular activation. The granules contain a crystalloid core composed of a major basic protein and a matrix composed of eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidise (Fig. 2.3).

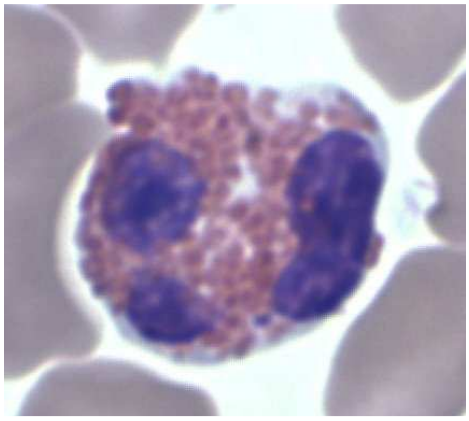

Figure 2.3
Activated eosinophil with evident secretory granules.

The migration of eosinophils from the circulation into tissues involves a stepwise interaction between eosinophils and endothelial cells. The steps are mediated by adhesion molecules on endothelial cells and counter-ligands on eosinophils and are followed by the passage of eosinophils through endothelial cells (see Chapter 1). Although the different types of leukocytes migrate into tissues in a similar way, their migration is mediated by different molecules. Eosinophils initially adhere to the endothelium by means of three selectins and their corresponding ligands. After cellular activation following exposure to chemoattractants, eosinophils adhere firmly to the endothelium through adhesion molecules of the integrin family. These include the CD18 family (β_2 integrins) and very late antigen 4

(VLA-4) molecules (β_1 integrins). The β_2 integrins interact with the intercellular adhesion molecule 1 (ICAM-1) on endothelial cells, whereas the β_1 integrins interact with vascular cell adhesion molecule 1 (VCAM-1). Although the CD18-ICAM-1 pathway is used by all leukocytes, the VLA-4-VCAM-1 pathway is used by eosinophils and mononuclear cells but not by neutrophils. ICAM-1 is induced by a variety of proinflammatory mediators, such as IL-1 and TNF-α, whereas VCAM-1 is induced primarily by IL-4. Resting eosinophils normally express β_1 and β_2 integrins, but the level of expression of these adhesion molecules and their affinity for their appropriate endothelial receptors is increased by chemoattractants.

Eosinophils, unlike neutrophils, can survive in tissues for prolonged periods (perhaps weeks), depending on the cytokines in the microenvironment. Only eosinophils and basophils have receptors for IL-3, IL-5, and GM-CSF present on both the precursor cells in bone marrow and the circulating cells. The lifespan of tissue eosinophils is not known, but IL-3, IL-5 and GM-CSF inhibit eosinophil apoptosis for at least 12 to 14 days *in vitro* and in explants of allergic sinus tissues. In contrast, eosinophils survive for less than 48 hours in the absence of these cytokines. Tissue eosinophils can also regulate their own survival through an autocrine pathway.

Moderate to severe eosinophilia occurs as a pathophysiologic response to infections with helminthic parasites. Eosinophilia induced by parasitic infection is dependent on IL-5 produced by Th2 lymphocytes. Eosinophils participate in the immune response against helminthic parasites by discharging their cytotoxic granular content onto the parasites, which kills them. However, depletion of eosinophils in mice with antibodies to IL-5 does not always increase their susceptibility to helminthic infections.

Since eosinophilia is a hallmark of atopic diseases and eosinophils are a major effector cells in these disorders, allergic diseases serve as a prototype for understanding the pathogenesis and consequences of eosinophilia. Patients with eosinophilia of any magnitude who have end-organ involvement should be treated with the goal of reducing eosinophil counts or blocking the effect of eosinophil products. Glucocorticoids, the most effective agents for reducing eosinophilia, suppress the transcription of a series of genes for inflammatory mediators, including the genes for IL-3, IL-4, IL-5, GM-CSF, and various chemokines. In addition, glucocorticoids inhibit the cytokine-dependent survival of eosinophils. In most patients, treatment with systemic or topical glucocorticoids causes a

rapid reduction in eosinophils, but a few patients have a resistance to glucocorticoids, with persistent eosinophilia despite high doses. The mechanism of resistance to glucocorticoids is unclear, but a reduced level of glucocorticoid receptors and alterations in activator protein 1, a transcription factor, are at least in part responsible in some patients. Patients with glucocorticoid resistance sometimes require other therapy such as myelosuppressive drugs (hydroxyurea or vincristine) or INF-α. The latest appears to be especially promising because it inhibits the degranulation and effector functions of eosinophils. Cyclophyllins (e.g. cyclosporine) have also been used because they block the transcription of numerous eosinophil-active cytokines (e.g. IL-5 and GM-CSF). Drugs that interfere with eosinophil chemotactic signals include recently approved leukotriene antagonists and inhibitors. The 5-lypoxygenase inhibitors (e.g. zileuton) block the rate-limiting step in leukotriene synthesis and inhibit the generation of the chemoattractant leukotriene B_4 and the sulfidopeptide leukotrienes, leukotrienes C_4, D_4 and E_4. These drugrs therefore decrease airway infiltration by eosinophils during the late-phase response. Drugs (e.g. zafirlukast) that block the receptor for leukotirene D_4, which is also a receptor for leukotriene E_4 and C_4, prevent the muscle contraction and increased vascular permeability mediated by eosinophil-derived leukotrienes. These drugs have been found to decrease exercise-induced bronchoconstriction and include base-line lung obstruction in patients with asthma. Some of the third generation anti-histamines (e.g. cetirizine) inhibit the vacuolization and accumulation of eosinophils after an allergen challenge and directly inhibit eosinophils *in vitro*. Cromolyn and nedocromyl inhibit the effector function of eosinophils, such as antibody-dependent cellular cytotoxicity. Phosphodiestare inhibitors raise intracellular cyclic AMP concentrations in eosinophils, and this in turn inhibits intracellular signalling, leading to decreased activation of eosinophils. The identification of molecules that specifically regulate the function or production of eosinophils offers new therapeutic strategies. Antibodies against IL-5 are especially promising because they have been effective in animal with allergic airway disease. Another molecular target interrupts the adhesion of eosinophils to the endothelium through the interaction of CD18 with ICAM-1 or VLA-4 with VCAM-1. In addition, phosphodiesterase inhibitors that are specific for leukocyte isoenzyme type IV are being developed.

2.7 Ocular allergy

In this thesis will be treated, more in depth, the aspects of ocular allergy, because of its very wide diffusion among the population and considering it as the model of allergy taken as an example.

The slow development of ocular and nasal allergic symptoms after exposure to plant products was originally noted by Blacklay in 1873 (see reference). Ocular allergy results from the exposure of the conjunctiva to an allergen and include a spectrum of clinical disorders that involve **different levels of immune activity** at the conjunctival or corneal interface. Each of these clinical entities is caused by an IgE-mediated sensitization to an antigen and the activation of **mast cells** and **eosinophils**. In the more chronic entities there is also an interaction of the allergen with T cells and a T helper 2 patter of cytokine release. The spectrum of ocular allergy is variable and notably *seasonal allergic conjunctivitis* (SAC) and *perennial allergic conjunctivitis* (PAC) are the most basic and least severe of the allergic inflammatory disorders. The seasonal incidence of SAC is closely linked with cycle of release of airborne plant-derived allergens, and specific IgE against grass and tree pollens has been found in the tears of patients with this condition (Allansmith et al., 1970). In SAC and PAC a type I hypersensitivity reaction appears to be responsible for the signs and symptoms. In SAC there are minimal pathological changes and only the early signs of cellular activation at the molecular level occur. In PAC the inflammatory markers become more pronounced with the increased duration of allergenic stimulation. In the more chronic forms of allergic conjunctivitis, namely *vernal keratoconjunctivitis* (VKC) in childhood and *atopic keratoconjunctivitis* (AKC) in adulthood, there is a mixed type I and type IV hypersensitivity reaction. There is a persistent state of mast cell, eosinophil and lymphocyte activation and this may result in the serious corneal complications that can occur with these diseases. *Giant papillary conjunctivitis* (GPC) that manifest in conctact lens wearers also tends to be chronic with a mixed hypersensitivity reaction but has no corneal significant involvement.

Conjunctival allergen challenge has been undertaken since 1932 (Peshkin, 1932). Conjunctival provocation tests (CPTs), which consist of instilling an offending pollen into the conjunctival sac, also produce the typical symptoms of hay fever conjunctivitis (Stegman et al., 1975; Friedlaender, 2003) and was the original method for evaluating allergic responses. Ocular challenge is used as a pharmacologic model for the evaluation of new

antiallergic medications and immunotherapy (Abelson et al., 1988). The positivity may be assessed by a **sign and symptom scoring system** that include subjective and objective signs such as conjunctival erythema, chemosis, tearing and pruritus. CPTs have also been shown to have a relatively good reproducibility in both eyes (Aichane et al., 1993). A CPT of ocular mast cells by way of opioid receptors has shown that 80% of normal patients reflect mast cell activation by detection of the release of histamine (7 versus 18 ng/L) and prostaglandin D_2 (0 versus 273 ng/L). The release of these mediators can be blocked by pretreating patients with cromolyn (Campbell et al., 1993).

It is estimated that ocular/conjunctival symtomatology is present in 40/60% of allergic individuals, and occasionally the ocular component may be the most prominent and disabling feature of their allergy. In a large study of 5000 allergic children, 32% had ocular disease as the single manifestation of their allergy (Marrache et al., 1978). Allergic conjunctivitis is encountered on a daily basis in general practice and its diagnosis and management are the responsibility not only of ophtalmologists, but also general practitioners, paediatricians, allergists and pharmacists. Conjunctivitis can vary from being seasonal, where some individuals are affected for only a few weeks to months, to perennial with symptoms that last throughout the year.

The eye presents unique immunological features since it is relatively isolated from the systemic immune system, and has an efficient local protection system in the structure of the external eye. The eye is constantly exposed to foreign substances, and the ocular tissuea have an array of immune cells to interact with these offending antigens. This represents an ideal site for hypersensitivity reactions because of the large number of mast cells in the conjunctiva and the potential for local synthesis of IgE (Bielory et al., 2000). Ocular allergy is not confined to the conjunctiva, but affects the entire ocular surface including conjunctiva, lids, cornea, lacrimal gland and tear film.

Ocular allergic conditions represent a spectrum of diseases that affect the ocular surface, from the acute and self-limited mild form of seasonal allergic conjunctivitis to the chronic, severe, sight-threatening atopic keratoconjunctivitis. Two acute disorders, SAC and PAC, and three chronic diseases, VKC, AKC, and GPC are described (Ono and Abelson, 2005).

Physicians in all specialties frequently encounter various forms of allergic diseases of the eye that present as "red eyes" in their general practice. However, allergists or clinical

immunologists are trained to uniquely understand and manage atopic disorders, because the eye is rarely the only target for an immediate allergic-type response. However, ocular signs and symptoms may be the initial and most prominent features of the entire allergic response that patients present to their physician.

Allergens and other ocular irritants are easily deposited directly on the surface of the eye. Many agents that are systemically absorbed also can be concentrated and secreted in tears, causing allergic conjunctivitis or an irritant form of conjunctivitis. Allergic diseases affecting the eyes constitute a heterogeneous group of clinicopathologic conditions with a vast array of clinical manifestations that range from single intermittent symptoms of itching, tearing, or redness, to severe sight-threatening corneal impairment. These conditions may be considered as part of an immunologic spectrum that affect the anterior surface of the eye with a variety of disorders that may overlap and may include seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC), and giant papillary conjunctivitis (GPC) (Hodges et al., 2007). In addition, tear film dysfunction (otherwise known as dry eye syndrome, DES) commonly complicates ocular allergy and its treatments, especially as the age of the patients increases and is included to reflect the spectrum of hypersensitivity responses from immunoglobulin E (IgE) mast cell hypersensitivity conditions to a mixture of mast cell and cell mediated disorders that involve different mechanisms, cytokines and cellular populations. For example, mast cell degranulation and histamine release play key roles with limited eosinophil involvement in the common forms of SAC and PAC, whereas AKC and VKC are characterized by more chronic inflammatory cellular infiltrates primarily composed of Th2 lymphocytes with an interplay with activated mast cells and eosinophils, and tear film dysfunction, a Th1 mediated disorder, commonly can overlap ocular allergy syndromes. Mast cell mediators such as histamine, tryptase, leukotrienes, and prostaglandins in the tear fluid have diverse and overlapping biologic effects, all of which contribute to the characteristic itching, redness, watering, and mucous discharge associated with both acute and chronic allergic eye disease. Histamine alone has been shown to be involved in the regulation of vascular permeability, smooth muscle contraction, mucus secretion, inflammatory cell migration, cellular activation, and modulation of T cell function. Histamine is a principal mediator involved in ocular allergy and inflammation. Large amounts of histamine are present in several mammalian ocular structures, including the

retina, choroid and optic nerve. Histamine receptors have been found on the conjunctiva, cornea, and ophthalmic arteries. Two separate histamine receptors, H1 and H2, have been identified in the conjunctiva. Histamine in the conjunctiva of allergic conjunctivitis patients can reach values greater than 100 ng/mL as compared with values of 5-15 ng/mL in controlled patients. Histamine can induce changes in the eye similar to those seen in other parts of the body. These changes include capillary dilation leading to conjunctival redness, increased vascular permeability leading to chemosis, and smooth muscle contraction.

Allergic conjunctivitis is caused by the direct exposure of the ocular mucosal surfaces to environmental allergens (such as pollens from trees, grasses and weeds) interacting with the pollen-specific IgE found on the mast cells of the eye. Of all the various pollens, ragweed has been identified as the most common cause of conjunctivo-rhinitis in the United States, approaching 75% of all cases of "hay fever" with variations of prevalence in different age groups in various regions of the world. Common conjunctival symptoms include itching, tearing, and perhaps burning. Clinical signs include include a milky or pale pink conjunctiva with vascular congestion that may progress to conjunctival swelling (chemosis). A white exudate may form during the acute state and become stringy in the chronic form. While ocular signs are typically mild, the conjunctiva frequently takes on a pale, boggy appearance that often evolves into diffuse areas of papillae (small vascularised nodules). These papillae tend to be more prominent on the superior palpebral conjunctiva. Occasionally, dark circles beneath the eyes (allergic shiners) are present as a result of localized venous congestion (Bielory, 2005). Symptoms usually consist of low-grade ocular and peri-ocular itching (pruritus), tearing (epiphora), burning, stinging, photophobia, and watery discharge. Redness and itching seem to be the most consistent symptoms (see table 2.4). The symptom of itching is strikingly characteristic of allergic conjunctivitis. In allergic patients, it is unusual to have conjunctival symptoms without nasal symptoms (Singh et al., 2007). The nasal mucosa is expected to react in the same fashion as the conjunctival mucosa.

In the **early-phase reaction**, an allergen binds to allergen-specific IgE on the mast cell. The mast cell Fc receptors are cross-linked by the allergens, sending signals via the cell membrane into the cytoplasm, activating the mast cells and resulting in the release of allergic mediators (Trocme et al., 2002). This early-phase reaction is immediate. More severe allergic reactions may demonstrate a **late-phase reaction**. These may be either sustained early-phase reactions or more discrete second peaks of response. The second-peak late

phase conjunctival reaction occurs from 2 to 9 hours after antigen exposure (Bonini et al., 1990).

Table 2.4

Signs	AC	VKC	AKC	GPC	DCS
Predominant Cell Types	Mast Cells, Eosinophils	Lymphocytes, Eosinophils	Lymphocytes, Eosinophils	Lymphocytes, Eosinophils	Lymphocytes
Chemosis	+	±	±	±	-
Discharge	Clear mucoid	Stringy mucoid	Stringy mucoid	Clear white mucoid	±
Lid Involvement	-	+	+	-	++
Itching	+	++	++	++	+
Burning	-	-	-	-	-
Gritty Sensation	±	±	±	+	-
Seasonal Variation	+	+	±	±	-

This occurs at a cellular level and does not correlate with a separate clinical late-phase response of allergic conjunctivitis. Usually, about 4-6 hours after allergen exposure, an influx occurs into conjunctival tissue of non-specific cells of the inflammatory response, including neutrophils, basophils, eosinophils and T lymphocytes (Trocme et al., 1988). It has been proposed (Gleich, 1982; Iliopoulos et al., 1990) that the late response explains the transition of the of the early acute inflammatory response into days or weeks of disease. The most striking piece of evidence in support of the clinical late phase response is the presence of two separate peaks of histamine in tears at 20 minutes and 6 hours after challenge, which have been found by several authors after ocular and skin challenge (Bacon et al., 2000; Abelson et al., 1990; Helleboid et al., 1991; Charlesworth et al., 1989). The late phase response represents, with its increase in inflammatory cells and adhesion molecules, the transition between a transient type I response and clinical disease, with a marked increase in tissue reactivity to challenge, contributing to the symptoms seen in asthma, atopic dermatitis, and allergic type disease. A conjunctival late phase response has been

described (Bonini et al., 1990; Bonini et al., 1988; Leonardi et al., 1990; Trocme et al., 1988). In the guinea pig model used by Leonardi and collegues (1990), the late phase response manifested in several forms, including a classic biphasic response (33%), a multiphasic response (25%), and a single prolonged response (41%). The histologic evaluation of the conjunctiva revealed the typical influx of non-specific cells of the inflammatory response, including neutrophils, basophils, and eosinophils. Tears collected from timed periods over the course of 6 hours after allergen challenge or CPT reflected the ability of mediators released during the late phase response to reproduce the influx of cells commonly seen after ocular allergen testing; however, clinical symptoms were not reproduced in this single patient. Direct application of leukotriene B_4 has been found to increase the number of eosinophils and neutrophils in rat conjunctiva (Trocme et al., 1988). Ocular challenge with PAF also resulted in an inflammatory response. The *substantia propria* was the primary site of vascular changes that included endothelial cell swelling, capillary dilation, and edema. Ocular challenge with histamine revealed vascular permeability, but not an inflammatory response, as measured by the epithelial expression of ICAM-1 (Ciprandi et al., 1993).

The eosinophils and T helper type 2 (Th2) lymphocytes and cytokines are primarily responsible for the late-phase reaction. The infiltration of eosinophils is paramount to the allergic response. Eosinophils are not ordinarily found in conjunctival scrapings from non-allergic individuals. The presence of even one eosinophil or esoinophil granule is considerable evidence in favour of a diagnosis of allergic conjunctivitis (Friedlaender et al., 1984). However, the absence of eosinophils should not rule out a diagnosis of allergy. Eosinophils are often present in the deeper layers of the conjunctiva and may be absent or undetectable in the upper layers. The frequency of eosinophils in the conjunctival scrapings from patients who have allergic conjunctivitis may vary from 20 to 80% (Abelson et al., 1983) depending on the patient population, the chronicity of the allergic condition, and the persistence of the examiner. Corneal infiltrates may occasionally be seen in severe allergic patients and tend to be nummular, subepithelial, and peripheral. Chemotactic factors, released during mast-cell degranulation, aid in eosinophil attraction and activation. The eosinophils release toxic proteins such as eosinophil major basic protein (MBP) and eosinophil cationic protein (ECP). These proteins have profound cytotoxic effects and stimulate further degranulation of mast cells, initiating a cascade of allergic events (Venarske et al., 2003).

The Th2 lymphocytes commonly release cytokines (e.g. IL-4, IL-5, IL-6 and IL-13) during the late-phase allergic inflammatory response and a recent study now indicates that mast cells may also be a source of Th2-type cytokines (Anderson et al., 2001). This study also shows that the phenotype of the conjunctival mast cell may further delineate which cytokines are released. For example, tryptase-positive mast cells secrete IL-5 and IL-6, whereas tryptase- and chymase-positive mast cells preferentially secrete IL-4 and IL-13. The tryptase- and chymase-positive mast cells tend to be the more prevalent phenotype in conjunctival tissue. The function of mast cells in SAC and PAC is clearly an important one and ongoing research continues to demonstrate their multifunctional role. The other factors that appear to play a crucial role in the inflammatory process of SAC and PAC include the above mentioned Th2-type cytokines, an increase in the ratio of Th1/Th2 cytokines, and an increased statement of adhesion molecules (Pesce et al., 2001).

The finding that after allergen challenge there is increased expression of E-selectin and ICAM-1 is of interest and would explain the recruitment of cells into the conjunctiva (Bacon et al., 2000). 6 hours after conjunctival challenge there is an increase in **E-selectin** (Bacon et al., 1998) and **ICAM-1** (Ciprandi et al., 1993) expression correlating with lymphocyte and granulocyte levels that would explain the increased infiltration of these cell types in the conjunctiva. **VCAM-1** induction is slower and has been shown to peak at 24 hours after conjunctival allergen challenge (Bacon et al., 2000). Increased E-selectin and ICAM-1 have been found at 6 hours, and increased VCAM-1 has been found at 24 hours after antigen challenge (Montefort et al., 1994; Bentley et al., 1993; Leung et al., 1991).

As our understanding of the underlying immunological mechanisms in allergic eye disease continues to grow future avenues for pharmacological targeting of different categories of allergic eye disease will be available. Appropriate treatment may be based on the specific immunopathology, and directed at the activated cell types primarily responsible for the disease process.

2.8 Treatment of ocular allergy

Mast cells are important effector cells in the conjunctiva. Several drugs used clinically for treating SAC (*azelastine, epinastine, ketotifen, olopatadine, levocabastine*) combine anti-histamine, mast cell stabilization and anti-inflammatory roles, do not penetrate the blood-brain barrier and exert effects on other cells. *Nedocromil* is a gold standard mast cell stabilizer

that has been used for many years in the therapy of SAC. Mast cells also synthesize and release cytokines of the T helper type 2 profile (IL-4, IL-5, IL-6, IL-8, IL-13, and TNF-α). These cytokines promote T cell growth and differentiation, class switching of B cells to produce IgE, adhesion molecule expression, inflammatory cell infiltration, and mediator release from activated cells. Release of histamine by mast cells also causes the pathognomonic itching associated with allergic eye disease and can result in chronic eye rubbing and further mechanical degranulation of mast cells (Siddique et al., 2007; Greiner et al., 1985; Raizman et al., 2000).

Some cases of ocular allergy cannot be explained by mast cell degranulation alone, especially in late-phase responses. This finding suggests that degranulation and mediator release of other cells such as basophils, eosinophils and neutrophils may trigger and perpetuate inflammatory responses observed in allergic conjunctivitis.

Structural components of the ocular surface also participate in the mechanisms of allergic inflammation. Fibroblasts have been shown to promote the formation of giant papillae as well as express adhesion molecules, extracellular matrix proteins, cytokines and chemokines. Mediators present in tear samples from ocular allergy patients have been recently found to upregulate eosinophil adhesion to human conjunctival epithelial cells *ex vivo*, providing further insight into which adhesion factors (e.g. β_2 integrins) may be responsible for eosinophilic infiltration to the conjunctiva (Cook et al., 2006).

- *Antihistamines*

  Antihistamines may be systemically to relieve allergic symptoms. These drugs may only partially relieve ocular symptoms, and patients often complain of side effects such as drowsiness and dryness of the eyes, nose and mouth. Antihistamine such as *antazoline* and *pheniramine* are available as eye drops and are usually combined with a topical vasoconstrictor such as naphazoline hydrochloride. These antihistamine-vasoconstrictor eye drops are useful in treating mild allergic conjunctivitis. They also relieve itching in most patients. Absorption of topical *levocabastine* is evident within 15 minutes after administration and persists for at least 4 hours, indicating high-speed and long-duration binding affinity of levocabastine for histamine H_1 receptors. In addition, it has been demonstrated that levocabastine

significantly downregulates ICAM-1 expression on blood vessel endothelial cells in the conjunctiva, which inhibits a late-phase response including recruitment of eosinophils and neutrophils (Bielory et al., 2005; Buscaglia et al., 1996).

- *Mast cell stabilizers*

Mast cell stabilizers have been a useful addition to the other drugs available for treating allergic conjunctivitis. Often, patients notice improvement within 24 to 48 hours. Mast cell stabilizers are most useful for relief of mild and moderate symptoms of allergic conjunctivitis. More severe cases may require the addition of topical corticosteroids. An extra-benefit of mast cell stabilizers is the relief of nasal symtoms caused by the drainage of tear fluid into the nasal passage. *Nedocromil sodium* is an example available in Europe and the Us.

Lodoxamine tromethamine 0.1% (Alomide) is a mast cell stabilizer that prevents the release of histamine and leukotrienes (Caldwell et al., 1992). Lodoxamine inhibits mediator release from mast cells, presumably by inhibiting calcium influx, thereby indirectly inhibiting increased vascular permeability. It is 2500 times more potent than *cromolyn* in inhibiting mediator release from mast cells; however, it appears to be roughly equivalent to cromolyn in controlling the symptoms of allergic conjunctivitis, vernal conjunctivitis, and giant papillary conjunctivitis.

Ketorolac tromethamine (Acular) belongs to the nonsteroidal anti-inflammatory drugs (NSAIDs) class and has been shown to relieve itching associated with allergic conjunctivitis. It also reduces levels of PGE_2 in tears. There may be some burning on instillation. It is unexpected that an NSAID would relieve itching, but research from Woodward and collegues (1996) suggested that some of the prostaglandins, particularly PGE_2 and PGI_2 , may be pruritogenic.

Olopatadine (Patanol, Pataday) inhibits mast cell degranulation and antagonizes histamine receptors to manage the itching, redness, chemosis, tearing, and lid swelling of the ocular allergic reaction. Its mast cell stabilizing ability has

been demonstrated *in vitro* (using human conjunctival mast cells) and *in vivo* (human clinical experience).

Ketotifen (Zaditor) is a benzacylcoheptathiophen derivative, approved for the temporary prevention of itching due to allergic conjunctivitis. It is a selective, non-competitive blocker of the H_1 histamine receptor. It inhibits inflammatory mediator release from mast cells, basophils and eosinophils. It inhibits chemotaxis and degranulation of eosinophils, type 1 hypersensitivity reactions, and leukotriene activity. In animal studies it decreases vascular permeability and leukocyte extravasation in rat and guinea pig models of anaphylaxis.

Nedocromil (Alocril) is a disodium salt of pyranoquinolone dicarboxylic acid approved for treatment of itching associated with allergic conjunctivitis. It inhibits histamine, LTC_4, and TNF-α. It decreases chemotaxis of neutrophils and eosinophils and renders them unresponsive to mediators. It blocks the expression of cell surface adhesion molecules involved in eosinophil chemotaxis and decreases vascular permeability induced by inflammation. It reduces itching and to a lesser extent redness associated with allergic conjunctivitis. It has an onset of action of 2 minutes after dosing and a duration of about 8 hours.

Pemirolast (Alamast) is a mast cell stabilizer with antihistamine properties. It is approved for the prevention of itching associated with allergic conjunctivitis. In SAC studies it decreased itching and, to a lesser extent, redness throughout the allergy season. It also decreased itching after conjunctival antigen challenge.

Azelastine (Optivar) is a phtalazinone derivative approved for the prevention or treatment of itching due to allergic conjunctivitis. It inhibits histamine release from allergen-stimulated mast cells and suppresses inflammation. It decreases expression of ICAM-1, reduces eosinophil chemotaxis, and inhibits PAF. It interfere with calcium influx in mast cells and inhibits the H_1 histamine receptor. It reduces itching and, to a lesser extent, redness in SAC, PAC, and after conjunctival antigen challenge.

Epinastine (Elestat) is a topically active, direct H_1 receptor antagonist and has affinity for the H_2, α_1, α_2, and 5-HT_2 receptors. It also inhibits histamine release from mast cells. Epinastine has a duration of action of at least 8 hours and it is administered twice a day. It is indicated for the prevention of itching associated with allergic conjunctivitis.

For a summary of the effects of these first two classes see Table 2.5.

Table 2.5

	IgE	*Epinastine*	*Olopatadine*	*Azelastine*	*Ketotifen*	*Nedocromil*
In vitro						
CBMC						
IL-5	-	↓	-	↓↓	↓↓	↓↓
IL-8	↑↑	↓↓	-	↓↓	-	-
IL-10	↑↑	↓	↓	-	-	-
TNF-α	↑↑	↓	↓	↓↓	↓	↓
TNF-β	↑↑	-	↓↓	↓↓	-	-
Histamine	↑↑	↓↓	↓	↓↓	↓↓	↓↓
Conjunctival Mast Cells						
IL-5	↑↑	↓	ND	ND	ND	ND
IL-6	-	↓	ND	ND	ND	ND
IL-8	↑	↓↓	ND	ND	ND	ND
TNF-α	↑	-	ND	ND	ND	ND
TNF-β	↑↑	-	ND	ND	ND	ND
Histamine	↑	↓↓	ND	ND	ND	ND
In vivo						
Edema	↑↑	↓	-	ND	ND	-
Tearing	↑↑	↓	-	ND	ND	↓
Redness	↑↑	↓	↓	ND	ND	↓
Neutrophils	↑↑	↓	-	ND	ND	-
Eosinophils	↑↑	↓	↓	ND	ND	↓

Note: CBMC = Human Cord Blood Stem-cell Derived

- *Corticosteroids*

Corticosteroids may be extremely effective in relieving symptoms of allergic rhinitis, but because the disease is a chronic, recurrent, benign condition, these drugs should be used only in extreme situations, commonly as a "burst" treatment for no more than 1 or 2 weeks. Topical steroids are associated with glaucoma, cataract formation, and infections of the cornea and conjunctiva. Any prolonged use (e.g. longer than 2 weeks) should

therefore be applied with the greatest caution, and the patient should preferably be monitored by an ophthalmologist. *Fluorometholone 0.1%* eye drops are often selected as a useful treatment of external ocular inflammation. This steroid is highly effective in allergic conjunctivitis. It appears that fluorometholone penetrates the cornea well, but is inactivated quickly in the anterior chamber. Thus, the complications of fluoromethone are rare. It may be that fluorometholone is inactivated before it has an opportunity to combine with trabecular meshwork or lens receptor. Thus, the incidence of glaucoma and cataract formation is expected to be lower than with prednisolone or dexamthasone. Two "modified" steroids have recently investigated for their efficacy in allergic conjunctivitis. *Rimexolone (Vexol)* is a derivative of prednisolone that is quickly inactivated in the anterior chamber. During a 4-week treatment period in patients who had uveitis, rimexolone caused an increase in intraocular pressure of 10 mmHg or more in 5% of patients, whereas prednisolone acetate 1% caused elevation in nearly 14% of patients. In a 6-week steroid-responder study, prednisolone 1% and dexamethasone 0.1% caused mean pressures to rise to 30 mmHg after 3 weeks. Rimexolone and fluorometholone caused mean pressures to rise to only 22 mmHg at 3 weeks and 24 mmHg at 6 weeks. Rimexolone has recently been approved for treatment of postcataract inflammation and for irititis.

Another modified corticosteroid that shows great promises is *loteprednol etabonate (Lotemax)*. It also seems to be highly effective in allergic conjunctivitis and is only rarely associated with a significant rise in intraocular pressure. A low-dose loteprednol etabonate *(Alrex)* has been approved for the relief of allergic conjunctivitis. Alrex is a useful treatment when mast cell stabilizers have been inadequate.

- *Other drugs*

Other antiallergic drugs are being investigated and show promising results in the treatment of allergic conjunctivitis, including *emedastine*, a selective blocker of the H_1 histamine receptor. *Cyclosporine*, a fungal antimetabolite that can be used as an anti-inflammatory drug, inhibits IL-2 activation of

lymphocytes. It is used systemically to prevent rejection of various solid-tissue transplants. It has been used as an eye drop in a variety of conditions including dry eye vernal keratoconjunctivitis and in high-risk corneal transplant patients. Cyclosporine appears to interfere with antigen processing and presentation of antigen to the uncommitted T lymphocytes. *Immunotherapy* has been successful in treating allergic conjunctivitis, and may alter the progression of atopic conditions.

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CHAPTER 3
THE INTEGRINS

3.1 Integrins in Immunity

A successful immune response depends on the capacity of immune cells to travel from one location in the body to another. Their ability to penetrate into tissues and make contact with other cells depends on intergin-like interactions with the surrounding environment.

Immune cells are collectively termed leukocytes and the key players are the B and T lymphocytes, the dendritic cells, the neutrophils and monocytes that differentiate into tissue macrophages, and the eosinophils. Leukocytes, especially T cells, migrate rapidly and a successful immune response depends on their capacity to quickly access lymph nodes and sites of injury and infection elsewhere in the body. Lymph nodes collect foreign antigens that drain into them from the body's tissues and thus act as the central immune-response centres or information hubs. It is in lymph nodes that T cells can screen antigen-presenting cells, such as dendritic cells or B cells, and is this event that initiates a standard immune response.

As a wound might occur anywhere in the body, lymphocytes migrate continuously through the widely distributed lymph nodes where they spend around 24 hours before exiting into the lymphatic circulation and back into the blood (Cyster, 2005; von Andrian and Mempel, 2003). This timing allows maximal exposure to any foreign antigen that is trapped on antigen presenting cells in the lymph nodes. Neutrophils and monocytes circulate in the blood until they are directed into wounded tissues by stimulants that are generated locally. Here they act as an early warning system for the lymphocytes that have been stimulated in the in nearby lymph nodes. These lymphocytes follow later and help to generate an immune microenvironment within the wounded tissue that can be very similar to that of a lymph node. Although the myeloid cells do not normally circulate through lymph nodes, they do so during infections. In fact, in some infections, large numbers of neutrophils enter lymph nodes through the lymph and are found in the T cell and dendritic cell regions of the node (Abadie et al., 2005; Chtanova et al., 2008). Therefore, the

movement of leukocyte from the blood into peripheral tissues is critical for immune surveillance and host defense. Further, aberrant leukocyte trafficking contributes to the pathogenesis of inflammatory and autoimmune diseases. Leukocyte trafficking is orchestrated and controlled by combinatorial inputs of adhesion and chemoattractant molecules located on both the leukocyte and the vascular endothelium. Numerous *in vitro* and *in vivo* studies have established that leukoctyes circulating in the blood are recruited to target organs by a series of sequential steps mediated initially by leukocyte and endothelial selectins and selectin ligands or subsets of leukocyte integrins and their endothelial ligands of the immunoglobulin superfamily (Springer, 1994; Butcher et al., 1995; Luster et al., 2005).

These functions of leukocytes depend greatly, but not exclusively, on the β_2 (**CD18**, **ITB2**) class of integrins. This class of four $\alpha\beta$ heterodimers consists of: **leukocyte function-associated antigen 1** (**LFA-1**, **CD11a/CD18**, $\alpha_L\beta_2$, **ITAL** antigen), **Mac-1** (**CD11b/CD18**, $\alpha_M\beta_2$, **ITAM** antigen), **p150,95** (**CD11c/CD18**, $\alpha_X\beta_2$, **CR4**, **ITAX** antigen), $\alpha_D\beta_2$ (**CD11d/CD18**, **ITAD** antigen). Leukocytes also express β_1 integrins (**CD29**) and $\alpha_4\beta_1$ (**CD49d/CD29**, **ITA4** antigen) is particularly important in immune responses.

Integrins on leukocytes are not constitutively active, but have their activity controlled by signalling through other membrane receptors that are active in an immune response. This is defined as *inside-out* signalling that convert integrins from an inactive to an active conformation, while the *outside-in* signalling that describes the signalling directed by integrins themselves once they are active.

3.2 Control of integrin activity

Integrins are normally in an inactive or non-ligand binding conformation when the leukocytes are circulating in the blood. Binding to other receptors on leukocytes, such as the chemokine receptors, antigen-specific T cell receptors or B cell receptors, triggers the inside-out signalling that activates the integrins. The end result of this signalling is a conformational change in the cytoplasmic and transmembrane domains of the integrin, which is relayed to the ectodomain, altering its ability to bind ligands (Kinashi, 2005).

In recent years, electron microscopic and crystallographic studies have identified three predominant β_2-integrin conformations that are thought to reflect different stages of

activation (Luo et al., 2007; Xiong et al., 2001). These are the **bent form**, the **extended form (intermediate affinity)** with a closed ligand-binding head that is of intermediate affinity for ligand, and the **extended form (high affinity)** in which the hybrid domain has swung out, enabling the ligand-binding I domain of the α-subunit to bind with higher affinity (Figure 3.1). These β_2 conformations can be distinguished by their interactions with specific antibodies, which react with the different epitopes that become exposed.

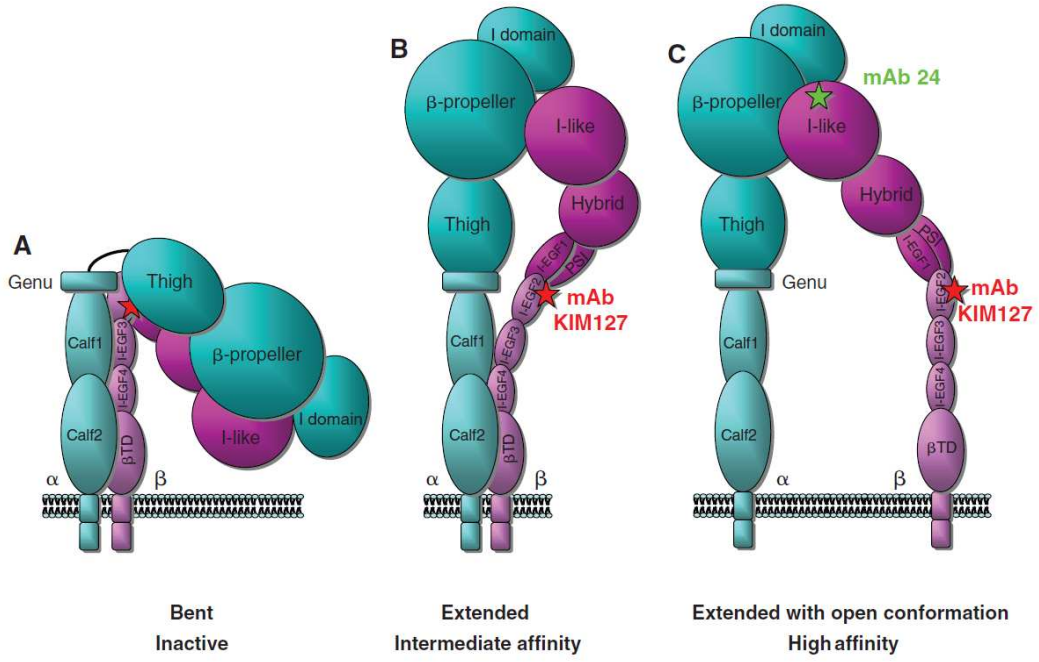

Figure 3.1
The three conformations of a β_2 integrin (ITGB2). Integrins are heterodimeric glycoproteins comprising non-covalently linked α- and β-subunits. Each subunit consists of a large extracellular region, a single hydrophobic transmembrane domain and a short cytoplasmic tail. (**A**) Bent – inactive. The inactive integrin is in a V shape with the ligand binding I domain close to the membrane. There is close association between the α- and β-subunits in the membrane-proximal region. (**B**) Extended – intermediate affinity. Inside-out signalling extends the integrin in a "switchblade-like" motion, orientating the I domain away from the membrane for optimal ligand binding. This epitope for the monoclonal antibody KIM127, which is located on I-EGF2 and obscured in the bent formation, becomes exposed. The KIM127 epitope, thus, serves as a marker for the extended β_2 integrin. (**C**) Extended with open conformation – high affinity. Local conformational changes within the α and β I domains, potentially generated by shear force, result in the hybrid domain swinging out and the subunit separation at the genu. This remodelling of the I domain ligand-binding site forms the epitope for monoclonal antibody 24 and causes increased affinity for ligand. (modified from Evans et al., 2009)

Chemokine receptor-signalling leads to extension of the bent low affinity form of LFA-1, and this happens in less than 0,4 seconds (Shamri et al., 2005). The interaction of intermediate-affinity LFA-1 with its ligand **intercellular adhesion molecule 1** (**ICAM-1**)

leads to the high affinity conformation that stabilizes adhesion. However, there is no clear evidence of how this switch happens. The purported mechanism is based on the capacity of mechanical forces to drive allosteric alterations in integrin conformation (Katsumi et al., 2004) and, for leukocytes, the **shear of blood flow** would be the major supplier of this force (Alon and Dustin, 2007).

The small GTPase **Rap1** is a key integrin regulator, and constitutively active mutant of Rap1 (such as Rap1V12) increase the *affinity* and *avidity* at the lymphocyte membrane (Katagiri et al., 2000; Sebzda et al., 2002). Impaired activation of Rap1 has been in some patients with the rare disorder *leukocyte adhesion deficiency III* (LAD-III) (see Chapter 1 and Kinashi et al., 2004). The leukocytes of the patients express normal levels of β_1, β_2, and β_3 integrins, but have faulty inside-out signaling (Alon et al., 2003; Kuijpers et al., 1997; McDowall et al., 2003). The LAD-III syndrome demonstrates the importance of integrin activation through this route. The patients suffer from severe and often life-threatening infections, because their leukocytes cannot migrate into tissues and their myeloid cells cannot use integrins to engulf pathogens. As for other GTPases, the GDP-GTP exchange cycle of Rap1 is regulated by guanine-exchange factors (GEFs). Certain Rap1 GEFs are activated by the second messenger Ca^{2+} and diacylglycerol (DAG), and one of the key Rap1 regulators in hematopoietic cells is *CalDAG-GEF1* (aka RasGRP2). *Rasgrp2*$^{-/-}$ mice have impaired integrin-mediated adhesion in platelets and neutrophils and are considered to be a model of human LAD-III (Bergmeier et al., 2007; Crittenden et al., 2004). This might be so because several LAD-III patients have a mutation in CalDAG-GEF1 that has been reported to affect splicing (Pasvolsky et al., 2007). These findings implicate this Rap1-GEF in the inside-out signaling of leukocyte integrins. Another route to Rap1 activation is through protein kinase C (PKC), which – similarly to CalDAG-GEF1 – is also responsive to Ca^{2+} and DAG. In platelets, rapid chemokine induction of Rap1 occurs via CalDAG-GEF1, whereas more sustained activation is via PKC (Cifuni et al., 2008). There also seems to be fast versus slow routes to Rap1 activation in lymphocytes, as chemokines activate Rap1 within seconds, which lasts a few minutes, whereas a more sustained level of Rap1 activation occurs through the T cell receptor (Katagiri et al., 2002; Shimonaka et al., 2003). Thus, there may be similarities between lymphocytes and platelets in the choice of pathways that lead to Rap1 activation that result in integrin activation. A downstream effector of PKC that leads to Rap1 activation is proposed to be protein kinase D1 (KPCD1; aka

PKD1, PKCμ), which forms a complex independently of its kinase activity, together with Rap1 and the β_1-integrin cytoplasmic domain, redistributing Rap1 to the membrane (Medeiros et al., 2005). However, PKC signaling does not always lead to Rap1 activation and Rap1 might not be involved in the activation of all classes of integrins. Ghandour et al. (2007) report that LFA-1 activation is dependent on CalDAG-GEF1 and Rap1, whereas VLA-4 integrin appears to be activated via a Rap1-independent PKC-mediated pathway. A conflicting report shows that the Rap1 inhibitor GTPase-activating protein (**GAP**) signal-induced proliferation-associated protein (**SPA1**) blocks both LFA-1 and VLA-4 integrin-dependent adhesion (Shimonaka et al., 2003).

A further set of proteins can bring Rap1 to the membrane and cause its activation. The Rap1-interacting adaptor molecule (**RIAM**) is expressed by T cells and activates Rap1 through TCR signaling (Lafuente et al., 2004). This pathway involves the adaptor adhesion- and degranulation- promoting adaptor protein (**ADAP**) and its binding partner SRC-kinase-associated protein of 55 kDa (**SKAP55**), which interact with RIAM (Kliche et al., 2006; Menasche et al., 2007).

The activation of Rap1 leads to the *redistribution of integrins* from the rear to the front of the cell. Associated with Rap1 are the adaptor protein **RAPL** (*regulator of cell adhesion and polarization enriched in lymphoid tissues*) and **Mst1** (*mammalian sterile 20-like kinase 1*) that are colocalized with LFA-1 in vescicular compartments (Katagiri et al., 2003; Katagiri et al., 2006). The implication is that the RAPL-Mst1 complex has a regulatory function in the intracellular transport of LFA-1. A final step requires the recruitment of **talin** by Rap1, which then binds to integrin causing the conformational shift that leads to an increase in affinity (Campbell and Ginsberg, 2004; Legate and Fässler, 2009) (Fig. 3.2).

The recruitment of talin and the ensuing integrin activation has become more complex with the identification of **kindlin-3** (**URP2**, **Mig2B**) as a haematopoietic-specific activator of integrins (Moser et al., 2008). Kindlin-3 belongs to an adhesion plaque protein family of three members, each with distinctive tissue distributions: kindlin-1 (FERM1), epithelial expression; kindlin-2 (FERM2), widespread expression; kindlin-3 (FERM3), haematopoietic cell-specific expression. Both talin and kindlin-3 have similar FERM (*protein 4.1*, *ezrin*, *radixin*, and *moesin*) domains that bind the β-subunit of integrins and the two NPxY sites (in which x denotes any amino acid) – talin at the membrane-proximal site and kindlin-3 at the membrane-distal site (Ma et al., 2008; Moser et al., 2008). Kindlin-3

knockout mice (*Ferm3*$^{-/-}$) have severe bleeding problems similar to the *Rasgrp2*$^{-/-}$ mice, suggesting that kindlin-3 is also a candidate gene involved in LAD-III.

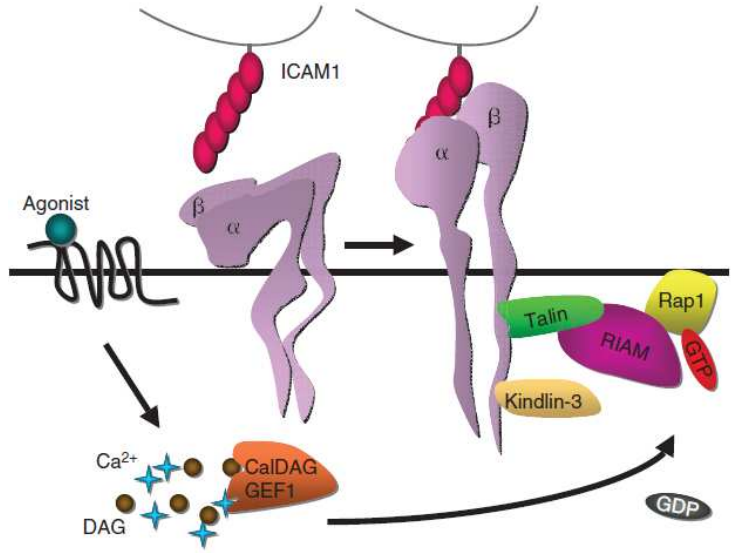

Figure 3.2
A model of inside-out signalling from agonist to integrin. For integrins to become activated, they need to be triggered by the inside-out signalling cascade. The key target of this pathway is the GTPase Rap1.

3.3 Outside-in integrin signalling

A further level of integrin-activity control is the regulation of **avidity**, and it is the outside-in signals from activated LFA-1 itself that appear to control the state of clustering. *Macroclustering* of LFA-1 on T cells follows on from the initial binding of LFA-1 to ICAM-1 in multivalent form, thus strengthening adhesion (Kim et al., 2004). On macrophages, inactive LFA-1 is randomly distributed, but **primed LFA-1** forms *nanoclusters* and then *macroclusters* when it binds to ICAM-1 (Cambi et al., 2006). Whether the nature of the interaction with the cytoskeleton also controls clustering is unclear. It has been shown that intermediate affinity LFA-1 on T cells is attached to the actin-binding protein α-actinin-1, whereas high affinity LFA-1 is attached to the cytoskeletal protein talin (Smith et al., 2005; Stanley et al., 2008). On macrophages, the LFA-1 macroclusters colocalize with talin. Considered together, the evidence suggests that the **high-affinity LFA-1-talin connection** favours the formation of stable clusters that further enhance adhesion.

It is well recognized that the successful functioning of T cells and other leukocytes depends on cytoskeletal remodelling (Billadeau et al., 2007; Vicente-Manzanares and Sanchez-Madrid, 2004). As outside-in signalling through leukocyte integrins causes cell spreading and migration, the implication is that cytoskeletal reorganization is downstream of the integrin-specific signalling. For T cells, LFA-1-mediated adhesion to ICAM-1 causes extensive F-actin remodelling, but little is understood of the integrin-proximal signalling that is involved. More detail is known about signalling in neutrophils and macrophages, which promotes β_2-integrin-mediated adhesion, spreading, chemotaxis, and other myeloid-specific functions, such as the production of reactive oxygen species and the release of cytokines and cytotoxic granules. The involvement of integrins in these activities is highlighted by the finding that neurophils from β_2-integrin-null mice are unable to carry out these functions.

The **Src** and **Syk** families of protein tyrosine kinases have been implicated in outside-in signalling in myeloid cells that express β_2 integrins, in platelets that express $\alpha_{IIb}\beta_3$, and also downstream of TCR and BCR in lymphocytes (Fig. 3.3).

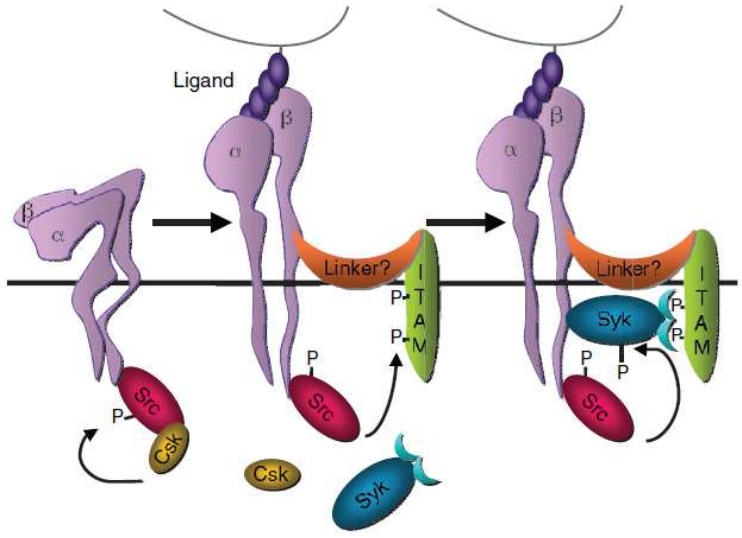

Figure 3.3
Ouside-in signalling associated with integrins on leukocytes. The inactive β_2 and β_3 integrins on myeloid cells and platelets, respectively, are constitutively associated with inactive Src kinase.

Syk is recruited by its tandem SH2 domains to an adaptor molecule containing an immunoreceptor tyrosine-based activation motif (ITAM) that is di-phosphorylated by Src kinase. By these means, Syk is brought within range of Src, which then phosphorylates it.

3.4 The role of integrins during extravasion

Three overlapping adhesion events mediate the migration of leukocytes out of the blood and into tissues. These sequential steps are required to enable the leukocytes to overcome the shear forces of blood flow. During the **first step**, circulating leukocytes are captured, become loosely attached to the vascular endothelium and begin to roll along using their **selectine** molecules. This rolling is mediated by *L-selectin* on the leukocyte and *E-* and *P-selectins* on the endothelium. The selectins bind to sialyl-Lewis^X moieties that decorate a number of glycosilated counter-receptors (Uchimura and Rosen, 2006). Rolling allows the leukocyte to examine the endothelial target for its repertoire of endothelial chemoattractants and integrin ligands.

The arrest of leukocyte rolling on target vascular beds involves rapid formation of shear resistant adhesions by specialized leukocyte integrins. The next step is mediated by **chemokines** that are constitutively produced in lymph nodes or induced in injured tissues and become tethered to proteoglycans on the vasculature. Most circulating leukocytes maintain their integrins in largely low affinity state (Carman and Springer, 2003). Leukocyte integrins must undergo *in situ* modulation to develop high avidity for their endothelial ligands to establish shear-resistant adhesion and firm leukocyte arrest on the target endothelial site. This dramatic change in integrin affinity is triggered when the rolling leukocyte encounters and rapidly responds to a proper chemoattractant signal presented on the apical endothelial surface. Lymphocytes and myeloid cells cease rolling and arrest on lymph node high endothelial venules as well as on peripheral tissues upon activation of at least one of the four major leukocyte integrins: VLA-4, $\alpha_4\beta_7$, LFA-1, and the myeloid-specific integrin, Mac-1 (Luster et al., 2005). New structural and functional data strongly suggest that bidirectional integrin signals involve rearrangements of their α and β subunit cytoplasmic tails and changes in the extracellular domain that follow binding of extracellular ligands.

Chemokines activate the integrins **VLA-4** and **LFA-1** by engaging the specific GPCRs (**inside-out signalling**) on leukocytes which leads to the third step of the cascade

of the integrin-mediated arrest of the leukocyte on the vessel wall. The integrin VLA-4 is important at this stage and can have a role in both leukocyte **rolling** and **arrest** (Tsuzuki et al., 1996). A new role of **VLA-4** in lymphocyte homing to lymph nodes has been recently investigated. In this scheme, the lipase **autotaxin**, which is expressed by HEVs, binds active integrin VLA-4, generating *lysophosphatic acid* that, in turn, act on GPCRs that then further amplifies the process of homing (Kanda et al., 2008). In light of the short time frame required for biologically significant integrin activation by chemokines, integrins may exist as preformed associations with different cytoskeletal adapter and membrane proteins (Liu et al., 2000), such as **tetraspanins**, CD47, CD98, and CD44 (Feigelson et al., 2003; Ticchioni et al., 2001; Suga et al., 2001; Nandi et al., 2004).

Chemokines also activate LFA-1, enabling leukocytes to firmly attach to the endothelial surface and to subsequently migrate across it. The crawling of leukocytes through the vasculature is known as **diapedesis**, and this can occur by both *paracellular* (through endothelial cell junctions) and *transcellular* (through the endothelial cell itself) routes (Carman and Springer, 2008).

Once a leukocyte establishes firm adhesion to the vascular endothelium, it undergoes a morphological change known as polarization. Subsequently, the leukocyte migrates over the apical endothelial surface toward interendothelial junctions, where it can then transmigrate into the subendothelial tissues. **Polarization** establishes the front (or leading edge) and the trailing edge (in leukocyte an extended rear projection, termed uropod, forms a specialized trailing edge) of a cell. After polarization is achieved, the cell is poised for directional movement. Depending on the adhesive and chemotactic cues, integrins on the polarized leukocyte generate highly dynamic adhesions. These integrins must constantly integrate both biochemical information from ligands and inside-out activation signals as well as mechanical signals in the form of external forces exerted by the shear stress experienced by the leukocyte at the endothelial contact site and internal foces generated by polymerized actin and actomyosin contractility (Alon and Dustin, 2007; Schwartz and Horwitz, 2006; Gupton and Waterman-Storer, 2006).

Polarization in leukocytes is primarily triggered by chemokines and involves reorganization of the actin cytoskeleton, Golgi, and microtubule-organizing center as well as redistribution of cell surface molecules. F-actin goes from being radially symmetric around the cell, to being focused in areas such as the leading edge. Chemokine receptors

redistribute to the leading edge, while other cell surface molecules, such as the adhesion molecule CD44, redistribute to the uropod. Integrin molecules also reorganize, forming **clusters** at the leading edge and the uropod. In addition, the activation state of the integrins can change in a spatially distinct manner during polarization, with high and intermediate affinity integrins being primarily localized to the leading edge (Shimonaka et al., 2003; Green et al., 2006).

Once a leukocyte establishes polarity on the endothelial surface, it can start the process of migrating across the endothelium to enter the underlying tissues. For the most part, the leukocyte relies on cues in the form of chemokines, adhesive integrin ligands, and shear flow to maintain later migration along the endothelial surface until it reaches the junction of two or more endothelial cells. Transendothelial migration involves spatiotemporal bidirectional signalling between the leading edge of the transmigrating leukocyte and specific junctional molecules that reversibly remodel the endothelial junction through contractility events that allow the leukocyte passage and the sealing of the contracted endothelial junction soon after the leukocyte has terminated its passage. The general model of cell motility involves repetitive cycles of new projections being sent out at the leading edge in the form of lamellipodia and filopodia. At these sites, new adhesions by integrins are laid down with the underlying substratum. This allow for traction as the bulk of the cell body is propelled forward, while at the same time adhesions are released at the rear of the cell. Integrins play key roles in cellular migration acting both as adhesive molecules that maintain locomotion over the apical endothelial surface and as signalling molecules, which, together with chemokine signals, maintain polarity and motility (Kinashi, 2005).

The signalling needed to coordinate migration is complex. Just as Rap1 activation plays a key role in integrin activation and leukocyte polarization, the activation of the small GTPase **Rac** has emerged as a key signalling event controlling cell motility on adhesive substrata (Srinivasan et al., 2003; Xu et al., 2003; Fenteany and Glogauer, 2004). Rac activation drives actin polymerization and lamellipodia formation (Hall, 2005). However, efficient migration requires this Rac activation to be spatially and temporally restricted to the leading edge of the cell. A recently defined signalling pathway provides for spatial and temporal regulation of Rac activation during VLA-4-integrin-dependent cell migration on both the vascular ligand **VCAM-1** and on the extracellular matrix protein **fibronectin**. This

VLA-4-dependent modulation of Rac activation is mediated by the reversible binding of **paxillin** to the α_4 cytoplasmic domain. The α_4 integrin subunits bind directly to the signalling adapter molecule paxillin (Liu et al., 1999), and this interaction is regulated by selective phosphorylation of the α_4 cytoplasmic domain at serine 988 in a protein kinase A-dependent manner. Phosphorylation at this position leads to release of paxillin from the α_4 subunit, while depshosphorylation promotes the α_4 integrin-paxillin interaction (Han et al., 2001). In a migrating cell, the phosphorylated α_4 integrins are localized to the leading edge of the cell, while dephosphorylated α_4 integrins are localized to the lateral and trailing edge of the cell. This **spatial regulation of α_4 integrin-paxillin binding** promotes effective leukocyte migration, as either disrupting or enforcing the association of α_4 with paxillin greatly impairs cell migration (Han et al., 2003). The α_4 integrin-paxillin interaction contributes to effective cell migration by spatially regulating Rac activity. Paxillin bound to α_4 integrin provides scaffolding for recruitment of additional signalling molecules to this site. One of these recruited signalling molecules is an adenosine diphosphate-ribosylation factor **(Arf)-GAP**, **GIT1**. GIT1 ultimately leads to inhibition of Rac activation by inhibiting the activation of another small GTPase, **Arf6**. Arf6 modulates Rac function through mechanisms involving changing in cellular distribution of Rac1 by endosomal trafficking and recruitment or Rac activators such as Rac-guanine nucleotide exchange factor (**GEF**), **DOCK180/ELMO** (Goldfinger et al., 2003; Han et al., 2003; Brown and Turner, 2004; Nishyia et al., 2005; Vitale et al., 2000; Santy et al., 2005). Thus, during α_4-dependent migration, the α_4 integrin-paxillin interaction contributes to the inhibition of Rac activation at the later and trailing edges through the recruitment of Arf-GAP.

α_4 integrins can also regulate Rac activation and cell migration through Src kinases, independent of the α_4-paxillin interaction. Thus, VLA-4 integrin-dependent activation of Rac at the leading edge can proceed through activation of Src kinases. Polarization and motility are also maintained by inhibition of Rac at the later and trailing edges mediated by the α_4-paxillin complexes that recruit an Arf6 inhibitor. These two integrin-mediated signalling pathways can be complemented by chemoattractive signals presented to the leukocyte on the apical junctional and subluminal compartments of the endothelial barrier. When these chemoattractant signals are robust, the VLA-4-Rac activation pathway may be less important for migration (Fig. 3.4). One unique feature of the α_4 cytoplasmic domain is that

it direcly binds to paxillin, and this interaction is negatively regulated by α_4 serine-988 phosphorylation. Therefore, as described by Hsia et al. (2005) the fibronectin binding integrins generate signals promoting cell migration through distinct mechanisms: a paxillin-mediated linkage to $\alpha_5\beta_1$ facilitates FAK activation, the formation of an activated FAK-Src complex, and increased cell motility, in part due to the tyrosine phosphorylation of p130Cas, generating signals leading to Rac activation and lamellipodium formation at the leading edge of migrating cells (Mitra et al., 2005; Schlaepfer and Mitra, 2004); even though Src activation is a common signalling component promoting $\alpha_4\beta_1$- and $\alpha_5\beta_1$-cell motility, **$\alpha_4\beta_1$-induced Src activity does not involve FAK**. Notably, phosphorylation of p130Cas by c-Src or a FAK-Src complex occurs via a shared pathway associated with enhanced Rac activation, lamellipodium formation, and cell motility (Fig. 3.4). Thus, the α_4 cytoplasmic domain connection to c-Src activation is a FAK-independent linkage to a common motility-promoting signalling pathway.

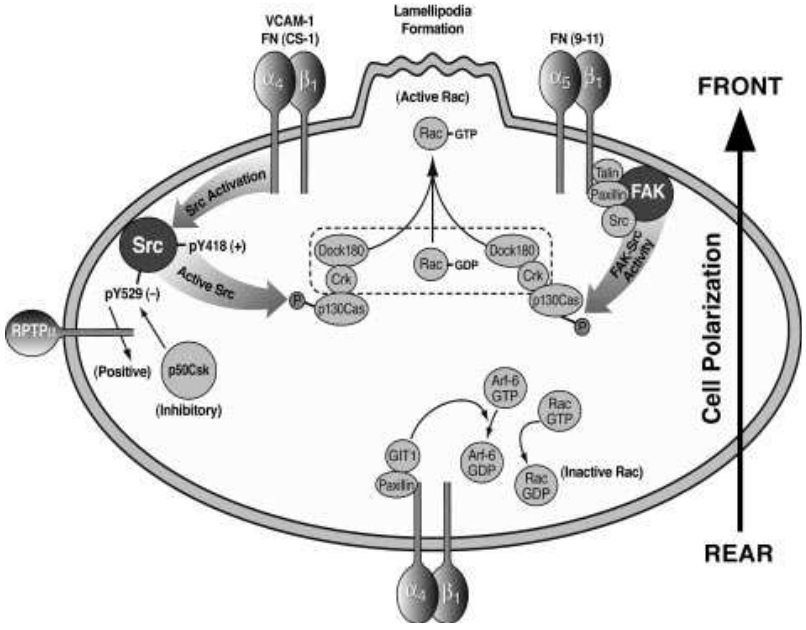

Figure 3.4
Model of receptor-proximal differences in $\alpha_4\beta_1$ and $\alpha_5\beta_1$ signalling events promoting p130Cas phosphorylation associated with Rac activation in migrating cells. $\alpha_4\beta_1$ binding to VCAM-1 or fibronectin (FN) (CS-1) stimulates c-Src-family PTK phosphorylation at Y418 and promotes Src catalytic activation in a FAK-independent manner. This linkage between α_4 and Src does not require paxillin binding to α_4, which has been shown to down-regulate Rac activation at posterior regions of migrating cells through a signalling linkage involving paxillin binding to the ADP ribosylation factor GTPase-activating protein (Arf6-GAP) GIT1. From Hsia et al., 2005

The above described paradigm for $\alpha_4\beta_1$ integrin-dependent regulation of Rac activation and migration is just one mechanism that contributes to leukocyte transendothelial migration. Certainly other cues that drive migration, such as chemoattractants and signals generated by mechanical force on the cell (such as shear flow), will come into play to regulate Rac activation and cell migration. The combined input of these cues will ultimately determine how and where leukocytes migrate. Furthermore, the relative importance of each of these cues may vary widely between different types of leukocytes. For example, while a fluid shear plays an important role in lymphocyte transendothelial migration, it plays a lesser role in neutrophil migration, especially on endothelium expressing high amounts of β_2 integrin ligands (Cinamon et al., 2001; Cinamon et la., 2004).

Not all forms of leukocyte migration are the same with respect to mechanics and signalling. In lateral migration, leukocytes migrate to reach the junction of endothelial cells, and in migration across the junction, the leukocyte moves between two endothelial cells. Junctional migration involves highly specialized adhesive interactions between the leukocyte and endothelial cells, which allows for the initial separation and reforming of endothelial junction as the leukocyte passes through. These differences in migration are reflected in different signalling pathways involved. For example, later migration of lymphocytes, driven by chemokines, requires DOCK2-dependent activation of Rac, while junctional migration of lymphocytes is DOCK2 independent.

Where and when leukocytes leave the blood and enter the peripheral tissues play key roles in immunity and inflammatory diseases. These locations are selected by repertoires of chemoattractants and adhesion molecules. The precisely regulated **expression** and **functional state** of these molecules allows for accurate specification of leukocyte trafficking throughout the body. Integrins are adhesion and signalling molecules involved in the adhesive interactions between leukocytes and the vascular endothelium needed to resist the physical forces of blood flow and mediate effective migration across the endothelium. To meet the challenge of adhesion under shear flow conditions, leukocyte integrins link to the cytoskeleton to resist detachment under shear flow condition and undergo rapid reversible modulation of their ligand-binding affinities within subseconds of stimulation by chemoattractants. In addition, integrin-mediated signal transduction must be both spatially

and temporally regulated in leukocytes to allow for effective migration across the endothelial barrier. Unravelling the molecular machineries that regulate adhesion, motility and transendothelial migration in different subsets of leukocytes may help to identify therapeutic targets to selectively modify leukocyte trafficking.

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CHAPTER 4
HIGH-THOUGHPUT SCREENING

4.1 Drug screening using cell lines

The rapid progress in combinatorial chemistry continues to yeald a myriad of potentially bioactive compounds everyday. With thousands of pharmaceutically valuable drugs at hand, there is an urgent need for engineered tissue equivalents that could serve as *in vitro* model systems during the initial stages of drug discovery, specifically during the preclinical stages of cell/tissue-based high-throughput screening (HTS).

The major research focus of the pharmaceutical and agrochemical industries is the fast and efficient detection of active compounds ("leads") with a pronounced developmental and market potential. During the past decades, remarkable efforts have been made to accelerate the lead discovery process (Fig. 4.1).

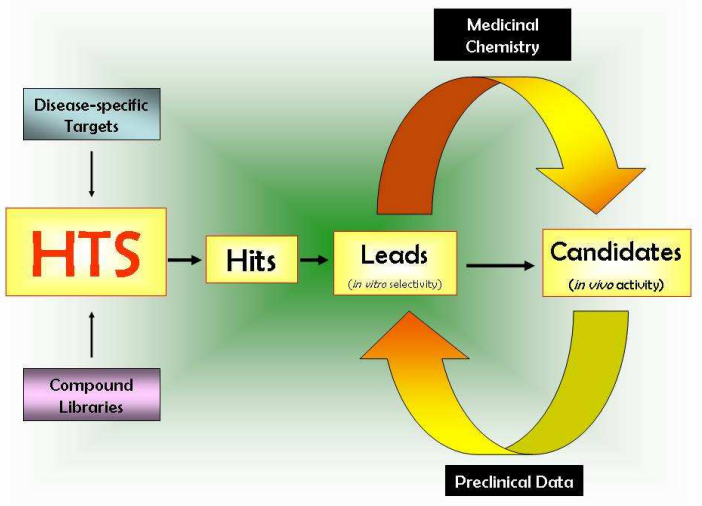

Figure 4.1
Scheme of the high-throughput procedure to screen compound libraries for the identification of suitable candidates for clinical studies.

Enabling technologies (e.g. robotics, high-throughput screening assay technologies as well as genomics and proteomics) were put into place throughout the industry to generate the necessary high-profile clinical and developmental candidates.

Another approach that parallels HTS technologies is the **virtual screening** (**VS**) that is one of the readily available technologies that may contribute to deliver clinical candidates more rapidly and economically. This technique can effectively identify biologically active compounds, or **hits**, from a pool of millions of virtual compounds (Waszkowycz, 2002; Shoichet, 2004).

A modern drug discovery program generally starts with the **target selection**, followed by **hit identification**, **hit-to-lead transition**, **lead optimization**, and **clinical candidate selection**. Hit identification, although it occurs at an early stage of the whole process, has a profound influence on the success of a program. HTS is the mainstream method widely applied in the pharmaceutical industry to identify hits, yet HTS suffers from many limitations – most importantly it is costly and time consuming. The basic costs of HTS include collecting and maintaining screening libraries of thousands to million of compounds, the reagents for the assay, and maintaining the robot systems. It usually takes months to complete an HTS, from designing and optimizing the assay, to cloning, expressing and purifying the target protein, to plating compounds, and to finally confirming the hits. In addition, experimental HTS inevitably suffers from misidentification or decision errors, i.e. "**false positives**" and "**false negatives**" (Malo et al., 2006). "False positives" and "false negatives" are frequently associated with technical or procedural problems during the assay, or result from the properties of the screened compounds themselves, such as low solubility or poor stability.

Both HTS and VS aim to identify a small subset of biologically active compounds from a vast number of compounds. Experimental HTS directly or indirectly detects the interactions between small molecules in an existing library and the target protein, while VS applies rule-based or property-based **pattern recognition** and **molecular docking** to filter a library of virtual compounds, which may not be synthesized yet.

Cell-based assays, if appropriately designed, can be used to rapidly identify molecular mechanisms of human disease and develop novel therapeutics. In the last 20 years, many gene that cause or contribute to diverse disorders, including cancer and neurodegenerative disease, have been identified. With such genes in hand, scientists have created numerous model systems to dissect the molecular mechanisms of basic cellular and developmental biology. Meanwhile, techniques for high-throughput screening that use large chemical libraries have been developed, as have cDNA and RNA interference libraries that

cover the entire human genome. By combining cell-based assays with chemical and genetic screens, we now have vastly improved our ability to dissect molecular mechanisms of disease and to identify therapeutic targets and therapeutic lead compounds.

A selected cell line for HTS must express the desired function in the correct signal context with a stable and reproducible signal window. The different cell types from which to choose have different advantages: **primary cells** best resemble the physiological situation, but cultivation and available cell numbers are critical issues, even when using techniques such as conditional immortalization or stem cell differentiation. A large panel of easy-to-cultivate **immortalized cell lines** is available with cell history, growth behaviour and functional information. These cells are ideal for functional screens as proliferation assays. **Recombinant cell lines** overexpressing the target are often used for screening, because cell lines with an optimal growth behaviour and signal window can be selected.

In order to obtain reproducible cell material for HTS, the favourite procedure is to generate a stable target-expressing cell line. This allows adjustment of the expression level by using different promoter elements and/or amplification with the methotrexate or glutamine synthase system combined with clone selection. In addition to stable cell lines, identical cell material can be produced by transient transfection of one large cell batch, followed by freezing the transfected cells in portions. Directly before HTS, the frozen cells are seeded into assay plates. For an optimal large-scale transient transfection, special standards must be fulfilled for the cell line, the expression plasmid, the medium, and the transfection reagent.

The ideal cell for HTS is *fast growing*, had *good adherence*, a *stable cellular phenotype*, a *broad tolerance against solvents*, and is *insensitive against culture modifications*. All of these parameters must be tested for each cell line and each assay, using HTS equipment and conditions early during cell development.

Adherence: most HTS are plate-based assays involving one washing or separation step, as well as the uncontrolled loss of cells is a frequent reason for low assay reproducibility. The degree of negative charges on the cell at physiological pH seems to be one important factor for good adherence. In addition, the present of bivalent cations (Mg^{2+}, Ca^{2+}) and the presence of extracelluar proteins determine the adherence of cells. Poor adherence can

be improved by coating the wells with charged substrates such as poly-L- or –D-lysine, or with components of the extracellular matrix.

pH and temperature: most cells grow best at a pH between 6.8 to 7.6, and the pH of most commercial media is 7.2 to 7.4. Media based on Earl's salt composition are buffered by bicarbonate/carbonic acid, and the pH is regulated by constant CO_2 delivery in an incubator. After 30 minutes at atmospheric pressure, the pH rises by about 0.1 to 0.2 pH units; after 4 hours the pH is raised by about 0.3 to 0.5 pH units. Media with Hank's salt composition are buffered by phosphoric acid and should be used at normal atmosphere. The addition of 10-20 mM HEPES to the media with a good buffering capacity between pH 7.2 and 7.4 helps to keep the pH constant. Depending upon the available instruments, screen conditions minimizing pH variations must be selected.

Media and additives: the medium is not only a food source but also represents the environment in which the cells live. Medium selection for the screen is dependent on the cell type and on the assay. The medium composition must support the desired cell function needed, and provide an optimal energy source as well as all important nutrients such as vitamins, growth factors, minerals, and essential lipids that the cells need. A media for cell production can differ from one used for plate generation, as the latter often contains antibiotics and HEPES. The optimal amount of foetal calf or bovine serum and ingredients such as glucose and glutamine can be different for the production phase compared to the plating medium. For fluorescence read-outs, as well as some reporter assays, the presence of phenol red in the medium enhances the background. All medium components such as FCS/FBS, growth factors, hormones, vitamins, ions, and nucleotides should be evaluated for possible interference with the assay signal. FCS contains different amounts of stress-protecting factors such as vitamin E and ATP, as well as calcium, which modulates cell function and influences cell adhesion, proliferation, and differentiation. Charges of media and additives must be checked for functionality for each assay, and should not be charged during HTS and development.

Solvent tolerance: Most compound libraries are dissolved in dimethyl sulfoxide (DMSO), the tolerance of which is dependent on the cell line and the duration of exposure. The solubility of a compound can also determine its useable concentration in the screen. Often-used cell lines such as CHO, BHK, NIH-3T3, and HEK293 show no growth inhibition and functional difference in the presence of up to 1% DMSO or ethanol, but primary cells are more sensitive in general.

Cell density: The optimal cell density must be determined. For transport and receptor function assays, a good confluent cell layer enhancing the target density is often advantageous, and cell numbers in the range of 8000 to 12000 per 384 wells should be considered. The influence of cell number and variations on the signal must also be monitored. Functions such as sensitivity to drugs, transcriptional activity, permeability, cells in mitosis or the differentiation state often change with cell density. For these assays, semi-confluent cultures are often preferable. Surface proteins (e.g. receptors) can be sensitive to proteases such as trypsin. Receptor function must be carefully checked if cells are to be used at different time-points after seeding. It is recommended that cells are harvested using nonenzymatic solutions. For image analysis, and for localization assays, a good separation of the single cells is needed, and aggregates must be avoided. Larger cells with a clearly separated, round nucleus and minimal morphological variation are ideal.

Unlike ordinary assay reagents, living cells are prone to change during their time in culture, in response to environmental changes, and they require between 4 and 24 hours after plating to adhere properly. Differences in cell density, metabolism and function on has to take into a consideration if using cells as reagents. Cells often function optimally at defined time points after plating or confluence. In particular, the signals of reporter assays are known to be sensitive to changes in cell number.

The use of **growth-arrested cells** for screening provides another possibility of enhancing flexibility and reproducibility. CHO cells which had been frozen after *mitomycin C-induced division arrest* in a calcium flux assay, and reported a stable signal-to-noise ratio for

four days after plating. Similarly, by using mitomycin C treatment it is possible to improve the robustness of a reporter assay to a Z'-value of 0.79, compared to 0.35 for normal cultured cells.

Differentiated cells reflect naturally growth-arrested cells by *keeping their functional phenotype stable* for several days in culture. Differentiated cells are more difficult to prepare, but this is acceptable if they provide reliable cell material for screening.

Maintaining plated cells under **hypothermic conditions** (4-8 °C) offers another possibility of enhancing flexibility in cell delivery. By using special solutions, normal coronary artery smooth cells, hepatic cells and skeletal muscle cells can be kept for between 2 and 7 days at 4-8 °C. This effect is due to the solutions balancing the altered cellular ion concentrations and counteracting cell swelling at low temperature.

4.2 General HTS assay prerequisites and set up

A certain level of automation is a prerequisite for an efficient HTS laboratory. In general the **microplate** has established itself as the major assay platform, starting with the 96-well plate which has been the first major step towards parallelization. The pressure to reduce the cost of screening, combined with technical improvements in pipetting equipment and plate manufacture, has led to increased use of higher-density plates with 384-1536 wells. Despite the clear advantage in reagent savings, the use of smaller volumes is more demanding on the equipment used for liquid handling and detection. Thus, the reduction of assay volumes can lead to compromised assay parameters, especially due to stronger evaporation effects and changed surface-to-volume ratios. Although the **number of screened wells per unit time** is an easily measurable and objective performance parameter, this does not directly define the desired output, which is the efficient identification of novel lead structures. As a consequence, further emphasis must be given to novel screening strategies and physiologically relevant screening approaches. Advanced screening approaches using cellular read-outs are one of the means by which this goal may be achieved.

Certain quality parameters must be met during the development of the assay. One of the basic parameters is to **outline the controls** used to define assay performance, and to calculate activities. Whereas *uninhibited samples* typically are used to serve as the **positive control**, the **negative** or **blank** control is often more difficult to define. In the best case, a

pharmacologically relevant standard can be used to define the level of maximal effect or the level of nonspecific signal blank (**NSB**). During assay development, it must be determined if there is sufficient faith that these control values are not too *artificial* to be reached by a pharmacologically active compound. In some cases the positive controls might require the addition of an agonistic compound to stimulate the system. Assays searching for agonistic activities might have accordingly reversed controls: for example, the blank value might represent the unperturbed control.

Assay performance and its **suitability** for HTS is usually calculated using the mean (M) and standard deviation (SD) of such control wells. The **screening window coefficient** (**z'**) is the most commonly used statistical parameter to describe the quality of an assay:

$$z' = 1 - \frac{3SD(positiveCTRLs) + 3SD(NSBcontrols)}{M(positiveCTRLs) - M(NSBcontrols)}$$

z' is a statistical tool that reflects the **assay's dynamic range** and the **variability** associated with the measurements. In recent years, the **z'-value** has been accepted as the most relevant parameter describing the assay robustness, and z'-values above 0.5 are considered as sufficient for screening campaigns. However, all of these parameters describe to a certain level only the robustness of the assay, and not whether the assay can detect the correct pharmacology and/or is capable of detecting hits with the desired sensitivity. In addition to the difficult quest for the correct pharmacology of novel targets, *target-unrelated effects* have been recognized as a major challenge for HTS operation. Such undesired assay interference leads to so-called **false positive or negative results**, and these can create a significant burden on resources during the hit validation phase. Phenomena such as the *inner filter effect*, which is caused by coloured compounds, the quenching of fluorescence by various mechanisms, autofluorescence of compounds, light scattering resulting from particles, and photo bleaching are common mechanisms of assay interference. Many compound-related assay interferences are concentration-dependent and this creates a barrier for the selection of high compound concentrations for screening. Another major disturbing effect is caused by *compound precipitation*; hence, exceeding a compound's solubility limits in HTS is not recommended but, due to the variety within the compound collection, this cannot always be avoided (McGovern et al., 2002). However, the most important parameter – the relevance

of the screening approach for the pathophysiological *in vivo* situation is the most difficult to appreciate *a priori*. One strategy to improve the level of confidence in the physiological nature of the screening is to present the target of choice within its cellular environment.

High-throughput and virtual screening are widely used to discover new lead compounds for drug design. These screening methods have discovered novel molecules, dissimilar to known ligands, that nevertheless bind to the target receptor at micromolar or submicromolar concentrations. Often screening hits are subsequently found to have peculiar inhibition properties: they act noncompetitively, show little relationship between structure and activity, and have poor specificity. These traits are non-drug-like and are undesirable in lead compounds. Accordingly, many attempts have been made to identify and remove molecules with these properties from screening databases. Such efforts have not been entirely successful because the underlying causes of these unusual behaviours are not completely understood. Consequently, screening hit lists continue to be populated, even dominated, by compounds that act with **atypical properties** that cannot be described by any existing model; such compounds are unlikely to be pharmaceutically useful. These properties are due to the biophysical characteristics of the compounds.

In creating a disease model, a researcher must balance the ease of working with the system against the relevance of the disease process. Typically, "relevance" is determined empirically when predictions made by a simple system (e.g. a cellular assay) are tested in a more complex system (e.g. a transgenic mouse model). The balance between tractability and relevance becomes even more challenging when attempting to create a cellular model that is amenable to HTS. It is reasonable that using a **discrete** and **quantifiable** molecular event that closely correlates with pathogenesis as the readout for cell-based HTS increases the likelihood of discovering compounds with activity in more stringent *in vivo* models. The value of a cell-based assay is greatly enhanced if the end point is specific to the disease mechanism. Most basic cellular phenotypes that can be readily measured (e.g. proliferation, death, and process extension) are under the control of multiple regulatory pathways. When such readout are used in screening assays to identify genetic or chemical modifiers, there is consequently a great chance that a nonspecific effect will account for the result. Conversely, when a highly specific molecular event (e.g. protein folding) is monitored within a cell, the odds are much lower that nonspecific processes could account for the result.

The choice of a library is critical to the success of a screening project. It is important to consider the size and the cost of the library, how to determine the mechanism of action of hits, and how to translate them in viable drugs. Because the cost of a library is high and the success rate is low, it is suggested that academic laboratories by screening small collections of biologically active molecules in cell-based assays. Ideally, compounds should be cell-permeant, nontoxic, and nonmutagenic and have good bioavailability. These characteristics will make them more effective in live cells and improve their likelihood of activity *in vivo*. Screening an established molecular space offers many advantages. These compounds are generally known to be biologically active, which increases their chance of modifying the molecular event of interest.

4.3 Shifting to high-content screening

The term "**high-content screening**" (**HCS**) describes the analysis of drug activities in cell-based assays. It is a technology platform designed to define temporal and spatial activities of genes, proteins, and other cellular constituents in living cells. The measured events can include **general effects** (e.g. cellular morphology, apoptosis, migration), **metabolic stimulation or inhibition**, and **specific effects on discrete target proteins**. From a biology-orientated perspective, the term "*phenotypic screening*" is also often used to describe complex cell-based compound screening. HCS is not a novel technique, but combines cellular manipulation, data acquisition and data processing within an integrated process.

In contrast to *target-based approaches*, assays which recapture generic cellular parameters deliver in-depth results with regard to the mode of action of screening compounds. Cellular assays with **multiparametric readouts** are desired when a multifaceted cellular phenomenon such as apoptosis is studied:

- They can provide access to targets not amenable to screening in conventional assays
- Multiplexed assays enable the direct identification of pharmacologically active compounds which can modulate the physiology or morphology of a cell.

These advantages must be reflected knowing that most successful drugs descend from natural products selected in intact biological systems, and many drugs show several

biological activities as nonsteroidal anti-inflammatory agents, cyclosporine, and antihistamines (Kubiniy, 2003). Even for the most popular drugs, not all of the targets and signalling pathways influenced in one cell are realized, much less the effect on the whole organism.

The minimization of biological complexity by screening isolated targets or individual pathways causes most emergent properties and all network responses of a cell to be missed. Hence, the future use of complex cell systems in combination with testing of several targets will allow the collection of information covering several disease parameters and pathways by selecting for cell-permeable, nontoxic drug candidates. Today, a series of new, more physiologically relevant cell culture methods have become available: tumour cells have been grown to three-dimensional spheroids and tested for their response against cytotoxic agents (Bartholoma et al., 2005), while embryonic stem cells allow the effects of compounds to be monitored in complex cell systems.

In future, the main challenges for complex cellular screens are:

- the identification of drug **targets**;
- the determination of **target-based structure-activity relationships** of the compounds;
- the **regulatory issues**.

The management and presentation of the growing amount of data as well as the integration of analyzed cellular parameters into a physiological context, represent a major challenge for cellular bioinformatics.

4.4 Scintillation proximity assay (SPA)

The recent advances in generating small molecules by a wide variety of combinatorial chemistry approaches has increased the pressure on screening methodology to rapidly evaluate the compound library data base and accordingly identify lead chemical structures. In order to screen such large numbers effectively the high throughput screening must be robust, automatable and precise.

Scintillation proximity assay is a radioisotopic homogeneous assay technology which allows the design of high-flux assays for a wide variety of biochemical and cellular targets. The method requires **no separation steps** and relies entirely on pipetting on a "mix and measure" format. The SPA technology is therefore easily automatable and precise and when

utilized with microtitre plate scintillation counters and robotics provides a highly versatile high throughput assay technology. The technique has been utilized effectively for radioimmunoassays, receptor binding assays, and a wide variety of enzyme assays. More recently, SPA has been applied to cellular adhesion molecule binding, protein-protein interactions, protein-DNA interactions, and cellular biochemistry assays.

The technique relies on upon the observation that a γ particle emitted from a radioisotope will only travel a limited distance in an aqueous environment. This path length is determined by the energy of the β particle. The β electron from [^{3}H] is emitted as a spectrum with an average path length of approximately 1.5 μm and the two monoenergetic Auger electrons emitted by [^{125}I] have path lengths of 1 μm and 17.5 μm respectively. In general these two isotopes are ideally suited for SPA.

In SPA the target of interest is immobilized to a small microsphere approximately 5 microns in diameter. The microsphere consists of a solid scintillant core which has been coated with a polyhydroxy film, which in turn contains coupling molecules such as antibodies, which allow generic links for assay design.

When a radioisotopically labelled molecule binds to the microsphere the radioisotope is brought into close proximity to the scintillant and effective energy transfer from the β particle will take place resulting in the emission of light. While the radioisotope remains in free solution it is too distant from the scintillant and the β particle will dissipate the energy into the aqueous medium and therefore remain undetected.

The technique has been successfully applied to radioimmunoassay by capturing specific antibodies onto SPA beads either through a secondary species specific antibody or protein A beads. Radiolabelled antigen binding may then be detected easily without the requirement for separating bound and free antigen. The SPA technique therefore replaces the requirement for charcoal or PEG precipitations.

For the assay of receptor-mediated binding events, an SPA bead has been designed which is coated with the lectin wheat germ agglutinin (WGA). This bead captures glycosilated cellular membranes and glycoproteins and has been used for a wide variety of receptor sources and cultured cell membranes. The receptor is immobilized onto the WGA SPA bead and a signal is generated on binding of an [^{125}I] or [^{3}H] labelled ligand. While the ligand is in free solution no signal is detected. As SPA is a homogeneous assay technology, it is possible to monitor in "real time" the binding event of interest without a need to

disturb the interaction with a separation step. SPA can therefore be used to monitor the time course of a binding event in "real time" by continuously monitoring a single assay.

All of these features of SPA make it an ideal rapid screening technology for all forms of combinatorial, chemical and natural product library screening programs.

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CHAPTER 5
AIM OF THE RESEARCH

5.1 To develop a prototype of high-throughput screening (HTS) based on SPA technology

One of the primary aims of this thesis was to develop a homogeneous high-throughput screening (HTS) assay for indentification of novel integrin antagonists for the treatment of ocular allergy, and to better understand the mechanisms of action of drugs that are already available.

Integrins are adhesion molecules ubiquitously expressed throughout the organism. Some of them are specifically expressed by immune cells and play pivotal roles in immune responses, directing leukocytes to the sites of a lesion or infection and helping their migration from the circulation to the injured tissues. Specifically, this thesis is focused on the actions of the $\alpha_4\beta_1$ integrin (VLA-4) in allergic inflammation. This integrin is expressed in activated leukocytes and interacts with its counter-receptor vascular cell adhesion molecule 1 (VCAM-1), enabling them to overcome the shear flow forces and localize at the level of inflammation. There is a series of undesired immune responses that, if non-treated, can result in harmful and permanent consequences; an example is the allergic inflammation. The study of compounds able to block cell adhesion mediated by VLA-4 could lead to the development of novel anti-inflammatory strategies. Therefore, an accurate and robust receptor-binding assay is highly desirable to screen a large collection of chemical entities. Scintillation proximity assay (SPA), because of its simplicity and high-throughput nature, has broad applications in measuring receptor-ligand interactions. Conventional filter-binding assays involve one or more separation steps and, thus, are laborious, non-homogeneous and not suitable for automation. SPA technology, on the other hand, provides a homogeneous screening approach that does not require post-reaction liquid handling steps and is well suited to automation.

In this study we propose a novel SPA method to assess specific binding properties of known selective VLA-4 ligands and of some anti-allergic drugs (such as levocabastine) indicated for the treatment of ocular allergy.

5.2 To better understand the role of integrins in ocular allergy

Once the ability of levocabastine – a second generation anti-histaminic agent – to bind to the integrins typically expressed in allergic eye disease was assessed, our research was aimed to demonstrate the contribution of this mechanism in the anti-allergic profile of this drug.

The ocular allergic response results from the exposure of the conjunctiva to an allergen. This event causes the mast cells to degranulate and release mediators such as histamine, prostaglandins and leukotrienes that induce itching, vasodilation and increased vascular permeability, that finally lead to tissue invasion of immune cells, particularly of eosinophils. Current guidelines for the treatment of ocular allergies advocate anti-histamines for occasional or intermittent symptoms, whereas in patients with moderate seasonal disease, and in patients with perennial disease, topical corticosteroids (with or without additional rescue anti-histamines) represents first-line treatment.

The movement of leukocytes from the blood into peripheral tissues plays a key role in immunity as well as in chronic inflammatory and allergic diseases. Therefore, pharmacological antagonism of VLA-4 is an attractive prospect for the treatment of predominantly eosinophil-mediated diseases such as allergic conjunctivitis.

In this study I investigated the ability of levocabastine to block the adhesion to immobilized VCAM-1 or to TNF-α activated HUVEC monolayers of human eosinophils or EoL-1 (human eosinophilic leukemia) cells.

Immune-modulation is coordinated by cytokines, which are chemical mediators with autocrine and paracrine effects that play a fundamental role in immune-modulation and in the perpetration of allergic inflammation. This thesis intends to ascertain whether levocabastine can modify the release of some eosinophilic cytokines that support allergic inflammation.

Finally, by means of an *in vivo* model of allergic conjunctivitis in guinea pig, the clinical symptoms of ocular allergy (redness, itching, swelling, lid reversion) was measured in animals treated and non-treated with levocabastine, in a time-dependent manner in order to

distinguish between the events involved in the early and the late phase responses. Excised conjunctivas were probed to weigh up the ability of levocastine to block eosinophil infiltration and/or activation.

CHAPTER 6
MATERIALS AND METHODS

6.1 Materials

Reagents

Levocabastine 0.05% solution eye drops containing cyclodextrins, without benzalkonium chloride; placebo containing cyclodextrins, without benzalkonium chloride; levocabastine 0.05% suspension eye drops containing benzalkonium chloride; levocabastine powder in a solution of sodium edetate, propylenglycol, disodium phosphate anhydrous, sodium dihydrogen phosphate monohydrate, hypermellose, polysorbate 80, purified water; BOL-303242 powder; BOL-303242-X emulsion; BOL-303242-X vehicle; were all provided by Bausch & Lomb (Rochester, NY, USA). Trimeton® (chlorpheniramine acetate) was obtained from Schering-Plough (Segrate, Italy). Decadron® (dexamethasone, 21 disodium phosphate) was obtained from Visufarma (Rome, Italy). Opatanol® (olopatadine hydrochloride) was from Alcon (Hemel Hempstead, UK). RPMI-1640 with L-glutamine, penicillin-streptomycin solution, AlexaFluor® 488 and 568 conjugated secondary antibodies, Hank's balanced salt solution, chloromethylfluoresceine diacetate (CellTracker Green CMFDA), G418, MagicMark™ XP western standard were purchased from Invitrogen (Carlsbad, CA, USA). PBS and FBS were purchased from Lonza Group Ltd (Basel, Switzerland). Recombinant human GM-CSF, soluble human vascular cell adhesion molecule 1 (VCAM-1) and soluble human tumour necrosis factor α (TNF-α) were purchased from R&D Systems (Minneapolis, MN, USA). IL-5, anti-CXCR4 antibody, ionomycin from *streptomyces conglobatus*, anti-β-actin antibody, lectin from *triticus vulgaris* (wheat germ agglutinin, WGA), calcimycin A-23187, fibronectin from human plasma, ovalbumin (OVA) grade V crystallized and lyophilized fraction, aluminium hydroxide [$Al(OH)_3$] gel, o-phenylendiamine, hydrogen peroxide (30%), triton-X-100, phorbol 12-myristate 13-acetate (PMA) and peroxidase acidic isoenzyme from horseradish, polyachrylamide/bispolyachrilamide, TEMED, ammonium persulphate, and sodium

dodecylsuphate (SDS) were obtained from Sigma-Aldrich (Steinheim, Germany). NE-PER™ extraction reagent, BCA protein assay, T-PER™ tissue protein extraction reagent were purchased from Pierce (Rockford, IL, USA). BIO1211 [(4-((2-methylphenyl)aminocarbonyl)acetyl-fibronectin CS-1 fragment (1980-1983))] was purchased from Bachem (Weil am Rhein, Germany). Monoclonal antibody mouse anti-human and rabbit anti-human α_4 subunit of human VLA-4 integrin were purchased from Calbiochem (Nottingham, UK) and Santa Cruz Biotechnologies Inc (Santa Cruz, CA, USA). Secondary antibody horseradish peroxidase-conjugated (goat anti-mouse and goat anti-rabbit), CruzMarker (sc-2035) and anti-annexin-1 antibody were purchased from Santa Cruz Biotechnologies Inc (Santa Cruz, CA, USA). Scintillation proximity assay (SPA) beads and Na[^{125}I] were obtained from Amersham Biosciences (GE Healthcare Europe) (Milan, Italy). Annexin-V-Fluos was obtained from Roche Applied Science (Monza, Italy). Protran™ nitrocellulose membrane was bought from Whatman® (Kent, UK). Anti-caspase-3 antibody was purchased from CellSignalling (Danvers, MA, USA). All plastic disposables were from Sarstedt (Verona, Italy) except form 96-well black clear bottom plates that were purchased from Corning Costar (Celbio, Euroclone) (Pero, Italy).

Cell lines

EoL-1, Jurkat clone E6.1, HEK293 cell lines and human umbilical vein endothelial cells (HUVEC) were obtained from Health Protection Agency Culture Collections (HPACC) (Salisbury, UK). HMC-1 cell line was a kind gift of Dr Pio Conti of the University of Chieti (Chieti, Italy). Human eosinophils, isolated from whole blood by density centrifugation followed by negative selection using immunomagnetic anti-CD16 beads (purity and viability were greater than 95%), were purchased from 3H Biomedical AB (Uppsala, Sweden) and cultured in complete cell culture medium (3H Biomedical AB) supplemented with IL-5 (1 ng/mL; R&D Systems) and granulocyte macrophages colony stimulating factor (GM-CSF) (10 ng/mL; R&D Systems) for 24 hours before being used in adhesion assays. The human T cell line Jurkat clone E6.1 was cultured in RPMI-1640 medium supplemented with L-glutamine, 10% heat-inactivated foetal bovine serum, antibiotic-antimycotic solution in a humidified incubator at 37 °C with 5% CO_2. EoL-1 (human eosinophilic leukemia) (Saito et al., 1985; Mayumi, 1992) and HMC-1 cell lines were maintained in RPMI-1640 medium with L-glutamine supplemented with 10% (v/v) FBS at

37 °C in a humidified atmosphere with 5% CO_2. All the lines were routinely grown in 75 cm^2 tissue culture flasks. Where indicated, 24 hours before the experiment 25 ng/mL PMA was added to the medium to induce eosinophil granulation and differentiation (Ohtsu et al., 1993; Zimmermann et al., 2000). Before steroidal treatment, EoL-1 cells were stabilized in serum-depleted medium (0.1% FBS) for 24 hours and then treated with dexamethasone (1 μM), levocabastine (1 mM), olopatadine (100 μM), BOL-303242-X (100 nM, 1 μM, or 10 μM), BOL-303242 (100 nM, 1 μM, or 10 μM) in a time-lapse between 24 and 96 hours to evaluate apoptosis or glucocorticoid-induced transactivation. Recombinant human GM-CSF (1 ng/mL) or IL-5 (1 ng/mL) were added at the beginning of incubation, where indicated for cytokine-mediated eosinophil survival assay. HEK293 cells stably transfected with the plasmid (pcDNA3.1, Invitrogen) containing the coding sequence for the α_4 integrin subunit gene (Fig. 6.1) were cultured in minimum essential medium (MEM) with L-glutamine, supplemented with 10% (v/v) FBS and non-essential amino acids, and kept at 37 °C in a humidified atmosphere (5% CO_2). Cells were transfected using a modified calcium phosphate procedure (Invitrogen) and selected with 400 μg/mL of G418 for at least three weeks. HUVEC were grown in endothelial cell growth medium (HPACC) and used within six passages in all experiments.

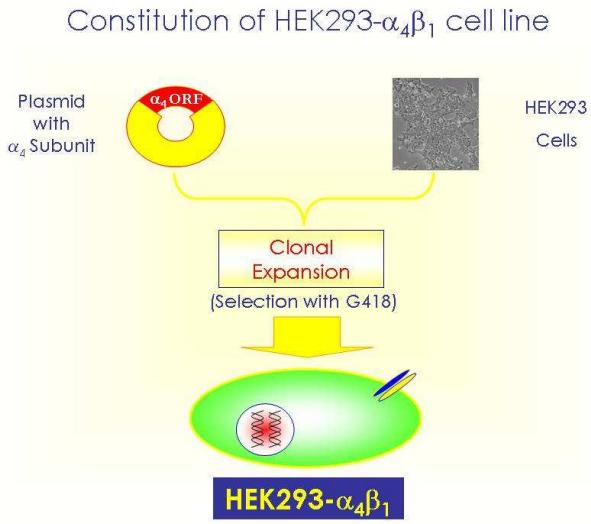

Figure 6.1
Descriptive image of the subcloning of the α_4 integrin subunit gene in naïve HEK293 cells. After transfection, cells were selected and expanded in the presence of G418 (400 μg/mL).

Animals

Male Dunkin-Hartley guinea pigs (250-300 g) were purchased from Charles-River Laboratories (Calco, Italy). Animals were handled in accordance with the Guiding Principles in the Care and Use of Animals Committee of the University of Bologna. Further, all experiments conformed to the ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research.

6.2 Methods

Cell dhesion assay

Adhesion of cultured cells was performed as described by Marcinkiewicz et al. (1996) (Fig.6.2). Briefly, 96-well plates were coated by passive adsorption overnight at 4 °C with 5 μg/mL of VCAM-1.

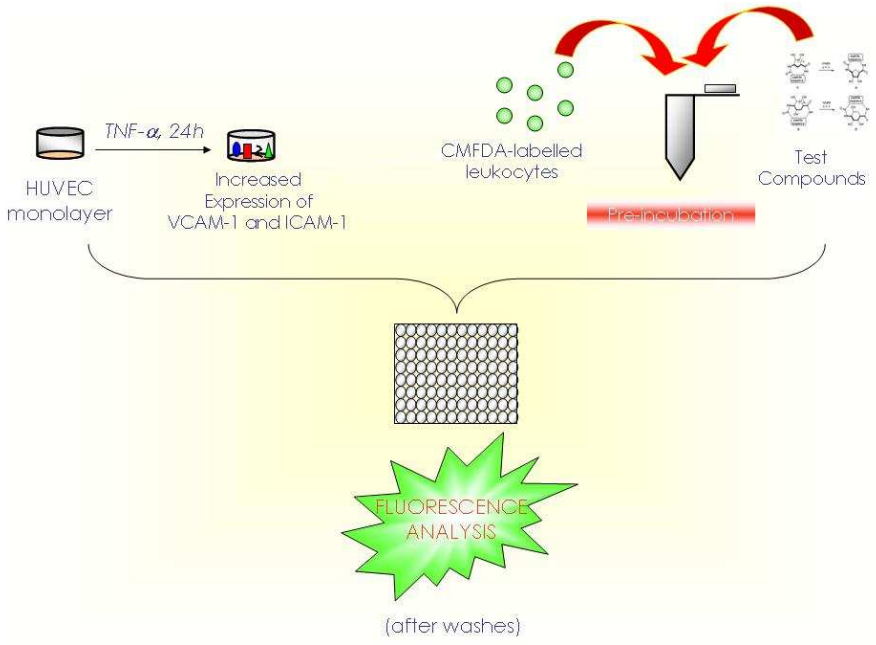

Figure 6.2
Schematic picture of a typical cell-adhesion assay. HUVEC cells (or human recombinant VCAM-1 or ICAM-1 can be adsorbed to the bottom of the well) are seeded and then treated for 12-24 hours with the pro-inflammatory cytokine TNF-α in order to make them express the adhesion molecules commonly expressed by the vascular endothelium during the inflammatory response. The day of the experiment, CMFDA-labelled leukocytes and test compounds are pre-incubated in microtubes to equilibrium, and then aliquoted in the wells with the activated-HUVEC monolayer. After washings with PBS to remove the non-specifically bound and unbound cells, the adherent leukocytes can be lysed to free the fluorescence that can be finally read.

A saturation curve for the ligand was achieved to establish the best signal-to-noise ratio (fig. 6.3). The non-specific hydrophobic binding sites were blocked by incubation with a 1% BSA/HBSS (w/v) solution for 30 minutes at 37 °C. On the day of the assay, the cells were counted and stained with 12.5 μM CMFDA for 30 minutes at 37 °C. After three rinses with HBSS/BSA in order to remove the excess of the dye, aliquots of 50000 cells were divided in a number of microcentrifuge tubes correspondent to the number of treatments.

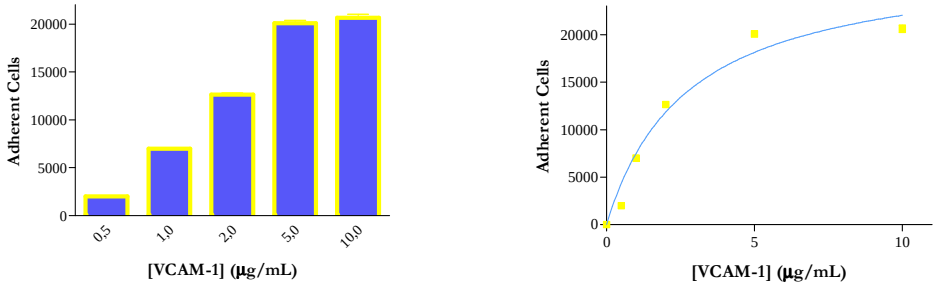

Figure 6.3
Saturation curve of EoL-1 adhesion to a 96-well plate coated with increasing concentration of VCAM-1, ranging from 0.5 μg/mL to 10 μg/mL. On the left the bar chart is shown, whereas the saturating hyperbola obtained by non-linear regression is displayed on the right. Each experiment was performed with eight replicates per point. The mean of three experiments was charted.

For inhibition experiments, cells were mixed with the drug and pre-incubated at 37 °C for 30 minutes to reach the equilibrium before being plated. After 30 minutes incubation at 37 °C in the coated wells, the non-specifically bound cells were washed away with the same HBSS/BSA solution. Bound cells were lysed by addition of a solution of PBS and Triton-X-100 0.5% (v/v) solution for 30 minutes at 4 °C. Fluorescence was measured by Victor^2 Wallac-1420 Multilabel Counter (Perkin Elmer) (Waltham, MA, USA) at excitation 485 nm/emission 535 nm, and the number of adherent cells was determined by interpolation with the standard curve. The efficacy of putative antagonists is determined by the decrement of adherent cells as compared to the control. Each experiment was performed in quadruplicate and the presented data represent the mean ± SEM of at least three independent assays.

Another version of the assay was performed with HUVEC cells, placed at the bottom of the wells, instead of the immobilized VCAM-1 ligand. 20000 HUVEC cells/well were grown to confluence in a 96-well plate and exposed for 12 hours with TNF-α which is a pro-inflammatory cytokine that induces the expression adhesion molecules typical of the

inflammatory process such as VCAM-1 and ICAM-1. Then, CMFDA-labelled EoL-1 cells (100000 cells/well) for 30 minutes at 37 °C in 5% CO_2 atmosphere. Integrin antagonists or anti-α_4 integrin subunit antibodies were administered to EoL-1 cells before their addition to HUVEC. After two hours incubation non-adhering EoL-1 cells were removed by gentle aspiration and wash with PBS. Cells were then lysed, and the signal was read as described above.

Flow cytometry analysis

FACS analysis were performed to characterize the specificity of action of the drugs to the integrin receptors. Jurkat clone E6.1 or EoL-1 cells (10^6 cells/sample) were incubated with a range of concentrations of the purported antagonist at 37 °C for 30 min in 1% BSA/HBSS. The excess of unbound drug was washed away and, then, all samples (except the negative controls) were incubated with a saturating concentration of the primary antibody anti-α_4 integrin subunit at 4 °C for 30 min. After two washes, cells were incubated with FITC-conjugated secondary antibody for 45 minutes in the dark. Finally, the excess of the unbound antibody was removed and the cells were resuspended in HBSS/BSA buffer, ready to be analysed in a EPICS® ELITE (Beckman Coulter®) flow cytometer (Marcinkiewicz C. et al.; 1996).

Moreover, FACS analysis were performed to measure the induction of surface expression of annexin I and the CXCR4 as a measure for transactivation. Eol-1 cells were double-stained with a red dye-conjugated secondary antibody to trace the variations in the expression of CXCR4 and annexin I, and a green dye-conjugated annexin V to exclude apoptotic cells from the analysis. The cells were counted and transferred to a 24-well plate (10^6 cells/well) where they were serum-starved (0.1% FBS) for 24 h. The cells were then exposed to dexamethasone, BOL-303242, levocabastine, olopatadine, and their vehicles, alone or in combination, for 24 or 72 h at 37 °C and 5% CO_2 atmosphere. At the end of the incubation time, the cells where harvested and each sample was divided into two tubes to perform a parallel test for annexin I and CXCR4 surface expression analysed by flow cytometry. After rinsing all samples with a HBSS solution with 1% BSA, the cells were incubated for 45 min on a shaker with anti-annexin I or anti-CXCR4 antibodies (1:200) on ice, with the exception of the negative control. The cells were, then, spun down and washed twice with the HBSS/BSA buffer before being exposed to the AlexaFluor® 568-conjugated

secondary antibody. The excess of unbound antibody was washed away, and all samples were incubated for 15 min in the presence of annexin-V-Fluos in a buffer and a dilution suggested by the manufacturer, as previously described. The cells were then rinsed and resuspended in HBSS/BSA buffer, ready to be analysed in a Biorad Bryte HS flow cytometry system (Biorad, UK). Electronic gates were set on annexin V negative cells and CXCR4 or annexin I positive cells.

Determination of eosinophil apoptosis

In order to assess glucocorticoid induced apoptosis and cytokine-induced eosinophil survival, EoL-1 cells were double-stained with annexin V-Fluos and propidium iodide. Annexin V-FLUOS was used according to manufacturer's instructions. Briefly, the cells were washed in PBS and suspended in Annexin V-Fluos labeling solution (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) added of propidium iodide (PI) (1 µg/mL). The cell suspension was incubated at room temperature for 10 min and analysed in a Biorad Bryte HS flow cytometry system (Biorad, UK). EoL-1 cells were gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. Apoptotic cells were defined as $Annexin^+/PI^-$ cells. A two-way dot plot was obtained to assess the percentage of apoptotic cells. $Annexin^-/PI^-$ cells were used as control, whereas $annexin^+/PI^+$ were considered necrotic.

Western blotting analysis

Eol-1 cells were pelleted and resuspended in 100 µL of CER I buffer (NE-PER™ Extraction reagent; Pierce). After 10 min incubation on ice, 5.5 µL of CER II buffer was added and the suspension was resuspended by vortex, incubated on ice for 1 min and then resuspended. The cytoplasmic fraction was separated by centrifugation at 16000 g for 5 min. The protein content was quantified using a BCA protein assay (Pierce). Proteins of the cytoplasmic extract (50 µg) were denatured at 95 °C for 3 min before being loaded and separated by 15 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We used a MagicMark™ XP Western Standard as molecular weight standard. Proteins were then transferred to Protran™ nitrocellulose membranes which were blocked with 5% non-fat milk in TBS (10 mM Tris-HCl, pH 8, containing 150 mM NaCl) plus 0.1% Tween 20 for 1 h at room temperature (25°C). The blots were then probed overnight at 4°C in TBS

containing 0.1% Tween 20, 5% non-fat milk and antibodies with a dilution of 1 : 1000 for caspase-3 monoclonal antibody, 1 : 5000 for β-actin antibody (used as loading control for cytoplasmic lysates). The membranes were incubated with peroxidase-conjugated secondary antibodies at a dilution of 1 : 2000. Blots were finally developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol (Pierce). Chemiluminescence was acquired using a luminescent image analyzer LAS-3000 (Fuji-Film).

Excised conjunctivas of immunized and non-immunized animals were homogenized in T-PER® reagent (Pierce) and protein were extracted following the manufacturer's protocol. The protein content was quantified using a BCA protein assay (Pierce). Proteins of the cytoplasmic extract (150 μg) were denatured at 99 °C for 4 min before being loaded and separated by 7.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We used a Cruz marker (sc-2035; Santa Cruz Biotechnology, Inc.) as molecular weight standard. Proteins were then transferred to ProtranTM nitrocellulose membranes (Schleicher & Schuell Italia, Legnano, Italy) which were blocked with 5% non-fat milk in TBS (10 mM Tris-HCl, pH 8, containing 150 mM NaCl) plus 0.1% Tween 20 for 1.5 h at room temperature (25°C). The blots were then probed for 1.5 h at room temperature in TBS containing 0.1% Tween 20, 5% non-fat milk and antibodies with a dilution of 1 : 800 for anti-VLA-4 integrin monoclonal antibody, 1 : 5000 for anti-β-actin antibody (used as loading control for cytoplasmic lysates). The membranes were incubated with peroxidase-conjugated secondary antibodies at a dilution of 1 : 10000. Blots were, finally, developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol (Pierce). Chemiluminescence was acquired using a luminescent image analyzer LAS-3000 (Fujifilm).

Scintillation Proximity-Binding Assay (SPA)

Fibronectin from human plasma (FN) was labeled with Na[^{125}I] as described by the manufacturer. The experiments were carried out with a ratio of 1 mg/50 μL anti-rabbit coated beads, 200 μg rabbit anti-VLA-4 antibody and 100 μg of VLA-4 receptor, extracted from HEK-293 cells stably expressing VLA-4, and purified by affinity chromatography following the procedure outlined by Solorzano et al. (2006) (fig. 6.4). The binding buffer was composed of Tris HCl 25 mM pH 7,5; $CaCl_2$ 1 mM; $MgCl_2$ 1 mM; $MnCl_2$ 1 mM; BSA 2% (w/v); PMSF 1 mM; aprotinin 1 μg/mL; leupeptin 50 μM. Firstly, we allowed the slow

interaction between the VLA-4 proteins and the anti-VLA-4 antibodies, incubating them together for 1 h at 4 °C; then the anti-rabbit antibody binding beads were added and the solution with the three components were incubated for 2 h at 4 °C in the dark. Beyond this point all incubations were conducted at room temperature. [^{125}I]FN was added to the vials (at least 10^5 cpm/tube) that were then incubated overnight on a shaker in the dark. Non-specific binding was determined in the presence of 100 μM Bio1211. The samples were read with a LS 6500 multipurpose scintillation counter (Beckham Coulter, Fullerton, CA).

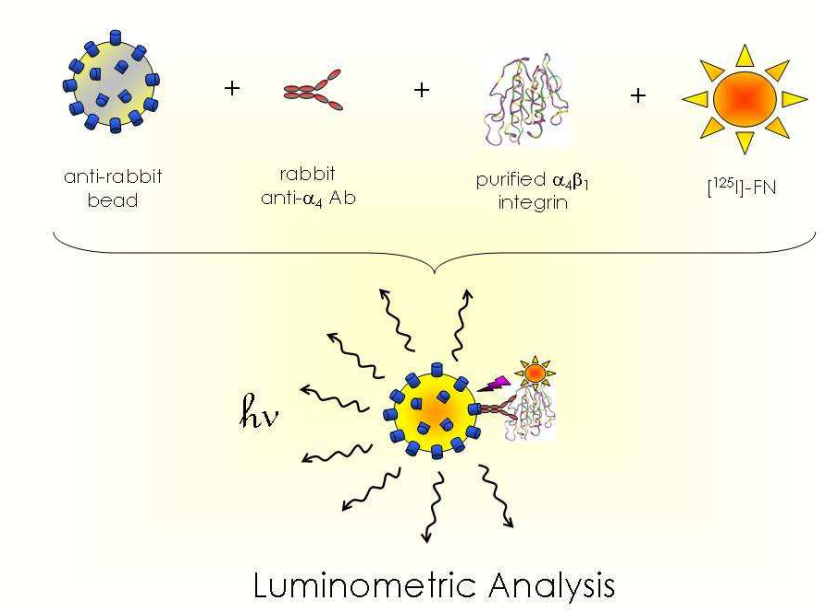

Figure 6.4
Scheme of the scintillation proximit assay (SPA) employed. α_4 transfected HEK293 cells represented our source of $\alpha_4\beta_1$ integrin which was possible to purify by affinity chromatography on a WGA column (see materials and methods)

Cytokine Assay

Half-million cells were aliquoted per point in a 24 well plate; each experiment was performed in triplicate and carried out in parallel with eosinophils differentiated and non differentiated with PMA (25 ng/mL, 24 h). Cells were suspended in low serum medium (RPMI-1640, 0,1% (v/v) FBS). TNF-α was used to induce cytokine secretion as previously described by Steube et al. (2000), and the net release was obtained by comparison to the basal (non-TNF-α treated). In the first experiment we evaluated the concentration and time

relation between TNF-α stimulation and cytokine release. Therefore, 5, 10, and 25 ng/mL of TNF-α was administered to the cells at 0, ½, 1, 2, 3, 6, 12 and 24 h (data not shown). An aliquot of 150 μL of supernate was collected for cytokines' analysis. Then, we considered whether levocabastine, an anti-allergic drug that demonstrated to be active not only as an H_1 receptor antagonist, could affect the release of these cell mediators. Differentiated and non-differentiated EoL-1 cells were exposed from 0,1 to 2,3 mM levocabastine, and aliquots of the supernates were collected after 12 and 24 h. The content of cytokines at time zero was estimated by treating the cells with 400 nM calcimycin (A-23187), which is a cytolytic agent that frees all the mediators from the cytoplasmic compartment. In addition, for each experiment we tested the effect of the vehicle, that was added to the wells in a concentration equal to the maximum amount used for drug dilution. Samples (supernates 12 and 24 h) in triplicate were shipped to Bausch & Lomb Optical Centre (Rochester, NY, USA) and cytokine levels measured using Luminex technology. Half-million cells were aliquoted per point in a 24 well plate; each experiment was performed in triplicate.

Animal experiments

The methods described by Khosravi E. et al. (1995) and Ebihara N. et al (1999) were applied. 20 male Dunkin-Hartley guinea pigs were divided into 3 groups of five animals: a control group (no immunization), a group immunized with ovalbumin i.p. (positive control) and a group pre-treated with levocabastine eyedrops. At day zero, 2 mL of a saline solution with 100 □g/mL ovalbumin (OVA) and 20 mg/mL $Al(OH)_3$ as adjuvant, were injected intra-peritoneally to each animal except for the negative control. Three weeks later all animals were challenged with 30 □L per eye of saline solution containing 100 mg/mL ovalbumin. Levocabastine was administered to the animals 60 and 30 minutes prior to the ovalbumin challenge. Pictures of both eyes were taken for the clinical score at 1, 2, 4, 6, and 24 hours after topical ovalbumin administration. The animals were sacrificed and the conjunctivas collected for the subsequent experiments.

32 male Dunkin-Hartley guinea pigs were divided into 8 groups of 4 animals: control group (no immunization), group immunized with ovalbumin i.p. (positive control), dexamethasone treated animals, BOL-303242-X group, BOL-303242-X/levocabastine

group, BOL-303242-X/olopatadine group, dexamethasone/levocabastine group, dexamethasone/olopatadine group. At day zero, 2 mL of a saline solution containing 100 μg/mL ovalbumin (OVA) and 20 mg/mL $Al(OH)_3$ as adjuvant, were injected intra-peritoneally (i.p.) to each of the animals, except for the negative control. Three weeks later all of the animals were challenged with 30 μL per eye of saline solution, containing 100 mg/mL ovalbumin. All the drugs were administered to the animals 60 and 30 minutes prior to the ovalbumin challenge. Pictures of both eyes were taken for the clinical score at 1, 2, 4, and 6 hours after topical ovalbumin administration. The animals were sacrificed and the conjunctivas collected for the subsequent experiments.

Eosinophil peroxidase assay

Eosinophil peroxidase assay was performed as previously described by Izushi K. et al.; 2002. Briefly, 24 h after conjunctivitis induction by antigen application, the guinea pigs were sacrificed by i.p. injection of Tanax® and the upper and lower parts of the conjunctiva were excised, cleaned, weighed and washed twice with ice-cold PBS. The tissues were homogenized with 50 mM Tris-HCl buffer (pH 8.0) using a Potter-Elvehjem glass/Teflon homogeniser (Wheaton, Millville, NJ, USA) on ice. The homogenates were placed in ice bath for 1 hour after addition of 350 μl of 50 mM Tris-HCl buffer and 150 μl of 0.1 % Triton X-100. Then, the substrate solution (400 μl of 50 mM Tris-HCl buffer containing 0.1 % Triton X-100, 1 mM o-phenylenediamine, and 0.5 mM hydrogen peroxide) was added to 200 μl of the sample and incubated at 37 °C for 10 minutes. The reaction was stopped with 200 μl of 4 M H_2SO_4. Absorbance at 490 nm was measured using a spectrophotometer (JASCO V-530, Jasco, UK). A standard curve was prepared using different concentrations of peroxidase diluted in 50 mM Tris-HCl buffer (pH 6.0) containing 1 mM o-phenylenediamine, and 0.5 mM hydrogen peroxide. Eosinophil peroxidase (EPO) activity was measured according to the method of Strath et al. (1985) which is based on the oxidation of o-phenylenediamine by EPO in the presence of hydrogen peroxide. One unit corresponds to one mmol of hydrogen peroxide decomposed in 10 minutes. Data are report as eosinophil peroxidase level (mU of enzyme/mg tissue).

Histological examination of eosinophil infiltration in conjunctival tissues

Twenty-four hours after antigen challenge, guinea pigs were sacrificed and the upper and lower parts of the eye conjunctiva were carefully excised and fixed in 10 % buffered paraformaldehyde solution. Samples were manipulated as follows: paraffin-embedded sections, 6 µm thick, carried out perpendicularly to the conjunctiva palpebral. Serial sections were stained with Hansel method modified to show the number of infiltrating eosinophils, which appear red. (Sheldon, Lovell and Mathews "A Manual of Clinical Allergy" published by W B Saunders Company, 1967).

Eosinophil peroxidase assay was performed as previously described by Izushi K. et al.; 2002: 24 h after conjunctivitis induction by application of the antigen, the guinea pigs were sacrificed by i.p. injection of Tanax®, and the upper and lower parts of the conjunctiva were excised, cleaned, weighed and washed twice with ice-cold PBS. The tissues were homogenized with 50 mM Tris-HCl buffer (pH 8.0) using a Potter-Elvehjem glass/Teflon homogeniser (Wheaton, Millville, NJ, USA) on ice. The homogenates were placed in ice bath for 1 hour, after addition of 350 µL of 50 mM Tris-HCl buffer and 150 µL of 0.1 % Triton X-100. Then, the substrate solution (400 µL of 50 mM Tris-HCl buffer containing 0.1 % Triton X-100, 1 mM *o*-phenylenediamine, and 0.5 mM hydrogen peroxide) was added to 200 µL of the sample and incubated at 37 °C for 10 minutes. The reaction was stopped with 200 µL of 4 M H_2SO_4. Absorbance was measured, using a spectrophotometer (JASCO V-530, Jasco, UK), at 490 nm.

Data analysis

To quantify the intensity of the bands image AIDA (Advanced Image Data Analyzer) software (Raytest) was used. All data are presented as mean ± SEM for the indicated number of experiments. Statistical significance was determined by Newman-Keuls test after ANOVA using GraphPad Prism (version 3.0; GraphPad Software Inc., San Diego, CA, USA). P-values < 0.05 were considered to be significant.

For the clinical score, each group was formed of five animals and the score was performed on changes at two hours after ovalbumin challenge for the main eye symptom sum score of itching, swelling, redness, and lid eversion. An analysis of variance (ANOVA) was performed using treatment as factor. The Newman-Keuls post hoc test was used to compare all pairs of groups.

To quantify the intensity of the bands an image AIDA (Advanced Image Data Analyzer) software (Raytest) was used. All data are presented as mean ± SEM, for the indicated number of experiments. Statistical significance was determined by Friedman test, after ANOVA, using GraphPad Prism (version 3.0; GraphPad Software, Inc., San Diego, CA, USA). P-values < 0.05 were considered to be significant.

For the clinical score, each group was formed of 4 animals and the score was performed on the changes that occurred at 1, 2, 4 and 6 hours after receipt of ovalbumin challenge. For the main eye symptom, a sum score of itching, swelling, redness, and lid eversion was used. An analysis of variance (ANOVA) was performed using treatment as factor, and the Dunnet's post hoc test was used to compare all pairs of groups.

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CHAPTER 7
RESULTS

7.1 Scintillation proximity assay optimization

For the setting up of the scintillation proximity assay (SPA) was initially performed a saturation curve for the [^{125}I]FN, labelled in our laboratory as described in detail in Appendix III.

Fibronectin from human plasma (FN) was labeled with Na[^{125}I] as described by the manufacturer (Pierce; Rockwell, IL, USA). The experiments were carried out with a ratio of 1 mg/50 µL anti-rabbit coated beads, 200 µg rabbit anti-VLA-4 antibody and 100 µg of VLA-4 receptor, extracted from HEK-293 cells stably expressing the VLA-4 integrin (see Appendix III for details), and purified by affinity chromatography (WGA column) following the procedure outlined by Solorzano et al. (2006). To ascertain the presence of $\alpha_4\beta_1$ integrin dimer, the fractions obtained were probed with both an anti- α_4 and anti-β_1 antibodies in a western blot analysis (fig. 7.1).

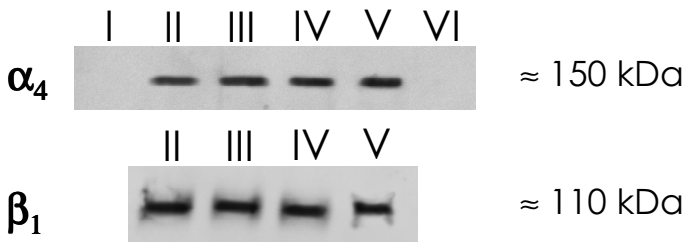


Figure 7.1
Western blot analysis on the fractions obtained by affinity chromatography purification of the $\alpha_4\beta_1$ integrin extracted from HEK293 cells stably expressing this protein. The fraction I clearly represents the empty volume of the column, in which no protein is detected. The fractions from II to V demonstrate the presence of both of the subunits of the dimmer, whereas the lane number VI is empty again because the protein had been already completely eluted.

Of the six collected fractions the presence of the α_4 subunit was confirmed in all except the first (void elution volume) and the last. The fraction from II to V were then assayed for the presence of the β_1 integrin subunit. Approximate molecular masses of the

α_4 and β_1 integrin subunits were compared to molecular mass standard, demonstrating similarities to the ones described by Takada et al. (1989).

After assessing the presence of the whole dimer, the fractions II-V were employed in a saturation binding by scintillation proximity assay (fig. 7.2).

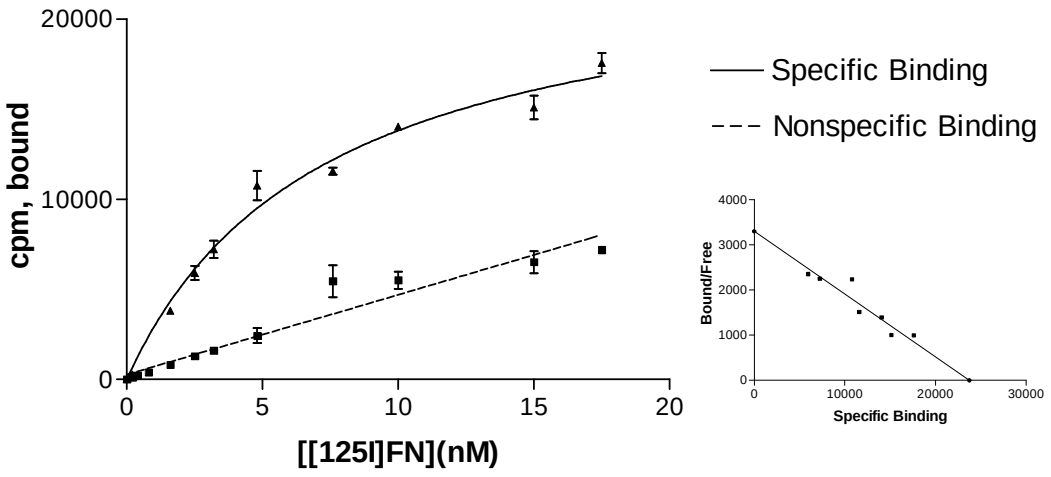


Figure 7.2
Saturation SPA binding of [^{125}I]FN to the purified $\alpha_4\beta_1$ integrin extracted and purified from HEK293 stably ex pressing this protein. The non-specific binding was determined in the presence of 100 μM BIO1211. (■) Non-specific binding; (▲) specific binding. Mean and ± SEM of three experiments are shown. As displayed the binding was concentration dependent and saturable. At the bottom right there is the Scatchard plot that allowed the calculation of the Kd (and Bmax.

The specific binding of ^{125}I-fibronectin to an SPA bead-associated VLA-4 integrin was time-dependent; the signal increased during the first 10 h then reached a plateau that remained constant for the remainder of the 24-h incubation time (data not shown). [^{125}I]FN was capable to bind to the anti-rabbit bead – anti-VLA-4 Ab – VLA-4 integrin complex in concentration dependent and saturable manner. The non-specific binding was evaluated in the presence of 100 μM BIO1211; it was non-saturable and directly proportional to [^{125}I]FN concentration. This technique had been extremely valuable for the possibility to set up the entire binding system in the reaction tube without the necessity to wash away the unbound. In fact, SPA is based on the principle of proximity: only if the radioligand is sufficiently close and for long enough to the microbead, its energy activate the scintillant leading to light emission. Because the emitted β particles or electrons can only travel short distances in the bulk solution, the microbead preferentially captures electrons from the bound

radiolabelled ligands. Finally, the amount of emitted light from the scintillant in the bead is directly proportional to the amount of radiolabelled ligand. Therefore, we might consider this novel method as "**one pot**" with no need of filtering passages and whose signal can be recorded either with a scintillation counter used as a luminometer or as a luminometer itself. Also the format can be reduced to multi-well plates and because of its predisposition to automatization SPA is extremely suitable for high-throughput screening. These assays give both qualitative and quantitative information. An adequate amount of beads is required to saturate the protein in order to achieve a maximal binding signal. However, excessive beads will lead to undesired exposure of bead surface to non-specific ligand binding. Therefore, the amount of bead applied should be kept to a minimum where protein saturation is still achievable. On the other hand, stable signal is highly dependent on protein stability and its coating ability on the beads. In comparison with conventional filter binding techniques, the SPA method omits steps such as pre-coating, pre-incubation, separation and washing, thus simplifying the assay protocol, mitigating labour intensity and reducing systemic errors.

7.2 SPA evaluation of levocabastine affinity to $\alpha_4\beta_1$ integrin

After SPA optimization and understanding of the binding characteristics through a saturation binding assay, the next step has been to try the functionality of this technique by the means of a competition binding with levocabastine. Levocabastine is a known antagonists of the H1 histamine receptor, indicated for the treatment of allergic conjunctivitis. Buscaglia et al. (1996) demonstrated that this compound is able to interfere with inflammatory adhesion molecules such as ICAM-1, widening the pharmacological profile of what was considered a simpli anti-histaminic. Therefore, we tried to appreciate direct competition of levocabastine to the binding to VLA-4 (fig. 7.3). It was learned from Gobel et al. (1999) that assay sensitivity can be improved by choosing a radiolabeled ligand concentration at or below its Kd to permit effective competition by an unlabelled ligand. In this study we selected a relatively higher concentration of [^{125}I]FN (able to produce 100000 cpm) in order to keep a high level assay sensitivity. Levocabastine caused concentration dependent inhibition of [^{125}I]FN binding to the SPA bead-associated $\alpha_4\beta_1$ integrin with an IC_{50} of 406.2 μM.

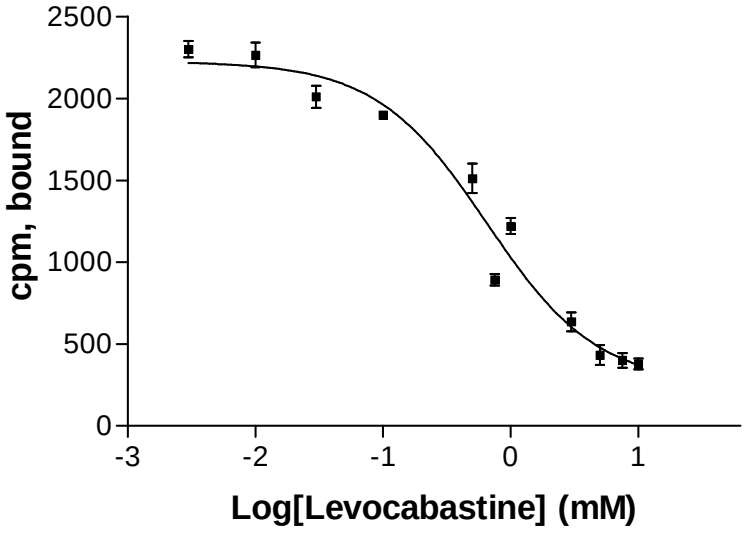


Figure 7.3
Competition binding of [^{125}I]FN and levocabastine to the VLA-4 integrin. Binding of [^{125}I]-FN to HEK-293 cell lysates was measured in the presence of increasing concentrations of levocabastine (0.003 – 10 mM). Non-specific binding was determined in the presence of 100 μM unlabeled Bio1211. Levocabastine displaced [^{125}I]FN binding to VLA-4 in a concentration dependent way (IC_{50}). The graph shows the mean obtained by three independent experiments. ± SEM is represented.

7.3 Levocabastine inhibition of EoL-1 and Jurkat E6.1 cell adhesion *in vitro*

Levocabastine is a potent anti-histaminic commonly used in ophthalmology for the treatment of allergic conjunctivitis. After the promising results obtained by SPA that confirmed the interaction of levocabastine with $\alpha_4\beta_1$ integrin, by the use of adhesion assays both on TNF-α-activated HUVEC cells and the adsorbed ligand VCAM-1, it was possible to perform a functional assay on EoL-1 cells. The results obtained were compared to the actions of chlorpheniramine, another anti-histaminic (fig. 7.4).

EoL-1 cells express the VLA-4 integrin (Higashimoto et al., 1996), and their exposure to increasing concentrations of levocabastine proved a dose-dependent decrease of adhesion to the immobilized ligand VCAM-1 (5 μg/mL). From the sigmoidal curve drew, it was possible to calculate an IC_{50} of 456.9 μM (fig. 7.4, panel A). Chlorpheniramine (fig. 7.4, panel B), instead, did not demonstrate a similar behaviour: the curve obtained is not convergent and it is, thus, not possible to calculate an IC_{50}.

Another line of leukocytes, Jurkat E6.1 cells, was assayed in the same way revealing an analogous behaviour (fig. 7.5, panel A and B).

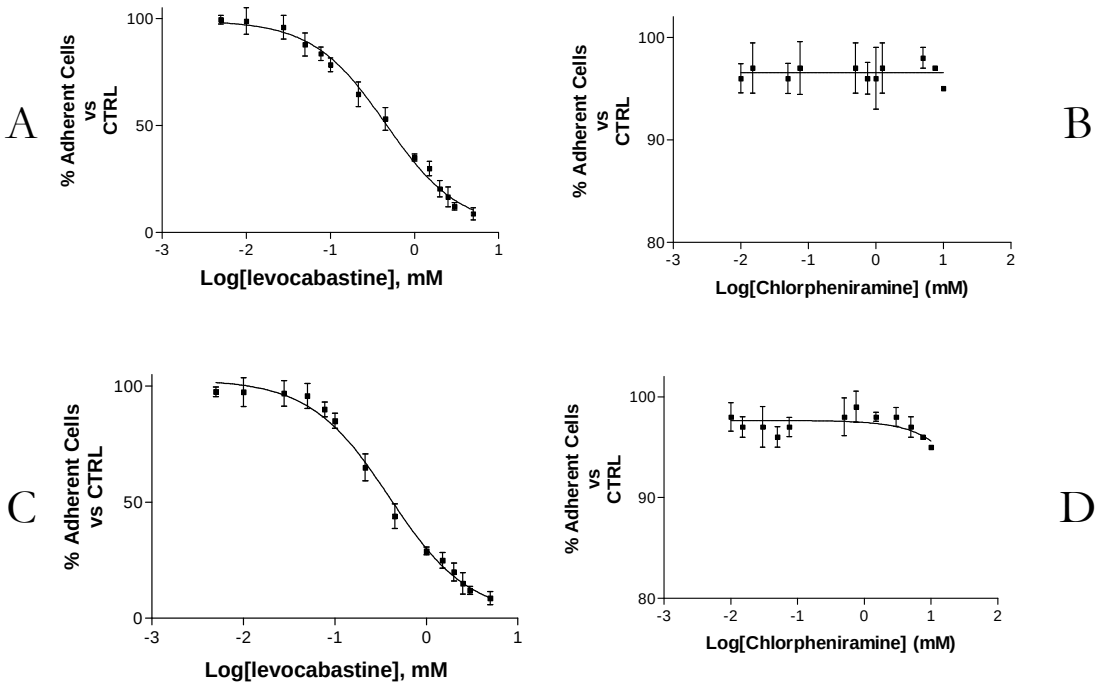


Figure 7.4
Dose-response curves of levocabastine and chlorpheniramine on EoL-1 and Jurkat E6.1 cell adhesion to VCAM-1 (5 μg/mL). Panel A: Levocabastine shows a concentration-dependent inhibition of EoL-1 cell adhesion to the immobilized ligand VCAM-1. The measured IC_{50} is 456.9 μM. Panel B: The anti-histaminic chlorpheniramine shows no activity on the adhesion of EoL-1 to VCAM-1. Panel C: Non-linear regression of the data of Jurkat E6.1 cell inhibition of adhesion mediated by increasing concentrations of levocabastine. (IC_{50} = 395.6 μM). Panel D: Chlorpheniramine shows not to be able to block Jurkat E6.1 cells to VCAM-1. Also in this case, the curve drew is not convergent. The fluorescence intensity of untreated cells adherent to VCAM-1 (positive control) was considered as 100%; non-specific adhesion was measured in control wells non-VCAM-1-coated and subsequently subtracted. The curve obtained is not convergent and it is impossible to calculate an IC_{50}. The data shown are the mean ± SEM of three independent experiments.

Moreover, Kwon et al. (2007) reported that HUVEC do not express VCAM-1 in the resting state, but the expression of this adhesion molecule is increased after exposure to TNF-α. Therefore, we investigated whether levocabastine influenced EoL-1 adhesion to TNF-α-stimulated HUVEC. As shown in figure 7.5, the number of EoL-1 cells adherent to HUVEC significantly increased after 12 hours exposure to TNF-α (25 ng/mL). Levocabastine (100 and 400 μM) reduced this increment in a concentration-dependent manner, as did the anti-α_4 integrin subunit antibody.

We therefore conclude that levocabastine, but not chlorpheniramine, includes the blockade of VCAM-1-mediated leukocyte adhesion to endothelial cells among its mechanisms of action.

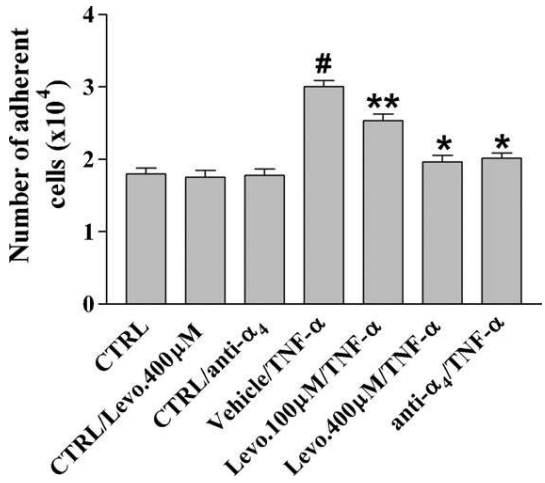


Figure 7.5
Levocastine and an anti-α_4 integrin subunit antibody reduce TNF-α-induced EoL-1 binding to HUVEC. HUVEC were treated for 12 hours with TNF-α (25 ng/mL) or with the vehicle alone (CTRL). EoL-1 cells pre-treated for 1 hour to the vehicle, levocabastine (100 or 400 μM) or an anti-α_4 integrin subunit antibody (5 μg/mL) and incorporated with CMFDA were co-cultured with HUVEC for 30 minutes. In control cells, levocabastine or anti-α_4 antibody did not influence EoL-1 cell adhesion. On the contrary, HUVEC pre-treatment with TNF-α caused EoL-1 firm adhesion. The data shown represent the mean ±SEM of three independent experiments performed in quadruplicate each. #$p<0.001$ vs CTRL; **$p<0.01$ vs vehicle/TNF-α-stimulated HUVEC; *$p<0.001$ vs vehicle/TNF-α-stimulated HUVEC.

Furthermore, we ascertained that the adhesion of human eosinophils (2 x 10^4 cells were added to each well) to VCAM-1 ranged from 0.9 to 2.2 x 10^4 cells. The adhesion of EoL-1, Jurkat E6.1 and human eosinophil cells to VCAM-1 (10 μg/mL) was inhibited by more than 92% after pre-treatment with 5 μg/mL anti-α_4 integrin subunit antibody and adhesion of Jurkat E6.1 and EoL-1 cells to fibronectin (10 μg/mL) was inhibited by 92% in the presence of this antibody. Neither drug up to 2 mM influenced cell viability, evaluated by trypan blue exclusion (data not shown). As shown in Table 7.0, levocabastine inhibited $\alpha_4\beta_1$ integrin-dependent adhesion of Jurkat E6.1 cells to VCAM-1 with an IC_{50} of 395.6 μM, and the adhesion of EoL-1 cells with an IC_{50} of 403.6 μM. Moreover, levocabastine inhibited adhesion of human eosinophils to VCAM-1 coated wells (IC_{50} = 443.7 μM). Levocabastine displayed a similar activity to inhibit $\alpha_4\beta_1$ integrin-dependent adhesion of Jurkat E6.1 and EoL-1 cells to fibronectin and also prevented $\alpha_L\beta_2$ integrin-

dependent adhesion of Jurkat E6.1 cells to ICAM-1 (IC_{50} = 33.3 μM). In contrast, chlorpheniramine, a potent blocker of H_1 histamine receptors (Sharif et al., 1996), did not affect Jurkat E6.1 cell adhesion to VCAM-1, fibronectin and ICAM-1 (Table 7.0).

Finally, we demonstrated that in these experimental conditions, the small peptide BIO-1211 behaves as a potent $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin antagonist that inhibited Jurkat E6.1 cell adhesion to VCAM-1, fibronectin and ICAM-1 coated wells (Table 7.0).

Table 7.0

Integrin-mediated Cell Adhesion	IC50 (mM)
Levocabastine	
$\alpha_4\beta_1$-mediated Cell Adhesion	
Jurkat/VCAM-1	*395,6*
EoL-1/VCAM-1	*403,9*
Human Eosinophils/VCAM-1	*443,7*
Jurkat/FN	*350,7*
EoL-1/FN	*360,6*
$\alpha_L\beta_2$-mediated Cell Adhesion	
Jurkat/ICAM-1	*33,3*
BIO-1211	
$\alpha_4\beta_1$-mediated Cell Adhesion	
Jurkat/VCAM-1	*$8{,}6 \times 10^{-3}$*
EoL-1/VCAM-1	*$7{,}2 \times 10^{-3}$*
Jurkat/FN	*$7{,}5 \times 10^{-3}$*
EoL-1/FN	*$6{,}6 \times 10^{-3}$*
$\alpha_L\beta_2$-mediated Cell Adhesion	
Jurkat/ICAM-1	*$8{,}4 \times 10^{-5}$*
Clorpheniramine	
$\alpha_4\beta_1$-mediated Cell Adhesion	
Jurkat/VCAM-1	*not active*
Jurkat/FN	*not active*
$\alpha_L\beta_2$-mediated Cell Adhesion	
Jurkat/ICAM-1	*not active*

7.4 Flow cytometric analysis of levocabastine-VLA-4 interaction

To prove the specificity of action of levocabastine on the VLA-4 integrin receptor, flow cytometry experiments were carried out on Jurkat E6.1 cells. Cells were pre-incubated with several concentrations of levocabastine and, then, exposed to an anti-VLA-4 primary antibody to verify the ability of the drug to compete with it to the binding to VLA-4. After a series of washings to eliminate the excess of unbound primary antibody, a fluorescein isothyocyanate-conjugated secondary antibody was added. As shown in fig. 7.6, levocabastine is able to shift the fluorescence peak to lower values depending on the increase of levocabastine concentration. In each image is reported the negative control, determined in the presence of the only secondary antibody.

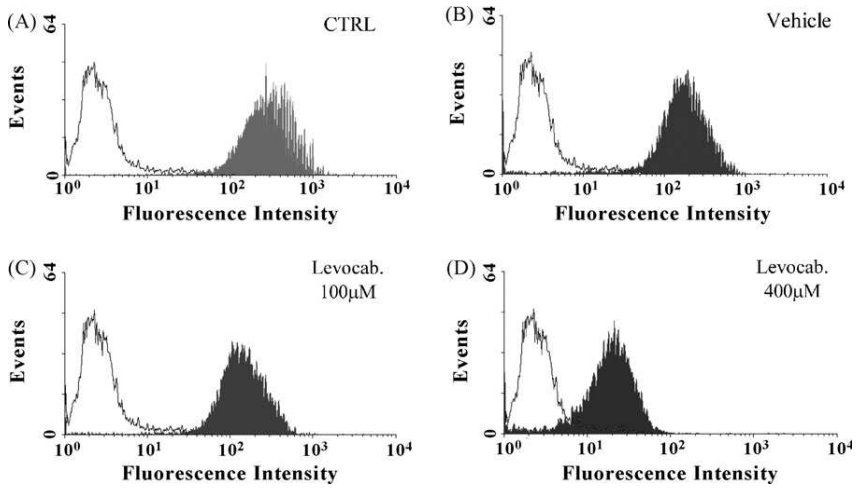


Figure 7.6
Levocabastine competes with a specific anti-α_4 integrin subunit in Jurkat E6.1 cells. Cells were incubated with an anti-human α_4 integrin subunit antibody (5 μg/mL) in the absence (CTRL; panel A) or presence of 100 μM (panel C) or 400 μM (panel D) levocabastine; or in the presence of the vehicle alone (panel B). The negative control is shown as the white filled curve.

7.5 Effect of levocabastine on EoL-1 cytokine release

Cytokines are autocrine and paracrine mediators that support inflammation acting on the vascular epithelium and modulating the activity of resident and circulating white blood cells. We evaluated the ability of levocabastine to reduce cytokine release from differentiated and non-differentiated (immature phenotype) EoL-1 cells using TNF-α as a

proinflammatory stimulus. Table 7.1 and Table 7.2 summarize the effects of levocabastine on 13 different cytokines at 12 and 24 h after TNF-α challenge; cells were or were not exposed to PMA, as indicated.

We received raw data from Bausch & Lomb Rochester, and we analyzed the data with statistical tools. EoL-1 cells were exposed to the vehicle in the same amount used to obtain the highest concentration of the drug.

Table 7.1

12h	TNF–α (positive ctrl) vs Basal	Vehicle vs Basal	TNF-α vs Lev 0.1 mM	TNF-α vs Lev 0.5 mM	TNF-α vs Lev 1.0 mM	TNF-α vs Lev 2.3 mM
Fractalkine (PMA)	ns	p<0.05	ns	ns	ns	ns
Fractalkine (NO PMA)	ns	p<0.01	ns	ns	ns	ns
IL-1α (PMA)	p<0.05	p<0.01	ns	ns	ns	ns
IL-1α (NO PMA)	p<0.05	p<0.001	ns	ns	ns	ns
IL-1β (PMA)	ns	p<0.001	ns	ns	ns	ns
IL-1β (NO PMA)	ns	ns	ns	ns	ns	ns
IL-1ra (PMA)	p<0.001	p<0.001	p<0.01	p<0.001	p<0.001	ns
IL-1ra (NO PMA)	p<0.001	p<0.001	ns	ns	ns	ns
IL-5 (PMA)	ns	p<0,05	ns	ns	ns	ns
IL-5 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-7 (PMA)	p<0.05	p<0.01	ns	ns	ns	ns
IL-7 (NO PMA)	p<0.001	p<0.001	ns	ns	ns	ns
IL-8 (PMA)	p<0.01	p<0.01	ns	ns	ns	ns
IL-8 (NO PMA)	ns	p<0.001	ns	ns	ns	ns
IP-10 (PMA)	p<0.01	p<0.001	ns	p<0.01	p<0.05	ns
IP-10 (NO PMA)	p<0.01	p<0.001	ns	ns	ns	ns
MCP-1 (PMA)	p<0.01	p<0.01	ns	ns	ns	ns
MCP-1 (NO PMA)	p<0.001	p<0.001	ns	ns	ns	ns
MIP-1α (PMA)	ns	p<0.01	concentration dependent increase of cytokine secretion p<0.001			
MIP-1α (NO PMA)	p<0.05	p<0.001	concentration dependent increase of cytokine secretion p<0.01			
MIP-1β (PMA)	p<0.001	p<0.001	ns	ns	ns	ns
MIP-1β (NO PMA)	p<0.001	p<0.001	ns	ns	ns	ns
RANTES (PMA)	p<0.001	p<0.001	ns	ns	ns	ns
RANTES (NO PMA)	p<0.05	p<0.001	increase of cytokine secretion p<0.001			
VEGF (PMA)	ns	ns	ns	ns	p<0.05	p<0.01
VEGF (NO PMA)	ns	ns	ns	ns	ns	ns

One-way analysis of variance (ANOVA) with Newman-Keuls post hoc test was used to compare all the pairs of treatments. Ns = non significant. All the values shown are intended as decrements, except where otherwise stated.

Table 7.2						
24h	TNF-α (positive ctrl) vs Basal	Vehicle vs Basal	TNF-α vs Lev 0.1 mM	TNF-α vs Lev 0.5 mM	TNF-α vs Lev 1.0 mM	TNF-α vs Lev 2.3 mM
Fractalkine (PMA)	ns	ns	ns	ns	ns	p<0.01
Fractalkine (NO PMA)	ns	p<0.001	ns	ns	ns	ns
IL-1α (PMA)	ns	p<0.01	ns	ns	ns	ns
IL-1α (NO PMA)	p<0.01	p<0.001	ns	ns	ns	ns
IL-1β (PMA)	p<0.01	p<0.05	p<0.05	ns	p<0.05	ns
IL-1β (NO PMA)	ns	ns	ns	ns	ns	ns
IL-1ra (PMA)	p<0.01	p<0.05	ns	ns	ns	ns
IL-1ra (NO PMA)	p<0.001	p<0.01	ns	ns	ns	ns
IL-5 (PMA)	ns	ns	ns	ns	ns	ns
IL-5 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-7 (PMA)	ns	ns	ns	ns	ns	ns
IL-7 (NO PMA)	p<0.05	p<0.01	ns	ns	ns	ns
IL-8 (PMA)	ns	ns	ns	ns	ns	ns
IL-8 (NO PMA)	p<0.01	p<0.01	ns	ns	ns	ns
IP-10 (PMA)	p<0.01	p<0.001	ns	ns	ns	ns
IP-10 (NO PMA)	p<0.001	p<0.001	p<0.05	p<0.05	ns	ns
MCP-1 (PMA)	p<0.001	p<0.001	ns	ns	ns	ns
MCP-1 (NO PMA)	p<0.001	p<0.001	ns	ns	ns	ns
MIP-1α (PMA)	p<0.01	p<0.01	ns	ns	ns	ns
MIP-1α (NO PMA)	p<0.01	p<0.001	Increase in cytokine release in a concentration dependent way p<0.001			
MIP-1β (PMA)	p<0.001	ns	ns	ns	ns	ns
MIP-1β (NO PMA)	p<0.001	p<0.001	ns	ns	ns	ns
RANTES (PMA)	ns	ns	ns	ns	ns	ns
RANTES (NO PMA)	ns	p<0.05	Increase in cytokine release in a concentration dependent way p<0.001			
VEGF (PMA)	ns	ns	ns	ns	p<0.05	p<0.01
VEGF (NO PMA)	p<0.01	ns	ns	ns	ns	p<0.01

One-way analysis of variance (ANOVA) with Newman-Keuls post hoc test was used to compare all the pairs of treatments. Ns = non significant. All the values shown are intended as decrements, except where otherwise stated.

Interestingly, levocabastine was able to significantly reduce the release of the proinflammatory cytokine IL-1β, and of IP-10, that is known to promote rapid transendothelial migration of effector cells of the immune system (Manes et al., 2006). Furthermore, we report a very important perturbation from the vehicle, that seems to stimulate cytokine release by itself. The placebo group, in fact, produced levels of cytokines similar to the one measured using calcimycin, which induces the release of all the vescicular content of the cells by lysis.

The placebo group, in fact, produced levels of cytokines similar to the one measured using calcimycin, which induces the release of all the vescicular content of the cells by lysis. Since

vehicle did not show toxicity, we speculate that this effect could be due to cyclodextrins which interfere with the plasma membrane and, specifically, with integrin functionality (Green, 1999; Pande, 2000; Berg, 2007). On this basis, we conclude that the effect of levocabastine 0.05% solution eye drops containing cyclodextrins without benzalkonium chloride on cytokine release should be further investigated in the absence of an interfering vehicle. An analysis of the data summarized in Table 7.1 and Table 7.2 indicates that levocabastine – at three different concentrations: 0.1, 0.5 and 1.0 mM – is capable to prevent the release of the following cytokines induced by TNF-α.:

- IP-10 in cells exposed for 12 h without PMA or differentiated with it. This cytokine is involved in inflammatory processes (Inukai Y et al, 2007).
- IL-1ra in cells exposed for 12 and differentiated with PMA. This cytokine seems to act as an antagonist of the inflammatory cytokine IL-1 and this may be a controversial result. Although, it should be pointed out that the effect of levocabstine was observed only after 12 h of exposure in cells treated with PMA.
- IL-1β in cells exposed for 12 h in cells differentiated with PMA. This cytokine is involved in the inflammatory response (Hallsworth et al 1998; Wong et al 2007). Levocabastine was effective in the PMA-treated group of cells exposed for 12 h.
- VEGF in cells exposed for 24 h in cells without PMA or differentiated with it. This cytokine is relevant for the inflammatory response in eosinophils (Solomon et al. 2003; Puxeddu et al., 2005). Interestingly, the release of this cytokine induced by TNF-α was blocked by levocabastine; however, the vehicle alone (contrary to what observed for the other cytokines) did not influence the release of VEGF.

An analysis of the data summarized in Table 7.3 and Table 7.4 indicates that levocabastine at the fixed concentration of 2 mM is capable to reduced the release of the following cytokines induced by three different concentrations of TNF-α (5, 10 and 20 ng):

- IL-12 P40 in cells exposed for 12 h without PMA or differentiated with it. This cytokine is involved in the inflammatory response in eosinophils (Wen et al. 2006).
- VEGF in cells exposed for 24 h without PMA or differentiated with it.
- IL-12-P40 in cells exposed for 24 h without PMA.
- VEGF in cells exposed for 24 h without PMA or differentiated with it.

- IL-8 in cells exposed for 24 h in cells differentiated with PMA. This cytokine is crucial for the inflammatory response in allergic diseases (Silvestri et al., 2006).

An analysis of the data summarized in Table 6 indicates that levocabastine (2 mM) is effective in reducing the release of the following cytokines induced by TNF-α (10 ng) for 24 h. This effect is not influenced by VCAM-1 or fibronectine:

- IL-P-40 in cells without PMA or differentiated with it.
- IL-1-ra in cells did not exposed to PMA.
- IL-6 in cells did not exposed to PMA Another cytokine relevant for the allergic response (Ghazizadeh, 2007; Fritz et al. 2006).
- IL-8 incells did not exposed to PMA.
- VEGF in cells cultured without PMA or differentiated with it.

Table 7.3

12 h	TNF-α 5ng vs Basal	TNF-α 10ng vs Basal	TNF-α 20ng vs Basal	TNF-α 5ng + Levocabastine 2mM vs TNF-α	TNF-α 10ng + Levocabastine 2mM vs TNF-α	TNF-α 20ng + Levocabastine 2mM vs TNF-α
Fractalkine (PMA)	ns	ns	ns	ns	ns	ns
Fractalkine (NO PMA)	ns	ns	ns	increase of cytokine secretion		
G-CSF (PMA)	ns	ns	ns	ns	ns	ns
G-CSF (NO PMA)	ns	ns	ns	increase of cytokine secretion		
GM-CSF (PMA)	P < 0.01	P < 0.01	P < 0.01	ns	ns	ns
GM-CSF (NO PMA)	ns	ns	ns	ns	ns	ns
IL-10 (PMA)	ns	ns	ns	ns	ns	ns
IL-10 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-12p40 (PMA)	P < 0.001	P < 0.001	ns	P < 0.001	P < 0.001	P < 0.001
IL-12p40 (NO PMA)	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001
IL-1α (PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
IL-1α (NO PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
IL-1β (PMA)	ns	ns	ns	ns	ns	ns
IL-1β (NO PMA)	ns	ns	ns	ns	ns	ns
IL-1ra (PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
IL-1ra (NO PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
IL-5 (PMA)	ns	ns	ns	ns	ns	ns
IL-5 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-6 (PMA)	ns	ns	ns	ns	ns	ns
IL-6 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-7 (PMA)	P < 0.001	P < 0.001	P < 0.001	ns	ns	ns
IL-7 (NO PMA)	P < 0.001	P < 0.001	P < 0.001	ns	ns	ns
IL-8 (PMA)	P < 0.001	P < 0.001	P < 0.001	ns	ns	ns
IL-8 (NO PMA)	P < 0.001	P < 0.001	P < 0.001	ns	ns	ns
IP-10 (PMA)	P < 0.001	P < 0.001	P < 0.001	ns	ns	ns
IP-10 (NO PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
MCP-1 (PMA)	ns	ns	ns	ns	ns	ns
MCP-1 (NO PMA)	P < 0.01	P < 0.001	P < 0.001	ns	ns	ns
MIP-1α (PMA)	ns	ns	ns	ns	ns	ns
MIP-1□ (NO PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
MIP-1β (PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
MIP-1β (NO PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
RANTES (PMA)	P < 0.01	P < 0.01	P < 0.01	increase of cytokine secretion		
RANTES (NO PMA)	P < 0.001	P < 0.001	P < 0.001	increase of cytokine secretion		
TGF-α (PMA)	ns	ns	ns	ns	ns	ns
TGF-α (NO PMA)	ns	ns	ns	ns	ns	ns
VEGF (PMA)	ns	ns	ns	P < 0.01	ns	ns
VEGF (NO PMA)	P < 0.05	P < 0.05	P < 0.05	P < 0.01	P < 0.01	P < 0.05

Table 7.4						
24 h	TNF-α 5ng vs Basal	TNF-α 10ng vs Basal	TNF-α 20ng vs Basal	TNF-α 5ng + Levocabastine 2mM vs TNF-α	TNF-α 10ng + Levocabastine 2mM vs TNF-α	TNF-α 20ng + Levocabastine 2mM vs TNF–α
Fractalkine (PMA)	ns	ns	ns	ns	ns	ns
Fractalkine (NO PMA)	ns	ns	ns	increase of cytokine secretion		
G-CSF (PMA)	ns	ns	ns	ns	ns	ns
G-CSF (NO PMA)	ns	ns	ns	increase of cytokine secretion		
GM-CSF (PMA)	ns	ns	ns	ns	ns	ns
GM-CSF (NO PMA)	ns	ns	ns	ns	ns	ns
IL-10 (PMA)	ns	ns	ns	ns	ns	ns
IL-10 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-12p40 (PMA)	ns	ns	ns	ns	ns	ns
IL-12p40 (NO PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
IL-1α (PMA)	ns	ns	$P < 0.01$	increase of cytokine secretion		
IL-1α (NO PMA)	$P < 0.05$	$P < 0.05$	$P < 0.05$	increase of cytokine secretion		
IL-1β (PMA)	ns	ns	ns	ns	ns	ns
IL-1β (NO PMA)	ns	ns	ns	ns	ns	ns
IL-1ra (PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	increase of cytokine secretion		
IL-1ra (NO PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.05$	$P < 0.01$
IL-5 (PMA)	ns	ns	ns	ns	ns	ns
IL-5 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-6 (PMA)	ns	ns	ns	ns	ns	ns
IL-6 (NO PMA)	ns	ns	ns	ns	ns	ns
IL-7 (PMA)	ns	ns	$P < 0.05$	ns	ns	ns
IL-7 (NO PMA)	$P < 0.01$	$P < 0.01$	$P < 0.01$	ns	ns	ns
IL-8 (PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
IL-8 (NO PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	ns	ns	ns
IP-10 (PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	ns	ns	ns
IP-10 (NO PMA)	$P < 0.001$	$P < 0.01$	$P < 0.001$	increase of cytokine secretion		
MCP-1 (PMA)	ns	ns	ns	ns	ns	ns
MCP-1 (NO PMA)	$P < 0.01$	$P < 0.01$	$P < 0.001$	ns	ns	ns
MIP-1α (PMA)	ns	$P < 0.05$	$P < 0.01$	ns	ns	ns
MIP-1α (NO PMA)	ns	ns	ns	increase of cytokine secretion		
MIP-1β (PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	ns	ns	ns
MIP-1β (NO PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	increase of cytokine secretion		
RANTES (PMA)	$P < 0.001$	$P < 0.001$	$P < 0.001$	increase of cytokine secretion		
RANTES (NO PMA)	$P < 0.01$	$P < 0.01$	$P < 0.01$	increase of cytokine secretion		
TGF-α (PMA)	ns	ns	ns	ns	ns	ns
TGF-α (NO PMA)	$P < 0.05$	ns	$P < 0.05$	$P < 0.05$	ns	$P < 0.05$
VEGF (PMA)	ns	ns	ns	$P < 0.05$	ns	$P < 0.05$
VEGF (NO PMA)	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Table 7.5

	TNF-α 10ng vs Basal	VCAM-1 vs Basal	VCAM-1 + Levocabastine 2mM vs VCAM-1	FN vs Basal	FN + Levocabastine 2mM vs FN	Levocabastine 2mM vs TNF-α	Vehicle vs Basal	Bio1211 vs TNF–α	CS-1 vs TNF-α
Fractalkine (PMA)	ns	ns	ns	ns	Increase	ns	Increase	ns	ns
Fractalkine (NO PMA)	ns	ns	ns	ns	ns	ns	ns	P < 0.05	P < 0.001
G-CSF (PMA)	ns	ns	Increase	ns	Increase	ns	Increase	ns	ns
G-CSF (NO PMA)	ns	ns	ns	ns	ns	ns	ns	ns	ns
GM-CSF (PMA)	ns	ns	ns	ns	ns	ns	ns	ns	ns
GM-CSF (NO PMA)	P < 0.001	P < 0.001	ns	P < 0.001	ns	ns	Increase	P < 0.05	P < 0.001
IL-10 (PMA)	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-10 (NO PMA)	ns	ns	ns	ns	ns	ns	ns	ns	P < 0.001
IL-12p40 (PMA)	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.01	P < 0.001	Increase	P < 0.05	P < 0.001
IL-12p40 (NO PMA)	P < 0.01	P < 0.05	ns	P < 0.05	P < 0.05	P < 0.01	ns	P < 0.05	ns
IL-1α (PMA)	P < 0.001	P < 0.001	Increase	P < 0.001	Increase	Increase	Increase	P < 0.05	ns
IL-1α (NO PMA)	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-1β (PMA)	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-1β (NO PMA)	P < 0.001	P < 0.05	ns	P < 0.001	ns	ns	Increase	ns	ns
IL-1ra (PMA)	P < 0.001	P < 0.001	Increase	P < 0.001	Increase	Increase	Increase	ns	P < 0.01
IL-1ra (NO PMA)	P < 0.001	P < 0.001	P < 0.01	P < 0.001	P < 0.001	P < 0.001	Increase	P < 0.001	P < 0.001
IL-5 (PMA)	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-5 (NO PMA)	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-6 (PMA)	ns	ns	ns	ns	ns	ns	ns	ns	Increase
IL-6 (NO PMA)	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	Increase	P < 0.05	P < 0.001
IL-7 (PMA)	P < 0.001	P < 0.001	Increase	P < 0.001	Increase	ns	Increase	ns	ns
IL-7 (NO PMA)	P < 0.001	P < 0.001	ns	P < 0.001	ns	ns	Increase	ns	P < 0.01
IL-8 (PMA)	ns	ns	Increase	P < 0.05	Increase	Increase	Increase	ns	Increase
IL-8 (NO PMA)	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	ns	Increase	P < 0.001	P < 0.001
IP-10 (PMA)	P < 0.001	P < 0.001	ns	P < 0.001	ns	ns	Increase	P < 0.001	ns
IP-10 (NO PMA)	P < 0.001	P < 0.001	ns	P < 0.001	ns	ns	Increase	P < 0.001	ns
MCP-1 (PMA)	P < 0.001	P < 0.001	Increase	P < 0.001	Increase	ns	Increase	P < 0.001	P < 0.001
MCP-1 (NO PMA)	P < 0.001	P < 0.001	ns	P < 0.001	Increase	Increase	Increase	ns	P < 0.001
MIP-1α (PMA)	ns	ns	Increase	ns	Increase	Increase	ns	ns	ns
MIP-1α (NO PMA)	P < 0.001	P < 0.001	Increase	P < 0.001	Increase	Increase	Increase	ns	Increase
MIP-1β (PMA)	P < 0.001	P < 0.001	Increase	P < 0.001	Increase	Increase	Increase	ns	P < 0.001
MIP-1β (NO PMA)	P < 0.001	P < 0.001	ns	P < 0.001	ns	ns	Increase	ns	Increase
RANTES (PMA)	ns	ns	Increase	ns	Increase	Increase	ns	ns	ns
RANTES (NO PMA)	ns	ns	Increase	ns	Increase	Increase	Increase	ns	ns
TGF-α (PMA)	P < 0.05	P < 0.05	ns	P < 0.05	ns	ns	ns	ns	P < 0.05
TGF-α (NO PMA)	P < 0.01	P < 0.05	ns	P < 0.05	ns	ns	P < 0.001	ns	P < 0.001
VEGF (PMA)	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	P < 0.001	ns	P < 0.05	P < 0.001
VEGF (NO PMA)	ns	ns	P < 0.001	ns	P < 0.001	P < 0.001	P < 0.001	ns	P < 0.001

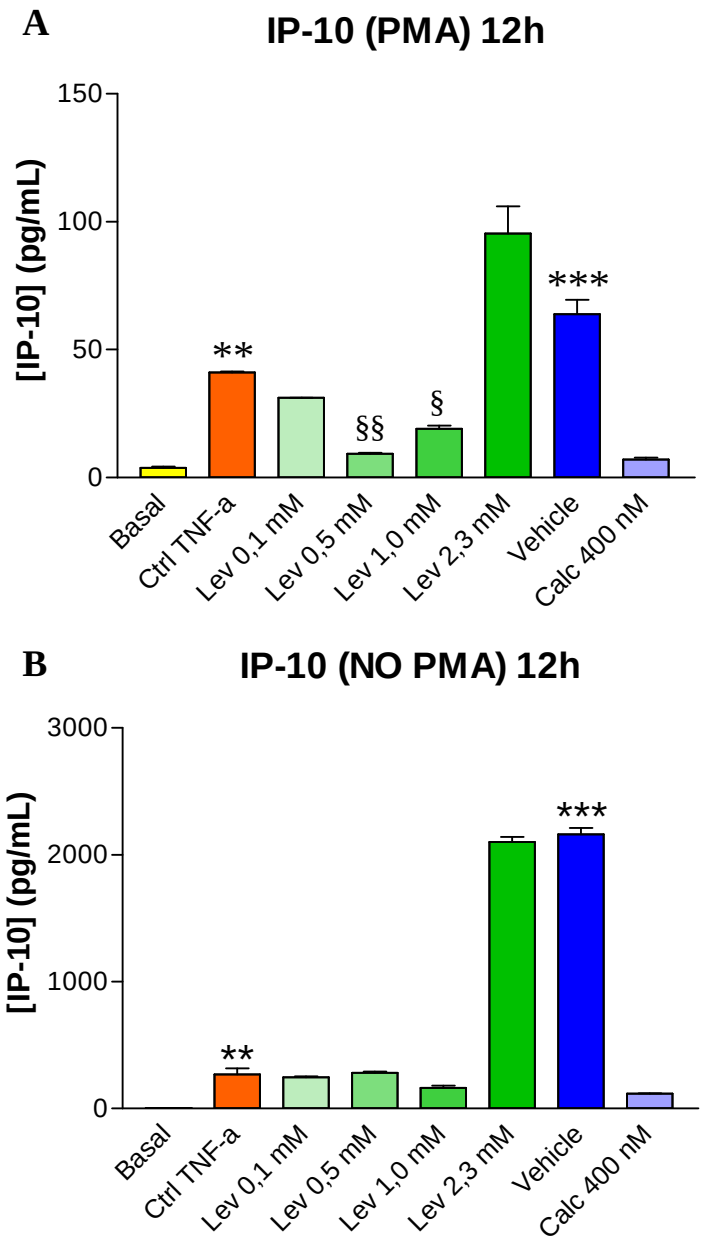


Figure 7.7
Effect of levocabastine with cyclodextrins on interferon-inducible protein-10 (IP-10) release after 12h of TNF-α challenge. In panel A EoL-1 cells have been differentiated with PMA 24h before the experiment, whereas panel B shows the effects on undifferentiated EoL-1 cells. ** $p < 0,01$ vs Basal; *** $p < 0,001$ vs Basal; § $p < 0,05$ vs TNF-α; §§ $p < 0,01$ vs TNF-α

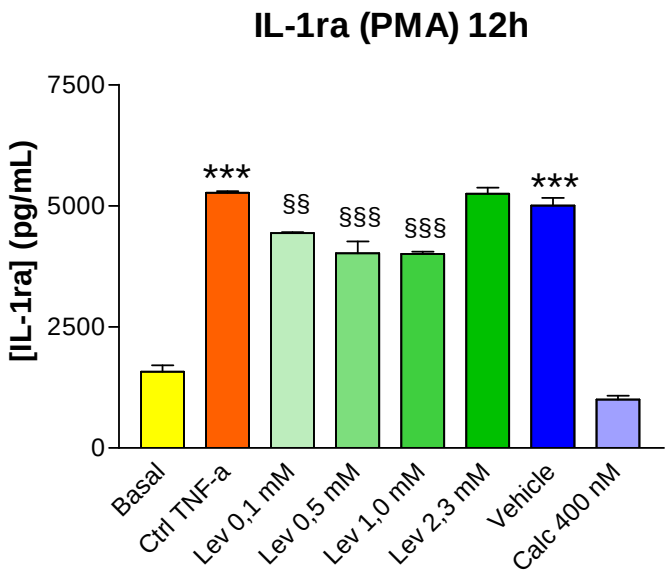


Figure 7.8
Effect of levocabastine with cyclodextrins on IL-1ra release after 12h of TNF-α challenge. EoL-1 cells have been differentiated with PMA 24h before the experiment. *** $p < 0,001$ vs Basal; §§ $p < 0,01$ vs TNF-α; §§§ $p < 0,001$ vs TNF-α

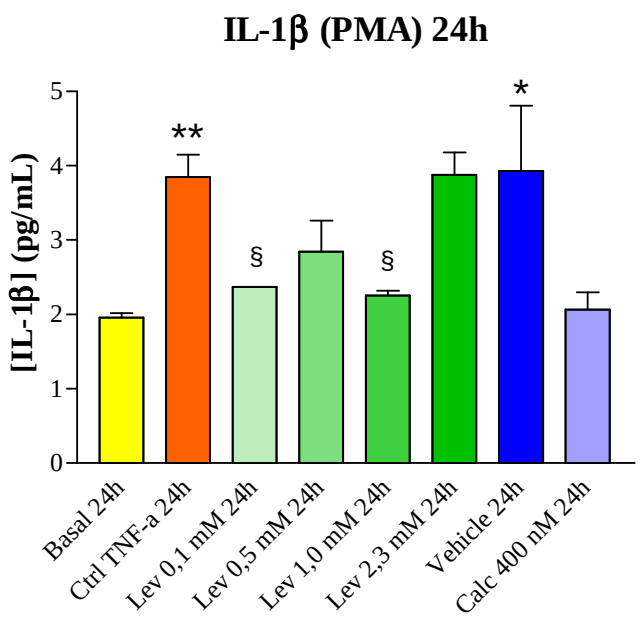


Figure 7.9
Effect of levocabastine with cyclodextrins on IL-1β released 12h after TNF-α challenge. EoL-1 cells have been differentiated with PMA 24h before the experiment. * $p < 0.05$ vs basal; ** $p < 0.001$ vs Basal; § $p < 0.05$ vs TNF-α;

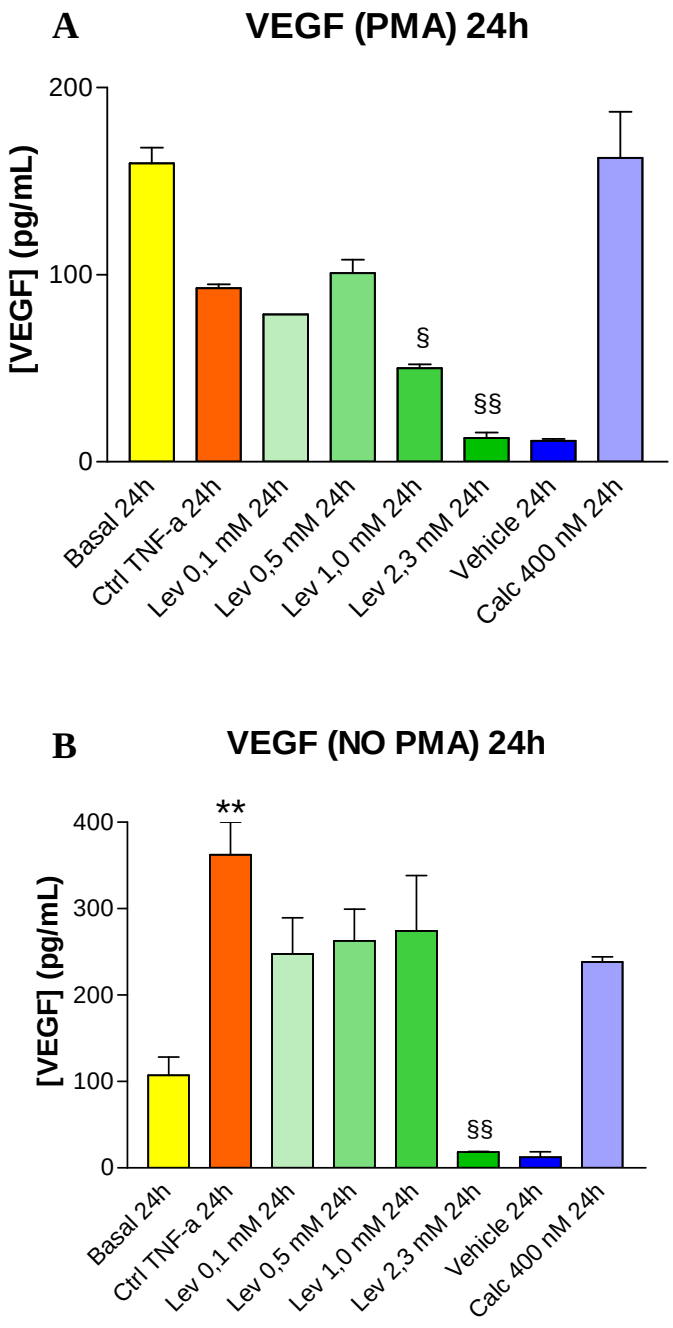


Figure 7.10
Panel A: Levocabastine with cyclodextrins is able to reduce the release of VEGF of EoL-1 cells differentiated with PMA 24h before the experiment. Panel B: Naïve EoL-1 cells challenged with TNF-α show a reduced release of VEGF after levocabastine with cyclodextrin pre-treatment. ** $p < 0.01$ vs Basal; § $p < 0.05$ vs TNF-α; §§ $p < 0.01$ vs TNF-α

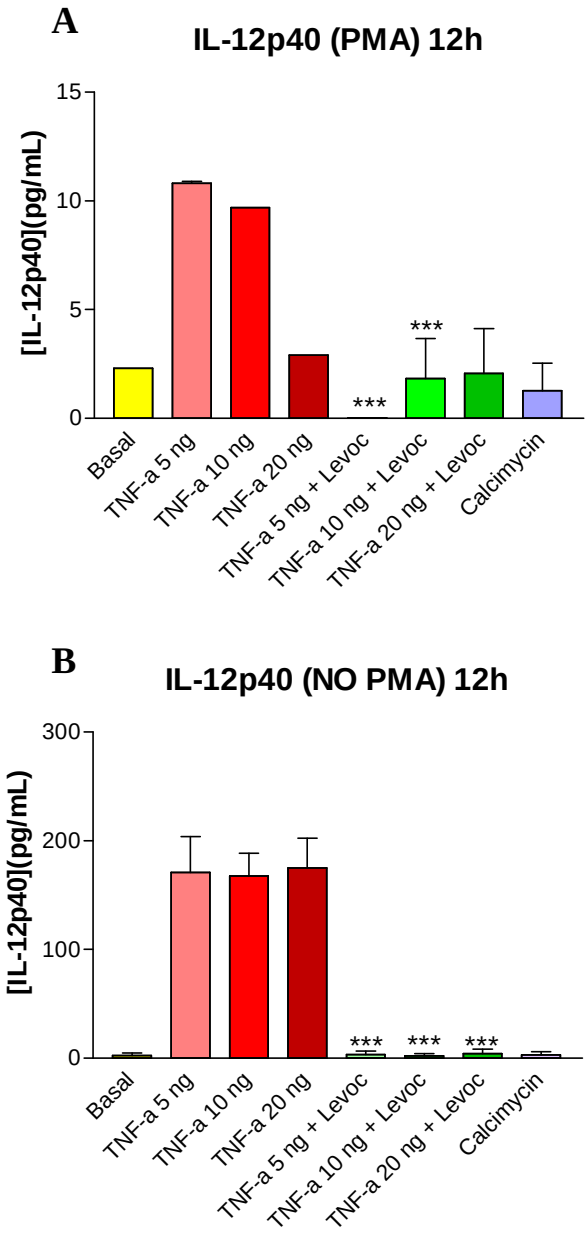


Figure 7.11
Panel A and B show the effect of levocabastine with cyclodextrins to reduce the release of IL-12p40 from, respectively, differentiated and undifferentiated EoL-1 cells 12h after TNF-α challenge. *** $p < 0.001$ vs TNF-α

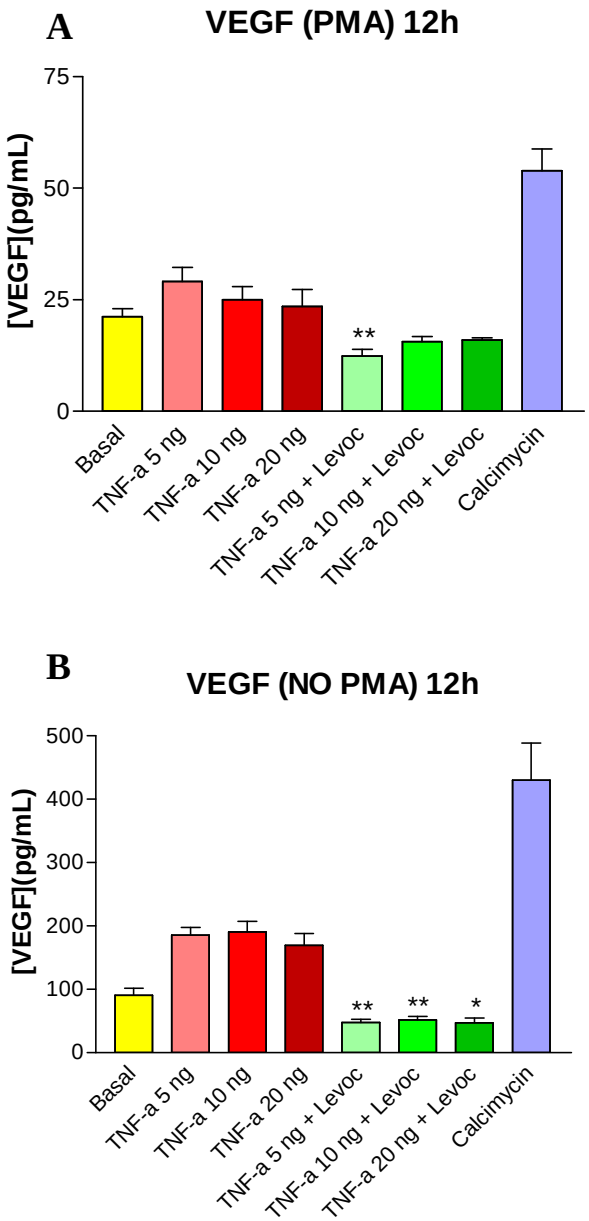


Figure 7.12
Panel A and B show the effect of levocabastine with cyclodextrins to reduce the release of VEGF from, respectively, differentiated and undifferentiated EoL-1 cells 12h after TNF-α challenge. * $p < 0.05$ vs TNF-α; ** $p < 0.01$ vs TNF-α

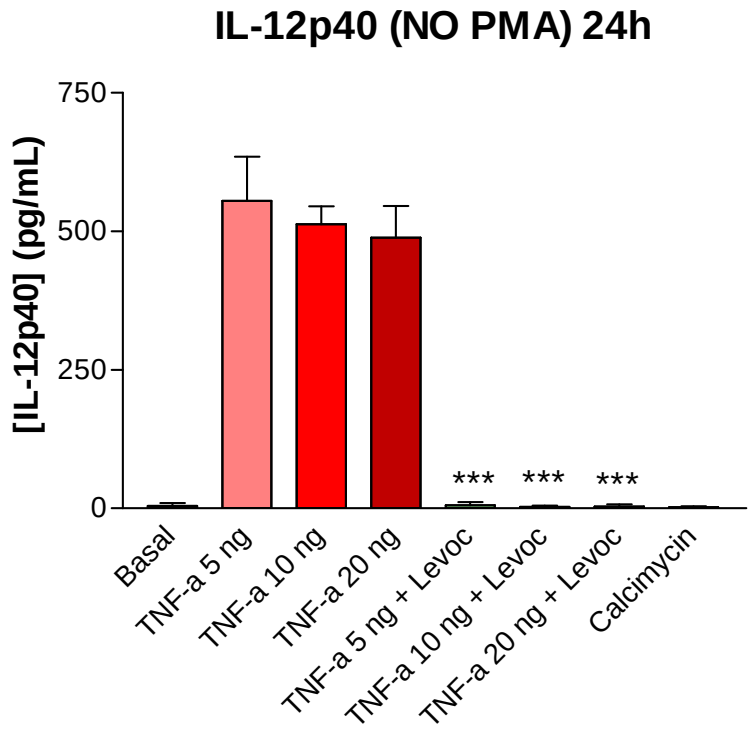


Figure 7.13
Effect of levocabastine with cyclodextrins on IL-12p40 release by undifferentiated EoL-1 cells. Supernatants were analysed 24h after TNF-α challenge. *** $p < 0.001$ vs TNF-α

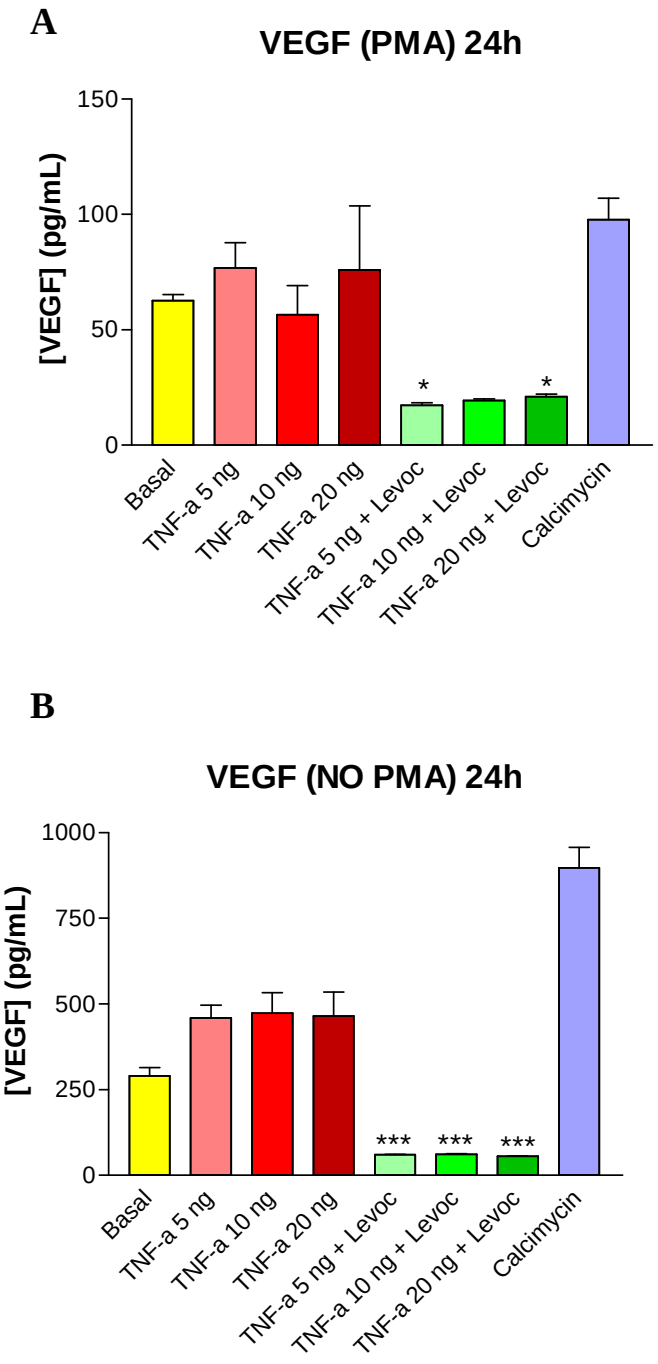


Figure 7.14
In panel A is shown the effect of levocabastine with cyclodextrins on VEGF release by PMA differentiated EoL-1 cells, whereas panel B represents the same experiment performed on naïve cells. Supernates were analysed 24h after TNF-α challenge. * $p < 0.05$ vs TNF-α; ** $p < 0.001$ vs TNF-α

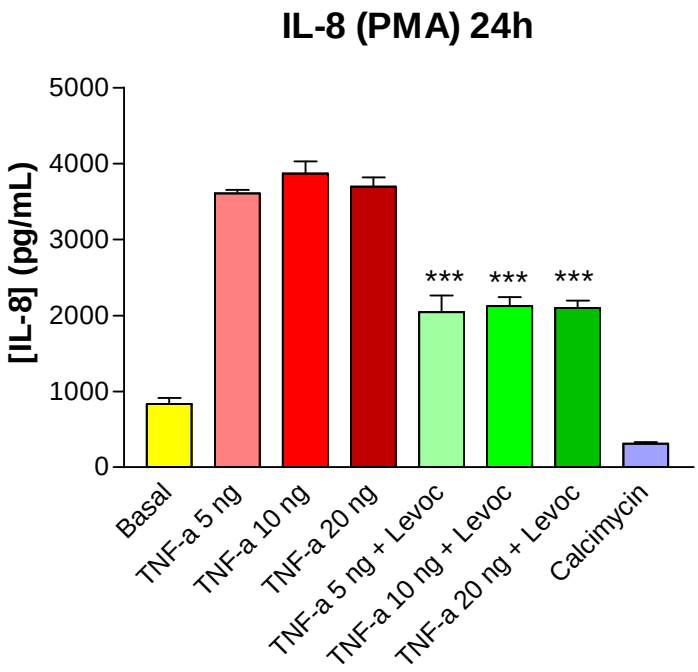


Figure 7.15
Levocabastine with cyclodextrins demonstrates to be able to reduce IL-8 release by PMA differentiated EoL-1 cells after TNF-α challenge. *** $p < 0.001$ vs TNF-α

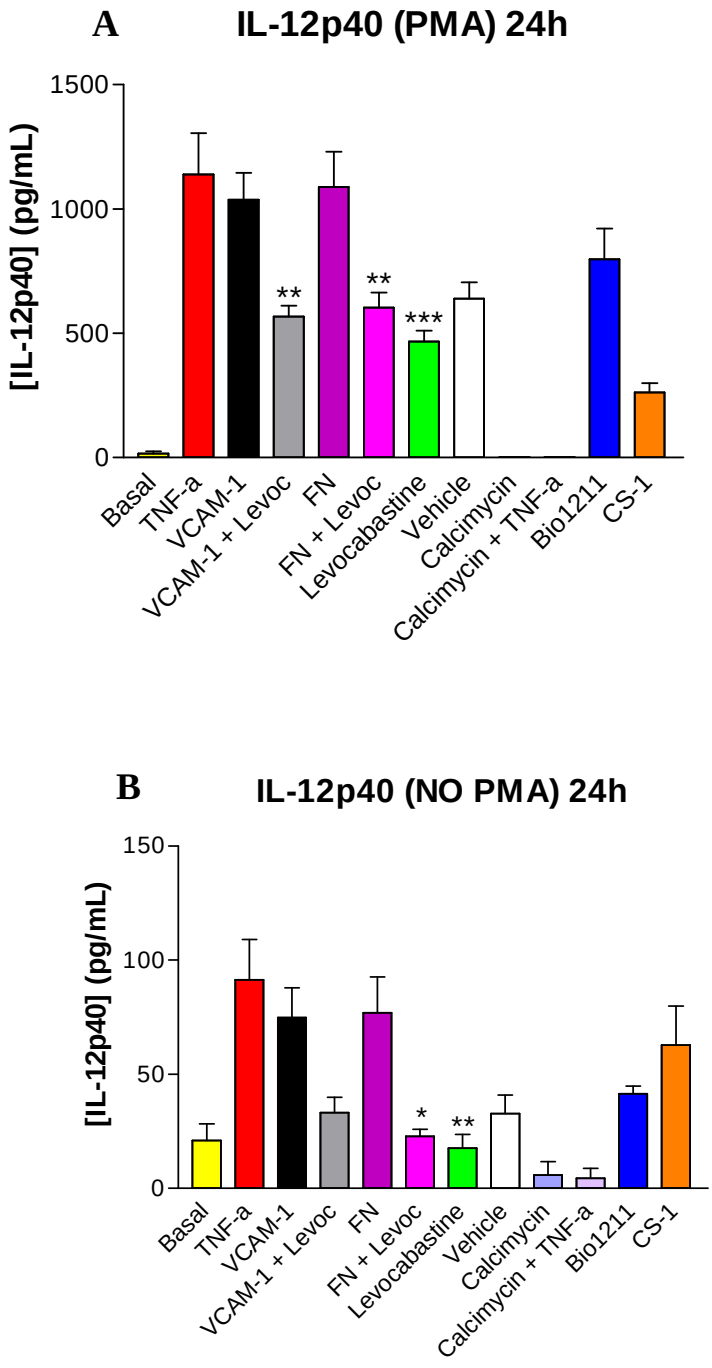


Figure 7.16
Effects of levocabastine with cyclodextrins on cytokine release of EoL-1 cells exposed to various ligands, after TNF-α exposure. Panel A: IL-12p40 analysis in PMA differentiated EoL-1 cells supernatants. Panel B: IL-12p40 presence in the supernatants of naïve EoL-1 cells. * $p < 0.05$ vs TNF-α; ** $p < 0.01$ vs TNF-α; *** $p < 0.001$ vs TNF-α

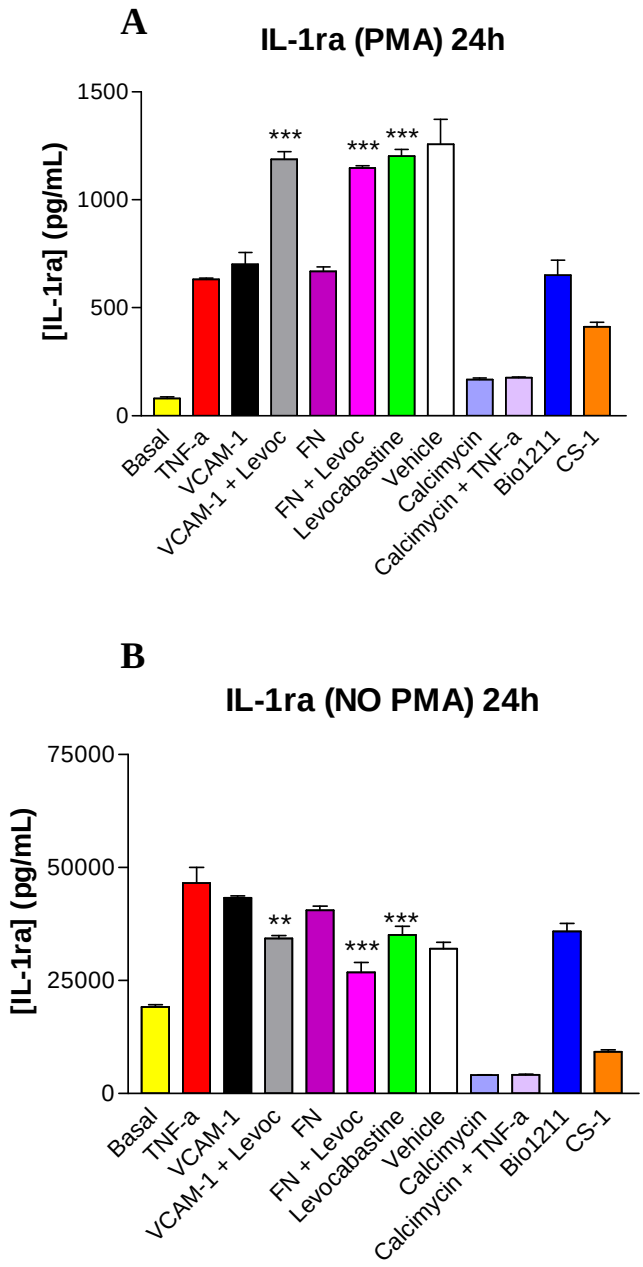


Figure 7.17
Panel A: IL-1ra analysis in PMA differentiated EoL-1 cells supernatants. Panel B: IL-1ra presence in the supernatants of naïve EoL-1 cells. ** $p < 0.01$ vs TNF-α; *** $p < 0.001$ vs TNF-α

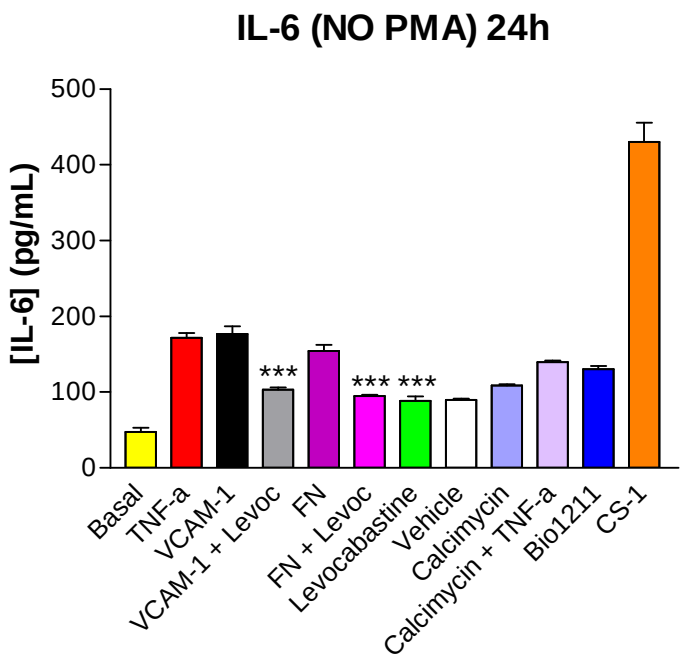


Figure 7.18
IL-6 release by non-differentiated EoL-1 cells is inhibited by levocabastine with cyclodextrins even in the presence of pro-inflammatory ligands such as VCAM-1 or fibronectin. *** $p < 0.001$ vs TNF-α

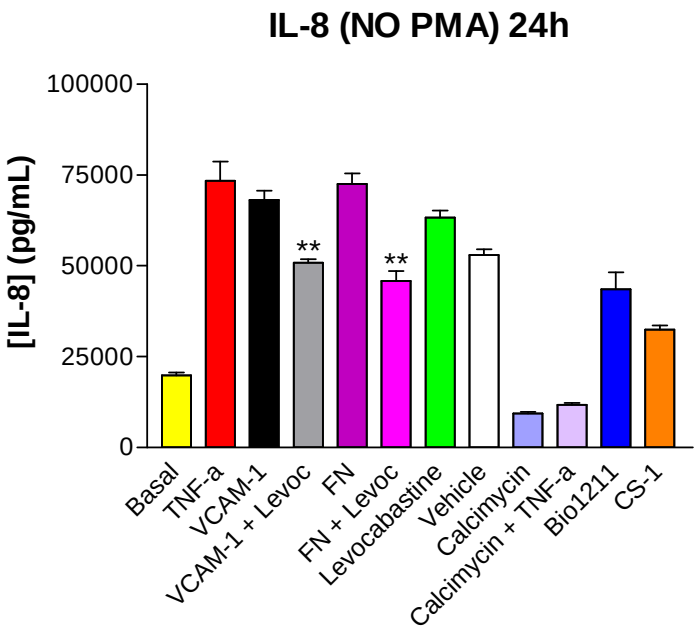


Figure 7.19
IL-6 release by non-differentiated EoL-1 cells is inhibited by levocabastine with cyclodextrins even in the presence of pro-inflammatory ligands such as VCAM-1 or fibronectin. *** $p < 0.001$ vs TNF-α

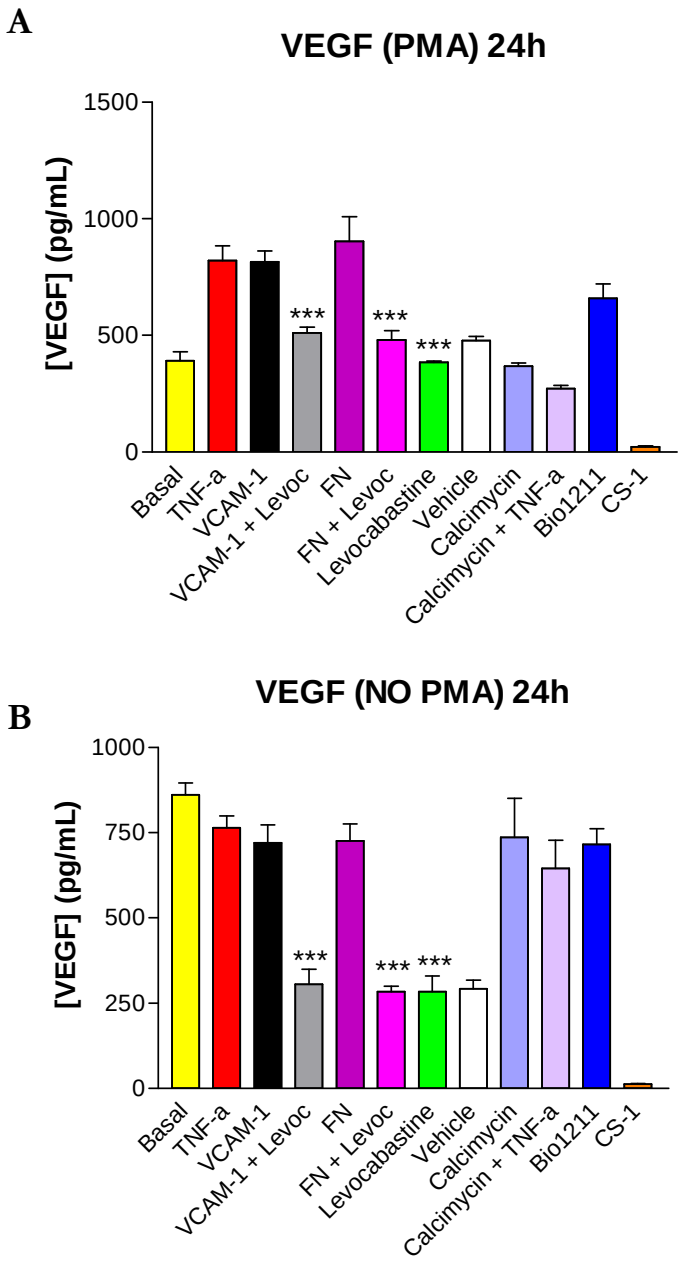


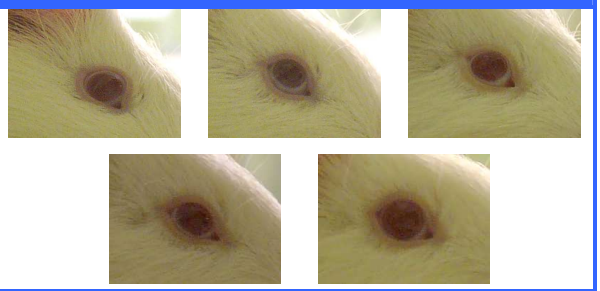
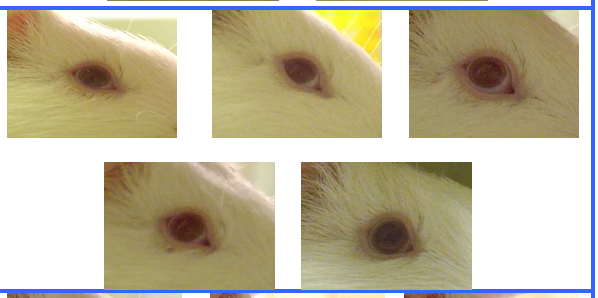
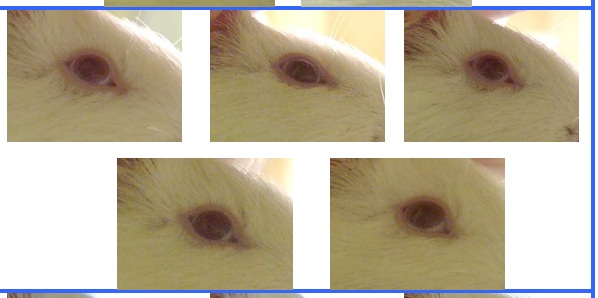
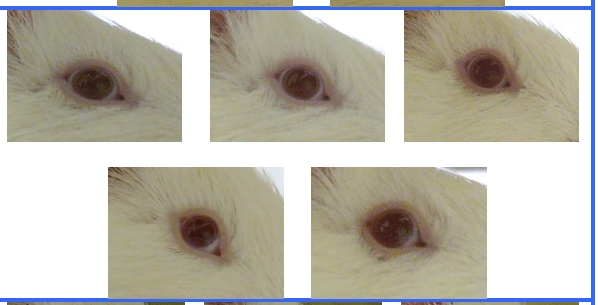
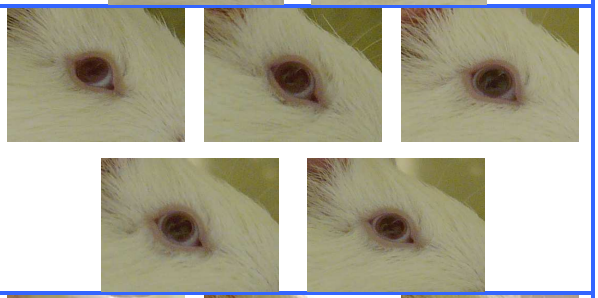
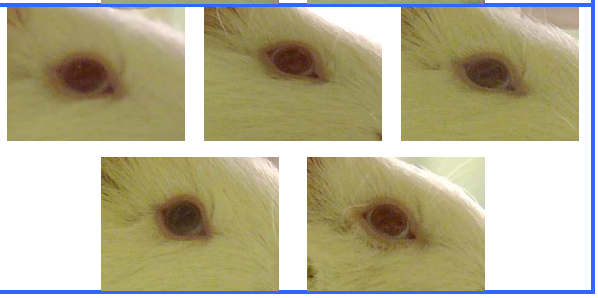
Figure 7.20
Measurement of VEGF levels in PMA differentiated and indifferentiated EoL-1 cells supernatants. Levocabastine with cyclodextrins is able to reduce VEGF release after TNF-α challenge. *** $p < 0.001$ vs TNF-α

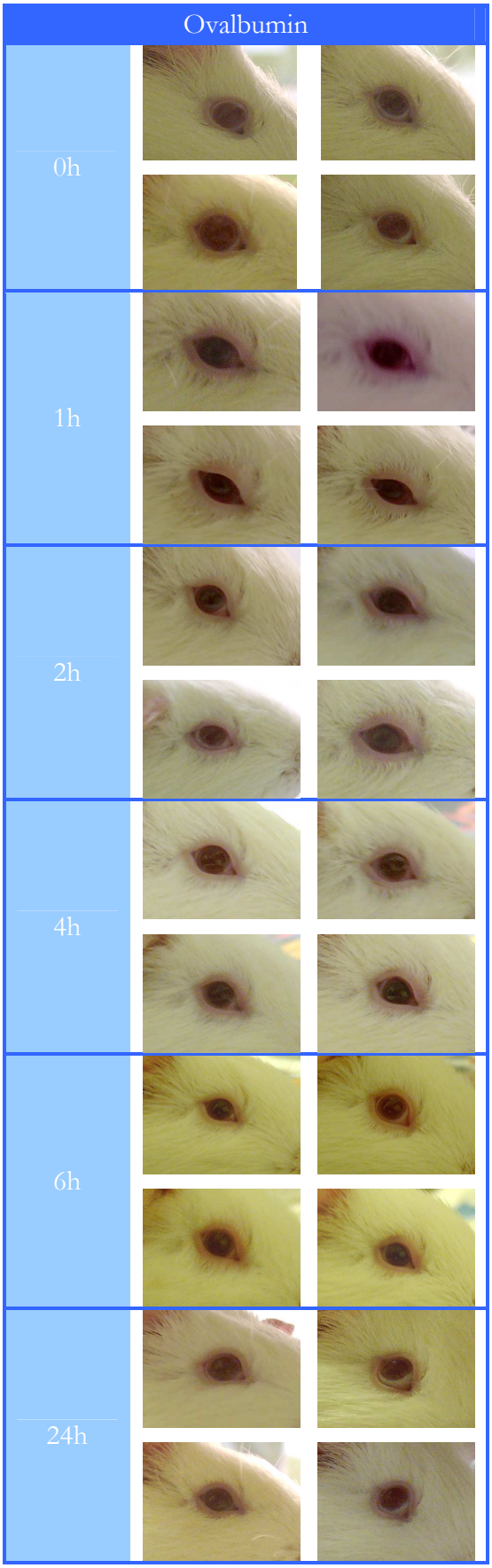
7.6 Levocabastine treatment in a guinea pig model of allergic conjunctivitis

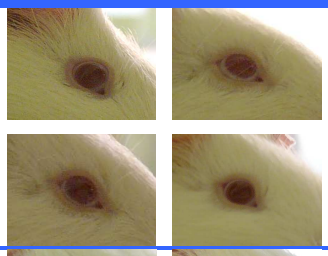
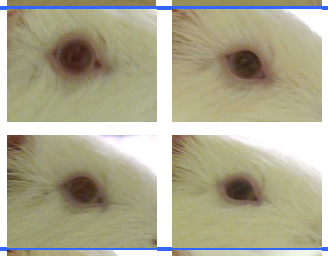
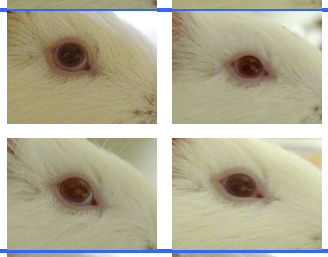
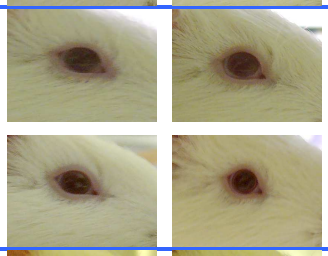
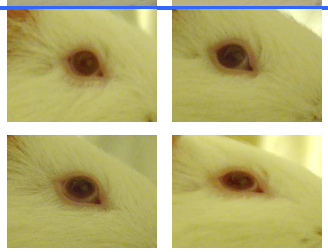
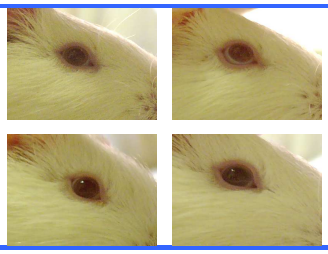
Giunea pigs were actively immunized by intraperitoneal injection of ovalbumin and, two weeks later, challenged with 30 μL of ovalbumin solution (100 μg/mL) in saline instilled into the conjunctival sac. Twenty minutes after the antigen challenge we registered swelling of the eyes, and chemosis was significantly enhanced in positive controls ($p<0.05$) as compared to negative controls. Levocabastine 0,05% eye drops pre-treatment (30 μL/eye 60 and 30 min before the challenge) significantly reduced the clinical symptoms of the induced allergic response up to four hours and six hours (figs below). Chlorpheniramine reduced the clinical symptoms induced by ovalbumin only up to 2 h after antigen challenge. One hour after challenge, during the ocular early phase reaction to ovalbumin, swelling of the eyelids and chemosis were more evident in treated animals than controls, but was significantly reduced by 0.05% levocabastine and 0.1% chlorpheniramine eye drops before treatment. However, only levocabastine pre-treated guinea pigs showed a significant reduction in the severity of conjunctival symptoms during the late phase reaction of allergic conjunctivitis 6h after ovalbumin challenge (Fig. 7.21).

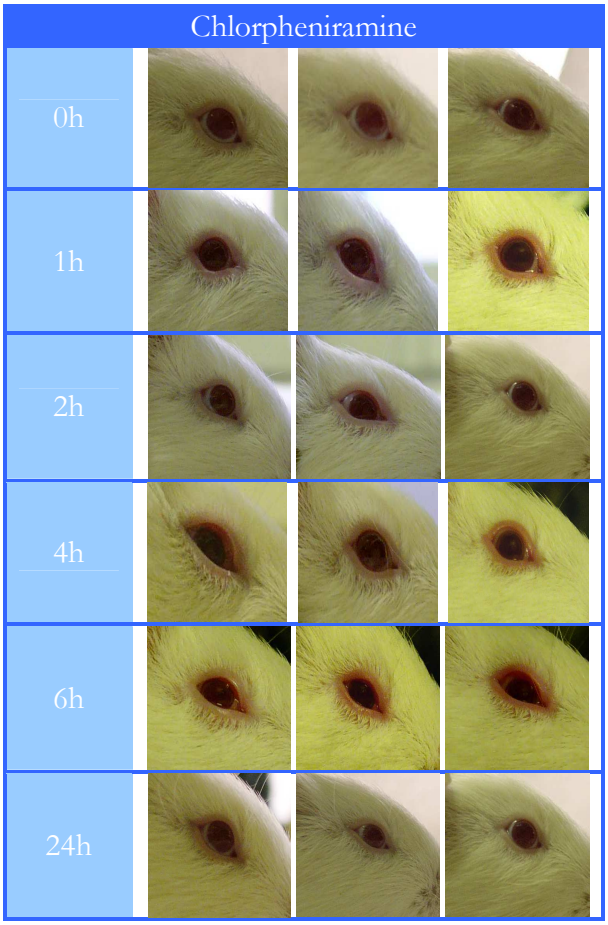
24 h later, guinea pigs were sacrificed and the conjunctivas excised. Protein were extracted for western blot analysis and to test the level of eosinophil infiltration, performed as described in the material and methods section. The histological examination of stained conjunctivas shows a clear reduction in eosinophil infiltration in the tissues when the animals are pre-treated with either of the three formulations of levocabastine, data confirmed by the count of eosinophil, whereas chlorpheniramine was ineffective (Fig. 7.22 and 7.23). Protein electrophoresis analysis clearly shows a reduced expression of VLA-4 in the tissues caused by the three levocabastine formulations but not by chlorpheniramine. Similarly, eosinophil peroxidase activity, taken as an index of eosinophil infiltration, was increased 24 h after antigen challenge in positive control giunea pigs, whereas a noteworthy reduction was observed in eosinophil peroxidase activity of levocabastine-treated guinea pigs but not in chlorpheniramine treated animals.

Exposure of actively immunized guinea pigs to the topical challenge of ovalbumin instilled into the conjunctival sac induced a significant increase, 24 h later, in α_4 integrin levels evaluated in conjunctival homogenates by western blot analysis (Fig. 7.24).

Placebo	
0h	
1h	
2h	
4h	
6h	
24h	


Ovalbumin
0h
1h
2h
4h
6h
24h

Levocabastine	
0h	
1h	
2h	
4h	
6h	
24h	



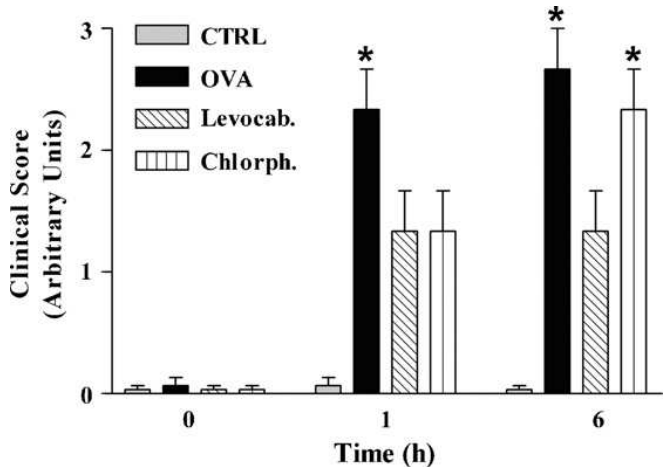


Figure 7.21
Effects of levocabastine and chlorpheniramine on conjunctival symptoms induced by ovalbumin in guinea pigs actively immunized by intraperitoneal injection of ovalbumin and, 2 weeks later, challenged with ovalbumin (30 µL of 2.5% solution) instilled into both eyes. Levocabastine 0.05% and chlorpheniramine 0.1% were instilled into both eyes (30 µL per eye) 60 and 30 minutes before ovalbumin challenge; controls received the vehicle alone and were not treated with ovalbumin. Each group comprised from three to five guinea pigs and the score was based on changes up to 24h after challenge for the eye symptoms of itching, swelling, redness, and lid eversion. The data show the mean ± SEM; *$p<0.01$ vs vehicle (Friedman test followed by Dunn's post hoc comparison.

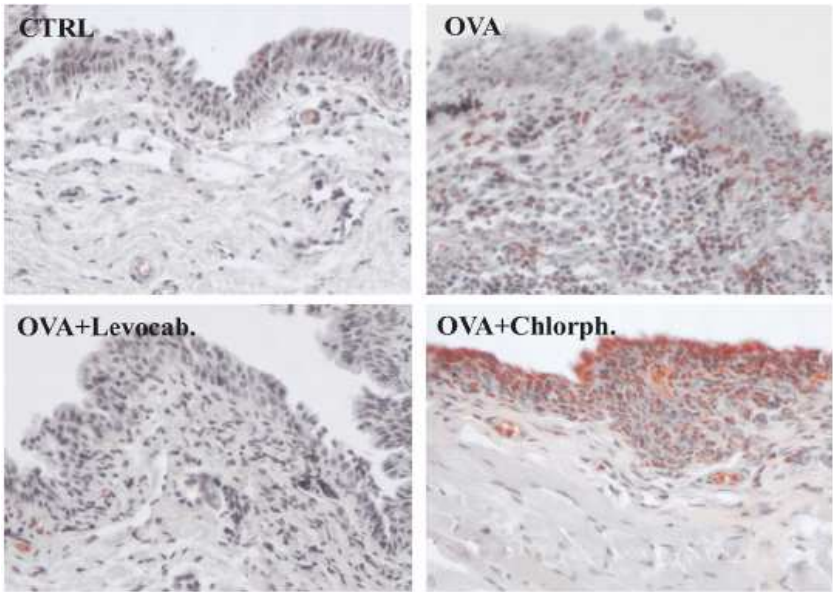


Figure 7.22
Photomicrographs of the conjunctiva 24h after topical challenge with ovalbumin. Substantial eosinophil infiltration is observed in ovalbumin-treated guinea pigs (OVA) in comparison to negative controls (animal non-immunized with ovalbumin) who received saline alone and were not challenged with ovalbumin (CTRL). In levocabastine treated guinea pigs there was much less eosinophil infiltration than in the ovalbumin treated group and in chlorpeniramine-treated animals.

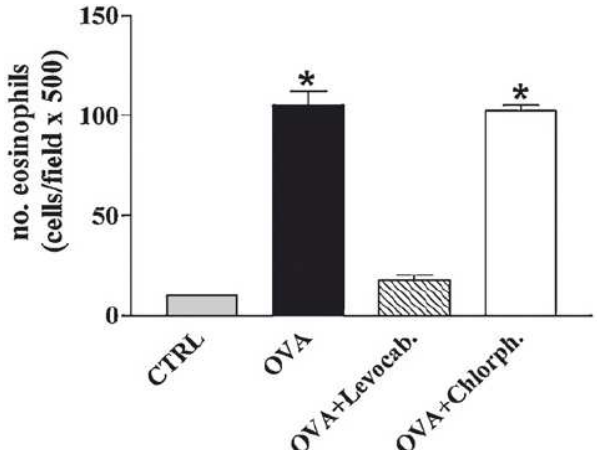


Figure 7.22
Effect of levocabastine and chlorpheniramine on eosinophil infiltration in the guinea pig conjunctiva 24 h after topical challenge with ovalbumin. The eosinophil in each field (500x) magnification were counted 24 h after antigen exposure. Controls received saline alone and were not challenged with ovalbumin (CTRL).

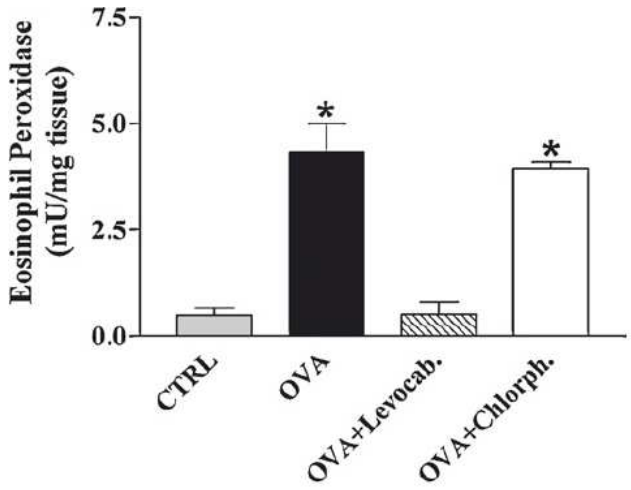


Figure 7.23
Effects of levocabastine and chlorpheniramine on conjunctival eosinophil peroxidase levels in the conjunctiva 24 h after topical challenge with ovalbumin.

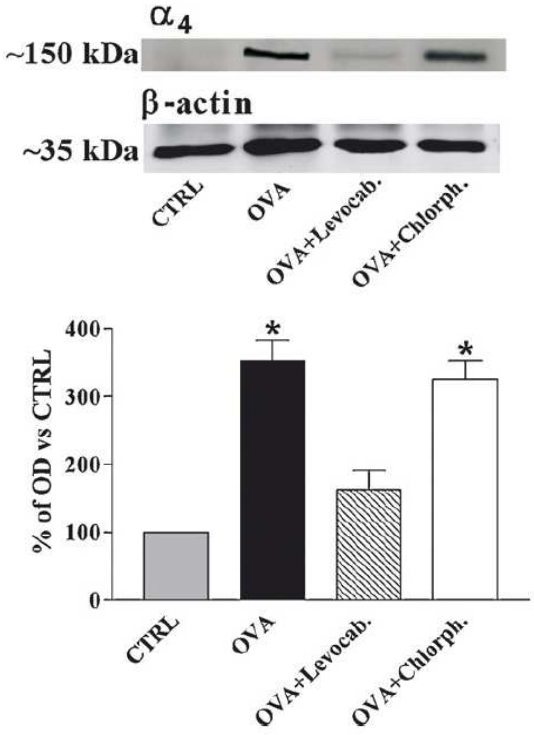


Figure 7.24
Effects of levocabastine and chlorpheniramine on conjunctival levels of α_4 integrin subunit 24 h after topical challenge with ovalbumin. Guinea pigs were euthanized, the conjunctiva was collected and protein extracted and used for western blot analysis. A respresentative western blot of three replicates and the densitometric analysis of the bands are shown. The approximate molecular mass of α_4 was determined by comparison to a molecular mass standard. OD refers to the relative optical density of each band, defined by normalization of the α_4 integrin subunit band to the β-actin bands (arbitrary units). Mean ±SEM (n=5). *$p<0.01$ vs CTRL (Newman Keuls test after ANOVA).

This increase was related to eosinophils and possibly other leukocytes infiltrating the conjunctiva and was significantly reduced by pre-treatment with levocabastine eye-drops but not by chlorpheniramine.

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CHAPTER 8
DISCUSSION

In this thesis a prototype of high-throughput screening for the identification of novel integrin antagonists was set up. This can be used to screen lead compounds in the development of drugs for the treatment of allergic inflammation. Moreover, these new technologies have been demonstrated to be extremely useful for the assay of drugs already used in therapy in order to better understand their mechanism of action and better specify their clinical use.

A scintillation proximity assay (SPA) allowed to determine the IC_{50} of levocabastine, a know H1 receptor antagonist, toward the $\alpha_4\beta_1$ integrin. The effectiveness of this approach was, then, tested in *in vitro* cell adhesion assays by measuring the ability of levocabastine to block the adhesion of EoL-1 and Jurkat E6.1 cell lines to multi-well plates coated with VCAM-1 or to monolayers of TNF-α activated HUVEC. Moreover, the levocabastine-mediated interference with leukocyte trafficking was corroborated by *in vivo* experiments on a guinea pig model of allergic conjunctivitis, that showed how pre-treating the animals with levocabastine eye drops before antigen challenge, significantly limits eosinophil tissue infiltration.

Tissue-specific recruitment of leukocytes is a requirement for immune surveillance in the body and plays a key role in the pathogenesis of various diseases. As a consequence, inhibition of immune cell trafficking between their source organ, blood and tissues have become a very attractive therapeutic approach. Given that leukocytes would not be depleted, impaired in their general function nor compromised to their capacity for circulation into the organs thus maintaining the physiological immune surveillance, such a strategy should be efficacious and, at the same time, should have little side effects. Therefore, targeting leukocyte recruitment is predicted to contrast systemic immunosuppressive therapies, which modify immune cell function or viability, resulting in the risk of severe side effects such as promotion of tumour development or infections.

Leukocyte tissue homing is generally regarded as a multistep process involving several adhesion molecules such as selectins, integrins and cytokine receptors. The very late antigen 4 (VLA-4, $\alpha_4\beta_1$ integrin) via its interaction with vascular cell adhesion molecule 1 (VCAM-1) appears to be of additional importance for leukocyte localization, whereas LFA-1 (CD11a/CD18, $\alpha_L\beta_2$) and Mac-1 (CD11b/CD18, $\alpha_M\beta_2$) bind to the intercellular adhesion molecule 1 (ICAM-1) contributing to leukocyte activation. Several lines of evidence have indicated that blocking leukocyte migration is efficacious in various animal models of inflammatory disorders. This has been demonstrated for all adhesive steps such as tethering and rolling, integrin activation by chemokines, firm adhesion, and finally transmigration of leukocytes through the endothelium. Indeed, natalizumab (Tysabri; Elan, Dublin, Ireland/Biogen Idec, Cambridge, MA, USA), a humanized monoclonal antibody targeting VLA-4, showed significant beneficial effects in controlled clinical trials of multiple sclerosis and Crohn's disease (Miller et al., 2003; Ghosh et al., 2003; Polman et al., 2006, Stüve and Bennett, 2007). Remission rates in Crohn's disease were up to 44% and response rates up to 71%, clearly indicating that therapeutic regimens targeting recirculation can be very effective. Unfortunately, soon after approval of natalizumab by the Food and Drug Administration (FDA), fatalities due to progressive multifocal leukoencephalopathy caused by reactivation of JC virus occurred, and Elan/Biogen Idec stopped sales of Tysabri. Given the multiple mechanisms through which VLA-4 may modulate immune function, it is incumbent upon future translational research to elucidate the pathways of natalizumab action that are relevant to the potential benefits and the risks observed during clinical development.

Another interesting approach is the one of combinatorial therapies in which a blocker of leukocyte trafficking can be associated with a cytokine receptor blocker. The pattern of the inflammatory response to an allergen is mediated by a number of different cells and a complex release of different inflammatory mediators. Generating small molecules against GPCRs is an established technology and, for some of the targets (CCR4, CCR10, CCR8, CXCR3), effective antagonists are already in clinical trials.

Ocular allergy management strategies are increasing exponentially. A growing list of clinically available agents is purposefully aimed at protecting the eye from inflammation. Concentrated focus on ocular allergy drug development is a natural derivative of an escalating number of allergy sufferers throughout the general population.

The $\alpha_4\beta_1$ integrin/VCAM-1 pathway is crucial for the firm adhesion and transmigration of eosinophils into the conjunctiva through vascular endothelial cells. The inhibition of conjunctival eosinophil by an integrin α_4 monoclonal antibody has been reported in a guinea pig model of allergic conjunctivitis (Ebihara et al., 1998). Treatment with anti-α_4 integrin and anti-VCAM-1 antibodies significantly suppressed the conjunctival eosinophil infiltration induced in mice by active immunization with ragweed-primed splenocytes (Fukushima et al., 2006). Whitcup et al. (1999) showed that antibodies against $\alpha_L\beta_2$ integrin and its counter-receptor ICAM-1 relieve both the clinical signs of immediate hypersensitivity and cellular infiltration into the conjunctiva, 24 h after antigen challenge in mice. Several antiallergic drugs, including levocabastine, down-regulate ICAM-1 expression (Bielory et al., 2005; Buscaglia et al., 1996; Ciprandi et al., 2003; Schultz, 2006; Ciprandi et al., 1996); Pesce et al. (2001) reported that in patients with allergic conjunctivitis, conjunctival endothelial cells expressed $\alpha_L\beta_2$ integrin. Therefore, blocking $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin-mediated cell adhesion may offer another strategy for the treatment of allergic conjunctivitis, as it prevents the development of the early and late phase responses.

$\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrins may act in concert to mediate eosinophil adhesion and signalling functions to meet the peculiar challenges these cells fare as they contact the vascular epithelium. "Cross-talk" between these integrins was proposed by Chan et al. (2000) who suggested that $\alpha_4\beta_1$ integrin, binding to VCAM-1, might also strengthen $\alpha_L\beta_2$-mediated adhesion of T lymphocytes to ICAM-1. With regard to eosinophils, Higashimoto et al. (1999) reported that adherence of EoL-1 to fibronectin was mediated by $\alpha_4\beta_1$ integrin and enhanced ICAM-1 expression, and this might contribute to increasing cell adhesion to the extracellular matrix and conjunctival eosinophil accumulation. Our data support this latter idea, as the blockade of eosinophil adhesion by levocabastine may prevent further eosinophil functional changes, including expression of adhesion molecules, such as ICAM-1 whereas levocabastine does not influence α_4 integrin expression. In fact, we observed that in the eosinophilic EoL-1 cell line levocabastine did not affect α_4 integrin expression in cells maintained for 12 h in fibronectin- or BSA-coated wells. In agreement with our data, Fukuishi et al. (2002) reported that the antiallergic drug olopatadine inhibited antigen-induced eosinophil infiltration and repressed IL-5-induced expression of $\alpha_L\beta_2$ integrin but not $\alpha_4\beta_1$ integrin. These *in vitro* data may help explain the reduction of the expression of

$\alpha_4\beta_1$ integrin that we observed in the conjunctiva of ovalbumin-sensitized guinea pigs treated with levocabastine. In fact, this effect might be a consequence of the reduction in recruitment and migration of eosinophils to the site of allergic inflammation by levocabastine rather than of levocabastine-induced $\alpha_4\beta_1$ down-regulation. Thus, hypothetically, levocabastine might exert its inhibitory effect on eosinophil adhesion binding to $\alpha_4\beta_1$ integrin, affecting its avidity as well as affinity for VCAM-1 and fibronectin. In fact, integrins increase their affinity or avidity to endothelial ligands upon exposure to chemokines and can undergo to conformational changes upon ligand binding apart from expression changes on the leukocyte surfaces (Broide and Sriramarao, 2001).

We confirmed the positive effect of levocabastine eye drops on early and late phase reactions induced by allergen specific-conjunctival challenge. Histamine and eicosanoids are responsible for the typical early phase reaction. However, mast cells also contribute to the synthesis and release of cytokines, chemokines and growth factors, triggering a cascade of inflammatory events on the surface of epithelial and endothelial cells that lead to the late phase reaction, with recruitment of eosinophils and neutrophils. Inflammatory cytokines may enhance the expression of E-selectin, ICAM-1 and VCAM-1 on the vascular endothelial cells and initiate the rolling of immune cells.

According to previous studies, levocabastine effect on the early phase response seems to be mediated by the blockade of histamine H1 receptors and mast cell stabilization (Dechant and Goa, 1991; Awouters et al., 1992). It reduces conjunctival eosinophil infiltration in the late phase response and, as proved in this study, affects $\alpha_4\beta_1$ integrin functions. However, it cannot be ruled out that levocabastine reduces inflammatory cell infiltration in the late phase response by affecting the release of cytokines and chemokines (Yanni et al., 1999; Noble and McTavish, 1995) from epithelial and mast cells.

Conjunctival epithelial cells do not express $\alpha_4\beta_1$ integrin (Fujihara et al., 1997) and its occurrence in the conjunctiva could be a consequence of eosinophil infiltration promoted by different mechanisms, independent of histamine binding to H1 receptors. This idea is further supported by experiments with the classical H1 antagonist chlorpheniramine. In fact, a 0.1% chlorpheniramine eye drops blocked only the early phase response of allergic conjunctivitis and did not reduce eosinophil infiltration or expression of the adhesion molecule $\alpha_4\beta_1$ in the conjunctiva of guinea pigs treated with ovalbumin. This drug did not modify Jurkat E6.1 or EoL-1 cell adhesion to VCAM-1 or fibronectin. First

generation anti-histmines like chlorpheniramine are weak inhibitors of histamine-stimulated cytokine synthesis (Yanni et al., 1999) while levocabastine is more potent (Dechant and Goa, 1991; Awouters et al., 1992). Furthermore, chlorpheniramine in an allergic model of allergic cutaneous antigen-induced inflammatory cell infiltration, did not significantly inhibit inflammatory cell infiltration in the late phase response (Charlesworth et al., 1992).

Up to now, investigations focused on levocabastine have ascertained the action of this compound on epithelial cells of the conjunctiva, while little is known on its effect on eosinophils. Kaisho and Akira (2006) have recently reported that the functional effects of some microbial products on eosinophils enhance their survival, can modulate the chemotactic migration and adhesion molecules expression. Focal adhesion kinase (FAK), a non-receptor protein tyrosine kinase 2 (PTK-2) involved in signalling downstream of integrins (Cary and Guan, 1999; Sieg et al., 2000) has been suggested to mediate inflammatory responses through the activation of MAPKs including p38 and ERK pathways (Neff et al., 2003; Chen et al., 1998). Cheung et al. (2008) finally speculated that FAK might participate in linking TLRs with the activation of MAPK cascade in human eosinophils, linking the action of adhesion molecules (integrins included) with degranulation and cytokine release of eosinophils (fig. 8.1).

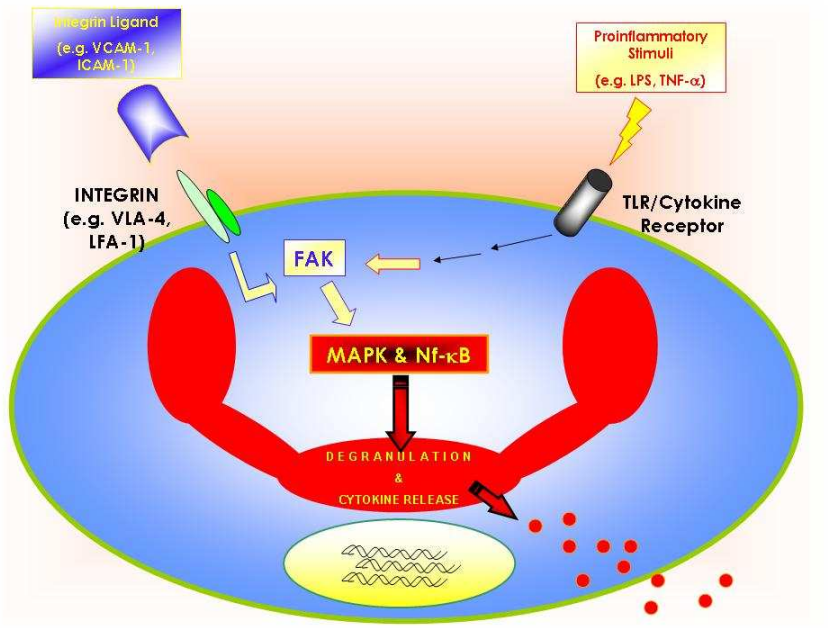

Figure 8.1
FAK-dependent activation of MAPK in eosinophil stimulation.

Levocabastine caused concentration-related blockad of adhesion of EoL-1 cells to VCAM-1 and fibronectin. The drug potency *in vitro* (IC_{50} around 400 μM) is important when this molar concentration is converted to a percentage weight/volume (400 μM equals about 200 μg/mL and is equivalent to a 0.02% solution). Because the conjunctiva is the target tissue for this compound, penetration and transport into the eye do not significantly affect drug delivery. For these reasons, and in view of the clinical efficacy of 0.05% levocabastine in allergic conjunctivitis models, the effect on $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin blockade appears acceptable. The analysis of the data related to cytokine release clearly shows that levocabastine is capable to cause in EoL-1 cells a statistically significant reduction of TNF-α-induced release of the cytokines IL-12p40, IL-8, VEGF. These cytokines are all involved, in different measure, in eosinophil survival and eosinophil-mediated inflammation (Choi et al., 2009; Gorksa et al., 2008; Meyts et al., 2006). Thus, levoacabastine seems to limit the autocrine and paracrine effects in support of the inflammatory process sustained by eosinophilic cytokine release (fig. 8.2).

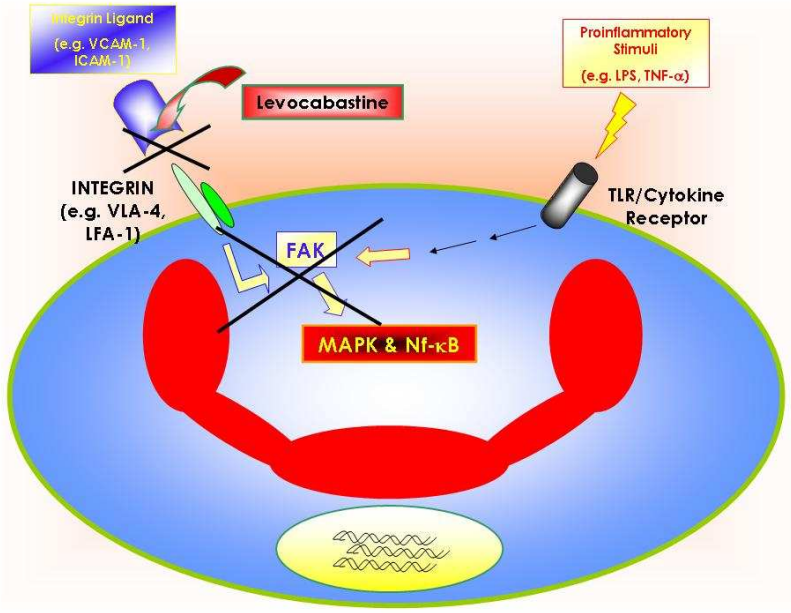

Figure 8.2
Levocabastine inhibition of eosinophil activation. The block of integrins interferes with the activation of focal adhesion kinases (FAKs), hampering the FAK-dependent activation of MAPK and Nf-κB an important event in eosinophil degranulation and cytokine release.

Levocabastine shows potent and sustained antiallergic activity after conjunctival administration. In ovalbumin-sensitized guinea pigs, the drug reduced or completely

prevented conjunctival symptoms and this effect lasted up to 24 h (Feinberg and Stokes, 1987). Complete inhibition of histamine-induced conjunctivitis persisted 24 h after topical levocabastine. Therefore, topical levocabastine may persist at an adequate conjunctival concentration for up to 6-8 h, long enough to interfere with eosinophil migration to the ocular surface.

Finally, we propose that levocabastine acts through different mechanisms as an antiallergic agent and some of the drug cellular targets may play a role in eosinophil adhesion. Topical levocabastine, by blocking integrin-mediated cell adhesion, may inhibit the development of the late phase response of allergic conjunctivitis. This compound easily reaches conjunctival cells and vessels when administered topically, and can prevent the recruitment and activation of eosinophils as well as other immune cells (e.g. neutrophils and other leukocytes).

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APPENDIX I
ARTERIES AND VEINS

Most of our knowledge about the vasculature is restricted to conduit vessels, namely the large arteries and veins. Arteries deliver well oxygenated (red-coloured) blood from the heart to the various tissues of the body, whereas veins collect and return deoxygenated blood (blue in color) back to the heart. Arteries have a thick muscular wall; veins have a thin, distensible wall. Arteries pulsate, whereas veins do not; large arteries are located deep within tissues, and are thus protected from traumatic injuries. Veins lie both deep and superficial. Capillaries are the macroscopically invisible blood vessels that connect *arterioles* to *venules*, thus creating a closed circulation, and at this level happens the exchange of gases, nutrients and catabolic substances. Their wide presence all over the organs and tissues of the body make them the vast majority of the surface area of the circulation. Capillaries have an extremely thin wall consisting of a single layer of **endothelial cells** surrounded to a variable degree by occasional **perycites** and extracellular matrix. The **endothelium** represents the cellular interface between circulating blood and underlying tissues: an healthy endothelium is *highly* ***active*** *and* ***adaptive***. In fact, each endothelial cell is analogous to a miniature adaptive input-output device. Input arises from the extracellular environment and may include biomechanical and biochemical stimuli; the nature of output depends on the level of organization and scale of investigation (Fig a.1). Single endothelial cells may undergo a change in cell shape or alteration in protein or mRNA expression, or they may proliferate, migrate or undergo apoptosis. *Monolayers of endothelial cells express barrier properties and may be assayed for adhesion and transmigration of white blood cells.* Other properties of the endothelium emerge only in the context of the blood vessel, whole organ or organism, such as endothelial-dependent regulation of *vasomotor tone*, *angiogenesis*, and *redistribution of blood flow*. The input-output device is not a black box, but rather contains a highly elaborate, non-linear array of dynamic signal transduction pathways that couple signals at the surface membrane to cellular response or phenotype.

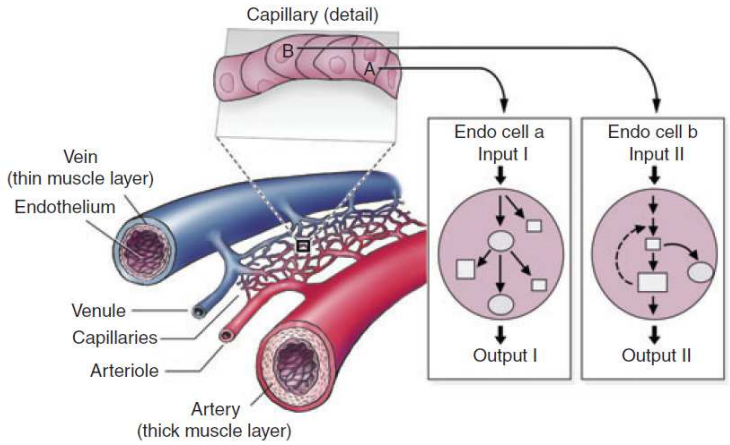

Figure a.1
Endothelial cells as input-output devices: as input changes in time and space, these cells adapt leading to phenotypic heterogeneity. (illustrated by Steve Moskovitz)

The input from the microenvironment change in space and time. For example, the blood-brain barrier endothelium is exposed to astroglial derived factors, whereas capillary endothelium in the heart is subject to paracrine signals from neighbouring cardiomyocytes. As input is coupled to output, variation in the extracellular environment across space and time leads to phenotypic heterogeneity, characteristic of an healthy endothelium (Fig. a.2).

Endothelial cells respond selectively to well defined stimuli, becoming *activated*. According to this view, quiescent endothelial cells express an anticoagulant, antiadhesive and vasodilatory phenotype, whereas activated endothelial cells express procoagulant, proadhesive and vasoconstrictory properties. This events are not simply occurring as an all-or-none phenomenon. In fact, endothelial cells' responses are graded on the basis of the strength of the stimulus, in a dose-dependent manner. Moreover, this pictures is further complicated by a site-specific expression of proteins that cannot be considered as markers of activation. For example, while P-selectin is considered a marker of endothelial cell activation, it is constitutively expressed in dermal microvessels of uninflamed skin. Another example is provided by thrombomodulin, whose expression is usually downregulated in endothelial cells during inflammation. However, the brain expresses little or no thrombomodulin and the toggle hypothesis would suggest that the blood-brain barrier is a chronic state of activation, which is not the case. The brain relies on other anticoagulants to

balance local hemostasis. Therefore, *endothelial cell activation must be adjudicated in an appropriate temporal and spatial context.*

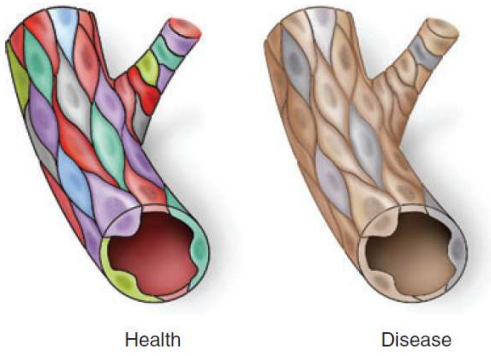

Figure a.2
In this hypothetical scheme, endothelial cell phenotype is represented by a color shade. On the right, according to a model of decomplexification (Golberger et al., 2002), a representation of a dysfunctional endothelium. (illustration by Steve Moskowitz)

Endothelial cells are strategically located at the interface between blood and tissues. In addition to responding rapidly (seconds to minutes) to agonists such as histamine and thrombin, vascular cells, upon exposure to cytokines, undergo profound alteration of function that involve gene expression and protein synthesis, requires hours to develop, and are relatively long-lasting. Vascular cells are both a target for and a source of cytokines. These soluble polypeptide mediators serve as communication signals with leukocytes as well as with diverse tissues and organs. The spectrum of responses elicited in endothelial cells by different cytokines is extremely vast and varied. Different cytokines activate distinct, largely non-overlapping, sets of functions that can be grouped into programs of activation/differentiation.

APPENDIX II
THE IMMUNE SYSTEM ELEMENTS

The cells of the immune system originate in the bone marrow, where many of them also mature. They then migrate to guard the peripheral tissues, circulating in the blood and in a specialized system of vessels called the lymphatic system. During this *recirculation*, they receive so-called **tonic signals** via their receptors that contribute to their survival.

All the cellular elements of blood, including the red blood cells, the platelets and the white blood cells derive ultimately from the **hematopoietic stem cells** in the bone marrow. As these stem cells can give rise to all the different types of blood cells, they are often known as pluripotent hematopoietic stem cells.

The **myeloid progenitor** is the precursor of the granulocytes, macrophages, dendritic cells, and mast cells of the innate immune system. Macrophages are one of the three types of phagocyte in the immune system and are distributed widely in the body tissues, where they play a critical part in innate immunity. They are the *mature form of* **monocytes**, which circulate in the blood and differentiate continuously into macrophages upon migration into tissues. **Dendritic cells** are specialized to take up antigen, process it, and display it for recognition by T lymphocytes.

Mast cells, whose blood-borne precursors are not well defined, also differentiate in the tissues. They mainly reside near small blood vessels and, when activated, release substances that affect vascular permeability. Although best known for their role in orchestrating allergic responses they are believed to play a part in protecting mucosal surfaces against pathogens.

The **granuolocytes** are so called because they have densely staining granules in their cytoplasm; they are also sometimes called polymorphonuclear leukocytes because of their oddly shaped nuclei. There are *three types* of granulocytes, all of which are relatively short

lived, and they are produced in increased numbers during immune responses, when they are produced in increased numbers during immune responses, when they leave the blood to migrate to sites of infection or inflammation. **Neutrophils** which are the third phagocytic cell of the immune system, are the most numerous and most important cellular component of the innate immune response: hereditary deficiencies in neutrophil function lead to overwhelming bacterial infection, which is fatal if untreated. **Eosinophils** are thought to be important chiefly in defense against parasitic infections, because their numbers increase when the body has a parasitic infection. The function of **basophils** is probably similar and complementary to that of mast cells.

The **common lymphoid progenitor** gives rise to the **lymphocytes**, and to the **natural killer cells** (NK) of innate immunity. In addition, it is also able to give rise to dendritic cells that appear indistinguishable form those derived from the common myeloid progenitor. However, as there are more common myeloid progenitors than there are common lymphoid progenitors, the majority of the dendritic cells in the body develop from common myeloid progenitors (fig. 1).

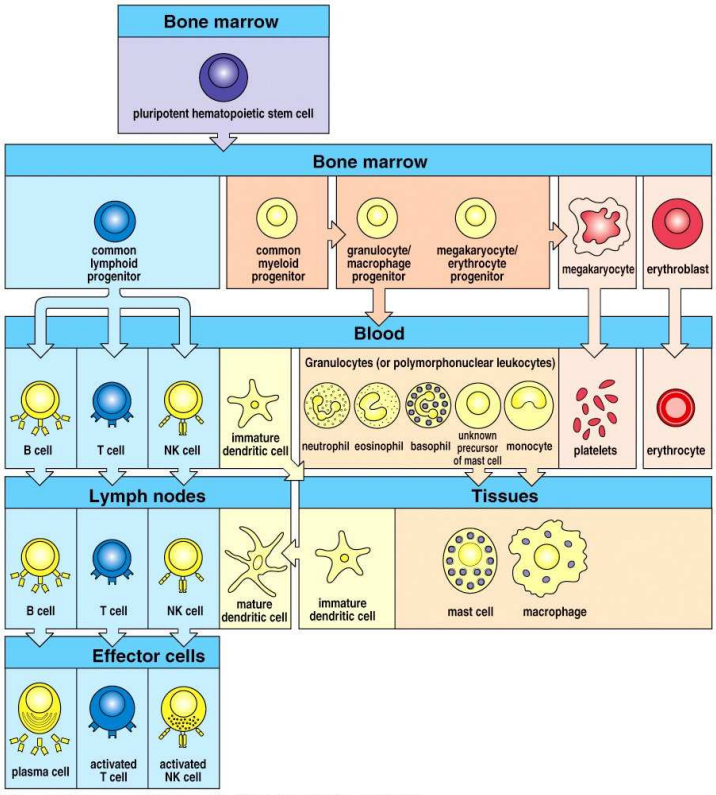

Figure 1
Immune cell differentiation from pluripotent hematopoietic stem cells.

Innate immunity is an essential prerequisite for the adaptive immune response, because the antigen-specific lymphocytes of the adaptive immune response are activated by co-stimulatory molecules that are induced on cells of the innate immune system during their interaction with microorganisms. The *cytokines* produced during these early phases also have an important role in stimulating the subsequent adaptive immune response and shaping its development. The most frequent sites of encounter between the body and microorganisms and other antigens are the mucosal surfaces. A mucosal immune system lines the airways, grastrointestinal tract, and urogenital system, and is the most extensive compartment of the immune system.

The **adaptive immune response** is required for effective protection of the host against pathogenic microorganisms. The response of innate immune system to pathogens helps to initiate the adaptive immune response, as interactions with these pathogens lead to the production of cytokines and the activation of dendritic cells to activated antigen-presenting cell status (fig. 2).

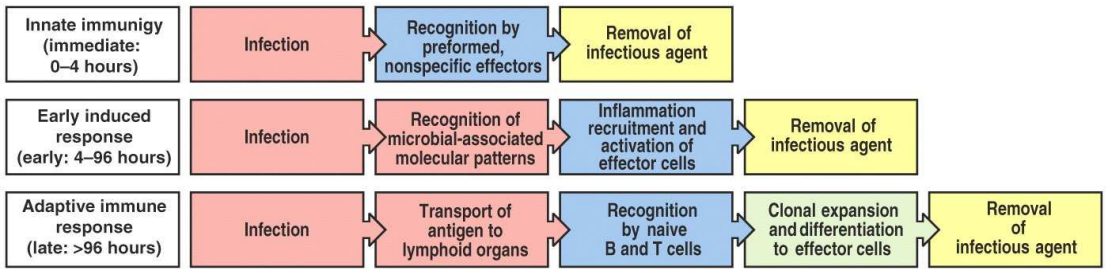

Figure 2
Innate and adaptive immune responses.

Allergic reactions occur when an individual who has produced IgE antibody in response to an **allergen**, subsequently encounters the same allergen. The allergen triggers the activation of IgE-binding cells, including *mast cells* and *basophils*, in the exposed tissue, leading to a series of responses that are characteristic to **allergy**. IgE is involved in protective immunity, especially in response to parasitic worms, which are prevalent in less economically developed countries. Allergy is one of a class of immune system responses that are termed **hypersensitivity reactions**. These are harmful immune responses that produce *tissue injury* and can cause *serious disease.*

IgE is produced by **plasma cells** located in lymph nodes draining the site of antigen entry and also locally, at the sites of allergic reactions, by plasma cells derived from

germinal centers developing within the inflamed tissue. IgE differs from other antibody isotypes in being predominantly localized in tissues, where it is tightly bound to mast-cell surfaces through the high-affinity IgE receptor known as **FcεRI**. Binding of antigen to IgE cross-links these receptors and this causes the release of chemical mediators from the mast cells, which can lead to the development of a **type I hypersensitivity reaction**. Basophils also express FcεRI; they can therefore display surface-bound IgE and also take part in the production of type I hypersensitivity reactions. The factors that lead to an antibody response dominated by IgE are still being worked out (fig. 3).

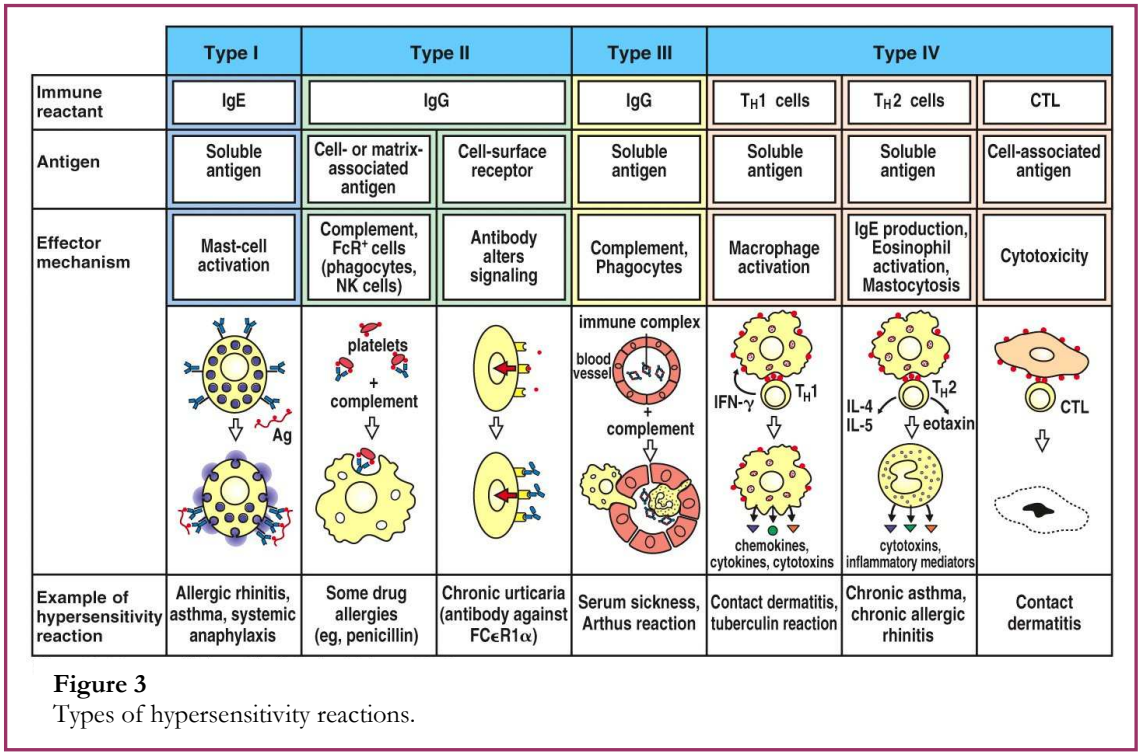

Figure 3
Types of hypersensitivity reactions.

As many as 40% of people in Western populations show an exaggerated tendency to mount IgE responses to a wide variety of common environmental allergens. This state is called **atopy**, has a strong familial basis, and is influenced by several genetic loci. Atopic individuals have higher total levels of IgE in the circulation and higher levels of eosinophils than their normal counterparts. They are more susceptible to allergic diseases such as *hay fever* and *asthma*. Genetic variations of the IL-4 gene has been associated with raised IgE levels in atopic individuals; the variant promoter directs increased expression of a reporter gene in experimental systems.

Eosinophils are granulocytic leukocytes that originate in the bone marrow. They are so called because their granules, which contain arginine-rich basic proteins, are colored bright orange by the acidic stain eosin. Only very small numbers of these cells are normally present in the circulation; most eosinophils are found in tissues, especially in the connective tissue immediately underneath respiratory, gut, and urogenital epithelium, implying a likely role for these cells in defense against invading oranisms. Eosinophils have to kind of effector function. First, on activation they release highly toxic granule proteins and free radicals, which can kill microorganisms and parasites but also cause significant tissue damage in allergic reactions. Second, activation induces the synthesis of chemical mediators such as **prostaglandins**, **leukotrienes**, and **cytokines**. These amplify the inflammatory response by activating epithelial cells and recruiting and activating more eosinophils and leukocytes.

The **activation** and **degranulation** of eosinophils is strictly regulated, as their inappropriate activation would be harmful to the host (fig. 4).

Class of product	Examples	Biological effects
Enzyme	Eosinophil peroxidase	Toxic to targets by catalyzing halogenation Triggers histamine release from mast cells
	Eosinophil collagenase	Remodels connective tissue matrix
Toxic protein	Major basic protein	Toxic to parasites and mammalian cells Triggers histamine release from mast cells
	Eosinophil cationic protein	Toxic to parasites Neurotoxin
	Eosinophil-derived neurotoxin	Neurotoxin
Cytokine	IL-3, IL-5, GM-CSF	Amplify eosinophil production by bone marrow Cause eosinophil activation
Chemokine	CXCL8 (IL-8)	Promotes influx of leukocytes
Lipid mediator	Leukotrienes C4, D4, E4	Cause smooth muscle contraction Increase vascular permeability Increase mucus secretion
	Platelet-activating factor	Attracts leukocytes Amplifies production of lipid mediators Activates neutrophils, eosinophils, and platelets

Figure 4
Chemical mediators and substances present in eosinophilic granules.

The first level of control acts on the production of eosinophils by the bone marrow. Few eosinophils are produced in the absence of infection or other immune stimulation. But when T_H2 cells are activated, cytokines such as IL-5, are released that increase the

production of eosinophils in the bone marrow and their release into the circulation. However, transgenic animals overexpressing IL-5 have increased numbers of eosinophils in the circulation but not in their tissues, indicating that the migration of eosinophils from the circulation into tissues is regulated separately, by a second set of controls. The key molecules in this case are CC chemokines, most which cause chemotaxis of several types of leukocytes, but three are particularly important for eosinophil chemotaxis and have been named **eotaxin 1** (CCL11), **eotaxin 2** (CCL24), and **eotaxin 3** (CCL26).

The eotaxin receptor on eosinophils, **CCR3**, is a member of the chemokine family of receptors. The eotaxins and these other CC chemokines also activate eosinophils. Identical or similar chemokines stimulate mast cells and basophils. For example, eotaxins attract basophils and cause their degranulation, and CCL2, which binds to CCR2, similarly activates mast cells in both the presence and the absence of antigen. CCL2 can also promote the differentiation of naïve T_H0 cells to T_H2 cells; T_H2 cells also carry the receptor CCR3 and migrate toward eotaxins.

A third set of controls regulates the state of eosinophil activation. In their nonactivated state, eosinophils do not express high-affinity IgE receptors and have a high threshold for release of their granule contents. After activation by cytokines this threshold drops, FcεRI is expressed, and the number of Fcγ receptors and complement receptors on the cell surface also increases. The eosinophil is now primed to carry out its effector activity – for example degranulation in response to antigen that cross-links specific IgE bound to FcεRI on the eosinophil surface.

In a local allergic reaction, mast-cell degranulation and T_H2 activation cause eosinophils to accumulate in large numbers and to become activated. Their continued presence is characteristic of chronic allergic inflammation and they are thought to be major contributors to tissue damage. **Eosinophils**, **mast cells**, and **basophils** can interact with each other. Eosinophil degranulation releases **major basic protein**, which in turn causes degranulation of mast cells and basophils. This effect is augmented by any or the cytokines that affect eosinophil and basophil growth, differentiation, and activation, such as IL-3, IL-5, and GM-CSF.

APPENDIX III
DETAILED EXPERIMENTAL PROCEDURES

Fibronection labelling with Na[^{125}I]

Fibronectin labelling was performed by the use of the IODO-GEN iodination reagent (1,3,4,6-tetrachloro-3α-6α-diphenylglycouril) by Pierce (Rockford, IL, USA). During oxidative reactions in the pre-coated tubes the iodination reagent remains adherent to the vessel because it is insoluble in typical aqueous media. This feature enables the sample to be decanted from the coated vessel to terminate the oxidative reaction.

1 mL of Tris Iodination Buffer (25 mM TrisHCl, pH 7.5; 0.4 M NaCl) was used to rinse the pre-coated tube, in which where then added 100 µL of Tris Buffer (25 mM TrisHCl, pH 7.5; 0.4 M NaCl; 5 mM EDTA). At this point 1.0 mCi of Na[^{125}I] (in a volume of around 10 µL) are put into the tube and gently mixed. After 6 minutes incubation (swirling the tube every 30 seconds) the iodide is activated and can be transferred to the fibronectin solution (1.0 nmol in 100 µL of Tris Iodination Buffer) where it is incubated for 6-9 minutes at room temperature, flicking the tube every 30 seconds. At the end of the incubation 50 µL of Scavenging Buffer (10 mg tyrosine/mL in Tris Iodination Buffer or PBS, pH 7.4) were added, mixed and incubated for 5 minutes with additional flicking at 1 and 4 minutes. Then, 1 mL of Tris/BSA Buffer (0.25% bovine serum albumin; 25 mM TrisHCl, pH 7.5; 0.4 M NaCl; 5 mM EDTA; 0.05% sodium azide) was added for blocking non-specific binding sites on the desalting column. A 5 µL aliquot was taken to assess the total amount of ^{125}I incorporated. The sample was then loaded on a 10 mL bed volume desalting column pre-equilibrated with 20 mL of Tris/BSA Buffer. At the end of the column the radioactive is recovered and the eluted fractions are scanned for radioactivity.

Determination of protein bound and free ^{125}I by standard trichloricacetic acid (TCA) precipitation procedure

5 µL of the eluted fraction were aliquoted in a glass tube to which were added 3 mL of 10% TCA/ 1% $Na_4P_2O_7$ and incubated on ice for 20 minutes. Then, class C/glass fiber

filters, pre-wet in 10% TCA/ 1% $Na_4P_2O_7$ solution, were placed on a filtering unit and the radioactive solution was decanted over them. After two washes with a 5% TCA solution, rinsing the tubes to increase the yield, filters were wet with absolute ethanol and kept under vacuum until complete dryness, ready to be counted.

Calculating the labelling yield

From the aminoacidic sequence of fibronectin (see image) it is possible to count 93 tyrosine residues, that represent the reactive moieties toward ^{125}I.

MLRGPGPGLLLLAVLCLGTAVPSTGASKSKRQAQQMVQPQSPVAVSQSKPGCYDNGKHYQINQQWERTY
LGNALVCTCYGGSRGFNCESKPEAEETCFDKYTGNTYRVGDTYERPKDSMIWDCTCIGAGRGRISCTIA
NRCHEGGQSYKIGDTWRRPHETGGYMLECVCLGNGKGEWTCKPIAEKCFDHAAGTSYVVGETWEKPYQG
WMMVDCTCLGEGSGRITCTSRNRCNDQDTRTSYRIGDTWSKKDNRGNLLQCICTGNGRGEWKCERHTSV
QTTSSGSGPFTDVRAAVYQPQPHPQPPPYGHCVTDSGVVYSVGMQWLKTQGNKQMLCTCLGNGVSCQET
AVTQTYGGNSNGEPCVLPFTYNGRTFYSCTTEGRQDGHLWCSTTSNYEQDQKYSFCTDHTVLVQTRGGN
SNGALCHFPFLYNNHNYTDCTSEGRRDNMKWCGTTQNYDADQKFGFCPMAAHEEICTTNEGVMYRIGDQ
WDKQHDMGHMMRCTCVGNGRGEWTCIAYSQLRDQCIVDDITYNVNDTFHKRHEEGHMLNCTCFGQGRGR
WKCDPVDQCQDSETGTFYQIGDSWEKYVHGVRYQCYCYGRGIGEWHCQPLQTYPSSSGPVEVFITETPS
QPNSHPIQWNAPQPSHISKYILRWRPKNSVGRWKEATIPGHLNSYTIKGLKPGVVYEGQLISIQQYGHQ
EVTRFDFTTTSTSTPVTSNTVTGETTPFSPLVATSESVTEITASSFVVSWVSASDTVSGFRVEYELSEE
GDEPQYLDLPSTATSVNIPDLLPGRKYIVNVYQISEDGEQSLILSTSQTTAPDAPPDPTVDQVDDTSIV
VRWSRPQAPITGYRIVYSPSVEGSSTELNLPETANSVTLSDLQPGVQYNITIYAVEENQESTPVVIQQE
TTGTPRSDTVPSPRDLQFVEVTDVKVTIMWTPPESAVTGYRVDVIPVNLPGEHGQRLPISRNTFAEVTG
LSPGVTYYFKVFAVSHGRESKPLTAQQTTKLDAPTNLQFVNETDSTVLVRWTPPRAQITGYRLTVGLTR
RGQPRQYNVGPSVSKYPLRNLQPASEYTVSLVAIKGNQESPKATGVFTTLQPGSSIPPYNTEVTETTIV
ITWTPAPRIGFKLGVRPSQGGEAPREVTSDSGSIVVSGLTPGVEYVYTIQVLRDGQERDAPIVNKVVTP
LSPPTNLHLEANPDTGVLTVSWERSTTPDITGYRITTTPTNGQQGNSLEEVVHADQSSCTFDNLSPGLE
YNVSVYTVKDDKESVPISDTIIPAVPPPTDLRFTNIGPDTMRVTWAPPPSIDLTNFLVRYSPVKNEEDV
AELSISPSDNAVVLTNLLPGTEYVVSVSSVYEQHESTPLRGRQKTGLDSPTGIDFSDITANSFTVHWIA
PRATITGYRIRHHPEHFSGRPREDRVPHSRNSITLTNLTPGTEYVVSIVALNGREESPLLIGQQSTVSD
VPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITV
YAVTGRGDSPASSKPISINYRTEIDKPSQMQVTDVQDNSISVKWLPSSSPVTGYRVTTTPKNGPGPTKT
KTAGPDQTEMTIEGLQPTVEYVVSVYAQNPSGESQPLVQTAVTTIPAPTDLKFTQVTPTSLSAQWTPPN
VQLTGYRVRVTPKEKTGPMKEINLAPDSSSVVVSGLMVATKYEVSVYALKDTLTSRPAQGVVTTLENVS
PPRRARVTDATETTITISWRTKTETITGFQVDAVPANGQTPIQRTIKPDVRSYTITGLQPGTDYKIYLY
TLNDNARSSPVVIDASTAIDAPSNLRFLATTPNSLLVSWQPPRARITGYIIKYEKPGSPPREVVPRPRP
GVTEATITGLEPGTEYTIYVIALKNNQKSEPLIGRKKTGQEALSQTTISWAPFQDTSEYIISCHPVGTD
EEPLQFRVPGTSTSATLTGLTRGATYNIIVEALKDQQRHKVREEVVTVGNSVNEGLNQPTDDSCFDPYT
VSHYAVGDEWERMSESGFKLLCQCLGFGSGHFRCDSSRWCHDNGVNYKIGEKWDRQGENGQMMSCTCLG
NGKGEFKCDPHEATCYDDGKTYHVGEQWQKEYLGAICSCTCFGGQRGWRCDNCRRPGGEPSPEGTTGQS
YNQYSQRYHQRTNTNVNCPIECFMPLDVQADREDSRE

Aminoacidic sequence of fibronectin. The 93 tyrosines are highlighted.

The important parameters that can be calculated are the following:

$$TotalCounts = (TotalCPM)x(Volume)$$

$$SpecificActivity = \frac{TotalCounts}{InitialFN(\mu g)}$$

$$LabellingYield = \frac{TCAcounts}{noTCAcounts}$$

Purification of $\alpha_4\beta_1$ integrin from HEK293 cells stably transfected with the α_4 subunit gene

Cells were harvested using a PBS based Cell Dissociation Buffer (GIBCO™) and the cell suspension obtained (10^8 cells/mL) was spun at 1200 rpm for 5 minutes. The pellet was washed once with PBS and resuspended in 5 volumes of lysis buffer (1% NP40; 25 mM TrisHCl, pH 7.5; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 2% BSA; 1 mM PMSF; 1 μg/mL aprotinin; 50 μM leupeptin) and homogenized on ice. After centrifuging at 10000g for 30 minutes at 4 °C. Load the supernatant sequentially on the affinity chromatography column (wheat germ agglutinin, WGA, lectin from *Triticum Vulgaris*) (Sigma-Aldrich) previously equilibrated with the Running Buffer (1% NP40; 25 mM TrisHCl, pH 7.5; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 1 mM $MnCl_2$; 2% BSA; 1 mM PMSF; 1 μg/mL aprotinin; 50 μM leupeptin). After 10 column volume washes, the proteins are eluted with by addition of 5% N-acetylglucosamine to the Running Buffer.

GLOSSARY

β-thalassemia	Form of thalassemia due to mutations in the HBB gene on chromosome 11 inherited in an autosomal recessive fashion
Acute Respiratory Distress Syndrome	Inflammation of the lung and parenchyma leading to impaired gas exchange with concomitant systemic release of inflammatory mediators
Affinity	The strength of binding between an individual integrin and its ligand. This can be altered by conformational changes in the integrin structure
Antigen-presenting Cells	A general term for all cells that display antigenic peptides in complex with major histocompatibility complex (MHC) molecules. The professional antigen-presenting cells are the dendritic cells, B cells and, occasionally, macrophages. They present antigen to lymphocytes
Avidity	A measure of the overall strength of binding of clustered integrins
Cachexia	General physical wasting and malnutrition associated with chronic disease
Chemosis	Swelling of the conjunctival tissue around the cornea
Chymases	Family of serine proteases found primarily in mast cells
Cryptococcus Neoformans	Causes a severe form of meningitis and meningo-encephalitis
Edema	Excess of fluid in interstitial or serous cavities; it can be either be exudate or transudate.
Eicosanoid Class Switching	Changes in production within the arachidonate-derived family – for example, prostaglandin and leukotriene to lipoxin – which initiate and/or are coincident with termination
Exudate	Inflammatory extravascular fluid that has a high protein concentration, cellular debris, and a specific gravity above 1.020
Exudation	Escape of fluid, proteins, and blood cells from the vascular system

	into the interstitial tissue or body cavities
Glomerulonephritis	Nephritis marked by the inflammation of the glomeruli of the kidney
Haptotaxis	Directional motility or outgrowth of cells, usually up a gradient of cellular adhesion sites or substrate-bound chemoattractants.
High Endothelial Venule	Specialized vasculature that is found only in lymph nodes
Immunological Synapse	The zone of contact between a lymphocyte or a natural killer cell and a target cell. On the lymphocyte side the immunological synapse receptors are organized to resemble a "bull's eye" with the T cell receptor or B cell receptor in the middle surrounded by a ring of LFA-1, with an outermost ring where the phosphatase CD45 is found
Immunoreceptor Tyrosine-based Activation Motif (ITAM)	A double YxxL motif that is separated by a 7-12 amino-acid linker. These motives are often found in immune-cell signalling molecules such as the CD3 ξ-chain of the T cell receptors, the γ-chain of the Fc receptor (and certain other receptors) and DAP12 proteins. Once phosphorylated, often by a Src kinase, the tyrosine residues act as a docking site for SH2 domains in other signalling kinases
Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM)	A conserved IVLxYxxIVL sequence found in the cytoplasmic part of many inhibitory receptors. Upon interaction of an inhibitory receptor with its ligand, the ITIM motifs become phosphorylated by Src family of kinases, allowing them to interact with phosphatases such as the phosphotyrosine phosphatases SHP-1 and SHP-2, or the inositol-phosphatase SHIP. The phosphatases recruited to ITIM motifs prevent activation of molecules involved in the signal transduction in the cell
Infection	Pathologic process caused by the invasion of normally sterile tissue or fluid or body cavity by pathogenic or potentially pathogenic microorganisms
Kawasaki Disease	Inflammation of the middle-sized arteries that affect many organs including the skin, mucous membranes, lymph nodes, and blood vessel wall
Killer-cell Immunoglobulin-like Receptors (KIR)	Family of cell surface glycoproteins expressed by all natural killer cells and a subset of T cells that interacts with MHC I molecules. KIR proteins are classified according to the number of the extracellular immunoglobulin domains (2D or 3D) and by the presence of either long (L) or short (S) intracellular domain. KIR proteins with the long cytoplasmic domain are capable of induction of inhibitory signals and early termination of natural killer cell

	activity upon ligand binding via one or two ITIM motifs located in their cytoplasmic domain. Less common KIR proteins with the short cytoplasmic tail, lacking the ITIM motifs, are involved in signalling activation
Leukocytes	Collective term for all immune cells such as T and B cells, dendritic cells, neutrophils, eosinophils, natural killer cells, and monocytes/macrophages
Lymphocytes	Refers only to T lymphocytes (T cells) and B lymphocytes (B cells)
Lytic Granules	A unique type of lysosomes present in natural killer cells and cytotoxic T cells that is secreted in a contact-dependent manner, in response to lymphocyte activation. Following the release of lytic granules at the cell-cell contact interface, the pore forming protein perforin, serine proteases (granzymes), and Fas ligand contained in the granules mediate the death of a target cell
Major Histocompatibility Complex (MHC)	A complex of genes encoding proteins involved in antigen presentation and immune responses. MHC proteins are classified as class I, class II and class III. Class I molecules (MHC I) are cell surface heterodimers consisting of a chain associated with β_2-microglobulin and are present on all nucleated cells. Class II proteins are cell surface heteromdimers comprising an a and b chain and are present on antigen presenting cells. Class III molecules are distinct from class I and class II and include complement components as well as tumor necrosis factor α and β
Margination	White cells assume a peripheral position along the endothelial surface
Nummular	Circular or oval in shape
Palmoplantar Pustulosis	Chronic recurrent pustular dermatitosis localized on the palms and the soles only, histologically characterized by intraepidermal vescicles filled with neutrophils
PSGL-1	P-selectin glycoprotein ligand 1
Pus	Purulent exudate: inflammatory exudate rich in leukocytes (mostly neutrophils), the debris of dead cells and, in many cases, microbes
Relapsing Fever	Infections caused by certain bacteria in the genus *Borrelia*.
Resolvins	Resolution phase interaction products. Endogenous local mediators possessing stereospecific and potent anti-inflammatory in addition to immunoregolatory actions
Sarcoidosis	Multisystem disorder characterized by non-caseating granulomas

Schistosoma Mansoni	Parasite of humans of the family of the trematodes. It causes schistosomiasis
Stasis	Concentration of red cells in small vessels and increased viscosity of the blood
Steel Locus	Encodes for a product which is essential for normal development of three distinct populations of stem cells: the neural crest-derived melanoblasts, germ cells, and blood cell precursors
T Cell Receptor and B Cell Receptor	The antigen-specific receptors, or immunoreceptors, on T cells and B cells, which recognise a wide array of different peptides that have been processed and displayed by antigen presenting cells
Toll-like Receptors (TLRs)	Are homologous to a *drosophila* protein called Toll, function to activate leukocytes in response to different types and components of microbes. Different TLRs play essential roles in cellular responses to bacterial lipopolysaccharide (LPS), other bacterial proteoglycans, and unmethylated CpG nucleotide, all of which are found only in bacteria, as well as double stranded RNA, which is produced only by some viruses. These receptors function by receptor-associated kinases to stimulate the production of microbicidal substances and cytokines in the leukocytes.
Transcellular Biosynthesis	Generation of new bioactive compounds that neither cell type can produce on its own
Transudate	Fluid with low protein content (most of which is albumin) and a specific gravity of less than 1.012 It is essentially an ultrafiltrate of blood plasma that results from osmotic or hydrostatic imbalance across the vessel wall without an increase in vascular permeability

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I would like to thank all the people that with their teachings and working examples have constantly guided my studying path.