Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN SCIENZE BIOCHIMICHE E BIOTECNOLOGICHE

XXVI CICLO

Settore Concorsuale di afferenza: 05/G1
Settore Scientifico disciplinare: BIO14

DISCOVERY AND PHARMACOLOGICAL CHARACTERIZATION OF A NOVEL SMALL MOLECULE ANTAGONIST OF INTEGRIN $\alpha_4\beta_1$: FOCUS ON ANTIALLERGIC ACTIVITY

Presentata da:

Dott.ssa Samantha Deianira Dattoli

Coordinatore Dottorato
Chiar.mo Prof.
Santi Mario Spampinato

Relatore
Chiar.mo Prof.
Santi Mario Spampinato

Anno Accademico 2012-2013

TABLE OF CONTENTS

1. ALLERGY AND INFLAMMATION: a complex immune origin disease

The term 'allergy' was coined by Clemens von Pirquet in 1906 to call attention to the unusual propensity of some individuals to develop signs and symptoms of reactivity, or 'hypersensitivity reactions', when exposed to certain substances. This term allergy can be used to refer to abnormal adaptive immune responses that either involve or do not involve allergen-specific IgE. Allergic inflammation occurs in disorders in which IgE is thought to participate.

An abnormal adaptive immune response directed against non-infectious environmental substances (allergens), including non-infectious components of certain infectious organisms. In allergic disorders, such as anaphylaxis, allergic rhinitis (hay fever), some food allergies and allergic asthma, these responses are characterized by the involvement of allergen-specific IgE and T helper 2 (TH2) cells that recognize allergen-derived antigens. There are two main types of allergen.

The first type encompasses any non-infectious environmental substance that can induce IgE production (thereby 'sensitizing' the subject) so that later re-exposure to that substance induces an allergic reaction. Common sources of allergens include grass and tree pollens, animal dander (sheddings from skin and fur), house-dust-mite faecal particles, certain foods (notably peanuts, tree nuts, fish, shellfish, milk and eggs), latex, some medicines and insect venoms. In some instances, allergen-specific IgE directed against foreign antigens can also recognize cross-reactive host antigens, but the clinical significance of this is unclear. The second type is a non-infectious environmental substance that can induce an adaptive immune response associated with local inflammation but is thought to occur independently of IgE (for example, allergic contact dermatitis to poison ivy or nickel).

A single allergen exposure produces an acute reaction, which is known as an early-phase reaction or a type I immediate hypersensitivity reaction. In many subjects, this is followed by a late-phase reaction. With persistent or repetitive exposure to allergen, chronic allergic inflammation develops, with associated tissue alterations. Early-phase reaction is an IgE-mediated type I immediate hypersensitivity reaction that can occur within minutes of allergen exposure. Reactions can be localized (for example, acute rhinoconjunctivitis in allergic rhinitis, acute asthma attacks, urticaria (hives) and gastrointestinal reactions in food allergies) or systemic (anaphylaxis).
In such reactions, IgE bound to FcεRI on mast cells and basophils is cross-linked by allergen, resulting in the release of the cells' diverse preformed and newly synthesized mediators. These events cause vasodilation, increased vascular permeability with oedema, and acute functional changes in affected organs (such as bronchoconstriction, airway mucus secretion, urticaria,

vomiting and diarrhoea). Some of the released mediators also promote the local recruitment and activation of leukocytes, contributing to the development of late-phase reactions.
Late-phase reaction is a reaction that typically develops after 2–6 h and peaks 6–9 h after allergen exposure. It is usually preceded by a clinically evident early-phase reaction and fully resolves in 1–2 days. Skin late-phase reactions involve oedema, pain, warmth and erythema.
In the lungs, these reactions are characterized by airway narrowing and mucus hypersecretion. They reflect the local recruitment and activation of TH2 cells, eosinophils, basophils and other leukocytes, and persistent mediator production by resident cells (such as mast cells). Mediators that initiate late-phase reactions are thought to be derived from resident mast cells activated by IgE and allergen or from T cells that recognize allergen-derived peptides (such T cells may be either resident at, or recruited to, sites of allergen challenge).
Environmental factors appear to be more important in determining whether an atopic individual develops a particular allergic disease, although genetic factors may exert an influence on how severely the disease is expressed and the extent of the allergic inflammatory response.

The inflammatory process has several common characteristics shared between different allergic diseases, including asthma, allergic rhinitis／rhinosinusitis, and atopic dermatitis (eczema). Allergic inflammation is characterized by IgE-dependent activation of mucosal mast cells and an infiltration of eosinophils that is orchestrated by increased numbers of activated CD4+ Th2 lymphocytes. However, in patients with more severe disease, other cells such as neutrophils, Th1, and CD8+ (cytotoxic) lymphocytes may also be involved. The clinical differences between these diseases are largely determined by anatomical differences and the interaction between allergic inflammation and structural cells, such as airway smooth muscle cells in the lower airways, resulting in bronchoconstriction, vasodilatation in the upper airways leading to nasal blockage and rhinorrhea and activation of keratinocytes in the skin. The fact that there are common characteristics of allergic diseases suggests that it may be possible to treat these common diseases with single agents, particularly, as they often coexist.

No single cell or mediator can account for all the features of allergic disease and different cells and mediators may be more important in one manifestation of allergic disease than another. For example, histamine clearly plays a key role in rhinitis yet has a relatively minor role in asthma, as judged by the differences in efficacy of antihistamines between these conditions.

In allergic diseases, the inflammatory response is activated inappropriately so that it becomes harmful rather than protective. Allergens, such as house dust mite and pollen proteins, activate mast cells and eosinophil inflammation, which has evolved to kill the invading parasites and would

therefore be self-limiting, but in allergic disease, the non-infective inciting stimulus persists and the normally acute inflammatory response becomes converted into a persistent inflammation that may have structural consequences in the airways and skin.

1.1 A COMPREHENSIVE VIEW OF ALLERGIC DISEASES

Over the past decades the increasing prevalence and incidence of allergic conditions among affluent societies have posed a heavy burden on health care systems. The estimated prevalence of allergic rhinitis among 13- to 14-year-old participants of the International Study of Asthma and Allergies in Childhood (ISAAC) in the United States is 12% to 22%.

Furthermore, the estimated prevalence of atopic sensitization among young adults in the United States is greater than 40% using the European Community Respiratory Health Survey (ECRHS) tools. Prevalence rates for children and adults in countries around the world differ widely. The ECRHS assessed geographic variations in asthma in 140,000 adults from 22 countries.

A six fold variation in the prevalence of current asthma was found among the different participating countries. A high prevalence (>7%) of asthma was described in Australia, New Zealand, United States, Ireland, and the United Kingdom. Asthma prevalences of < 4% were found in Iceland, parts of Spain, Germany, Italy, Algeria and India. The ISAAC study provides the most extensive information for asthma in children around the world. There seems to be agreement between the different estimates, and the prevalence in some countries is similar to the one mentioned in the ECRHS study [Rigoli *et al*, 2011].

Allergic diseases have dramatically increased in the last decades. A single cause of this increase cannot be pinpointed and experts are therefore considering the contribution of numerous factors, including genetics, air pollution in urban areas, pets, and early childhood exposure [Leonardi *et al*., 2007]. The associated costs have increased substantially as more of the population require treatment for allergies [Friedlander *et al*., 2011].

- **Respiratory Allergy**

Rhinitis and asthma pose an important health problem with a wide range of etiologies. Allergic rhinitis is defined as a clinical symptomatic nasal inflammatory reaction induced by IgE-mediated allergen after exposure of the membranes of the nasal surface. It involves the following

symptomatology: itch, nasal discharge, sneezing, and nasal stuffiness. Asthma is a chronic inflammatory disorder of the airways with participation of various types of cells. It leads to recurrent episodes of wheezing, breathlessness, chest tightness and cough, usually accompanied by variable airflow obstruction, usually reversible with medication, as well spontaneously, and bronchial hyperresponsiveness against different stimuli. Allergic and infective causes are most common and have a substantial socioeconomic impact.
Allergic rhinitis and asthma significantly impair quality of life and affect both school and work performance.

A proper diagnostic of asthma and rhinitis is basic for the implementation of an appropriate treatment plan. Treatment of allergic rhinitis and asthma has four cornerstones: education of the patient, allergen avoidance measures, symptomatic treatment and allergen immunotherapy. Studies on the effectiveness of avoidance measures and reduction of allergens in the home environment,
as well as the different meta-analysis conducted on these studies, have shown that, while sanitation and avoidance measures are effective in reducing levels of allergens, it is not accompanied by a substantial reduction in symptoms.
In addition to avoidance and environmental control measures, patients should start an individualized pharmacological treatment. The treatment should be consistent with the severity of symptoms.

1.2 OCULAR ALLERGIES

Ocular allergy represents one of the most common ocular conditions encountered in clinical practice.

Allergic conjunctivitis is an inclusive term that encompasses seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC), and atopic keratocongiuntivitis (AKC). However, AKC and VKC have clinical and pathophysiological features quite different from SAC and PAC, in spite of some common markers of allergy [7]. Seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC) are caused by IgE-mediated environmental airborne allergens such as grass and tree pollens, mites, molds, and animal dander. A minority of allergic eye diseases are of the cell-mediated type, which may manifestsolely as blepharitis or blepharoconjunctivitis. The usual causes of the cell-mediated type are cosmetics, nickel, or topical eye medication.

Allergic eye inflammation is a localized allergic condition that is frequently associated with rhinitis and occasionally with asthma but often observed as the only or prevalent allergic manifestation. The symptoms and signs can manifest alone in the eyelids as atopic blepharitis or contact blepharitis.
Lid symptoms may occur together with conjunctival symptoms. However, allergic conjunctivitis is the most common manifestation alone or together with lid symptoms. The eye is red and itchy, and there is lacrimation and slight discharge. Rubbing of the eyes, light sensitivity, redness, lacrimation, and lid or conjunctival edema may indicate ocular allergy. Visual acuity is usually normal. However, in some severe forms of allergic conjunctivitis, the cornea may be affected by atopic keratoconjunctivitis and vernal keratoconjunctivitis (VKC) [Kari *et al*., 2012].

- ***ETIOLOGY***

- **Role of Mast Cell and T-Lymphocyte in Allergic Conjunctivitis**

Conjunctiva is the most common site for allergic reactions. It is a vascularized tissue consisting of a large number of dendritic cells and macrophages. These cells regulate innate and adaptive immunity of the conjunctival epithelium. Antigen presenting cells such as dendritic cells, B cells and macrophages play a key role in antigen recognition and processing by T- lymphocytes. B lymphocytes produce the immunological response by expressing receptors for antigen recognition on the cell membrane. Macrophages are another important component that regulates the production of major cytokines and removal of pathogens. Dendritic cells are involved in the T-helper type 2 (Th-2) mediated allergic responses. These cells present antigen to memory T-lymphocytes, which produce Th-2 cytokines and also regulate the production of IL-12. In addition, conjunctival epidermal dendritic cells, commonly known as langerhans cells are also involved in the antigen processing and presenting function. Two major cell types involved in the allergic immune-pathological response are mast cell and T-cells.
The normal conjunctival epithelium is composed of CD4+ and CD8+ T lymphocytes. In a chronic allergic state memory T lymphocyte increases dramatically.

- **Role of Mast Cells in Allergic Eye Diseases**

Two different types of mast cells exist in humans, classified as MCT and MCTc subtypes on the basis of granule and protease content. MCT subtype contains single neutral protease or typtase, whereas MCTc carries both typtase and chymase. MCT subtype rises in acute inflammation while

an increased number of MCTc subtype is found in chronic inflammatory symptom. Mast cell mediated acute allergic eye diseases such as SAC and PAC are categorized as type-1 hypersensitivity reactions. These reactions occur due to the exposure of allergens on the conjunctival mucus membrane and follow three phases, i.e. sensitization, early and late phases.

In sensitization phase, allergens are processed and localized by antigen presenting cells (APC) as a complex of peptide fragments with MHC class-II molecules on their surface. The allergen-MHC class II complexes on the APC then promote the maturation of T-helper (TH) cells into Th2 cells, which result in the production of cytokines. These cytokines further promote the synthesis of IgE from B-cells. IgE binds to FcεRI receptors on mast cell, which upon subsequent exposure to allergens results in the cross linking of these antibodies. It changes the mast cell membrane permeability and results in the release of inflammatory mediators. Such inflammatory mediators include histamine, serotonin, leukotriene (LTC4), prostaglandin, carboxypeptidase A, platelet activating factor (PAF), cathepsin G, neutrophil and eosinophil.

These inflammatory mediators contribute to the early phase response that begins within seconds of allergen exposure. The early phase clinical symptoms are itching, tearing, swelling, edema and redness. In the last phase response aggregation of FcεRI receptor initiates the breakdown of membrane phospholipids into inflammatory mediators such as prostaglandin, leukotrienes and thromboxanes that lead to late phase response via recruitment of additional inflammatory cells. The late phase response involves the infiltration of eosinophils, neutrophils and basophils.

- **Role of T Lymphocytes in Allergic Eye Diseases**

Conjunctiva is a common site of allergic reactions. It is well vascularized tissue that contains large numbers of dendritic/ Langerhans cells and macrophages. Lymphocytes—both T and B cells—are important cells in allergic reactions. These are divided into CD^{4+} T-helper (Th) cells and CD^{8+} killer T cells. CD^{4+} Th cells are important in the regulation of allergic reactions. These cells can be further divided into Th1 and Th2 cells. Th1 cells are activated in the classic delayed-type hypersensitivity reaction and in some autoimmune diseases.

The activation of Th2 cells leads to the anaphylactic type of hypersensitivity reaction. The subclasses of Th cells (Th1, Th2) require different kinds of cytokines to be activated. T cells start and regulate the immune response. Interleukin (IL)-4 has an essential role in differentiating Th cells into Th2 cells. The activated Th cells regulate the maturation of B lymphocytes to mature, antibody-producing B cells and plasma cells. The maturation of B lymphocytes into IgE-producing cells requires, in addition to IL-4, direct contact between pre-B and Th cells.

The prerequisite for the immune reaction is that the antigen must be recognized and presented to the cells, which are responsible for the development of the immune response.
The antigen-presenting cells in the conjunctiva are the Langerhans cells, which present the antigen to T lymphocytes. In the cell-mediated type of reaction (delayed type or type IV), T cells recognize the antigen and start the inflammatory reaction. Typical delayed-type reactions are contact dermatoconjunctivitis and Mantoux (tuberculin) reaction. Macrophages activated by Th cells destroy intracellular bacteria but also cause other delayed inflammatory reactions, including the contact hypersensitivity of the skin and tuberculin reaction. These reactions reach their peak in 24 to 72 hours [Mishra *et al*., 2011].

1.3 CELLULAR TYPES INVOLVED IN ALLERGIC REACTIONS

- *EOSINOPHILS*

Eosinophils were first described in 1879 by Paul Ehrlich, who noted their unusual capacity to be stained by acidophilic dyes. Interestingly, our appreciation of this unique property of eosinophils is clear and steadfast, but a comprehensive understanding of the function of these cells in health and disease remains elusive. Some basic characteristics of eosinophils are established and accepted. It is clear that eosinophils are granulocytes that develop in the bone marrow from pluripotent progenitors. They are released into the peripheral blood in a phenotypically mature state, and they are capable of being activated and recruited into tissues in response to appropriate stimuli, most notably the cytokine interleukin-5 (IL-5) and the eotaxin chemokines. Eosinophils spend only a brief time in the peripheral blood (they have a half-life of ~18 hrs) before they migrate to the thymus or gastrointestinal tract, where they reside under homeostatic conditions. In response to inflammatory stimuli, eosinophils develop from committed bone marrow progenitors, after which they exit the bone marrow, migrate into the blood and subsequently accumulate in peripheral tissues, where their survival is prolonged [Hogan, S. P. *et al*.,2008].
Relatively few mature eosinophils are found in the peripheral blood of healthy humans (less than 400 per mm^3), but these cells can be readily distinguished from the more prevalent neutrophils by virtue of their bilobed nuclei and large specific granules (Figure 1.1).

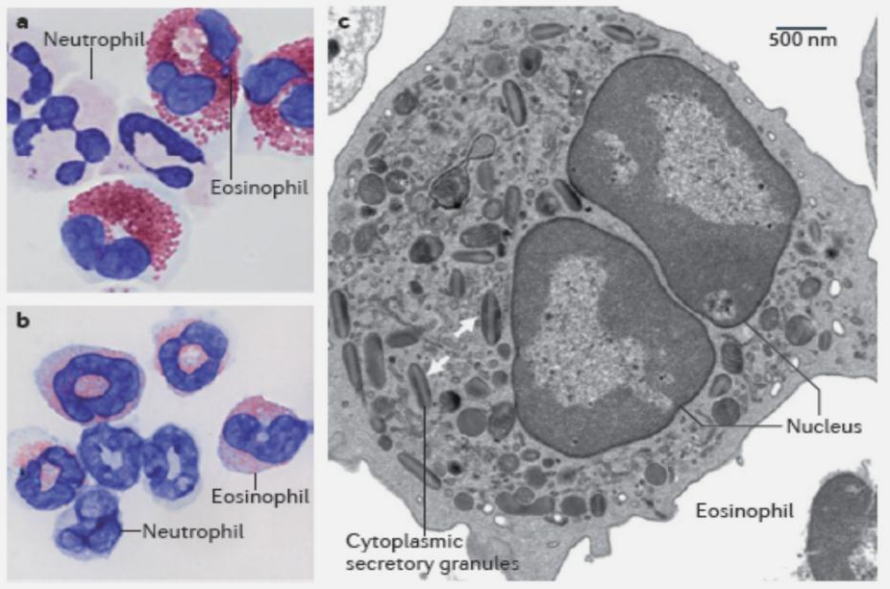


Figure 1.1 *The eosinophil.* ***a*** *| Human eosinophils from peripheral blood stained with modified Giemsa exhibit characteristic bilobed nuclei and large red-stained cytoplasmic secretory granules. Original magnification, ×100.* ***b*** *| The image shows eosinophils and neutrophils isolated from the spleen of a Cd2-interleukin-5-transgenic mouse and stained with modified Giemsa.* ***c*** *| The image shows a transmission electron micrograph of a mouse eosinophil. Cytoplasmic secretory granules are indicated by the arrows; the central core of these granules contains cationic major basic protein, and their periphery contains the remaining major cationic proteins, cytokines, chemokines, growth factors and enzymes. Original magnification, ×6,000.*

Human eosinophil granules contain four major proteins: eosinophil peroxidase, major basic protein (MBP) and the ribonucleases eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN). The granules also store numerous cytokines, enzymes and growth factors. Other prominent features of eosinophils include primary granules that contain Charcot–Leyden crystal protein (also known as galectin 10 and eosinophil lysophospholipase) and lipid bodies, which are the sites of synthesis of cysteinyl leukotrienes, thromboxane and prostaglandins.

Eosinophils have been identified and characterized in all vertebrate species, but their morphology, repertoire of cell-surface receptors and intracellular contents vary significantly between species. Eosinophils express surface receptors for ligands that support growth, adhesion, chemotaxis, degranulation and cell-to-cell interactions. Of particular note, there are several crucial differences between mouse and human eosinophils that must be taken into account when interpreting mouse model studies of human disease (Figure 1.1).

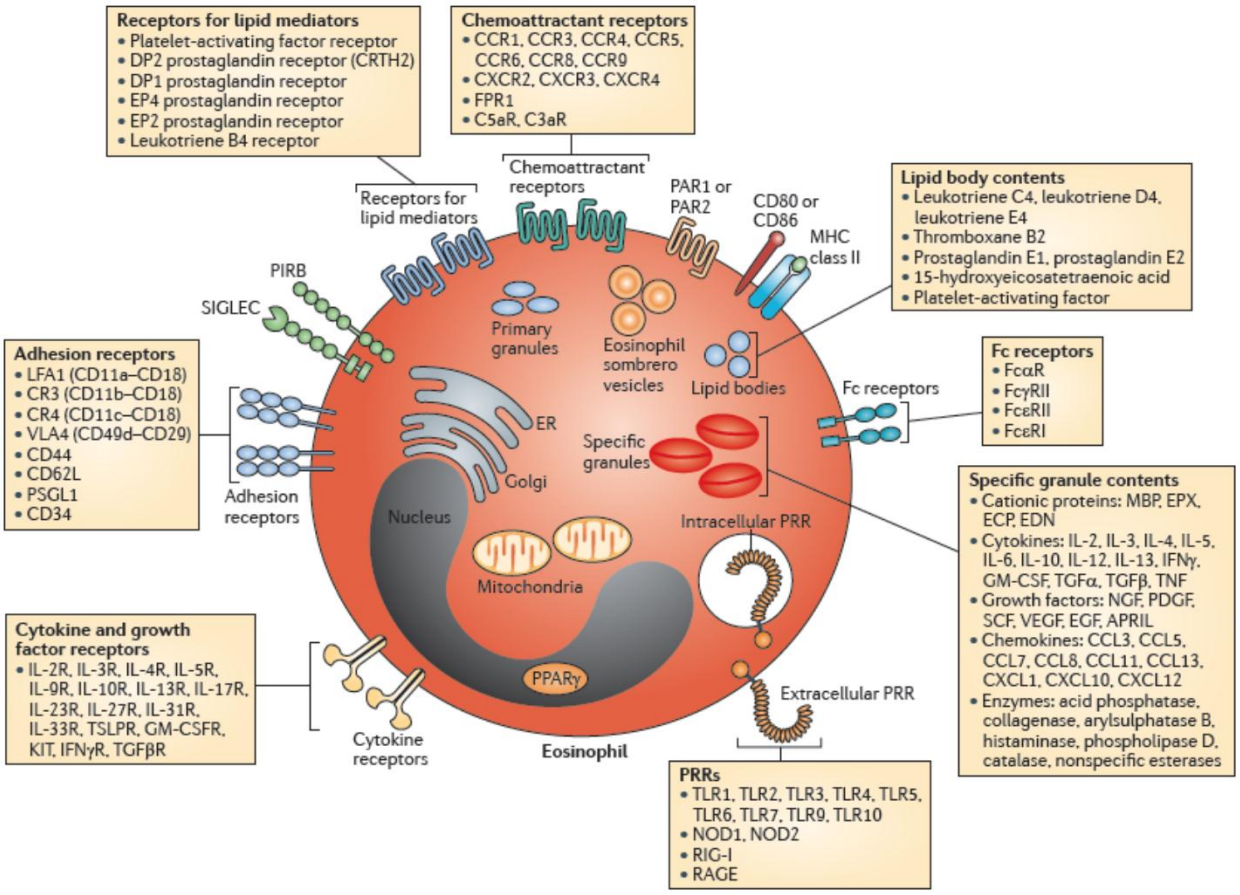


Figure 1.2 *Eosinophils are equipped with features that promote interactions with the environment. In one such interaction, eosinophils release the contents of their specific granules in response to external stimuli. Some of these granule contents are released via membrane-bound vesicles known as eosinophil sombrero vesicles. Eosinophils also synthesize lipid mediators for release in cytoplasmic lipid bodies and store Charcot–Leyden crystal protein (CLC) in primary granules. Although not highly biosynthetic, mature eosinophils have minimal numbers of mitochondria and a limited endoplasmic reticulum (ER) and Golgi, as well as a nucleus. Eosinophils express a wide variety of receptors that modulate adhesion, growth, survival, activation, migration and pattern recognition. Mouse eosinophils do not express CLC or Fcε receptor 1 (FcεR1) and have divergent homologues of sialic acid-binding immunoglobulin-like lectin 8 (SIGLEC-8) and the granule ribonucleases eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP)12. APRIL, a proliferation-inducing ligand; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CXCL, CXC-chemokine ligand; CXCR, CXC-chemokine receptor; EGF, epidermal growth factor; EPX, eosinophil peroxidase; FPR1, formyl peptide receptor 1; GM-CSF, granulocyte–macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; MBP, major basic protein; NGF, nerve growth factor; NOD, nucleotide-binding oligomerization domain protein; PAR, proteinase-activated receptor; PDGF, platelet-derived growth factor; PIRB, paired immunoglobulin-like receptor B; PPARγ, peroxisome proliferator-activated receptor-γ; PRR, pattern-recognition receptor; PSGL1, P-selectin glycoprotein ligand 1; RAGE, receptor for advanced glycation end-products; RIG-I, retinoic acid-inducible gene I; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumour necrosis factor; SCF, stem cell factor; VEGF, vascular endothelial growth factor.*

The life cycle of the eosinophil is divided into bone marrow, blood, and tissue phases. Eosinophils are produced in bone marrow from pluripotential stem cells. The stem cells differentiate into a progenitor, which is capable of giving rise to mixed colonies of basophils and eosinophils, pure basophil colonies, or pure eosinophil colonies. Among various hematopoietic factors, those important for eosinophil proliferation and differentiation are interleukin (IL)-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-5. IL-3 and GM-CSF are relatively nonspecific and stimulate proliferation of neutrophils, basophils, and eosinophils. In contrast, IL-5 potently and specifically stimulates eosinophil production.

Although the eosinophil is a formed element of the peripheral circulation, it is primarily a tissue-dwelling cell. In healthy individuals, most eosinophils are found in the gut (but not the esophagus), mammary gland, uterus, thymus and bone marrow; the gastrointestinal eosinophil is the predominant population of eosinophils. At baseline condition, eosinophils are present in the gastrointestinal tract independent of adaptive immunity and enteric flora, and the eosinophil levels are regulated by the constitutive expression of eotaxin-1 and eosinophil chemokine receptor, CCR3. Eosinophils also home into the thymus, mammary gland, and uterus, as controlled by eotaxin-1.

Eosinophils also interact with and modulate the functions of other leukocytes.

- ***Interaction with lymphocytes***

Eosinophils clearly respond to signals (such as IL-5) that are provided by T cells. Two recent studies indicate that T cells also respond to signals provided by eosinophils. Although not ‘professional’ antigen-presenting cells, eosinophils can express cell-surface components that are required for antigen presentation (such as MHC class II molecules and the co-stimulatory molecules CD80 and CD86). Indeed, eosinophils can process antigens and stimulate T cells in an antigen-specific manner, resulting in T cell proliferation and cytokine release. In addition, eosinophils release preformed cytokines (such as IL-4, IL-13 and IFNγ) that promote either TH2 or TH1 cell responses.

Eosinophils also promote humoral immune responses. Indeed, they are capable of priming B cells for the production of antigen-specific IgM43. Eosinophils communicate extensively with tissue-resident mast cells. Eosinophils and mast cells are found in close proximity to one another under homeostatic conditions in the gut, and they also colocalize in the allergic lung and in the inflamed gut in patients with Crohn’s disease. The bidirectional signalling that occurs between eosinophils

and mast cells involves several immunomodulatory mediators. These include stem cell factor (also known as KIT ligand), granule proteins, cytokines (such as granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-3, IL-5 and tumour necrosis factor (TNF)), nerve growth factor and mast cell proteases. Actual physical coupling of eosinophils and mast cells has been observed both *in vitro* and *in vivo*, and this interaction prolongs eosinophil survival.

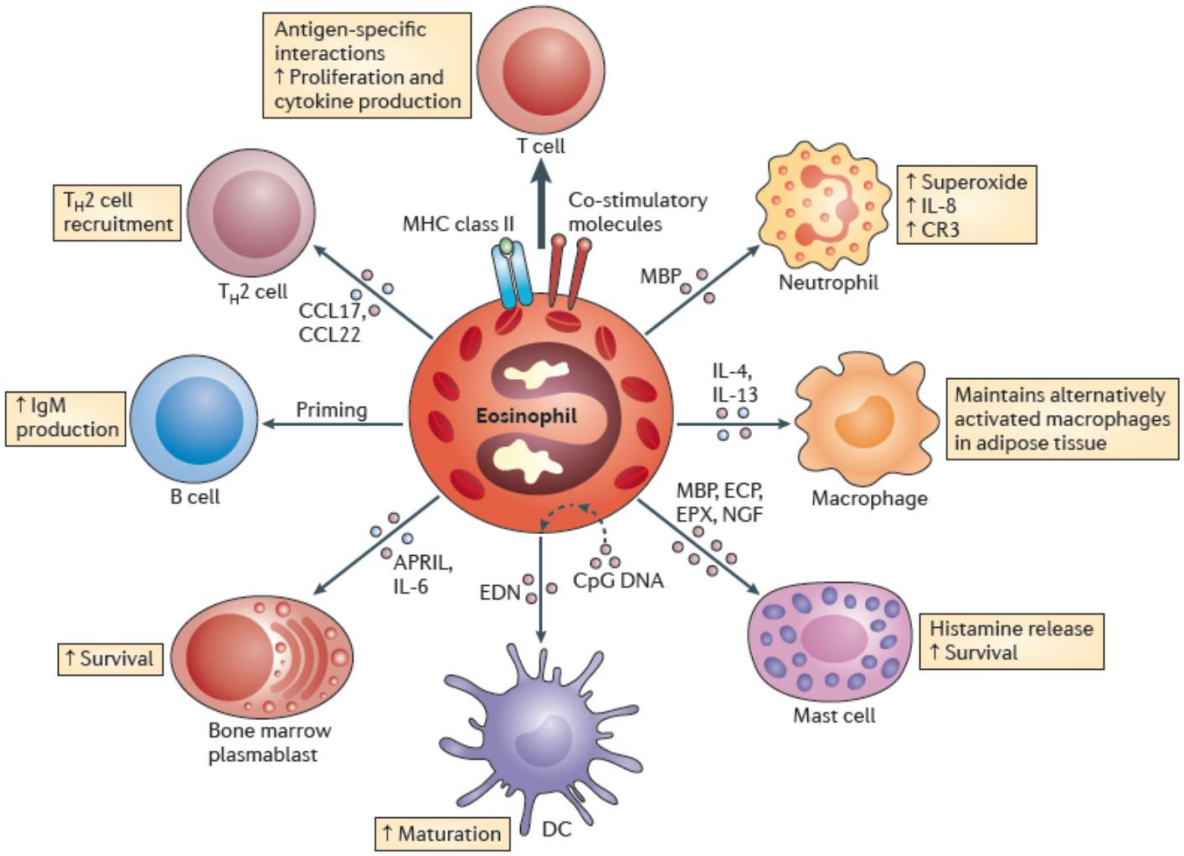


Figure 1.3 *Eosinophils not only respond to signals, but also have a definitive impact on the actions of other leukocytes. Eosinophils can express MHC class II and co-stimulatory molecules, process antigens and stimulate T cells to proliferate and produce cytokines in an antigen-specific manner. Furthermore, acting together with dendritic cells (DCs), eosinophils regulate the recruitment of T helper 2 (TH2) cells in response to allergen sensitization and challenge by producing CC-chemokine ligand 17 (CCL17) and CCL22. Eosinophils also prime B cells for antigen-specific IgM production and sustain long-lived plasma cells in mouse bone marrow via the production of a proliferation-inducing ligand (APRIL) and interleukin-6 (IL-6). Eosinophils that are stimulated by CpG DNA induce DC maturation. Major basic protein (MBP) released from eosinophils activates neutrophils, causing them to release superoxide and IL-8 and increase their expression of the cell-surface integrin complement receptor 3 (CR3). Eosinophils also maintain alternatively activated macrophages in adipose tissue by producing IL-4 and IL-13. The eosinophil granule proteins MBP, eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX) activate mast cells, resulting in the release of histamine. Likewise, eosinophil-derived nerve growth factor (NGF) prolongs mast cell survival.*

- *MAST CELLS*

Mast cells are members of the innate immune system that develop from CD34+ hematopoietic precursor cells in the bone marrow and circulate in the blood in an immature form. Only after they have established residency in a particular tissue, do they complete their tissue-specific differentiation and maturation.

Both the regulation of mast-cell survival and proliferation and the modulation of important phenotypical characteristics of mast cells — including their susceptibility to activation by various stimuli during innate or adaptive immune responses, their ability to store and/or produce various secreted products, and the magnitude and nature of the secretory response of mast cells to specific activation stimuli— can be finely controlled or 'tuned'. The main survival and developmental factor for mast cells is stem-cell factor (SCF; also known as KIT ligand), but many growth factors, cytokines and chemokines can influence the number and phenotype of mast cells, including interleukin‑3 (IL‑3), which is of particular importance in mice, TH2-cell-associated cytokines (such as IL‑4 and IL‑9), CXCL12 (also named stromal cell-derived factor-1, or SDF-1) [Lin *et al*., 2000; Godot *et al*., 2007], nerve growth factor (NGF) and transforming growth factor-β1 (TGF-β1).

Mast cells are often classified according to their location or protease content. In mice, MCs can be classified into two subpopulations: mucosal type MCs and connective tissue-type MCs [Galli *et al*., 1984]. In humans, MCs are subcategorized into MC_T, which express high levels of the MC-specific protease tryptase but not of chymase, and MC_{TC}, which express both tryptase and chymase.

- *PHYSIOLOGICAL AND PATHOLOGICAL ROLE*

Mast cells are considered first line defenders against infections because of their prevalence in tissues such as the skin, gut, respiratory tract and urinary tract that form the barriers between self and the environment. They are also found in close association with blood vessels, lymphatic vessels and nerves. These anatomical locations license mast cells to contribute to a multitude of protective and pathologic events including angiogenesis, wound healing and the exacerbation of inflammation. While the numbers and activation of MCs increase in many human diseases, proof of MC involvement in these diseases has been derived mostly from animal models developed in various strains of MC-deficient mice [Grimbaldeston *et al*., 2005; Tsai *et al*., 2005; Reber *et al*., 2012; Rodewald and Feyerabend, 2012]. Studies in these animal models suggest that MCs play

important roles in a variety of immune and inflammatory reactions, including a central role in allergies, defense against some pathogens, resistance to venoms, and development or exacerbation of certain autoimmune diseases. Mast cells can also influence many aspects of the biology of immune cells, including granulocytes, monocytes/macrophages, dendritic cells (DCs), T cells, B cells, natural killer (NK) cells and NKT cells. In this case, the effects of mast cells on the recruitment, survival, development, phenotype or function of immune cells are referred to as 'immunomodulatory' functions.

Through their effector and/or immunomodulatory functions, mast cells can promote the initiation and increase the magnitude of inflammation, tissue remodelling and, in some cases, tissue injury associated with the innate or adaptive immune response.

- *MAST CELLS ACTIVATION*

Mast cells are famous for their role in hypersensitivity reactions where they are activated by cross-linking of the high affinity IgE receptor (FcεRI).

Depending on the type, property, strength, and combination of the stimuli they receive, MCs secrete a diverse and wide range of biologically active products that can trigger, direct,or suppress the immune response. Mast cells soluble products can be divided into two categories: (a) preformed mediators, such as histamine, proteoglycans, and neutral proteases and certain cytokines, in particular tumor necrosis factor-alpha (TNF-α), that are rapidly and instantaneously released upon activation; (b) newly synthesized mediators, such as cytokines, chemokines, lipid mediators, growth and angiogenic factors that start to be synthesized after MC activation. Although these products are all important in both innate and acquired immunity, the rapid release of mast cells mediators is crucial for the initiation of the immune response at the site of infection since they are able to modulate the immune-cell trafficking and to provide co-stimulatory signals for cell activation. Tissue mast cells are the major resident cell population expressing FcεRI and IgE-antigen cross-linking results in the release of several preformed molecules that are stored in mast cell granules including histamine, serotonin, tryptase, chymase as well as the lipid-derived mediators prostaglandin D2 (PGD2) and leukotriene B4 (LTB4). These mediators affect many aspects of the early-phase allergic reactions such as vasodilation, local or systemic increases in vascular permeability, constriction of smooth muscle and mucus secretion. Such activation also leads to the release of newly synthesized mediators, which can initiate more severe and prolonged late-phase allergic responses.

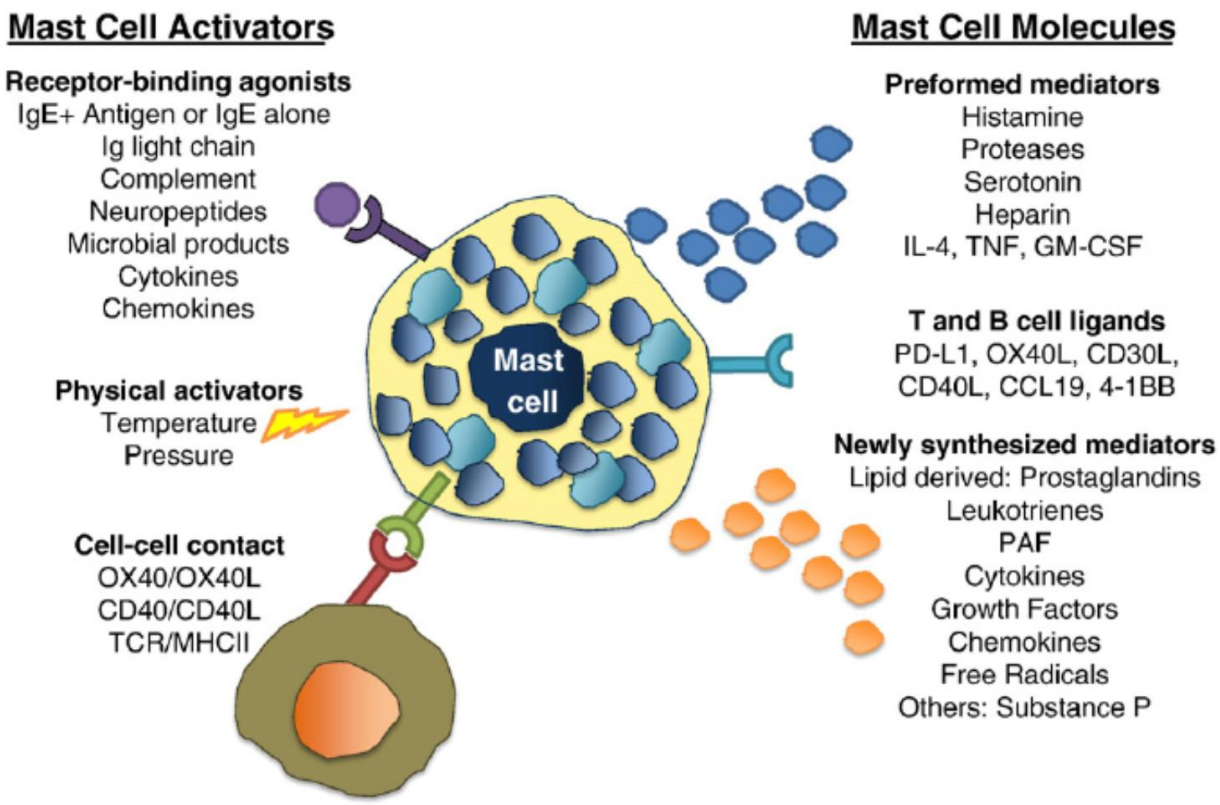


Figure 1.4 *The mast cell, the "jack of all trades" immune cell. The multitude of activators and the many modes of mast cell response account for their ability to impact a variety of physiological and pathogenic processes. Receptor-binding agonists, physical activators and cell–cell contact can all activate mast cells.*

2. INTEGRINS

Integrins are a major family of cell surface-adhesion receptors that are expressed in all metazoans. In vertebrates, 18 α subunits and 8 β subunits have been discovered, which combine into 24 different heterodimers that recognize overlapping but distinct sets of extracellular ligands (Figure 2.1) [Humphries JD *et al*., 2006; Hynes RO, 2002] .

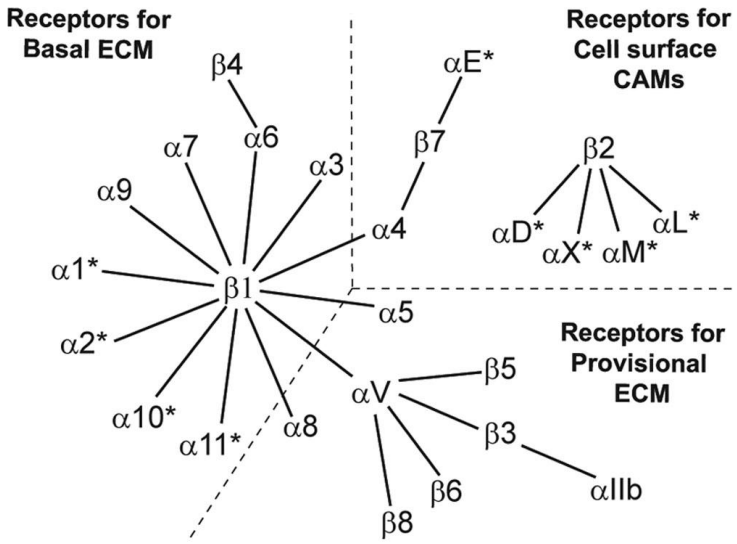


Figure 2.1 *Integrin family. Integrins are loosely grouped into three classes that bind basal extracellular matrix (ECM), provisional ECM and cell surface adhesion molecules (CAMs), respectively. Basal ECM mainly includes fibrinogen, fibronectin, vitronectin, cryptic collagen and von Willebrand factor.*

As adhesion molecules, integrin can mediate cell-cell, cell-matrix and cell-pathogen interactions [Askari JA *et al*., 2009]. Integrin-mediated adhesion and signaling events are important in normal physiological responses including immune response, tissue morphogenesis, wound healing, hemostasis, cell survival and cell differentiation. Conversely, dysregulation of integrins are involved in the pathogenesis of many diseases, including cancer metastasis, auto-immune disease and thrombotic vascular diseases.[Garmy-Susini B. *et al*., 2008; von Andrian UH *et al*., 2003]

2.1 Integrin Overall Structure

Integrins thus are able to communicate binding to the extracellular matrix or ligands on other cells to the actin cytoskeleton, to discriminate against soluble ligands, and to bind only with high affinity to cell surface or matrix-bound ligands [Luo *et al*., 2007; Springer and Dustin, 2012].

Integrins are type I trans-membrane proteins with large ectodomains, short trans-membrane and cytoplasmic domains (except for β_4 subunit with exceptional long cytoplasmic domain of ~1,000 amino acids). There are four extracellular domains, β-propeller, thigh, calf-1 and calf-2, present in all α subunits. In addition, half of the α subunits incorporate an additional autonomous folding domain of ~200 amino acids termed the inserted domain (I domain) (Figure 2.2). Thus, integrins can be classified into two subfamilies, the α I-containing integrins and α I-less integrins.

The β subunit ectodomain contains eight domains, β I domain, hybrid, PSI, β-tail and four integrin-EGF domains. Electron microscopy and X-ray crystal structure studies of several integrins ($\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_X\beta_2$) have demonstrated independently that the overall shape of integrin ectodomain is that of a large "head" on two long "legs" with flexible "knees" (Fig. 2.2, panel B and C) [Xie C. *et al*., 2010; Xiong JP *et al*., 2009]. The α head is composed of β-propeller domain and α I domain in α I-containing integrins, while a single β-propeller forms a head in α I-less integrins. The β I domain, sharing the same overall fold as the α I domain, is present in all β subunits and forms β head. α I domain is the ligand binding domain in α I-containing integrins whereas the β I domain forms a major ligand binding pocket in α I-less integrins.

Integrin affinity regulation is highly related to its global and local conformational rearrangements. It has been demonstrated that integrins exist in at least three conformational states: the bent conformation with closed headpiece, the extended conformation with closed headpiece and the extended conformation with open headpiece, which are corresponding to the low-, intermediate- and high-affinity states, respectively (Fig. 2.2, panel B and C). Correspondingly, it is proposed that integrin activation is accompanied with a switchblade-like opening of the headpiece-tailpiece interface, which extends the ligand-binding headpiece of the integrin heterodimer away from the plasma membrane.

The α subunit β–propeller and thigh domains and the β subunit β I, hybrid, PSI (plexin, semaphorin, and integrin), and I-EGF-1 domains form the ligand binding headpiece, the head and the upper legs. The α subunit calf-1 and calf-2 and the β subunit I-EGF-2 to I-EGF-4 and β tail domains form the lower legs [Xiong *et al*., 2001; Zhu *et al*., 2008].

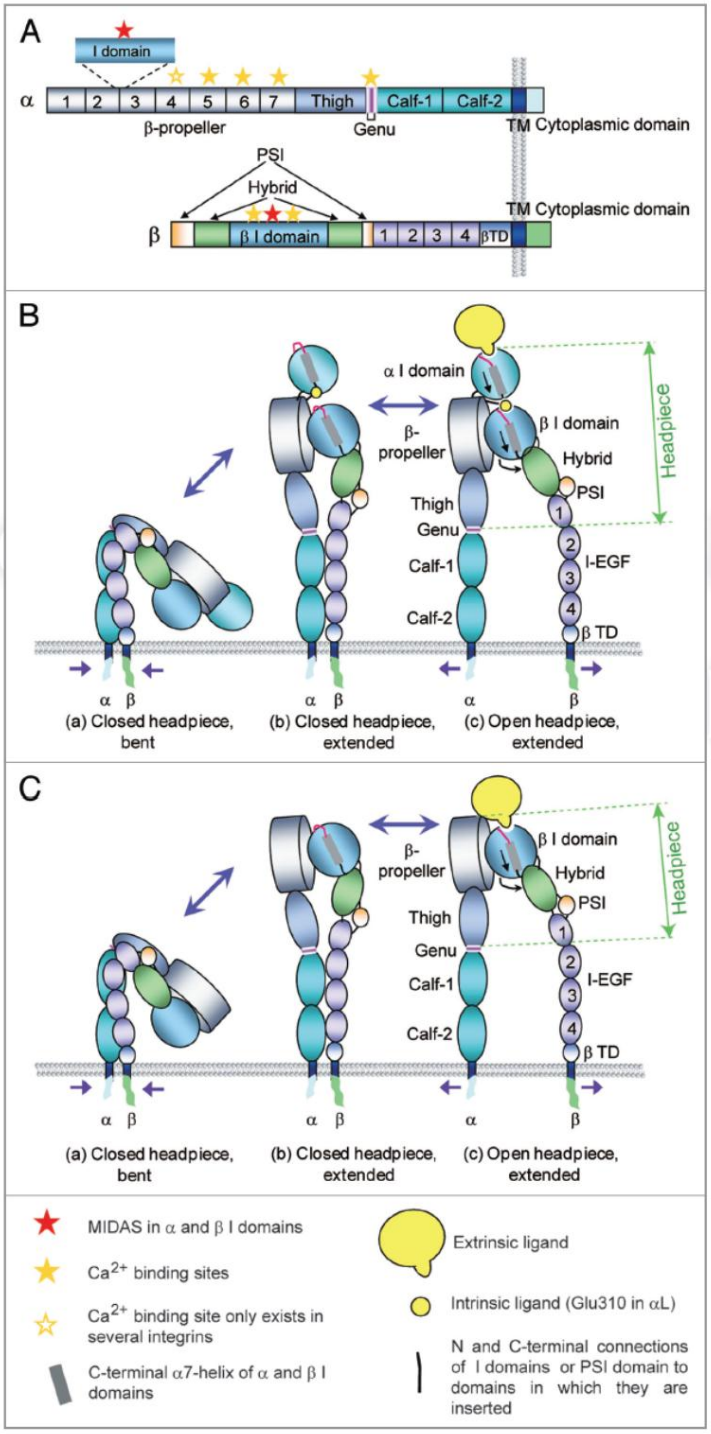


Figure 2.2 *Schematic representation of integrin structure and conformational rearrangements. Panel A, organization of domains within the primary structure. α I domain inserted in β-propeller is denoted by dash lines. Yellow and red asterisks denote Ca^{2+} and Mg^{2+}-binding sites, respectively. Open asterisk denotes the Ca^{2+}-binding site in the forth repeat of β-propeller domain in some α subunits. Panel B and C, conformational rearrangements of αI-containing (B) and α I-less integrins (C) during activation.*

Two distinct types of global conformational changes occur in integrin extracellular domains. Extension at the knees releases integrins from a compact bent conformation (Figure 2.3, A, B, D, and E). In integrin headpiece opening, the hybrid domain swings out, the β I domain changes from closed to open conformation, and affinity for ligand increases [Luo *et al*., 2007].
β I domains, present in all integrin β subunits, transmit conformational change from their interface with the swinging hybrid domain to a ligand binding site at an interface with the α subunit β -propeller domain (Figure 2.3, A–F).

The β I domain divides the hybrid domain into N- and C-terminal sequence segments. Activation at the metal ion-dependent adhesion site (MIDAS) in the β I domain ligand binding site is communicated to the opposite end of the β I domain by α7 helix pistoning at the C-terminal connection to the hybrid domain.

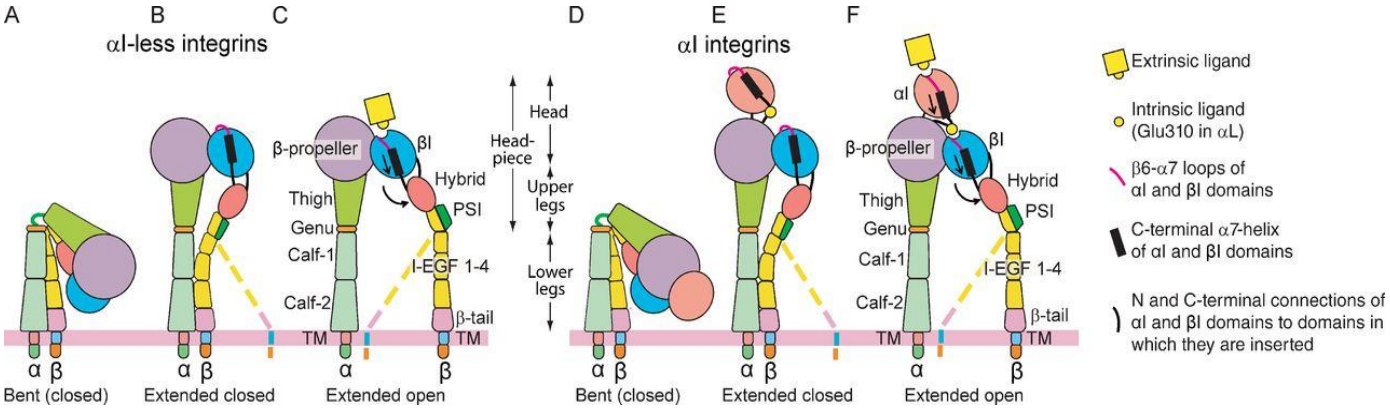


Figure 2.3 *Integrin domain organization and conformational states. Two lower β leg conformations (one with a dashed line) are shown for the extended states because the lower β leg is highly flexible, and these states can exist with the α and β TM domains either associated or separated. However, signal transmission through the membrane, both in the inside-out and outside-in directions, requires TM and cytoplasmic domain separation.*

2.2 Role of divalent cations in integrin function

Most integrins are not constitutively active and often expressed on cell surface in an inactive state, in which they neither bind ligands nor signal. The basal low adhesiveness of integrins is very important for their biological functions, such as integrins on circulating blood cells.

Cell surface integrins are in the equilibrium among these conformational states which can be shifted by certain stimuli, such as extracellular metal ions. Divalent cations are essential for integrin functions, from stabilizing integrin structure to mediating its interaction with the ligand and modulating integrin-ligand binding in either an enhancing or a suppressing way. The removal of divalent cations by EDTA completely inhibits integrin-ligand binding. And the formation of integrin heterodimer complex is also dependent on the presence of at least micromole levels of divalent cations.

Physiologically, most integrins are in the resting state with the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+} in body fluid. The millimole level Ca^{2+} often has an inhibitory effect on integrin-ligand binding [Dransfield *et al*., 1992; Humphries MJ, 1996]. By removal of Ca^{2+}, the remaining Mg^{2+} alone can promote ligand binding in most integrins. Studies also indicate that micromolar Ca^{2+} can synergize with suboptimal concentration of Mg^{2+} to further activate integrins. As a non-physiological stimulus, Mn^{2+} can shift integrins into high affinity conformations (detected by special activation-dependent mAbs) and strikingly activate integrins, even with the presence of millimolar Ca^{2+}.

Three kinds of binding sites for Ca^{2+} have been identified in $\alpha_4\beta_1$, which differ in Ca^{2+}-binding affinity [Chen *et al*., 2001]. The first kind is a moderate-affinity site (ED50 50–100 mM) that only can be rapidly occupied after integrin binds ligand; the second kind is a ligand binding-independent high-affinity site (ED50 20 mM); the third kind has the low-affinity for Ca^{2+} and its occupancy can inhibit integrin-ligand binding. Integrin crystal structures have provided new insights into the metal ion-binding sites, and their functions are further revealed by mutagenesis studies. To date, eight divalent cation binding sites have been discovered in integrin ectodomains with five in a subunit and three in β subunit.

2.3 Integrin signalling

In addition to their structural role, integrins are highly involved in signal transduction processes. Integrin signaling has often been divided into 'inside-out signaling' and 'outside-in signaling'. The former refers to the fact that integrins can exist in activated, inactive or intermediate states, in response to external cues. The solution of the crystal structure of the external domain of the $\alpha_v\beta_3$ integrin [Xiong *et al*., 2001], followed by further progress on structure and function, has been very helpful in understanding integrin activation. Thus, in the inactive state the integrin external domain is thought to be in a bent configuration while the cytoplasmic tails are closely juxtaposed by charge-charge interaction. In the active form the external domain is extended and the tails separate.

The association of the cytosolic protein talin with the beta subunit tail is the key final step in integrin activation [Kim *et al*., 2011; Margadant *et al*., 2011]. However, a multiplicity of other proteins, particularly the small GTPase Rap1, are involved in the regulation of **'inside-out signaling'.**

- *TALIN*

Talin is a 270 kDa protein which is composed of a 50 kDa N-terminal globular head domain and a 220 kDa C-terminal rod domain. The talin head domain contains three FERM (protein 4.1, ezrin, radixin, moesin) subdomains: F1, F2, and F3, and a non-homologous F0 domain. The PTB (Phosphotyrosine Binding) domain is located in the F3 subdomain and binds to the conserved membrane-proximal NPxY motif of β integrin cytoplasmic tail, and binding of the F3 domain is sufficient to activate integrins [Calderwood *et al*., 2002].

THD has two binding sites in the β integrin cytoplasmic domain. It can bind strongly to NPxY motif and weakly to the membrane proximal part of β3 integrin [Wegener *et al*., 2007].

Mutations that interrupt the strong integrin–talin interaction (NPxY-talin interaction) totally abolished the ability of talin to activate integrin whereas disrupting the weak talin–integrin interaction showed little effect on talin affinity but reduced integrin activation [Tanentzapf and Brown, 2006; Wegener *et al*., 2007]. This interaction is important in precise talin–integrin positioning and interrupting the interaction resulted in defected integrin activation [Anthis *et al*., 2009]. Binding of talin to the β integrin tail may cause TM domain separation (Fig. X), leading to integrin activation [Ye *et al*., 2011]. An in vitro reconstruction of integrin activation showed that binding of THD alone is sufficient to trigger integrin global extension and activation in lipid nanodiscs.

The rod domain of talin contains multiple binding sites for actin, vinculin, and an autoinhibitory binding site to its own integrin activating THD [Calderwood, 2004; Goksoy *et al*., 2008; Moser *et al*., 2009].

The C-terminal rod of talin also has essential role in integrin signaling. Over expression of THD cannot restore integrin clustering and focal adhesion formation in talin depleted cells and a more recent research implicated a potential role of talin rod domain in cell proliferation regulation, indicating the importance of this domain in integrin outside-in signaling [Zhang *et al*., 2008; Wang *et al*., 2011].

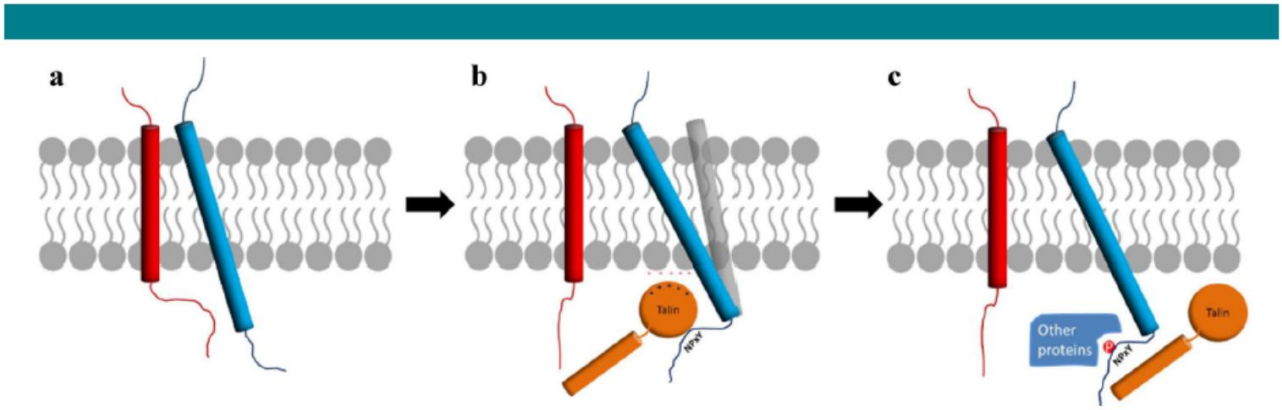


Figure 2.4 *Talin and integrin signaling. A, in resting state, TM domains of α (red) and β (blue) subunits are associated together. B, talin binding to β integrin cytoplasmic tail NPxY motif and membrane proximal part causes change of β integrin TM domain tilt angle (from gray to blue) resulting in longer helix embedment and induces TM domain separation. C, talin displacement from β cytoplasmic tail in outside-in signaling. Phosphorylation on the tyrosine residue (red sphere) of NPxY motif decreases talin binding affinity, resulting in displacement of the protein by other β integrin-binding proteins.*

- *KINDLIN*

Another integrin binding protein kindlin also plays a critical role in integrin activation. The kindlin family is consisted of three members: kindlin1, kindlin2, and kindlin3. Kindlins are well conserved in animals and defects on family members can cause serious diseases.

Kindlins have three C-terminal FERM domains, similar to talin, but the F2 domain is split by an inserted Pleckstrin Homology domain. While talin strongly binds to membrane proximal region of the β integrin cytoplasmic domain, kindlin F3 domain interacts with the membrane distal NxxY motif [Moser *et al*., 2008; Harburger *et al*., 2009].

2.4 Outside-In Signaling

In physiological conditions, only a fraction of integrins on the cell surface may adopt an extended conformation with high affinity for ligands. However, exposure to extracellular matrix (ECM) ligands results in stabilizing of high affinity state and integrin clustering, which in turn increases ligand binding valency or avidity. Clustered integrins not only provide stable connections to ECM but also transfer extracellular information into corresponding intracellular reactions by recruiting effectors to integrin cytoplasmic tail. This outside-in signaling of integrin regulates cell shape, migration, growth, and survival. It has been shown that both lateral association (clustering) of integrin heterodimers and the conformational change of the receptors are required for conveying outside-in signals [Luo *et al*., 2007; Zhu *et al*., 2007; Wang *et al*., 2010, 2011b).

Integrin engagement with ECM can also modulate the classic Erk/MAP Kinase pathway downstream from RTK activation [Juliano *et al*., 2004]. This involves regulation at the level of the Raf-1 kinase [Edin and Juliano, 2005; Lin *et al*., 1997] and also effects on the trafficking of activated Erk to the nucleus [Aplin *et al*., 2001].

Integrins can also signal directly via activation of tyrosine kinases. The discovery that cell adhesion or integrin clustering could activate tyrosine phosphorylation via focal adhesion kinase (FAK) was a key event in understanding the function of integrins [Kornberg *et al*., 1991; Schaller *et al*., 1992].

FAK recruitment to integrin cytoplasmic tail is an early event in outside-in signaling [Parsons, 2003; Schlaepfer and Mitra, 2004]. This non-receptor tyrosine kinase is highly conserved and FAK knock-out is embryonic lethal in mice. FAK is consisted of an N-terminal FERM domain which functions as an activation switch and adaptor, a central catalytic domain, a proline-rich region and a C-terminal FAK Adhesion Targeting domain. Under resting state, the FERM domain interacts with its own catalytic domain and therefore blocks accessibility of FAK substrates [Cooper *et al*., 2003; Dunty *et al*., 2004; Lietha *et al*., 2007]. Early evidence indicated that interaction between the FAK FERM domain and β integrin tail releases this autoinhibition, causing FAK autophosphorylation and activation [Schaller *et al*., 1995]. Displacement of the FERM domain from its catalytic domain results in autophosphorylation on tyrosine 397, which leads to Src activation. Then activated Src further phosphorylates and promotes FAK activity. FAK activation is closely associated with focal adhesion formation and cell migration.

Recruitment of Src leads to additional tyrosine phosphorylations with several consequences including recruitment of the SH2-adaptor Grb2 in complex with SOS,a GEF for Ras, leading to activation of the Ras-MAP Kinase cascade. Further, SH3 domains mediate binding of GRAF and ASAP1, which are GAPs for the Rho and Arf 1/6 small GTPases, leading to effects on the actin

cytoskeleton. Additionally the adaptor protein Cas and its partner Crk recruit the DOCK180/ELMO complex which acts as a GEF for the Rac GTPase and thus to further effects on the cytoskeleton. Finally, the adaptor protein paxilin binds to a sequence in the c-terminal of FAK and serves both as a structural protein linking to actin and also influence the ERK pathway via Raf-1.

Thus FAK serves as the hub of a multi-protein signal transduction complex that influences cell cycle, cytoskeletal organization, and cell motility.

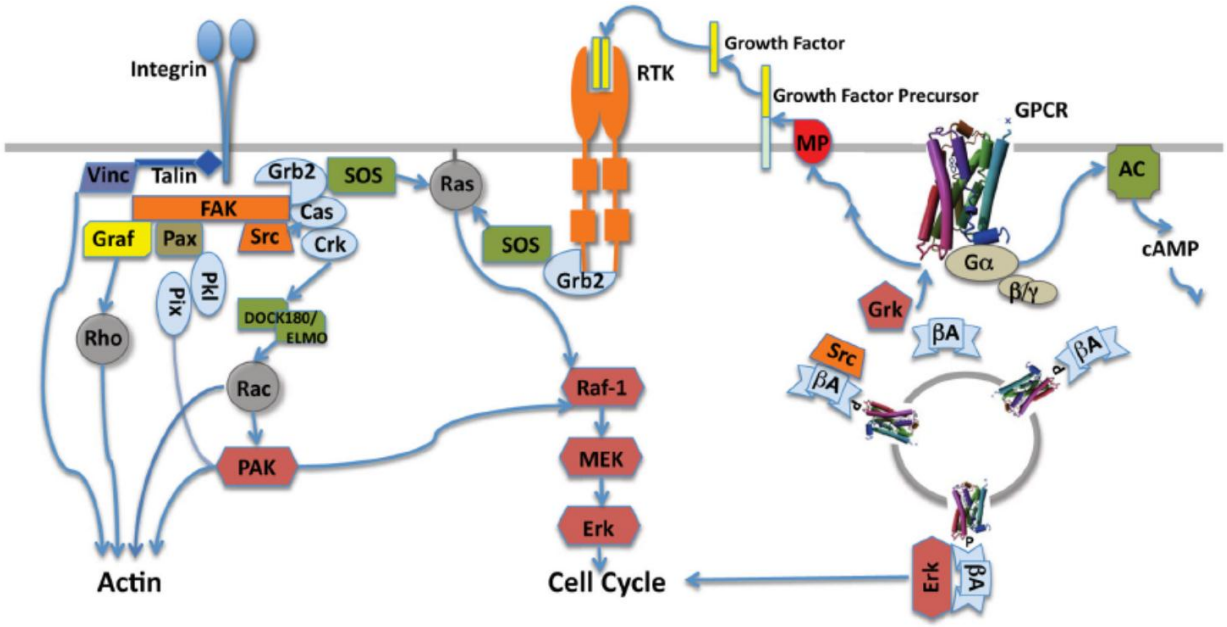


Figure 2.5 *Signaling by Integrins, RTKs and GPCRs. This illustrates highly simplified versions of the downstream signaling pathways regulated by these three families of receptors as well as some of the interconnections between pathways. Cytoskeletal proteins-Talin, Vinculin (Vinc), Paxilin (Pax); Adaptor proteins-Grb2, Cas, Crk, Pkl, Pix, beta-arrestin (βA); Small GTPases-Ras, Rac, Rho; Heterotrimeric GTPase (Gα/βγ) Tyrosine kinases-Focal Adhesion Kinase (FAK), c-Src, RTK; Serinethreonine kinases-PAK, Raf-1, MEK (dual specificity), Erk, Grk; Guanine nucleotide exchange factors-SOS,DOCK180/ELMO; Metalloprotease (MP).*

3. THE LEUCOCYTE RECRUITMENT CASCADE IN INFLAMMATION

The circulation of leukocytes including lymphocytes, monocytes, macrophages, dendritic cells, and granulocytes from the peripheral circulation (blood pool) to other tissues including the gut mucosa is an important aspect of immune surveillance [Delves and Roitt, 2000b; Delves and Roitt, 2000a; McIntyre et al., 2003]. A crucial feature of any inflammatory response is a rapid recruitment of leukocytes from the blood to the site of inflammation.

Leucocyte recruitment not only plays a role in the acute response to pathogen infection or tissue injury but also represents a major pathophysiological component of almost any inflammatory pathology, including atherosclerotic vascular disease, or autoimmune diseases, such as rheumatoid arthritis, psoriasis or multiple sclerosis.

Leukocyte recruitment into inflamed tissue follows a well defined cascade of events, beginning with capturing of free flowing leukocytes to the vessel wall, followed by rolling, adhesion to endothelial cells, postadhesion strengthening, crawling, and finally transmigration (Figure 3.1). During these steps, different integrins have to be activated via inside–out signaling. Each step necessitates a different set of integrins that differs among leukocyte subsets. Similarly, different activating stimuli trigger distinct signaling pathways during every step.

Leukocyte expressed integrins include the α_4 integrins (CD49d), $\alpha_4\beta_1$ (VLA4) and $\alpha_4\beta_7$ (LPAM) and the β_2 integrins $\alpha_L\beta_2$ (LFA-1, lymphocyte function-associated antigen-1, CD11a/CD18) and $\alpha_M\beta_2$ (Mac-1, macrophage antigen-1, CD11b/CD18).

Besides mediating firm leukocyte arrest, $\alpha_4\beta_1$ which binds to VCAM-1 (vascular cell adhesion molecule-1), and $\alpha_4\beta_7$, interacting with VCAM-1 and MAdCAM-1, are able to mediate leukocyte rolling independent of selectins. The endothelial counter-ligands of leucocyte integrins also include several receptors of the immunoglobulin superfamily, such as the intercellular adhesion molecules (ICAM)-1-5, whereby the most studies exist on ICAM-1 and ICAM-2. Both ICAM-1 and ICAM-2 can specifically bind to LFA-1 and Mac-1.

Capturing and rolling. Capturing of leukocytes from the bloodstream is initially mediated by selectins displayed on the endothelial luminal surface and their corresponding counter-receptors on leukocytes. This initial contact triggers a signaling cascade leading to the activation of integrins on the rolling leukocyte to an intermediate-affinity conformation effectively slowing down the rolling velocity of the cells (hence the term “slow rolling”) [Kuwano et al, 2010; McEver et al., 2010].Integrin activation in leukocyte rolling is best understood in neutrophils rolling on E-selectin. The importance of integrins in monocyte and T cell rolling is less well studied. In vitro studies

suggest a predominant role of VLA-4 for monocyte rolling, as blockade of b2-integrins by mAbs had no effect on rolling flux and cell adhesion on IL-4–activated HUVECs [Luscinskas *et al*., 1994]. CD4+ T cells were also found to roll on P- and E-selectin [Alon *et al*., 1994]. CD8+ T cells roll on TNF-a– and IFN-g–treated vessels in a VLA-4–dependent manner. VLA-4 seems to be the predominant integrin mediating lymphocyte rolling [Nandi *et al*., 2004]; however, physiological relevance of monocyte and T cell rolling for recruitment to peripheral tissues and involved signaling pathways affecting integrin affinity and avidity are still unclear.

Adhesion and postadhesion strengthening. In most tissues rolling or tethering is insufficient to permanently target the leukocyte to the vessel wall: without further stimuli the cell detaches. During rolling, however, leukocytes pick up inflammatory signals presented on the endothelial cells that may trigger arrest. On a cellular level, leukocyte arrest is mediated by the activation of GPCRs that mediate activation of integrins to their extended, high-affinity conformation, resulting in binding of ligands such as ICAM-1 or VCAM-1 [Ghandour *et al*., 2007]. Lateral movement of integrins on the cell surface following arrest results in an accumulation of integrins at the site of endothelial ligand presentation, forming an integrin "cluster" at the leukocyte/endothelial border. This increase in avidity may represent a trigger event for outside–in signaling, thus marking a crucial event for postadhesion strengthening. VLA-4 and LFA-1 seem to be the predominant integrins mediating T cell adhesion, whereas monocytes adhere in a VLA-4– or β_2-integrin–dependent manner and neutrophil adhesion is LFA-1 dependent.

Intravascular crawling. Crawling was first observed in vitro and initially termed "locomotion": monocytes that adhered to HUVECs were found to actively move to cellular junctions to start transmigration within seconds. However, the molecular events involved in the transition from adhesion to crawling are still largely elusive.

Transmigration and detachment. At the preferred site of transmigration, integrin binding to ICAM-1 and VCAM-1 constitutes central players in the orchestra of protein interactions involved in both para- and transcellular transmigration [Williams *et al*., 2007]. Activated endothelium forms ring-like membrane structures enriched with ICAM-1 and VCAM-1 that are maintained for the duration of transmigration.

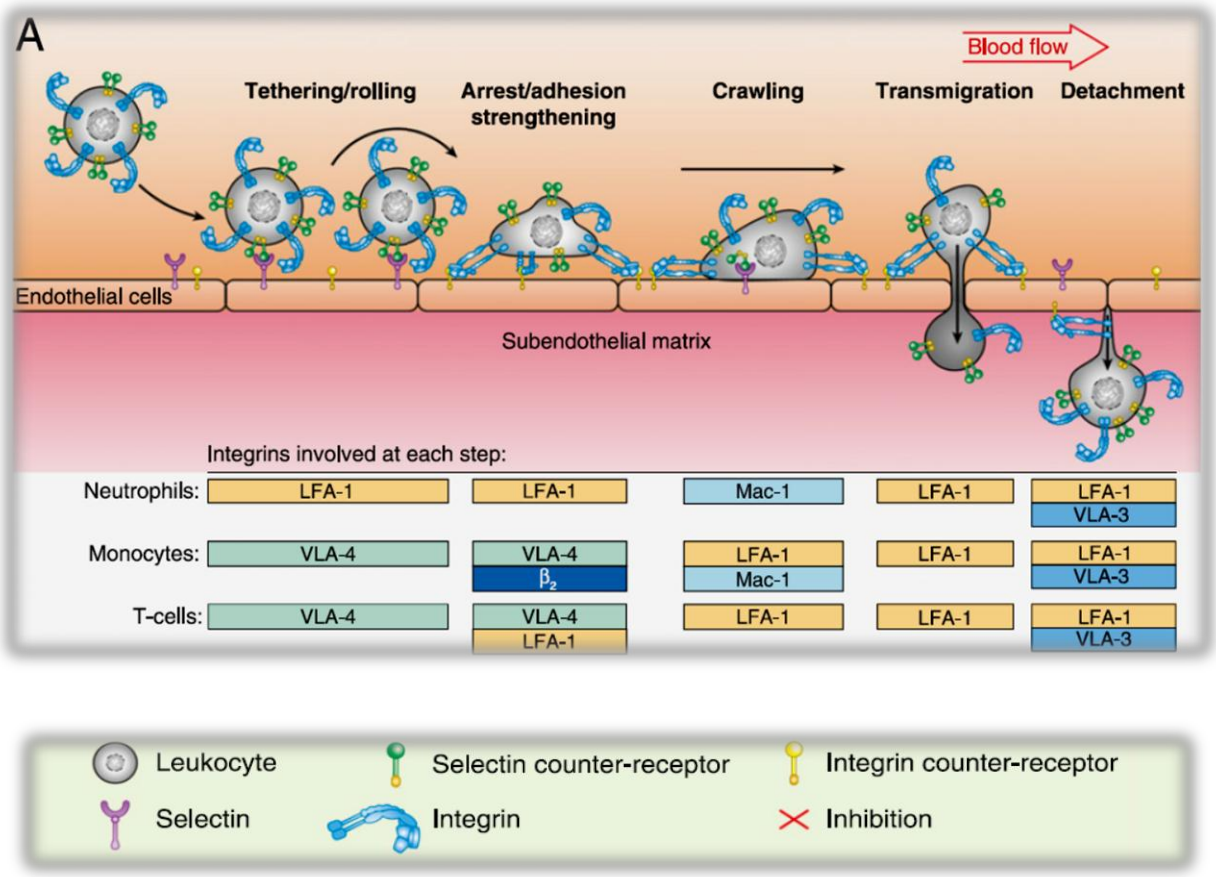


Figure 3.1. *A, Integrins in leukocyte recruitment. In each step of the leukocyte recruitment cascade, integrins play a crucial role. During rolling, integrins mediate interaction with the endothelium. After this contact, integrin activation mediates cell arrest, and accumulation of integrins at the endothelial/leukocyte bordermarks postadhesion strengthening. Leukocytes then crawl over the endothelium before they transmigrate and finally detach. Integrins found to be involved in each step are listed below.*

3.1 ASPECTS OF VLA4 FUNCTION AND REGULATION

According to the UniGene EST profile the overall expression patterns of integrin alpha 4 subunit (ITGA4) and integrin alpha L subunit (ITGAL) are very similar. These integrins are expressed in tissues associated with blood and lymphatic tissues. Blood, bone marrow, lymph, lymph nodes, spleen, and thymus are primary sites of expression. However, VLA-4 integrin is strongly expressed on CD^{34+} early hematopoietic stem progenitor cells (HSPCs). Its expression is critical for homing and retention of HSPCs, since blocking VLA-4-specific interactions using mAbs or small molecule antagonists is sufficient to induce cell mobilization into peripheral blood [Coulombel *et al*., 1997; Oostendorp and Dormer, 1997; Gazitt, 2004; Chigaev et al., 2011d]. This observation is also confirmed by the fact that the expression of VLA-4 is more pronounced in "germ cell tumors."

First, it directly participates in cell arrest under flow, where firm adhesion is mediated by activated (high-affinity, unbent) integrins. Second, VLA-4 contributes to cell-cell interactions that are critical for immune system responses. For example, it plays a part in the formation of immunological synapse, and participate in cell co-stimulation.

The discovery that in addition to firm adhesion, VLA-4 can mediate cell tethering and rolling [Alon *et al*., 1995] represents the first indication of a functional difference between VLA-4 and LFA-1. More detailed analysis revealed that VLA-4 supports a number of adhesive interactions that are directly related but not limited to the maintenance of immune cells through hematopoiesis [Imai *et al*., 2010], as well as intrinsic immune responses. Thus, VLA-4 participates in chemokine-dependent cell arrest on endothelium, NK, and MKT cell recruitment to bone marrow, cell recruitment in response to bacterial infections, bacterial killing.

3.2 CONFORMATIONAL ANALYSIS OF INTEGRIN VLA-4 STATES

Taken together, the overall scheme of the VLA-4 conformation regulation can be generalized as follows (Figure 3.2). At rest, the low affinity bent conformation prevents cell tethering and rolling because of the positioning of the ligand binding site. If ligand engagement occurs, it would have a very short lifetime. However, it is also possible that a series of engagements of integrins or other receptors [selectins for example [Kuwano *et al*., 2010] could provide a signal resulting in molecular extension.

This could lead to rolling on an extended low affinity integrin. Rapid activation by Gαi-coupled GPCR induces a short-lived high affinity extended state (seconds to minutes), followed by a sustained extended low affinity state. If during the short period that VLA-4 engages its counter-structure, a long-lived tether will form. Under shear and external force this interaction can potentially be sustained for a longer period of time because of mechanical (catch bond) or signaling/cytoskeletal events. If no engagement of the integrin occurred, a low affinity extended state could be ideally suited for rolling under shear.

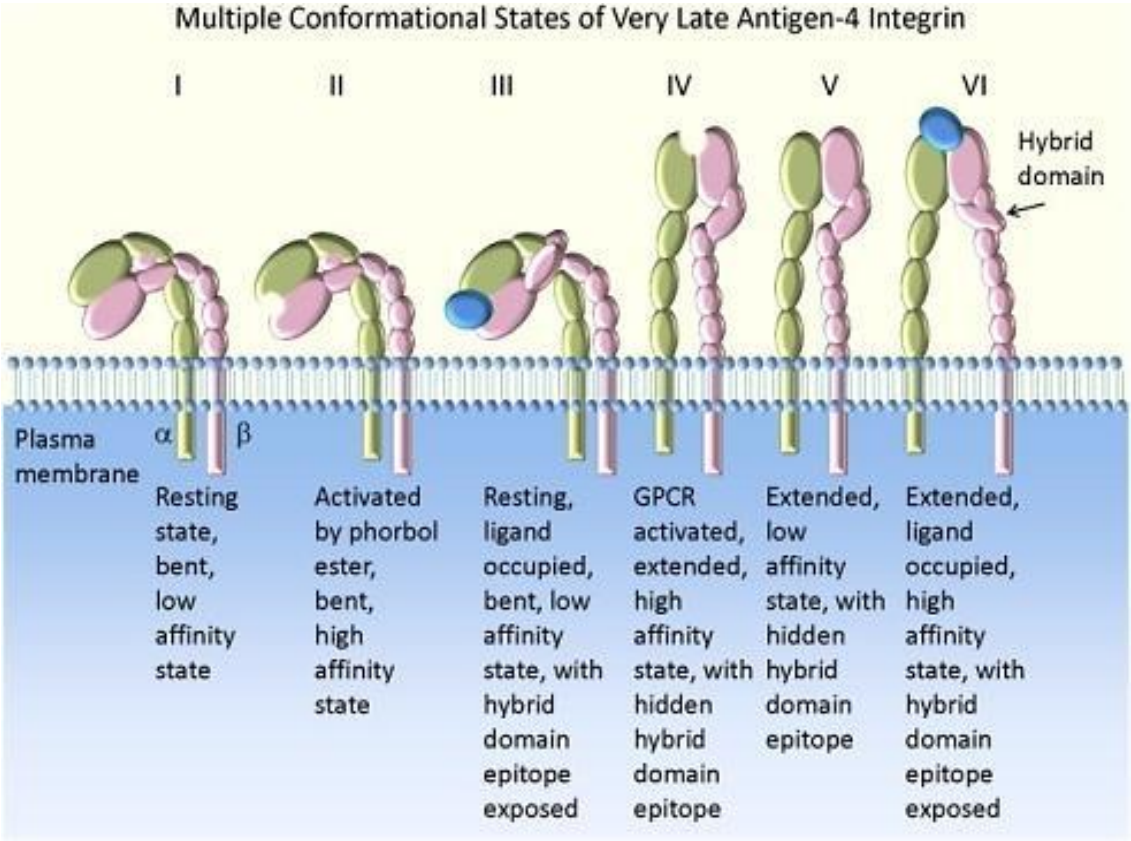


Figure 3.2 Model of VLA-4 integrin conformation and affinity. The bent low affinity state is observed on resting cells (I). Activation by phorbol ester creates a high affinity state lacking molecular extension as detected by a FRET-based approach (II). This conformation results in the slow accumulation of cell aggregates in suspension, with a low tether capture frequency but a long tether duration in the rolling assay. The addition of VLA-4 specific ligand to resting cells leads to exposure of a hybrid domain (LIBS) epitope (III). This state is bent (or at least not fully extended) because a further molecular extension can be detected with a FRET-based approach. Activation through a wild type Gαi-coupled GPCR induces a high affinity extended conformation (IV). This conformation results in the rapid accumulation of cell aggregates in suspension , with high tether capture frequency and long tether duration. The low affinity extended (or at least partially extended) conformation (V) can be detected for several minutes after signaling from wild type Gαi-coupled GPCR, because of relatively faster desensitization of the ligand binding affinity than relaxation of the conformation. Conformation V may also result from consecutive stimulation through Gαi-coupled and Gαs-coupled receptors .The ligand occupied and extended high affinity state (VI) was detected after Gαi-coupled GPCR activation in the presence of ligand.

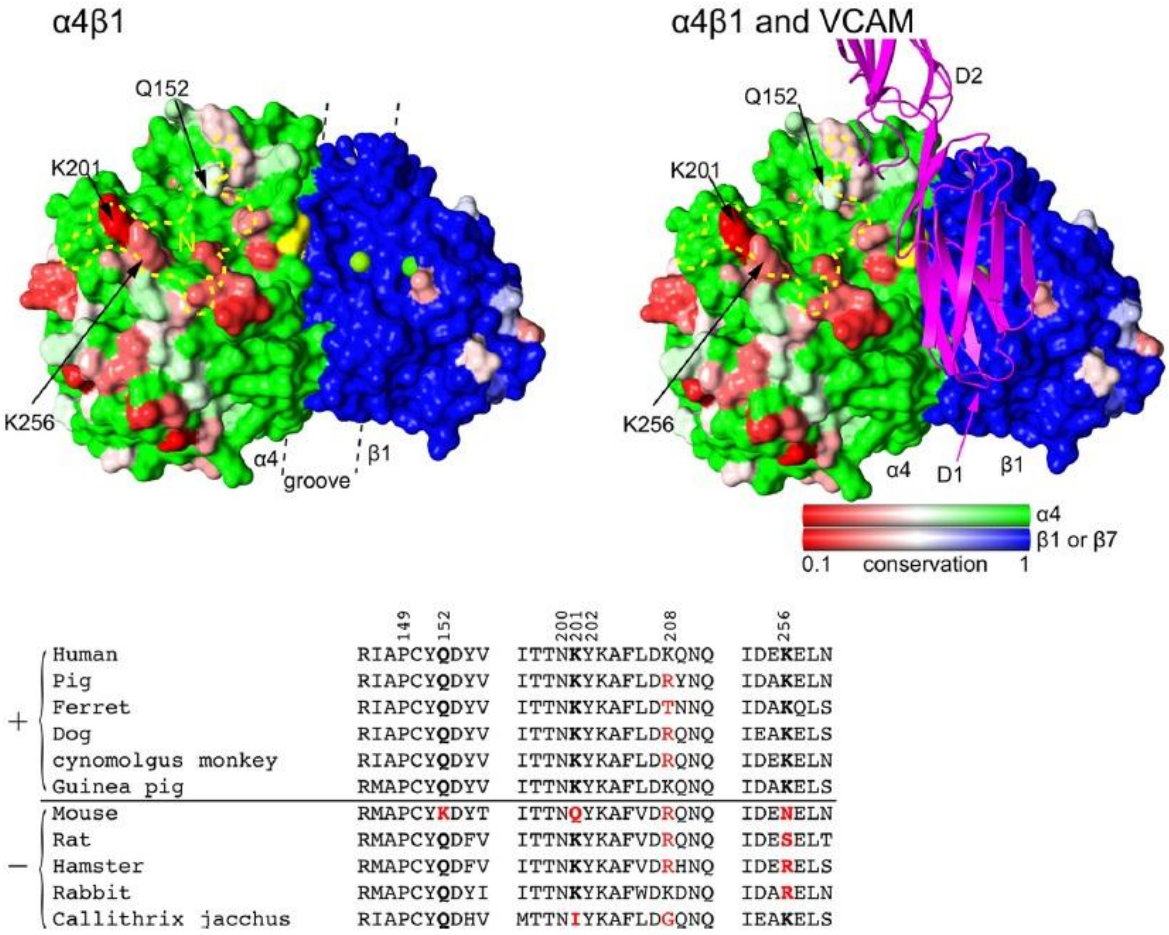
3.3 INTEGRIN PHYSIOLOGY AND ITS IMPLICATION FOR DRUG DISCOVERY

Natural ligands of VLA4 are, for example, the vascular cell adhesion molecule-1 (VCAM-1) and fibronectin with its alternatively spliced connecting segment CS-1 domain as binding motif. Fibronectin is recognized by VLA4 via the Leu-Asp-Val (LDV) binding epitope and VCAM-1 interacts via QIDSPL recognition sequence. Integrin $\alpha_4\beta_1$ is mainly expressed on the surface of lymphocytes, eosinophils, monocytes, basophils, and mast cells, whereas $\alpha_4\beta_1$ is present on subpopolations of T- and B-lymphocytes and on eosinophils.

Diseases associated with $\alpha_4\beta_1$ and $\alpha_4\beta_7$ are mainly inflammatory and autoimmune diseases which go along with a pathological accumulation of activated leucocytes in the affected tissues [inflammatory bowel disease (IBD), rheumatoid arthritis (RA), asthma, Crohn's disease (CD), multiple sclerosis (MS)]. Their involvement in disease processes prompted several pharmaceutical companies to pursue α_4 integrin antagonists as potential anti-inflammatory agents.

Natalizumab (Elan and Biogen, Inc.) was the first humanized monoclonal antibody targeting the α_4-integrin subunit. And was approved by the FDA for the treatment of MS and CD in 2004. The application of the drug caused severe side effects in three patients resulting in an infection with PML, but a re-evaluation of the FDA brought it back to the market in 2008 under the trade name of Tysabri for patients with moderate to severe CD after an inadequate response to conventional therapies and patients suffering from a severe form of MS with lack of alternative therapies.

Natalizumab recognizes an α_4 region with significant sequence variation among human and mammals commonly used in disease models and toxicology (Figure 3.3). By contrast, the long narrow binding groove for small molecule antagonists and the biological ligands MAdCAM and VCAM is highly conserved in $\alpha_4\beta_7$ and invariant in $\alpha_4\beta_1$ (Figure 3.3). Domain 1 of VCAM can be docked in this groove with some precision by placing the Asp side chain of its rigid, integrin-binding loop in the same β I domain MIDAS Mg^{2+}-coordinating position as the carboxyl group of a co-crystallized antagonist.



		149	152	200	201	202	208	256
+	Human	R	I	A	P	C	Y	Q	D	Y	V	I	T	T	N	K	Y	K	A	F	L	D	K	Q	N	Q	I	D	E	K	E	L	N
	Pig	R	I	A	P	C	Y	Q	D	Y	V	I	T	T	N	K	Y	K	A	F	L	D	R	Y	N	Q	I	D	A	K	E	L	N
	Ferret	R	I	A	P	C	Y	Q	D	Y	V	I	T	T	N	K	Y	K	A	F	L	D	T	N	N	Q	I	D	A	K	Q	L	S
	Dog	R	I	A	P	C	Y	Q	D	Y	V	I	T	T	N	K	Y	K	A	F	L	D	R	Q	N	Q	I	E	A	K	E	L	S
	cynomolgus monkey	R	I	A	P	C	Y	Q	D	Y	V	I	T	T	N	K	Y	K	A	F	L	D	R	Q	N	Q	I	D	E	K	E	L	N
	Guinea pig	R	M	A	P	C	Y	Q	D	Y	V	I	T	T	N	K	Y	K	A	F	L	D	K	Q	N	Q	I	D	A	K	E	L	S
−	Mouse	R	M	A	P	C	Y	K	D	Y	T	I	T	T	N	Q	Y	K	A	F	V	D	R	Q	N	Q	I	D	E	N	E	L	N
	Rat	R	M	A	P	C	Y	Q	D	F	V	I	T	T	N	K	Y	K	A	F	V	D	R	Q	N	Q	I	D	E	S	E	L	T
	Hamster	R	M	A	P	C	Y	Q	D	F	V	I	T	T	N	K	Y	K	A	F	V	D	R	H	N	Q	I	D	E	R	E	L	S
	Rabbit	R	M	A	P	C	Y	Q	D	Y	I	I	T	T	N	K	Y	K	A	F	W	D	K	D	N	Q	I	D	A	R	E	L	N
	Callithrix jacchus	R	I	A	P	C	Y	Q	D	H	V	M	T	T	N	I	Y	K	A	F	L	D	G	Q	N	Q	I	E	A	K	E	L	S

Figure 3.3 *Species-specific differences around the integrin α4β1 ligand-binding sites. Conservation on the solvent accessible surface is displayed from invariant (1, green or blue) to low (0.1, red) for both panel using the species shown in bottom. Mouse-human sequence is shown as invariant (green or blue) or different (red). Invariant α and β subunit residues are shown in green and blue, respectively, to visualize the subunit interface. Additionally, invariant VCAM-binding α4 residues Tyr-187 and Trp-188 are in yellow and β subunit MIDAS and ADMIDAS metal ions are shown as green spheres. Antibody footprints are outlined in yellow dashes (N for natalizumab) and key antigenic residues are labeled. VCAM is shown in ribbon representation in the orientation found previously in docking to $\alpha_4\beta_7$. The $\alpha_4\beta_1$ model was made by superimposing the β1βI domain from $\alpha_5\beta_1$ onto $\alpha_4\beta_7$. The location of the ligand binding groove is marked with dashed lines. Sequence conservation was calculated by AL_2CO with species equally weighted, using the sum of pairs measure with the BLOSUM62 matrix with normalization of the scoring matrix. The bottom panel represents sequence variation in the natalizumab epitope. Sequences are of species found to be positive (+) or negative (-) for natalizumab reactivity in European Medicines Agency filings. Residues in the epitope are numbered. Residues most important for species reactivity are in bold. Residues that differ from human are red.*

The orientation between D1 and D2 of VCAM-1 is variable among crystal structures (Figure 3.4). D1 of docked VCAM does not clash with natalizumab Fab; however, where D2 of VCAM emerges from the groove, it clashes in some orientations (Figure 3.4, panel *A* and *B*). This docking model thus predicts a noncompetitive mechanism of antagonism where natalizumab would lower affinity of $\alpha_4\beta_1$ by limiting the number of VCAM conformations accessible for binding (Figure 3.4, panel *B*).

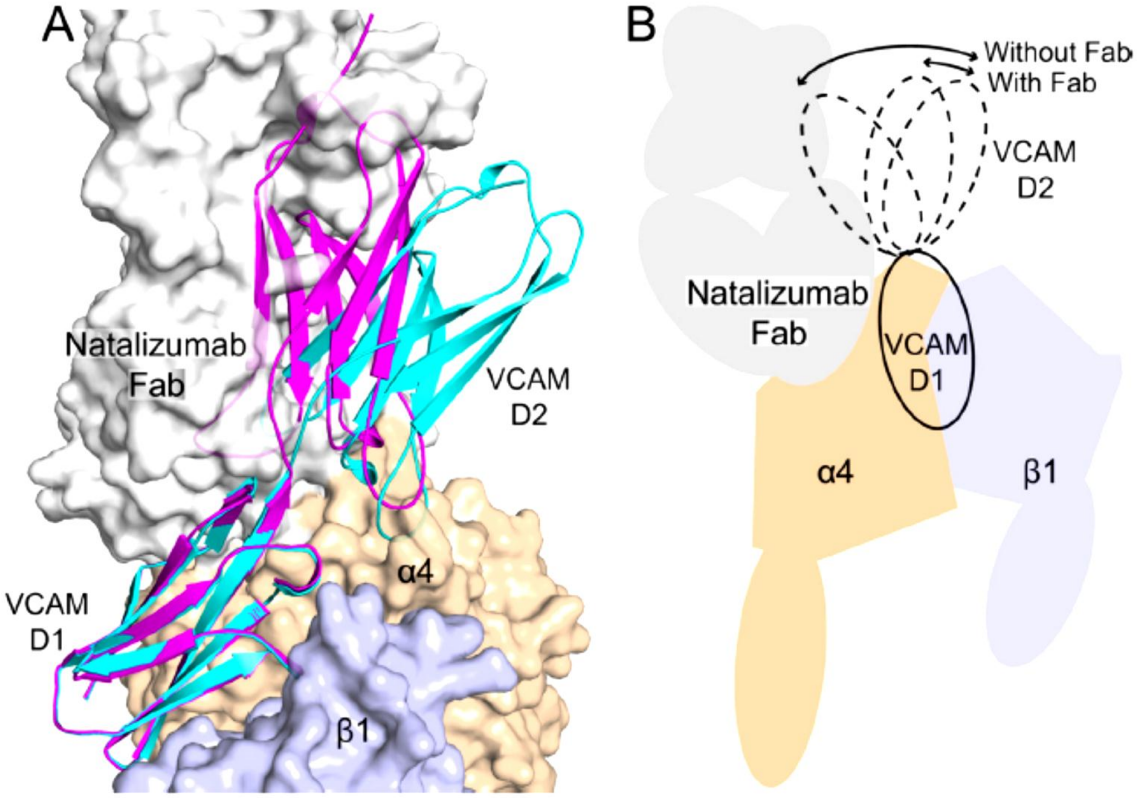


Figure 3.4 *Model of VCAM binding to a natalizumab Fab-$\alpha_4\beta_1$ complex. Transparent solvent accessible surfaces are shown of the α_4 β-propeller domain (wheat), β1βI domain (light blue), and natalizumab Fab (gray). Two examples of VCAM D1D2 crystal structures that differ the most in D1-D2 orientation are shown in ribbon representation.*

The noncompetitive inhibition mechanism described is a departure from previous concepts on how antibodies block function, particularly for integrins, which are known to undergo conformational change. Natalizumab clearly binds to a different site on $\alpha_4\beta_1$ than VCAM, making the noncompetitive mechanism conceptually easy to appreciate. A change in the conformational space accessible to D2 of VCAM that is imposed by natalizumab is the simplest explanation of the structural and ligand competition data.

Because ligand-binding sites are well conserved across species, the finding here that a function-blocking, species specific antibody binds beside, rather than in, a ligand-binding site may be more common than not. Another species-specific therapeutic antibody, vedolizumab, also binds to the edge of the ligand-binding site, but on the opposite, β_7 side. Vedolizumab inhibits binding of MAdCAM, but not VCAM, to integrin $\alpha_4\beta_7$. This unexpected result appears to be a consequence of binding close enough to the ligand-binding site to inhibit binding of MAdCAM, but not quite close enough to inhibit binding of VCAM. Similarly, efalizumab to LFA-1 binds to the αL βI domain outside the binding site for D1 of ICAM-1, in a position where it would clash with or require bending away of D2 of ICAM-1. The greater presence of species-specific differences outside of the ligand-binding site has thus skewed the current generation of therapeutic antibodies toward previously unanticipated mechanisms of action, which include noncompetitive antagonism and inhibition of binding of some and not other ligands. Currently, technology is maturing to generate synthetic antibody libraries that are devoid of tolerance to self.

The severe side effect is blamed to the immunosuppressive action of the antibody and is a prominent side effect observed with many α_4 antagonist since this leukocyte cell adhesion receptor is directly involved in immune response.

- *Peptidic and Non-Peptidic Ligands*

A great variety of small peptidic and non-peptidic ligands interfering with the VCAM-1/VLA-4 interactions have been extensively described in the literature. For instance, the combination of a LDVP sequence and N-terminal o-methylphenylureaphenylacetyl group (MPUPA) leads to the potent selective BIO1211. However, residual peptidic nature of this molecule and its related compounds resulted in rapid enzymatic hydrolysis and clearance in vivo. Linear and cyclic peptide derivatives are predominantly based on the LDV-binding motif, whereas privileged scaffolds for small-molecule antagonists are phenylureido-LDV-peptidomimetics (relatively selective for $\alpha_4\beta_1$) and acylphenylalanine derivatives (biselective for $\alpha_4\beta_1$ and $\alpha_4\beta_7$). The most advanced small-molecule drug candidate targeting alpha4 is the orally bioavailable AJM-300 (Ajinomoto) wich is currently in clinical Phase III for the treatment of IBD, UC, and CD.

The small molecule HMR-1031 (Aventis Pharmaceuticals) is currently in Phase II trials for arthritis and was formerly investigated for the treatment of asthma. HMR-1031 was investigated together with IVL984 and IVL745 in a systematic teratogenicity study and showed that the teratogenic risk is corresponding to the affinity of the ligand for the inactivated integrin state. It was observed that ligands binding both the activated and inactivated integrins with high affinity showed a significantly

higher risk of toxicity and embryonic defects in a whole rat embryo culture when compared to compounds binding only to the activated state. The α_4-subtype targeting $\alpha_4\beta_1$ and $\alpha_4\beta_7$ ligands TRK-170 (Toray Industries: CD) and Firategrast (GlaxoSmithKline/Tanabe: IBD, MS, RA, asthma, CD) are currently actively developed in clinical Phase II, all showing good safety profiles and promising data in preclinical animal models.

A majority of compounds specific for VLA-4 and several other integrins, including $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, are competitive (direct) inhibitors (formally agonists that promote LIBS). Until recently, no VLA-4-specific allosteric antagonists had been described [Chigaev *et al*., 2011d]. For a competitive drug, the ligand binding site location is very close (or overlaps) with its natural ligand binding site. Therefore, direct competition with the integrin natural ligand can be observed.

For integrins possessing a ligand binding site that is exposed at rest (such as VLA-4), binding of competitive inhibitors would occur at any time, and binding site occupancy would simply depend on binding affinity and drug concentration. Is it possible to identify allosteric antagonists for integrins with an exposed ligand binding site? Using an approach that relies upon the exposure of the Ligand Induced Binding Site epitope (LIBS) to distinguish VLA-4 competitive antagonists [Njus et al., 2009], several VLA-4-specific allosteric antagonists were identified [Chigaev et al., 2011d]. These molecules, although not competing directly with VLA-4-specific ligands, blocked VLA-4-dependent cell adhesion.

4. AIM OF THE RESEARCH

Inflammation is a key pathophysiological process in a large number of common diseases. The motility of its cellular components is a key feature of the immune system since it allows the selective recruitment of cells to defined places and niches not only to combat invading pathogens but also to pass through tightly controlled programs of immune cell development and maturation. The selective recruitment of a particular T cell subset into tissues during inflammation is now understood to be a tightly orchestrated multistep adhesion cascade, regulated by selectins, integrins, chemokines, and chemoattractant lipids, that specifically directs the trafficking of leukocytes into sites essential for their function. It depends on the type of inflammatory response and involves interactions between specific ligands expressed on the T cell surface (selectin ligands and integrins) with their respective adhesion molecules expressed on the vascular endothelium (selectins and intercellular and vascular adhesion molecules ICAM-1/ICAM-2 and VCAM-1) in combination with appropriate chemokines.

When activated, α_4-family integrins mediate cell–cell adhesions crucial to immune function [Kinashi, T., 2012]. $\alpha_4\beta_1$ (VLA-4) binds to fibronectin splice variants at the CS-1 domain, and to vascular cell adhesion protein 1 (VCAM-1) which is expressed on activated endothelial near sites of inflammation, whereas $\alpha_4\beta_7$ binds to mucosal vascular addressin cell adhesion molecule 1 (MAd-CAM) to capture antigen-presenting cells in post-capillary venules of peripheral lymphoid tissue [Villablanca, E.J. *et al*., 2011].

α_4-family integrins have been extensively targeted by drugs selective for one or both receptors. The inappropriate activation of leukocytes in tissues by cytokines drives many autoimmune diseases, including multiple sclerosis (MS), Crohn's disease, and inflammatory bowel disease (IBD). Targeting integrin $\alpha_4\beta_1$ for pharmacologic blockade may allow for the selected modulation or inhibition of the migration of immune cells that traffic to an affected organ and cause disease.

Despite some obstacles, this idea is quickly becoming reality as an array of drugs that inhibit or modulate cell migration are actively being studied in clinical trials. The humanized monoclonal antibody Natalizumab, which recognizes α4 integrins expressed on the surfaces of certain lymphocytes, is an effective therapy for multiple sclerosis (MS) and Crohn's disease (CD). Natalizumab blocks binding of $\alpha_4\beta_1$ integrin to vascular cell adhesion molecule 1 (VCAM-1), which is expressed on endothelial cells in nervous tissue undergoing multiple sclerosis flare. Natalizumab also blocks binding of $\alpha_4\beta_7$ integrin to mucosal addressin cell adhesion molecule 1 (MadCAM-1), which is up-regulated on endothelial cells in the gut, in patients with active CD.

Such an improved understanding of the underlying mechanisms involved, has resulted in the identification of an array of potential drug targets aimed at modulating cell migration in order to treat a broad range of autoimmune and inflammatory diseases. Small molecules of different nature, peptidic, non peptidic and peptidomimetic, that inhibit binding between α4 integrins and their ligands have been the subject of much research in recent years [Dattoli *et al*., 2013]

Based on these promises, we analyzed a library of peptidomimetic inhibitors of $\alpha_4\beta_1$ integrin constituted by a rigid central scaffold, β-amino acid residues, and partially modified retro-inverso sequence. To determine inhibitory activity of these compounds, first screening were performed in vitro through cell adhesion assays in Jurkat E6.1 cells and a binding radioligand assay (SPA). Both methods were optimized and set up by us. Scintillation proximity assay (SPA) is a homogeneous assay technology which is bead based and removes the need for a filtration step to separate bound from free ligand in a receptor binding assay. SPA is routinely used for radioligand binding assays, particularly in drug screening applications where high throughput is required. After this first step, further analysis concentrated on the most potent compound of the library, named DS70. The overall project was developed on two principal branches, defined by *in vitro* and *in vivo* assays.

We aimed to characterize *in vitro* its inhibitory activity against α4β1 integrin expressed in different cell lines, representatives of all key typologies of cells recruited in an inflammatory process: leucocytes, eosinophils and mast cells. This goal was achieved by cell adhesion assays. We also confirmed its specificity for α_4 family integrin, employing several cell lines expressing different subtypes of integrin receptors.

Outside-in signaling mediated by natural ligands of extracellular matrix also involves the activation of kinases in response to integrin-mediated cell adhesion. Therefore, the combination of lateral aggregation of integrins in the membrane and integrin ligand binding leads to recruitment and activation of FAK (focal adhesion kinase). This process is not entirely understood, but a conformational change leading to FERM domain displacement in FAK is involved. This change is associated with increased phosphorylation of tyrosine (Tyr)397, which along with an exposed PxxP motif forms a binding site for the Src homology 2 (SH2) and SH3 domains, respectively, of the Src kinase. Src then phosphorylates other tyrosines contributing to full activation of FAK. This active FAK/Src complex mediates a number of important signaling cascades downstream of integrins. Key examples include the control of the ERK MAP kinase pathway, the phosphatidylinositol-3 kinase (PI3K)/AKT pathway, and Rho GTPase activities. Therefore, we focused our attention on the possibility to modulate levels of FAK and ERK1/2 phosphorylation using different concentration of DS70. We definitively tempted to analyze antagonist effects on integrin $\alpha_4\beta_1$ signaling pathways.

Another mechanism we planned to explore is the possible involvement of integrin $\alpha_4\beta_1$ and the consequent effects of its blockade during activation of mast cells, triggered by activated T cells or calcium ionophores (PMA and calcimycin).

According to the literature, it is known that the classical view of mast cells as mere cellular effectors of type 2 adaptive immune responses is overcame, arguing in favor of a broader role for mast cells including their interaction with different subsets of cells. If it is well established that mast cells modulate T-cell responses, it is also known that helper T-cell-derived factors affect mast cell biology.

Th cells have been reported to influence mast cell activation. It has been shown that mouse T cells (activated either by PMA or by anti-CD3 antibodies) enhance FcεRI mediated degranulation in mast cells [Inamura N., *et al*., 1998]. This effect is contact dependent and is mediated at least in part by LFA1/ICAM-1 interaction. Similarly, human Jurkat T cells triggered by anti-CD3 antibodies stimulate HMC 1.1 cells to secrete β-hexosaminidase, TNFα and matrix metalloproteinase.

Thus, direct contact between surface molecules on mast cells and on activated T cells was found to provide the stimulatory signal in mast cells necessary for degranulation and cytokine release independent of T cell intracellular function. Indeed, separation of the two cell populations by a semi-permeable porous membrane prevented this pathway of mast cell activation [Bhattacharyya S.P. *et al*., 1998]. Finally, morphological analysis of inflamed tissues has often provided data compatible with the existence of functional cross-talks between these two cell subsets. For instance, mast cells and T cells have been shown in close proximity in human tonsils and in a mouse model of contact hypersensitivity [Nakae S. *et al*., 2005].

With regards to *in vivo* assays, we chose a classical model of ocular inflammation, represented by the already validated allergen challenge-model closely mimicking allergic conjunctivitis, guinea pigs pre-immunized through peritoneal administration of ovalbumin. From previous studies in our laboratory [Qasem A.R. *et al*., 2008], it was known that in a model of allergic conjunctivitis, levocabastine eye drops reduced the clinical aspects of the late-phase reaction and the conjunctival expression of $\alpha_4\beta_1$ integrin by reducing infiltrated eosinophils. We also propose that blockade of integrin-mediated cell adhesion might be a target of the antiallergic action of DS70 and may play a role in preventing eosinophil adhesion and infiltration in allergic conjunctivitis. To further characterize its mechanism of action, we evaluated at different times several parameters of inflammation assessed in conjunctival samples and in tears, after pre-treatment with compound DS 70 0.1% (30 and 10 minutes before allergen challenge): clinical score resolution, conjunctival eosinophilic infiltration in tears and in conjunctiva, conjunctival mRNA levels of CCL5, CCL11,

interleukin (IL)-1β and interleukin-8. We also performed immunohistochemical staining to detect changes in expression levels of α_4 subunit, and Giemsa staining to observe effects on mast cell degranulation.

5. MATERIALS AND METHODS

5.1 IN VITRO EXPERIMENTS

5.1.1 Materials

Cell culture media, phosphate-buffered saline (PBS) and fetal bovine serum (FBS) were from Lonza (Euroclone S.p.A, Milan, Italy); HBSS and chloromethylfluorescein diacetate (CMFDA) were from Invitrogen (Carlsbad, CA, USA). Lectin from Triticus vulgaris, soluble fibronectin (FN) from human plasma were purchased from Sigma-Aldrich (Steinheim, Germany).

Black and white 96-well clear-bottom plates were purchased from Corning Costar (Celbio, Milan, Italy). Soluble human VCAM-1 and MAdCAM-1 was purchased from R&D Systems (Minneapolis, MN, USA). BIO-1211(N-[[4-[[[(2-methylphenyl)amino]carbonyl]amino]-phenyl]acetyl]-L-leucyl-L-aspartyl-L-valyl-L-proline) was purchased from Bachem (Weil am Rhein, Germany). Tissue Protein Extraction Reagent (T-PER) was purchased from Pierce (Rockford, IL, USA).

Rabbit anti-human monoclonal antibodies against the α_4 subunit of $\alpha_4\beta_1$ integrin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-human monoclonal antibody against β_1 subunit (MAB 2000) was obtained from Chemicon (Millipore, Billerica, MA, USA). Secondary antibody (goat anti-mouse or goat anti-rabbit) were purchased from Santa Cruz Biotechnology Inc. Polyacrylamide gel, N,N,N0,N0-tetramethy-lethylenediamine (TEMED), ammonium persulfate (APS) and SDS were purchased from Sigma-Aldrich. Hybond-ECL Nitrocellulose Membrane was from Amersham Biosciences (GE Healthcare Europe, Milan, Italy). $Na^{125}I$ was obtained from PerkinElmer Inc., Waltham, Massachussets, USA). Polyvinyltoluene (PVT) anti-rabbit binding beads were supplied by Amersham Biosciences as a powder and reconstituted in distilled water.

All other reagents were of analytical grade or the highest purity available, purchased from Sigma.

5.1.2 Cell culture

D283 human medulloblastoma cells originally obtained from the American Type Culture Collection (Rockville, MD, USA) were kindly donated by professor Giovanna Cenacchi (Department of Biomedical and Neuromotor Sciences, “Alma Mater” University of Bologna).

The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, USA) containing L-glutamine, added with 20 % (v/v) fetal bovine serum (FBS, Hyclone), 1% non-essential aminoacids (Gibco) and a mixture of antibiotics and antimycotics 100x (100 units of penicillin, 100 µg of streptomycin, 0,25 µg of amphotericin B/ml medium plus 0,85% of Fungizone®) (Gibco).

SK-MEL-24 is one of a series of melanoma cell lines established from patient-derived tumor samples. This cell line expresses wild type B-Raf and wild type N-Ras. This cell line was established from a metastatic site (lymph node) in a 67-year-old Caucasian male with malignant melanoma. SK-MEL-24 cells (ATCC) were routinely grown in minimum essential medium (EMEM, Lonza) supplemented with 10% fetal bovine serum (FBS), non-essential aminoacids, sodium pyruvate and and a mixture of antibiotics and antimycotics.

The cell line K-562 (ECACC) was established from the pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises. The cell population has been characterized as highly undifferentiated and of the granulocytic series. Studies conducted by Anderson, et al., on the surface membrane properties led to the conclusion that the K-562 was a human erythroleukemia line. K-562 blasts are multipotential, hematopoietic malignant cells that spontaneously differentiate into recognizable progenitors of the erythrocytic, granulocytic and monocytic series.

Jurkat cells E6.1 (ECACC) are an IL-2 producing T lymphocyte cell line, commonly used to study T cell signaling. Established from the peripheral blood of a 14 year old boy with T cell leukemia, Jurkat cells are also useful for studying acute T cell leukemia. Jurkat cells are also useful for analysis of T cell signaling, including p38 MAPK, JNK signaling, and NF-kB signaling.

EoL-1 cells (ECACC), a recently established human eosinophilic leukemia cell line, have cytological features of myeloblasts under normal culture conditions, and differentiate not only phenotypically but also functionally into eosinophils by a number of stimuli. EoL-1 cells are particularly useful for analyzing leukemic cell differentiation and the properties of malignant eosinophils. EoL-1 cells are also a useful in vitro model for studying human eosinophil functions and their regulation.

Jurkat E6.1, K-562 and EoL-1 were maintained as a stationary suspension culture in RPMI-1640 with 10% fetal bovine serum (FBS) and a mixture of antibiotics and antimycotics.

TF-1 cell line was a kind gift by professor Giorgio Gallinella (Department of Pharmacy and Biotechnology, University of Bologna, S. Orsola-Malpighi Hospital). The TF-1 cell line was established in October 1987 from a heparinized bone marrow aspiration sample from a 35 year old Japanese male with severe pancytopenia. The cells are completely dependent on interleukin 3 (IL-3) granulocyte-macrophage colony-stimulating factor (GM-CSF) for long term growth. The morphological and cytochemical features, and the constitutive expression of globin genes, indicate the commitment of the cells to the erythroid lineage. Hemin and delta-aminolevulinic acid induce hemoglobin synthesis, and TPA induces dramatic differentiation of the TF-1 cells into macrophage-like cells. The TF-1 cell line is unique because of its responsiveness to multiple cytokines. The base medium for this cell line is RPMI-1640 (Lonza), with the following components: 2 ng/ml recombinant human IL-3, fetal bovine serum to a final concentration of 10% (FBS) and a mixture of penycillin/streptomycin 1%.

RPMI 8866 cell line has a lymphoblastoid morphology; they were a kind gift from professor Santoni Angela (Laboratory of Molecular Immunology and Immunopathology, Department of Molecular Medicine, "Sapienza" University of Rome). This cell line has been established from the peripheral blood of a 51-year-old American woman with chronic myelogenous leukaemia in May 1966. The B lymphoid cell line expresses a relatively high amount of calcitonin receptors, membrane bound Ig and soluble IgE-binding factor (Fc epsilon RII/CD23). Culture medium is RPMI 1640 (Lonza) with 10% Fetal Bovine Serum (FBS),enriched with Hepes 5 mM and sodium pyruvate 0,5 mM. Cells are usually kept in 50 mL of culture medium and they form large clumps.

HMC 1.1 cells were kindly provided by professor Butterfield Joseph. H. (Mayo Clinic, Division of Allergic Diseases, Rochester, Minnesota, USA). The growth factor-independent human mast cell line, HMC-1.1 was grown in IMDM medium (Iscove's medium with 25 mM Hepes) supplemented with sodium carbonate (3,02 g/L), l-glutamine (2 mM), 1,2 mM alphathioglycerol (Sigma), FBS (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml). HMC-1.1 is a factor-independent, immature mast cell line derived from the peripheral blood of a patient suffering from mast cell leukemia. In fact, two subclones, HMC-1.1 and HMC-1.2, have been characterized. HMC-1.1 has a KIT V560G mutation, whereas HMC-1.2 has two KIT mutations (V560G and D816V).

Adherent cells were detached with trypsin or with a cell scraper and viability was determined using trypan blue staining. Cells were kept at a temperature of 37 °C, a minimum relative humidity of 95% and an atmosphere of 5 % CO2 in air. For all experiments, cells were used between passage 3 and 15.

5.1.3 Cell adhesion assays

Cell adhesion assays were performed with all cell lines previously described. Briefly, 96-well plates were coated at 4°C overnight with different concentrations of ligands:

	FN	VCAM-1	ICAM-1
Jurkat E6.1	10µg/mL	2 µg/mL	2 µg/mL
Eol-1	25µg/mL	5 µg/mL	/
HMC 1.1	/	2 µg/mL	/
TF-1	10µg/mL	1 µg/mL	/
RPMI 8866	10µg/mL	2 µg/mL	/
K562	10µg/mL	/	/
SK-MEL-24	10µg/mL	/	/
D283	10µg/mL	/	/

The employ of different ligand agonist concentrations was justified by different levels of $\alpha_4\beta_1$ integrin expression on cell surfaces. Cell adhesion assays with Jurkat E6.1, Eol-1, HMC1.1, TF-1 and RPMI8866 were done as described below. Non-specific hydrophobic binding sites were blocked by incubation with a BSA 1%/HBSS (w/v) solution for 30 minutes at 37°C. The day of the assay, the cells were counted and stained with 12.5 µM CMFDA (30 minutes at 37°C). After three rinses with BSA/HBSS to wash away the excess dye, different aliquots of cells were divided among a number of tubes corresponding to the number of treatments. For inhibition experiments, cells were mixed with the drug and pre-incubated at 37°C for 30 minutes to reach equilibrium before being plated. After 30 minutes incubation at 37°C in the coated wells, the non-specifically bound cells were washed away with BSA/HBSS solution. Adherent cells were lysed by the addition of 0.5% Triton X-100 in PBS (30 minutes at 4°C). Released CMFDA was quantified by fluorescence imaging at Ex485 nm/Em535 nm (Wallac ARVO 1420 multilabel counter) and adherent cells was counted by interpolation on a standard curve. The number of adherent cells was calculated by comparison with a standard curve prepared in the same plate using known concentrations of labeled cells.

K562, SK-MEL-24 and D283 cell adhesion assays were carried out in a slight different way. Cells were counted with a hemocytometer and pre-incubated with various concentrations of each compound for 30 minutes at room temperature to reach a ligand–receptor equilibrium. At the end of the incubation time, the cells were plated (50000 cells per well) and incubated at room temperature for 1 hour. All the wells were then washed with PBS to remove non adherent cells, and 50 μL hexosaminidase [4-nitrophenyl-N-acetyl-β-d-glucosaminide dissolved at a concentration of 7.5 mM in 0.09 M citrate buffer (pH 5) and mixed with an equal volume of 0.5% Triton X-100 in H_2O] was added. This product is a chromogenic substrate for β-N-acetylglucosaminidase, whereby it is transformed into 4-nitrophenol; absorbance was measured at 405 nm. The reaction was blocked by the addition of 100 μL of a stopping solution [50 mM glycine and 5 mM EDTA (pH 10.4)], and the plates were read in a Victor^2 Multilabel Counter (PerkinElmer, Waltham, MA, USA).

The efficacy of putative antagonists (at least eight different concentrations were used) was determined by the reduction in adherent cells compared to the untreated control. Each experiment was conducted in quadruplicate and the data are presented as the mean ± S.E.M. of at least three independent assays. Data analysis and IC_{50} values were calculated using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

5.1.4 Scintillation proximity assay (SPA)

The radioligand binds to the $\alpha_4\beta_1$ integrin and the close proximity of the isotope to the scintillant incorporated in the beads allows the radiation energy to transfer to the scintillant where it can be detected as counts per min (cpm). FN was labeled with Na[^{125}I] using the IodogenKit, as specified by the manufacturer (Pierce). ^{125}I-FN was purified from unincorporated iodine by gel filtration chromatography on PD-10 columns; trichloroacetic acid-precipitable radioactivity was 6.30 x 10^{10} μCi/mol. The experiments were carried out in scintillation vials; in each vial 1 mg/50 μl anti-rabbit-coated beads, 400 ng of rabbit anti-α_4 integrin antibody and a portion of cell eluate (containing approximately 100 μg of $\alpha_4\beta_1$ integrin) were added. The $\alpha_4\beta_1$ integrin was extracted from Jurkat cells (cultured in RPMI 1640 medium containing L-glutamine, 10% FBS, antibiotic-antimicotycotic solution and kept in a humidified incubator at 37°C in a 5% CO_2 atmosphere). The cells were collected and then cytoplasmic proteins were extracted with the T-PER extraction buffer and $\alpha_4\beta_1$ integrin was purified by affinity chromatography. For Western blot experiments on extracts we used antibodies against α_4 and β_1 integrin subunits to confirm that both integrins partitioned to the cell lysate collected from the affinity column. The binding buffer contained Tris-HCl 25 mM pH 7,5;

$CaCl_2$ 1 mM; $MgCl_2$ 1 mM; $MnCl_2$ 1mM; BSA 2% (w/v); phenylmethanesulfonyl fluoride (PMSF) 1 mM; aprotinin 1 mg/ml; leupeptin 50 mM.

First, we allowed for the slow interaction between the α_4 integrin protein and the compound under examination by incubating together for 1,5 hours at room temperature. Then we added the rabbit anti-human α_4 integrin antibody, followed by an incubation time of 2 hours at 4°C. From this point on, all incubations were conducted at room temperature.

Then the anti-rabbit antibody binding beads were added, and the solution containing the four components was incubated for 2 hours at room in the dark. ^{125}I-FN was added to the vials, which were then incubated overnight on a shaker in the dark. The samples were read using a LS 6500 multipurpose scintillation counter (Beckham Coulter, Fullerton, CA, USA). The SPA procedure was optimized in preliminary experiments.

5.1.5 Western Blotting

Total protein fractions were obtained using the commercial T-PER Tissue Protein Extraction Reagent (Pierce): lysis and resuspension buffer contained a mixture of protease inhibitors composed of 0,5 mg/mL Benzamidine, 2 µg/mL Aprotinin, 2 µg/mL Leupeptin, 2 mM phenylmethlysulfonyl fluoride, and a cocktail of phosphatase inhibitors 100x (Sigma).

The first step is the addition of appropriate amount of T-PER Reagent to each sample; cells are strikingly resuspended to permit lysis. After 10 minutes of gently agitation at 4 °C, samples were centrifuged at 10,000 g for 10 minutes to collect supernatants. Protein samples were then kept at –80°C, and protein concentration was determined using the Lowry method [Lowry *et al*., 1951].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed according to standard procedures: proteins from the total extract (100 µg) were separated by SDS-PAGE at different polyacrylamide concentration: 12% for detection of phosphoERK 1/2 and 7,5% for detection of phosphoFAK and integrin subunit α_4.

Briefly, all gels were transferred onto a Hybond-ECL nitrocellulose membrane (GE Healthcare) after three hours of run at constant mA. As a molecular weight standard, it was used the Magic Marker XP (Invitrogen).

All blots were blocked with 5% non-fat milk in TBS 1X (10mM Tris-HCl, pH8, containing 150mM NaCl) plus 0,1% Tween-20 for 1 hour at room temperature, except for total ERK (as loading control) that required a 1 hour incubation with TBS 1X plus 1% BSA at 37 °C in gentle agitation.

To detect phosphoERK 1/2, phosphoFAK and total FAK membranes were incubated overnight at 4°C with specific rabbit polyclonal IgGs (Cell Signaling), used at a dilution of 1:1000 in TBS 1X, 0,1% Tween-20 and BSA 5%.

For detection of total ERK (as loading control), primary antibody (Promega) was used at a recommended diluition of 1:5000 in TBS 1X, 0,1% Tween-20 and BSA 0,1%, for two hours at room temperature.

For detection of integrin subunit α_4 and $\beta_{1,}$ it was used, respectively, a 1:200 and 1:500 primary antibody dilution in blocking buffer for 1,5 hours at room temperature.

All the membranes were incubated with peroxidase-conjugated secondary antibodies at a dilution of 1:8000 (goat anti-Rabbit HRP conjugated IgG; Santa Cruz), for 1,5 hours at room temperature, except for integrin subunit α_4 and phosphoFAK that required the same antibody, with a diluition of 1:4000. For integrin subunit $\beta_{1,}$ instead, a goat anti-mouse HRP conjugated IgG (Santa Cruz) was used at 1:4000 diluition for 1,5 hours at room temperature.

Detection of immunoreactive bands was done by the enhanced chemiluminescent method (Immobilon Western Chemiluminescent HRP Substrate – Millipore) and a FUJILAS3000 acquisition camera.

5.1.6 β-hexosaminidase release assays

Degranulation from HMC 1.1 cells was monitored by measuring the activity of a granule enzyme β-hexosaminidase released in the cell supernatants after appropriate treatments.

In the first set of experiments, Jurkat T cells ($1x10^5$ cells for each well) were pre-incubated for 30 minutes in the presence of phorbol myristate acetate (PMA; 50 ng/mL) at 37 °C in normal culture medium, washed three times with RPMI 1640, and co-coltured with an equal number of mast cell previously treated with different concentration of DS70 at 37 °C for 60 minutes in a volume of 100 µL. Medium used for co-colture is composed by IMDM (Iscove's Modified Dulbecco's Medium), L-glut (2mM) and BSA 0,1%. After 22 hours, 96-well plate is centrifuged for 2 minutes at 3000 rpm; then, aliquots of 50 µL of the supernatants of each well were transferred to a separate 96-well plates. Fifty microliters of substrate solution ([4-nitrophenyl-N-acetyl-β-d-glucosaminide dissolved at a concentration of 7.5 mM in 0.09 M citrate buffer (pH 5) and mixed with an equal volume of 0.5% Triton X-100 in H_2O]) were added to each sample and the plates were incubated for 90 minutes at 37 °C. The reaction was stopped by addition of 100 µL of stopping solution [50 mM glycine and 5 mM EDTA (pH 10.4)], following by reading the absorbance at 405 nm. Each sample

was evaluated in triplicate. Degranulation is expressed as percentage of the cells' total mediator content obtained by lysis of cells with Triton X-100 0,2%.

In another set of experiments, $1x10^5$ HMC 1.1 cells per each well were pretreated at 37 °C for 60 minutes with different concentrations of compound DS70 in a final volume of 100 µl . All the assay was performed in sterile Tyrode's buffer (NaCl 137 mM; KCl 2,7 mM; $CaCl_2$ 1,8 mM; $MgCl_2$ 1mM; glucosio 5,6 mM; Hepes 20 mM; BSA 0.1%, Ph 7.4).

Positive controls include treatments with PMA 100 nM e calcimycin 1 µM at 37 °C for 30 minutes. Calcimycin, also known as ionophore A23187, is a polyether antibiotic from Streptomyces chartreusensis. It binds and transports calcium and other divalent cations across membranes. The substance is used mostly as a biochemical tool to study the role of divalent cations in various biological systems.

Controls included cells mixed with 0.2% Triton X-100 to measure total β-hexosaminidase release, and samples mixed with Tyrode's buffer (vehicle control) as a measure of background β-hexosaminidase release. Each sample was run in triplicate. After incubation with controls or test samples, each well is assayed for the presence of β-hexosaminidase as previously described.

Percent (%) degranulation was calculated using the following equation: (experimental β-hex release – vehicle control β-hex release) / (Triton X-100 β-hex release–vehicle control β-hex release) x 100.

5.2 IN VIVO EXPERIMENTS

5.2.1 Animals

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

5.2.2 Animal experiments

The method used has been described by Khosravi and colleagues in 1995 and by Ebihara and colleagues in 1999.

40 male Dunkin-Hartley guinea pigs were divided into 4 groups of 6 animals each. 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 intraperitoneally (i.p.) to each of the animals, except for the negative control. Treatment was repeated two weeks and three weeks later.
Four weeks after the first OVA immunization, all animals were challenged with 30 μL per eye of saline solution, containing 100 mg/mL ovalbumin. DS70 powder was dissolved, at the moment, in a vehicle containing 10% PEG 3350/1%PS80, Phosphate Buffer pH 7.0.
One month after the first immunization, compound DS70 (0.1% weight/volume) eye drops were instilled into the conjunctival sac (30 μl/eye) of the treated guinea pigs: 30 and 10 minutes before the animals were challenged with 30 ml/eye of saline solution, containing 100 mg/ml OVA, instilled into the conjunctival sac. Negative controls received saline alone.

Conjunctival clinical symptoms were rated blind on both eyes using the following scale: 0, no symptoms; 1, slight conjunctival redness with or without tears; 2, mild conjunctival redness with tears and mild chemosis; 3, mild conjunctival redness with tears and moderate chemosis; 4, severe conjunctival redness with tears and partial lid eversion; 5, severe conjunctival redness with tears and lids more than half closed. Pictures of both eyes were taken to evaluate the clinical score 1, 2, 4, 6 and 24 hours after topical ovalbumin administration. The animals were euthanized 24 hours after OVA challenge by i.p. injection of Tanax® (3 ml/kg; Hoechst AG, Frankfurt-am-Main, Germany). Tarsal conjunctiva of both eyes, was collected to carry out the experiments thereafter described.

5.2.3 Collection of ophthalmic lavage fluid (OLF)

The collection of OLF was carried out as follows: a 30-µl aliquot of assay buffer consisting of 0.3 M sucrose in 50 mM sodium acetate buffer pH 5.4 with 10 units/ml heparin was applied to the eye using a micropipette, without touching the eye. After two or three forced blinks, the 30 µl of lavage fluid was collected. The lavage was repeated 3 times in each eye (90 µl /animal), and was conducted at 1, 2, 4, 6 and 24 hours after topical ovalbumin administration.

5.2.4 Measurement of eosinophil peroxidase (EPO) activity in tears

EPO activity in the OLF was measured according to the method of Tagari and colleagues. Briefly, following the centrifugation (4°C, 300 g, 15 minutes) of the fluid, thirteen µl of sample was added to 80-µl aliquots of sucrose assay buffer (see collection of OLF) with or without 3 mM (final) KBr, in a 96 well plate. 3,3',5,5'- Tetramethylbenzidine (TMB) liquid substrate system liquid (Sigma) was added in a volume of 40 µl and mixed. All solutions were previously warmed to 21°C.

After 10 minutes of incubation, the peroxidase reaction was stopped by the addition of 60 µl of 2 N H_2SO_4. Absorbance at 450 nm was measured.

5.2.5 Eosinophil peroxidase assay

Eosinophil peroxidase assay was performed as previously described by Izushi and colleagues in 2002: 24 hours after conjunctivitis induction, by application of the antigen, the guinea pigs were sacrificed and the upper and lower parts of the tarsal 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 homogenizer (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.

5.2.6 Quantitative PCR analysis of cytokines and chemokines in tarsal conjunctiva

Tarsal Conjunctiva specimens were stored in RNAlater at -20°C. Total RNA was extracted from homogenized conjunctivae with TRI Reagent and measured using a spectrophotometer. For each conjunctival sample, 1 μg of total RNA was treated with RNase-free DNase for 15 minutes at 25°C, followed by incubation at 65°C for 10 minutes to inactivate DNase. The RNA samples were then converted into cDNA using oligo(dT), random primers, and the High-Capacity cDNA Reverse Transcription Kits according to the manufacturer's instructions. Real time PCR was performed using GoTaq® qPCR Master Mix. The protocol consisted of denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C denaturation (20 s) and 58°C annealing (1 min). No-template controls and DNA melting curve analysis were used as controls to ensure the lack of contaminating DNA in the RNA preparations and to rule out primer-dimer formation, respectively. Induction of mRNA was determined from the threshold cycle (C_t) values normalized for GAPDH expression and then normalized to the value derived from conjunctivae of not immunized guinea pigs. Real time PCR primers for guinea pig genes evaluated in this project are listed in Table 1. Primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), interleukin-1β and eotaxin (CCL11) were published by Kubo and colleagues in 2005; primer sequences for RANTES (CCL5) were published by Lacy and colleagues in 2011. All primers were synthesized by Sigma-Aldrich.

Table 1. Primers for real-time PCR

mRNA targets	Sense primer	Antisense primer
GAPDH	CCGGCCAAATACGATGACAT	TGTAGCCCAAGATGCCTTTGAG
IL-1β	CATGAGCTTCGTACAAGGAGAAAG	CAGGTACAGATTCTTCCCCTTGA
Eotaxin (CCL11)	TCTGCACACTGCACCATGAA	AAGCAGAGACTGTGAGCAGCA
RANTES (CCL5)	CTGGCCCACTGCTTAGCAAT	CCTTGCTTCTTTGCCTTGAAA
IL-8	CCTTGGATTCCCCTTTATTCCT	CGTATGTCCCCATGACATTGTG

5.2.7 Immunostaining for α_4 subunit

Tissues were fixed in formaldehyde and embedded in paraffin before cutting, and staining was performed by using a peroxidase-based method. Briefly, sections of 6 μm in thickness were

deparaffinated in xylene, dehydrated in ethanol, and washed in water. Heat mediated antigen retrieval was performed with citrate buffer (pH 6.0) for 20 minutes and the sections were washed 3 times in TBS-T (TBS + 0.025% Tween-20). Immunohistochemical staining was made using Rabbit specific HRP/DAB detection kit (Abcam) following manufacturer's instructions. Briefly, specimens were incubated with Protein Block solution for 5 minutes at room temperature to block nonspecific background staining. After washing once in TBS-T, tissue sections were incubated with antibodies that recognized human integrin α_4 (diluted 1:500) overnight at 4°C. The specimens were washed 4 times in TBS-T and incubated with Biotinylated goat anti-mouse IgG for 30 minutes at room temperature.

Slides were then developed with streptavidin-biotin complex/horseradish peroxidise (HRP) and diaminobenzidine substrate (DAB), according to the manufacturer's instructions. Haematoxylin was used as background staining. Sections were examined with standard light microscope (Nikon Eclipse E800) in a blinded manner and photomicrographs were taken with a 20x objective.

5.2.8 Giemsa staining for mast cells degranulation

Gustav Giemsa was born in Germany in 1867, worked mainly as a chemist, and died in 1948. The staining method, which carries his name, was designed primarily for the demonstration of parasites in malaria, but it was also employed in histology because of the high-quality staining of the chromatin and the nuclear membrane, the metachromasia of some cellular components, and the different qualities of cytoplasmic staining depending on the cell type. The use of methylene azure and its mixture with methylene blue to form an eosinate made stable the stain and its results.

Giemsa Stain is a buffered thiazine-eosinate solution designed to provide coloration of blood cells similar to the original product described by Giemsa. Nuclei will be varying shades of purple. Cytoplasmatic staining will be varying shades of blue to light pink. Fine reddish to lilac granules may be present in cytoplasm of some cell types. Basophils will demonstrate dark blue black granules in the cytoplasm. Eosinophils will demonstrate bright orange granules in the cytoplasm. Red blood cells should be pink to orange.
Sections are dewaxed, rinsed in alcohol and then in water. Staining with Giemsa (diluited 1:10 in bidistilled water) last two hours at room temperature. After one wash in water, blots were dried out, rinsed briefly in alcohol, and mounted to be examined with standard light microscope.

6.1 IN VITRO RESULTS

6.1.1 Binding affinity of library compounds to human $\alpha_4\beta_1$ integrin by SPA and $\alpha_4\beta_1$/VCAM-1-mediated cell adhesion in vitro

Jurkat cells, expressing human $\alpha_4\beta_1$ integrin, were used to measure peptidomimetic binding to $\alpha_4\beta_1$ integrin by SPA. To purify receptor proteins, an affinity chromatography column was used. The presence of glycosidic groups in subunit α_4 allows a specific interaction with WGA (wheat-germ agglutinin) columns. Once the interaction is established, proteins are eluted with a concentrated solution of N-acetylglucosamine (200 mM), which allows the removal of proteins specifically bound to the resin. Western blot analysis, performed with specific antibodies for α_4 and β_1 integrin subunits confirmed that both subunits are present in all fractions of eluate employed for the SPA (Figure 7.1, panel A). Specific binding of ^{125}I-FN to an antibody-captured $\alpha_4\beta_1$ integrin was time-dependent; the signal increased during the first 10 hours, then reached a plateau and remained constant for the remainder of the 24 hours incubation (data not shown). The relatively slow kinetics of the SPA may require the establishment of an equilibrium between the different components. ^{125}I-FN specific binding, measured after overnight incubation, was concentration-dependent (Figure 7.1, panel B) and was blocked in the presence of 100 μM BIO-1211 (a potent $\alpha_4\beta_1$ and $\alpha_L\beta_2$ integrin antagonist) to capture the integrin complex (data not shown). The straight sequence MPUMP-β^2-Pro-Asp-OBn **BL31** was not able to inhibit ^{125}I-FN binding. On the contrary, the retro compounds **BL16**–**DS45** caused a concentration-dependent inhibition of ^{125}I-FN binding (Table 1). The sequence BnCO-Asp-β^2-Pro-AMPUMP **BL16** showed IC_{50} and Ki values (M) of 1.0×10^{-7} and 6.0×10^{-8}, respectively. The analog of **BL16, named DS64,** containing *iso*Asp, has an IC_{50} of 2.9×10^{-7} M and Ki of 1.8×10^{-7} M. **DS45** equipped with the 2,6-dicholorobenzoyl group revealed a modest affinity, in the micromolar range, while **BL25**, analog of **DS64** with the 2,6-dicholorobenzoyl instead of the phenylacetyl (BnCO) group, did not displace ^{125}I-FN significantly. The same behaviour is observed comparing **RDM355** and its analog **RDM416:** the first shows an interesting value of IC_{50} equal to 34×10^{-9} M, instead **RDM416** looses its activity. **DS23** share some characteristic structures with **DS70**, as the introduction of beta proline to confer rigidity to molecule, but the fundamental carbossilic moiety is beared by a malonate group. However, it shows a noticeable IC_{50}, equal to 48×10^{-9} M.

DS70 results the most potent one: it caused concentration-dependent inhibition of ^{125}I-FN binding to the SPA bead-associated $\alpha_4\beta_1$ integrin (Figure 6.1, panel C) with an IC_{50} of 22×10^{-9} M.

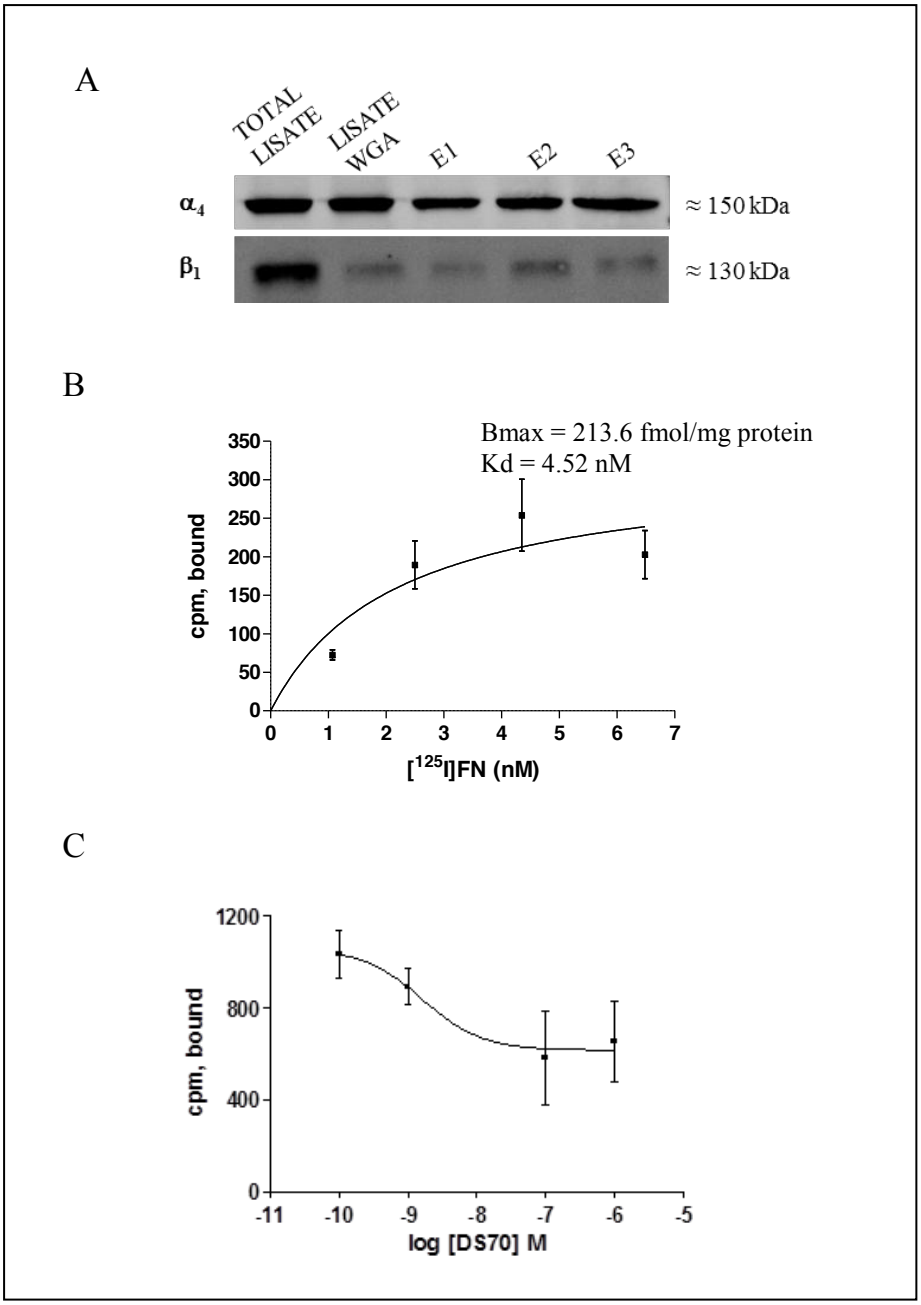


Figure 6.1 *A, Representative autoradiogram of a Western blot experiment, evaluating the α_4 and β_1 integrin subunits in cell lysates fractions (E1, E2, E3) of Jurkat cells, expressing $\alpha_4\beta_1$ integrin. The lysates were purified by affinity chromatography and three fractions of approximately 150 µl were collected; the presence of α_4 integrin was confirmed in all the fractions. These fractions were then assayed to confirm the presence of the β_1 integrin subunit. Approximate molecular masses of the α_4 and β_1 integrin subunits were determined by comparison with molecular mass standards. B, Saturation SPA binding of [^{125}I]-FN to the purified $\alpha_4\beta_1$ integrin extracted from Jurkat cells and incubated with increasing concentrations of the radioligand (cpm). Non specific binding was determined in the presence of unlabeled fibronectin. Specific binding obtained by subtraction of the non-specific binding from total binding counts. C, Inhibition of [^{125}I]-FN binding by DS70 in the SPA. Binding to Jurkat E6.1 cell lysates was measured in the presence of increasing concentrations of compound and of a fixed amount of [^{125}I]-FN (100.000 cpm) as described under materials and methods. Mean ± S.E.M. of three experiments done in triplicate.*

We assayed the ability of a small library of compounds capable to inhibit cellular adhesion of $\alpha_4\beta_1$ integrin-expressing Jurkat cells to VCAM-1. In a first set of experiments, we ascertained that adhesion of these cells to 96-well plates coated with human recombinant VCAM-1 was concentration-dependent and was not observed in BSA-coated wells.

As shown in Table 1, peptidomimetics **BL31**, **BL25 and DS45** did not affect cell adhesion to any significant extent. Interestingly, the retro compounds BnCO-Asp-β^2-Pro-AMPUMP **BL16** and BnCO-*iso*Asp-β^2-Pro-AMPUMP **DS64** inhibit cell adhesion with an IC_{50} of 0,16 and 0,20 $\times 10^{-6}$ M, respectively. On the contrary, **DS45** fails to behave as antagonist, despite the micromolar affinity revealed by the SPA (Table 1). DS70 also in this case shows the most potent $IC_{50,}$ equal to 2 x 10^{-9} M (Figure 6.2).

Finally, neither compounds (up to 2 mM) influences cell viability, evaluated by Trypan blue exclusion (data not shown).

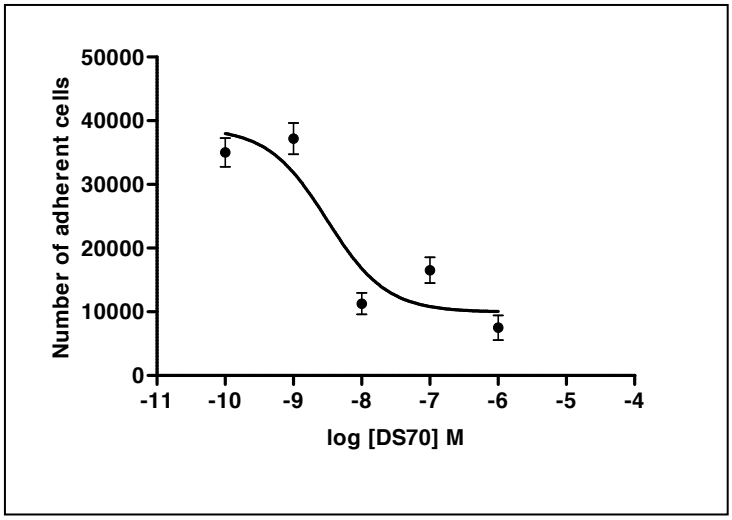
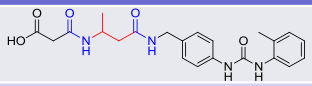
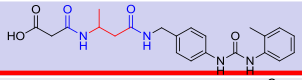
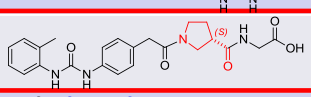
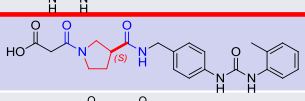
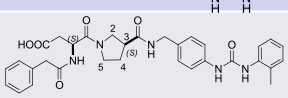
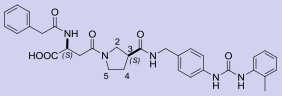
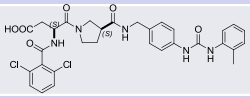
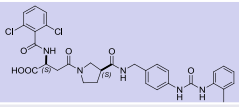
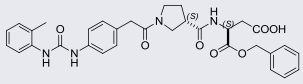


Figure 6.2 *Concentration–response curve showing the effects of DS70 on Jurkat E6.1 cell adhesion VCAM-1 mediated. Results are expressed as the number of cells attached ± S.E.M from quadruplicate wells and repeated at least three times.*

Table 1 *SPA binding to bead-associated $\alpha_4\beta_1$ integrin and IC_{50} inhibition values of Jurkat E6.1 cell adhesion VCAM-1 mediated of peptidomimetic library compounds. Mean ± S.E.M. of at least five experiments done in triplicate.*

Compound	Structure	SPA IC_{50} (μM)	SPA Ki (μM)	Adhesion assay - α4β1 integrin-mediated adhesion on VCAM-1 - IC_{50} (μM)
RDM355		0,034 ± 0,005	0,021 ± 0,004	0,011 ± 0,004
RDM416		n.a.	n.a.	1,5 ± 0,7
DS70		0,022 ± 0,003	0,014 ± 0,002	0,0020 ± 0,0005
DS23		0,048 ± 0,004	0,030 ± 0,003	0,040 ± 0,007
BL16		0,18 ± 0,06	0,11 ± 0,03	0,16 ± 0,01
DS64		0,63 ± 0,02	0,38 ± 0,04	0,20 ± 0,05
DS45		4,30 ± 0,48	2,72 ± 0,24	n.a.
BL25		n.a.	n.a.	n.a.
BL31		n.a.	n.a.	n.a.

6.1.2 DS70 inhibits $\alpha_4\beta_1$ integrin-mediated cell adhesion in different cell lines

To determine efficacy and potency of DS70, we have developed cell adhesion assays using different cell lines and different ligands.

Once confirmed blockade of Jurkat cells adhesion to VCAM-1, we proceeded to analyze its selectivity within the family α_4: we tested compound DS70 in a cell line expressing integrin $\alpha_4\beta_7$, RPMI8866 (Figure 6.4). Inhibition curve shows that this compound maintains a good capacity of inhibition of cellular adhesion to fibronectin (10 μg/ml). The IC_{50} value is equal to 340×10^{-9} M: it is lower than observed towards the integrin $\alpha_4\beta_1$.

Adhesion assays carried out on cell line Jurkat E6.1 using the ligand agonist ICAM-1 confirmed that inhibition of cell adhesion determined from DS70 is mediated by $\alpha_4\beta_1$ or $\alpha_4\beta_7$ integrin, not by $\alpha_L\beta_2$ integrin, both expressed on these cells.

DS70 was found to inhibit the cellular adhesion in all cell lines expressing $\alpha_4\beta_1$ integrin: in HMC 1.1 cell line IC_{50} value is equal to 218×10^{-9} M; in Eol-1 cell line, values are equal to 26×10^{-9} M, and 36×10^{-9} M for FN-mediated cell adhesion; in TF-1 cell line, IC_{50} values are equal to 72×10^{-9} M for VCAM-1 mediated cell adhesion, and 218×10^{-9} M for FN- mediated one.

We can, therefore, conclude that DS70 behaves as a $\alpha_4\beta_1$ or $\alpha_4\beta_7$ dual antagonist. To investigate any selectivity of molecule, adhesion assays on other cell lines expressing the major subtypes of integrins were carried out.

As regards to integrin $\alpha_9\beta_1$, DS70 acts as a partial agonist of the cellular adhesion, with a value of EC_{50} equal to 3.8×10^{-9} M. Towards integrin $\alpha_v\beta_3$ and $\alpha_5\beta_1$, on the other hand, this compound fails to show any activity (Figure 6.4).

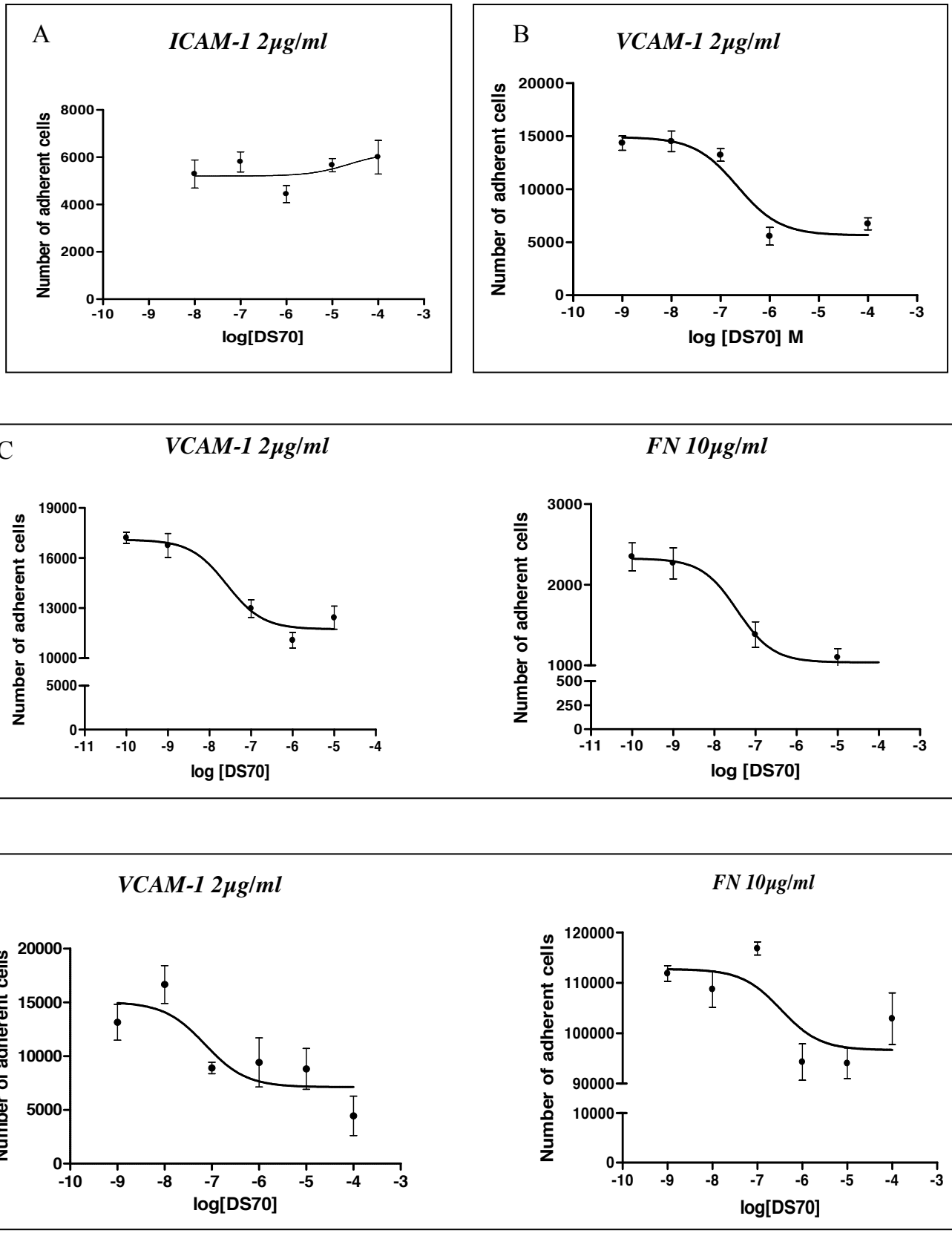
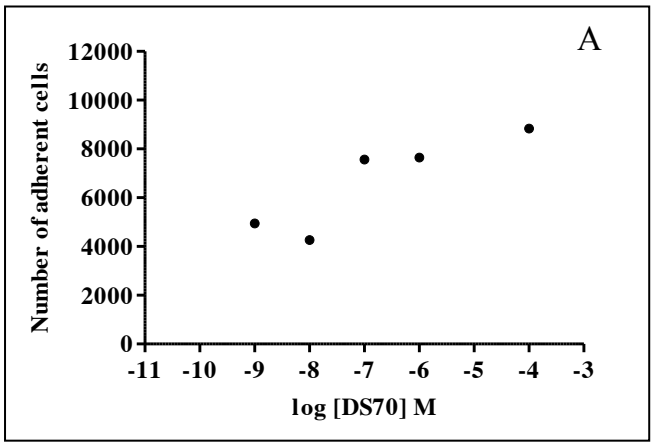
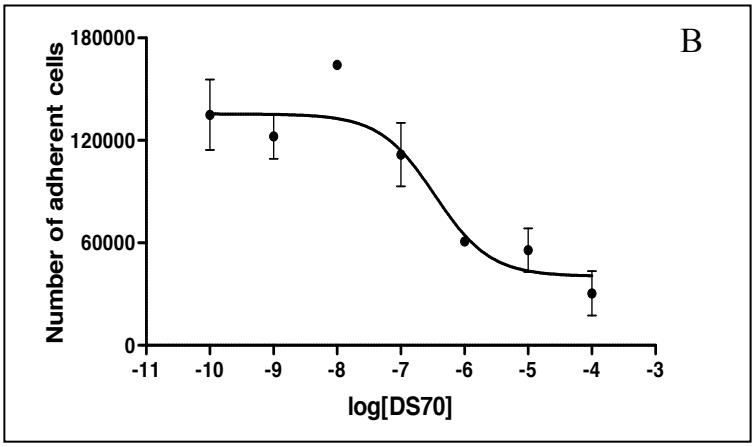


Figure 6.3 *Schematic representation of characteristic sigmoidal curves of cell adhesion inhibition in all cell lines expressing $\alpha_4\beta_1$ integrin induced by different agonists: panel A, Jurkat E6.1 cell line, panel B, HMC 1.1; panel C, Eol-1 cell line; panel D, TF-1 cell line. Mean ± S.E.M. of three experiments done in triplicate.*

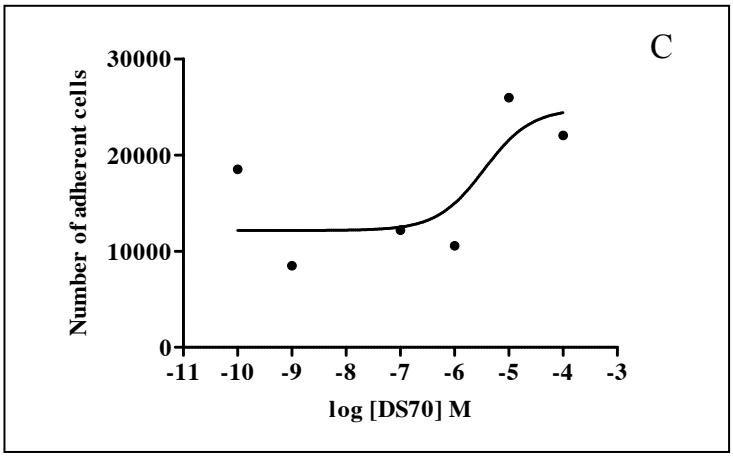
K562 cell line (chronic myelogenous leukemia)



RPMI 8866 (lymphoblastoid leukemia)



SK-MEL-24 cell line (human melanoma)



D283 cell line (medulloblastoma)

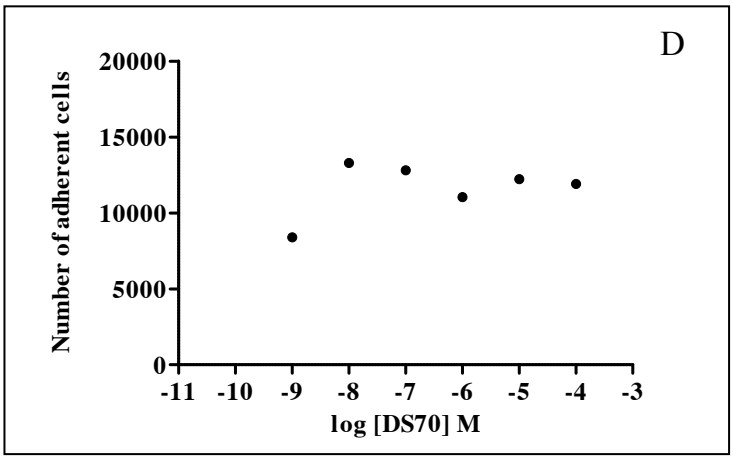


Figure 6.4 *Analysis of cellular adhesion mediated by fibronectin in the presence of increasing concentrations of DS70 compound carried out in three different cell lines: panel A, K562 cells, which express integrin $\alpha_5\beta_1$;panel B, RPMI8866, which express integrin $\alpha_4\beta_7$; panel C, SK-MEL-24 cells, that express integrin $\alpha_v\beta_3$; panel D, cells D283, expressing integrin $\alpha_9\beta_1$. Mean ± S.E.M. of three experiments done in triplicate.*

6.1.3 Effect of DS70 on fibronectin and VCAM-1-induced ERK 1/2 and FAK phosphorylation in different cell lines

Integrins activate intracellular signaling pathways through the recruitment of Src-family kinases (SFKs). Most integrins recruit focal adhesion kinase (FAK) through their β subunits. FAK functions as a phosphorylation-regulated scaffold to recruit Src to focal adhesions. FAK also activates extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) by recruiting the RAP1 guanine nucleotide-exchange factor (GEF). RAP1 then activates ERK/MAPK through B-Raf. Alternatively, FAK can activate ERK/MAPK by recruiting the growth-factor-receptor-bound-2 (GRB2) and son-of-sevenless (SOS) complex. We chose to evalutate DS70 ability to modulate integrin signaling, performing Western blot analysis in several cell lines expressing $\alpha_4\beta_1$ integrin.

All cell lines, 24 hours before treatment, were cultured in medium containing 1% fetal bovine serum (FBS), in order to reduce any interference of serum components.

The first step was a pretreatment of 30 minutes (Jurkat cells) or 60 minutes (all others cell lines) at 37 °C with DS70 at a different concentrations; subsequently, soluble VCAM-1 (2 μg/ml for Jurkat cells), FN at concentration of 25 μg/ml for Eol-1 cell line and 1 μg/ml for TF-1 cell line were added.

To assess the expression of phosphorylated FAK in the Jurkat cell line, we used a sensitive antibody to detect the phosphorylation of tyrosine 197, showing a band with apparent molecular weight of approximately 125 kDa. As can be seen in figure 6.5, VCAM-1 is able to significantly increase the levels of phosphorylation of FAK, confirming activation of integrin $\alpha_4\beta_1$ intracellular signaling. Preincubation of cells with DS70 10^{-6} M significantly reduces FAK phosphorylation.

Similar effects were observed as regards to levels of phosphorylation in Jurkat cells: VCAM-1 was able to stimulate phosphorylation of ERK1/2, while DS70 is capable of reversing this positive effect.

Interestingly, I observed an unusual behavior of DS70: at lower concentrations it significantly reduces phosphorylation levels of ERK1/2, wherease it was less effective at higher concentrations. This phenomenon deviates from normal trend of a classical drug, which may be represented by a sigmoid dose-dependent. In this case, the maximum magnitude of cellular response was observed with pre-incubation with DS70 at 10^{-7} M. A possible explanation of this phenomenon occurs in the observation that higher concentrations may cause a loss of efficacy due to the tight interaction of

DS70 with the integrin. So, DS70 seems to possess a different behaviour in this cell line: I suggest a bell-shape trend.

Treatments of Eol-1 cell line was performed as previously described: I investigated a wide range of DS70 concentrations. Fibronectin was chosen as ligand agonist because of its efficacy to activate integrin mediated signaling. In figure 6.6 is reported ERK 1/2 phosphorylation levels induced by fibronectin, and the efficacy of DS70 to act as an antagonist. 10^{-6} M resulted the most efficacious concentration, reconfirming a peculiar characteristics of DS70 already highlighted in Jurkat E6.1 cell line.

In TF-1 cell line, according to preliminary studies, I used a lower concentration of the agonist ligand fibronectin (1 µg/ml) as this cell line exhibits an higher adhesive ability. Consequent to this are reduction of agonist concentrations used in this study, and viceversa increase of peptidomimetic concentrations analyzed. In these experimental conditions, DS70 blocked fibronectin mediated cell adhesion at 10^{-7} M (Figure 6.8).

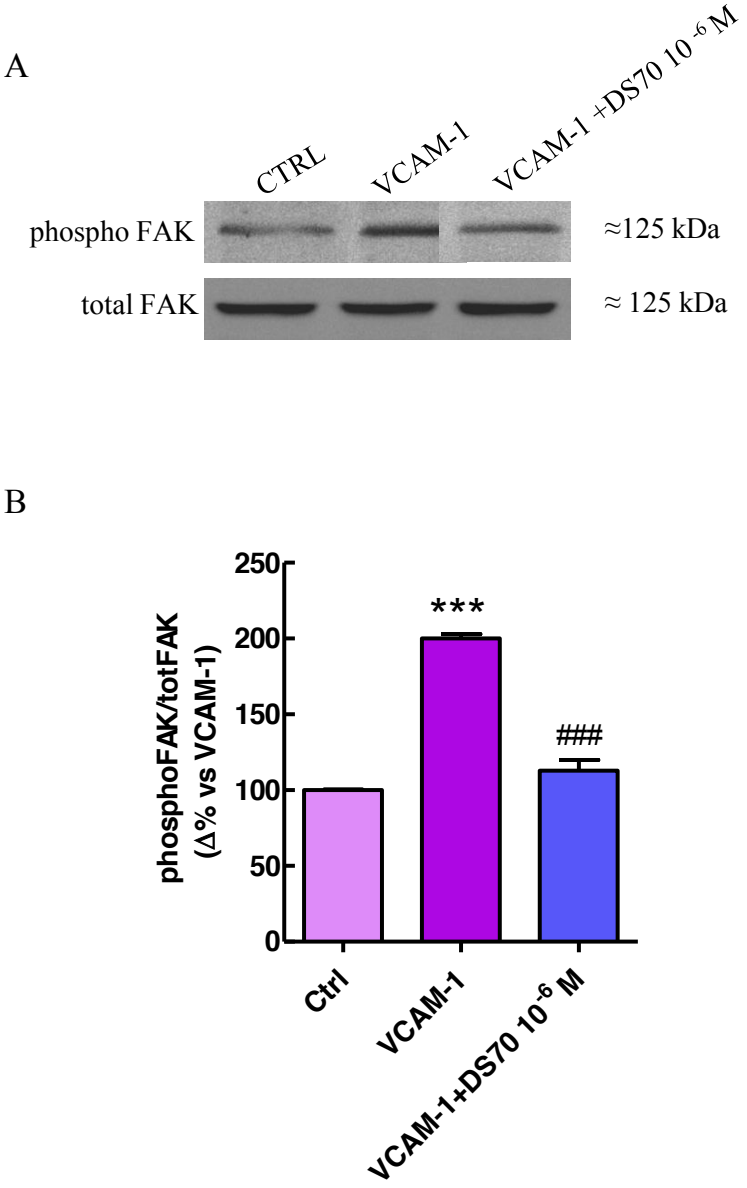


Figure 6.5 *DS70 antagonizes VCAM-1 induced phosphorylation of FAK. Phosphorylation levels of FAK protein in Jurkat cells E6.1 exposed to VCAM-1 2 µg/ml for 120 minutes in the presence or absence of the compound DS70 at a concentration of 10-6 M are shown. Panel A, autoradiogram of a representative experiment. Panel B, quantification of the optical density of the individual bands. # # # p<0.001 vs CTRL; *** p<0.001 vs VCAM-1 120' (Newman-Keuls test after ANOVA) [Mean ± S.E.M.; n=6].*

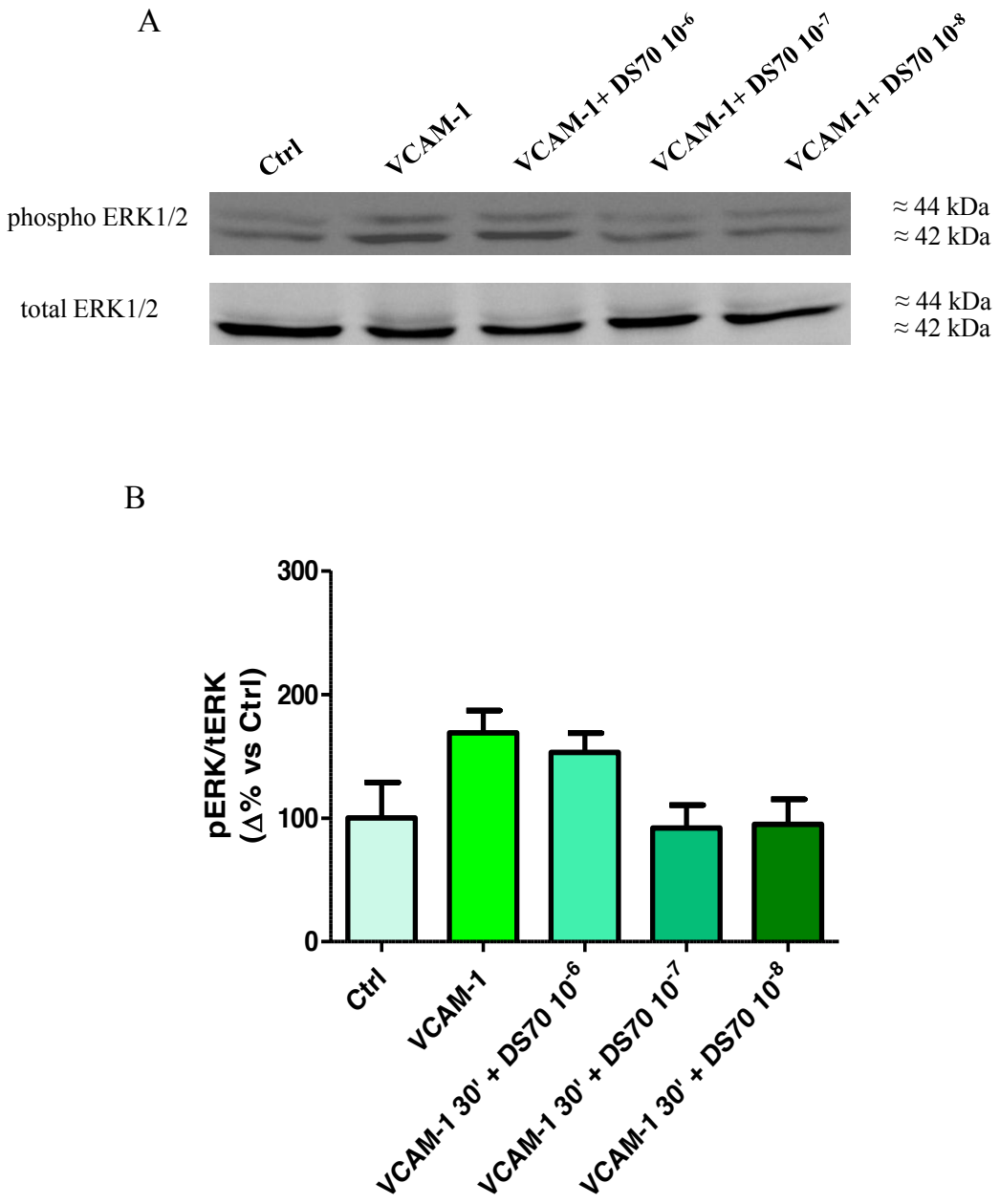


Figure 6.6 *Expression of phosphorylation levels of ERK 1/2 proteins in Jurkat cells E6.1 exposed to VCAM-1 2 µg/ml for 30 minutes in presence of compound DS70 at a concentration of 10^{-6}, 10^{-7} and 10^{-8} M. Panel A, autoradiogram of a representative experiment. Panel B, quantification of the optical density of the individual bands. No significance was observed with Newman-Keuls test after ANOVA. Mean ± S.E.M.; n=6.*

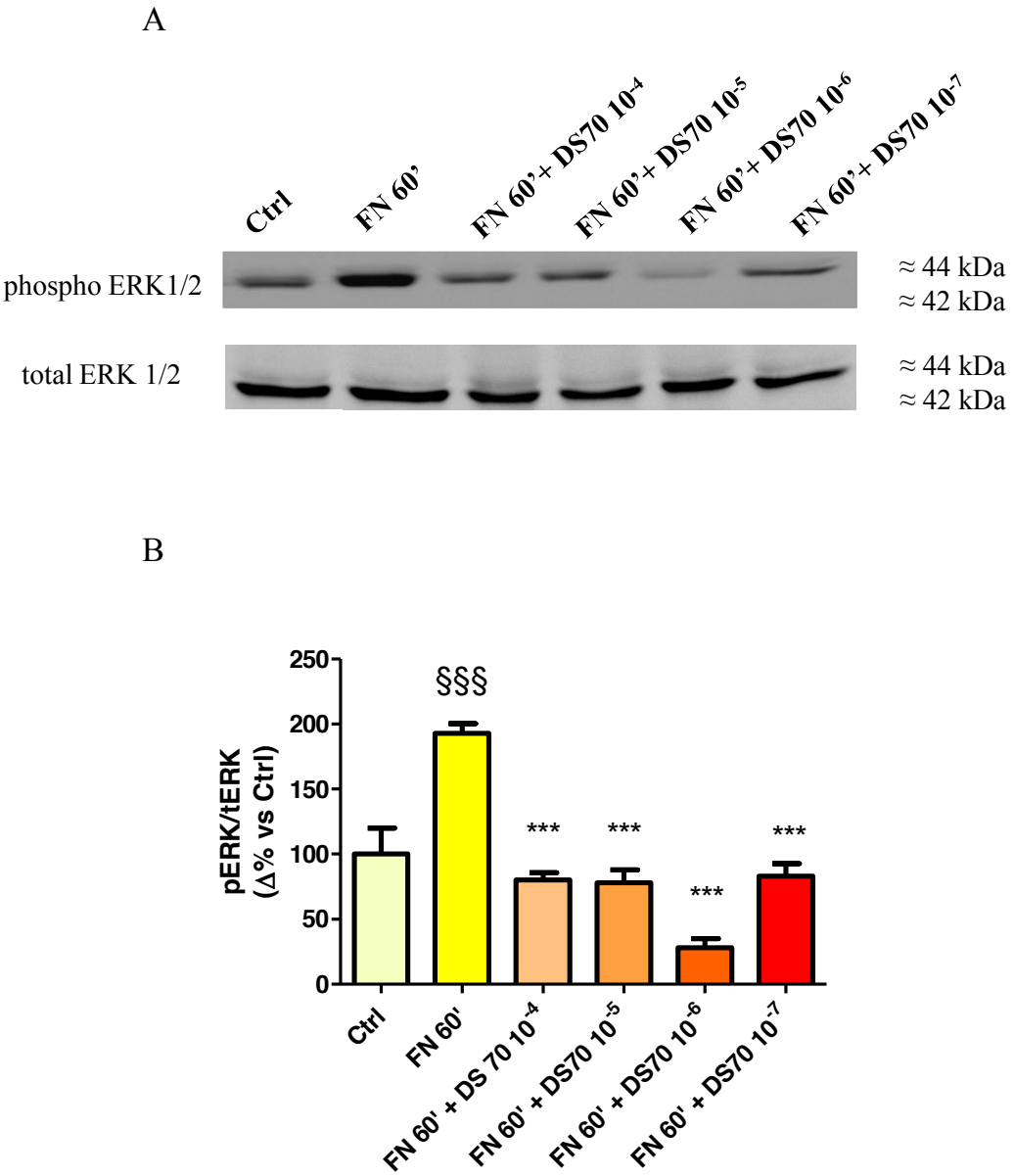


Figure 6.7 *Expression of phosphorylation levels of ERK 1/2 proteins in cells EOL-1 exposed to fibronectin (FN) 25 µg/ml, for 60 minutes, in the presence of the compound DS70 at a concentration of 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} M. Panel A, autoradiogram of a representative experiment. Panel B, quantification of the optical density of the individual bands. §§§ p<0.001 vs Ctrl; *** p<0.001 vs FN 60' Newman-Keuls post test after ANOVA. Mean ± S.E.M.; n=6.*

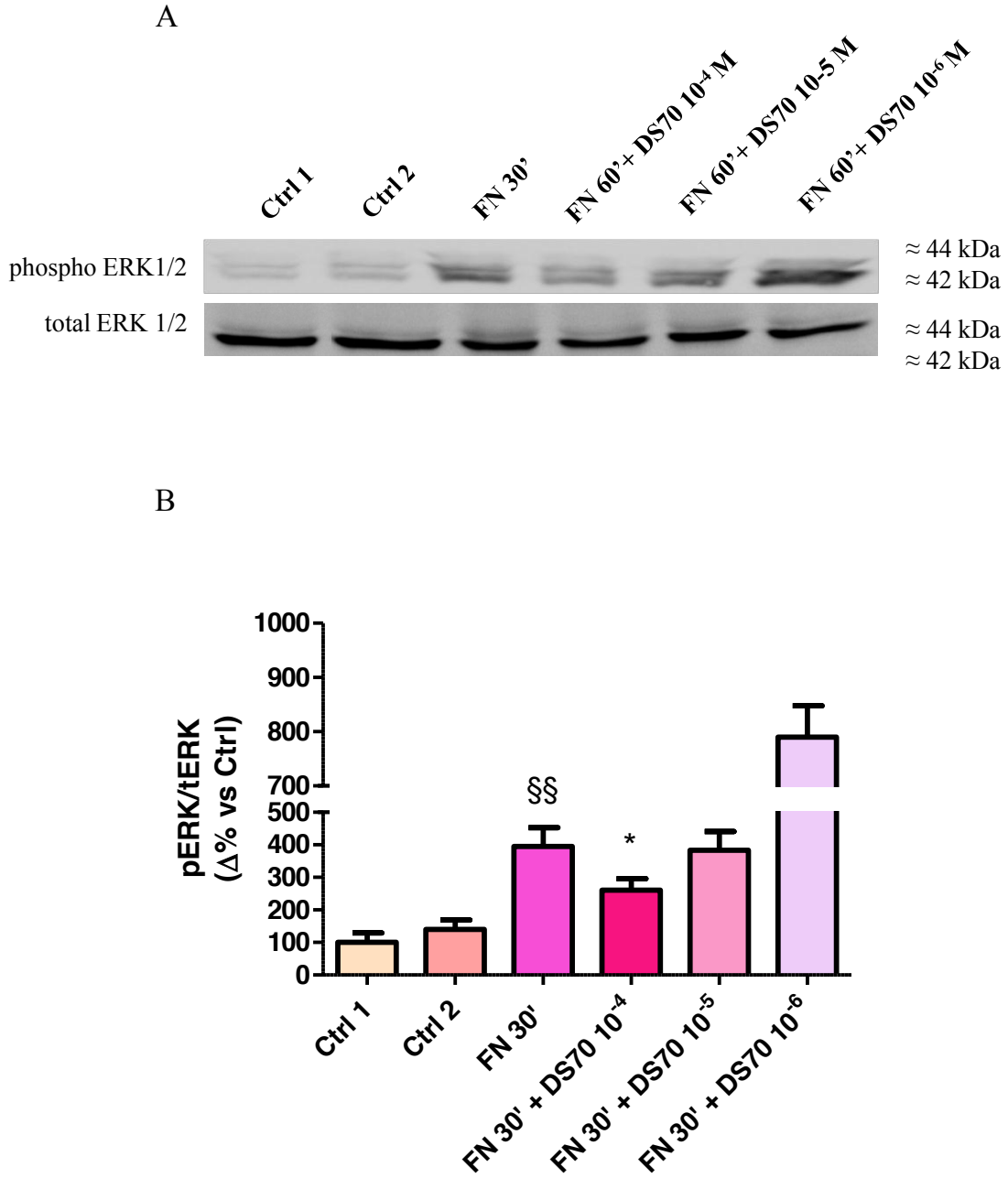


Figure 6.8 *Expression of phosphorylation levels of ERK 1/2 proteins in cells TF-1 exposed to fibronectin (FN) 1 µg/ml for 30 minutes, in the presence of the compound DS70 at a concentration of 10^{-4}, 10^{-5} and 10^{-6} M. Panel A, autoradiogram of a representative experiment. Panel B, quantification of the optical density of the individual bands. §§ P<0.01 vs Ctrl; * p<0.05 vs FN 30' Newman-Keuls post test after ANOVA. Mean ± S.E.M.; n=6.*

6.1.4 Analysis of proteins ERK 1/2 expression in cells HMC-1.1 exposed to fibronectin, PMA, calcimycin and DS70

The MAPK pathway plays a crucial role in inflammatory induced responses in mast cells: thus modulating this system we can act directly on mechanisms regulating inflammation. It is known that Nf-κB activation is modulated by MAPK, which induce phosphorylation and subsequent degradation of the inhibitory protein IκB, endogenous inhibitor that prevent NF-κB activation in cytoplasm, by keeping it in inactive form. Therefore, increase of intracellular calcium levels is an essential element for release of mediators from cytoplasmic granules, in addition to the activation of MAPK.

Treatment with PMA and calcimycin (A23187) causes activation of cells HMC-1.1 *in vitro*: I confirmed that this event activates MAPK phosphorylation. Addition of DS70 to cells prior to these activators counteracts changes in ERK1/2 phosphorylation. As shown in figure 6.9, western blot analysis confirms an increase of ERK1/2 phosphorylation as consequence of the treatment with PMA and calcimycin. Concomitant addition of fibronectin further increases phosphorylation levels, implying a some way additive mechanism of stimulation. Pre-treated cells with DS70 at the concentration of 10^{-5} M showed a reduction of ERK1/2 phosphorylation (Figure 6.9).

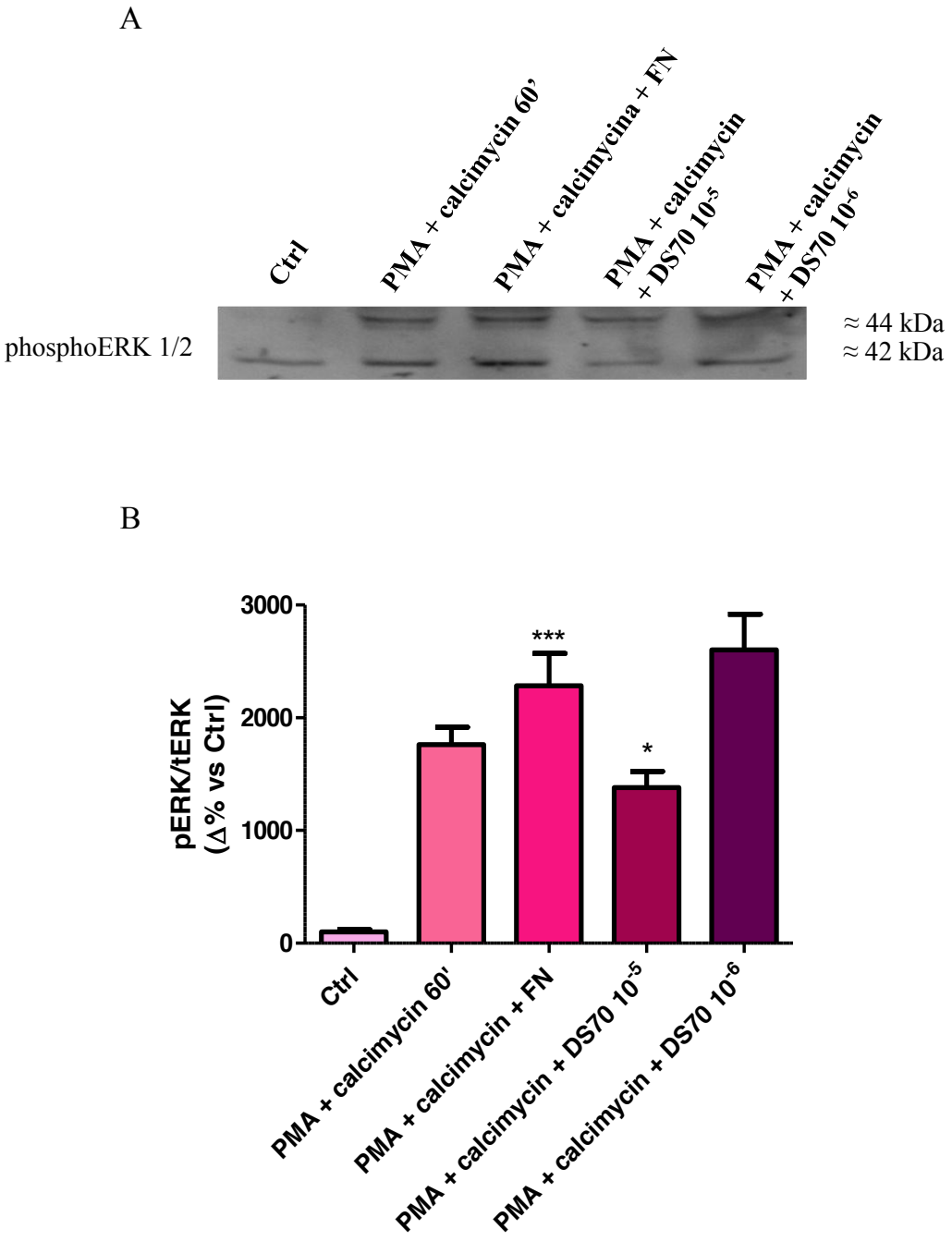
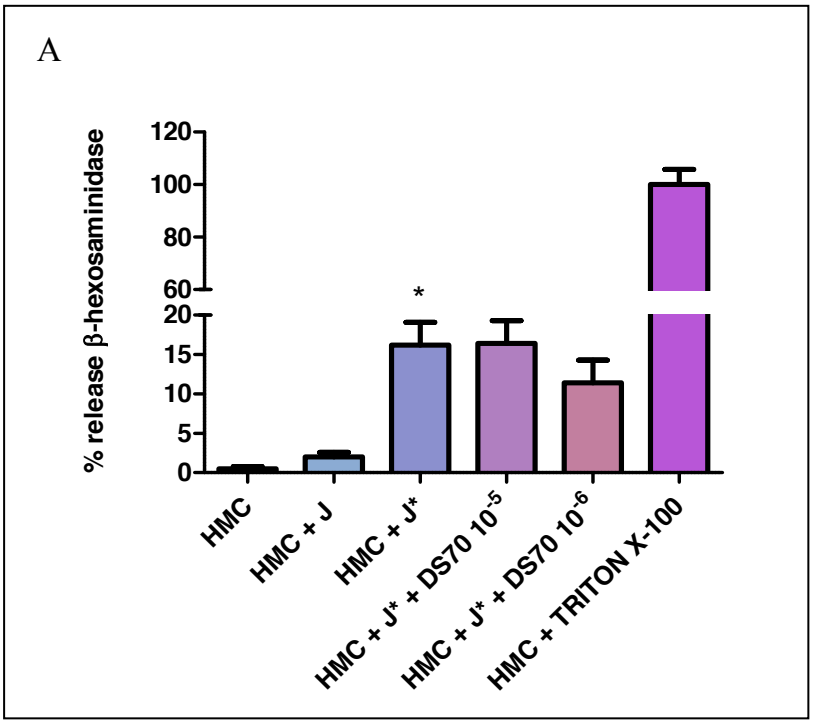


Figure 6.9 *ERK 1/2 phosphorylation in HMC-1.1 cells exposed to PMA 100 nm and calcimycin 1 µM in the presence or absence of FN or compound DS70 at a concentration of 10^{-5} M and 10^{-6} M. Panel A, autoradiogram of a representative experiment. Panel B, quantification of the optical density of the individual bands. *** P<0.001 vs CTRL (Newman-Keuls test after ANOVA). * P < 0.05 vs PMA, calcimycin and fibronectin. Mean ± S.E.M.; n=5.*

6.1.5 Analysis of T-cell or PMA/calcimycin induced mast cell degranulation levels after exposure to DS70

In agreement with other studies, I observed a significant increase in the percentage of β-hexosaminidase release following Jurkat T-cell contact, reaching a value of 16.2 %. DS70 (10^{-6}M) reduces this release to 11.4 %. This effect seemed to be lost at final concentration equal to 10^{-5} M (Figure 6.10, panel A).

With regards to PMA and calcimycin mediated degranulation, cells were pre-treated for 30 minutes at 37 °C with DS70 at concentrations 10^{-5}M, 10^{-6}M and 10^{-7}M, and then exposed to PMA and calcimycin for 60 minutes at 37 °C. Data reported in the graph below can be explained as it follows: percentage of degranulation relative to positive control (only PMA and calcimycin) is around 44 %. After pre-treatment with DS70, the entity of the degranulation is reduced following a trend concentration-dependent; in fact there is a value equal to 25.7 % for DS7 10^{-5} M, to 23.4 % for DS70 10^{-6} M and to 20.2 % for DS70 10^{-7} M (Figure 6.10, panel B).



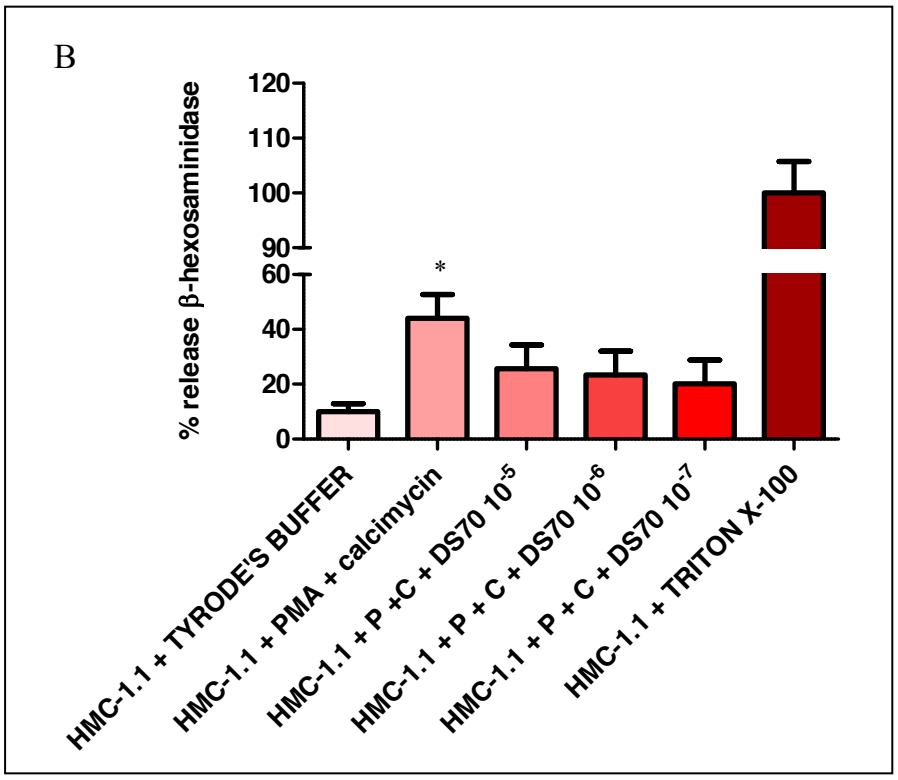


Figure 6.10 *Panel A, Effect of PMA activated T cells (J*) on β-hexosaminidase release by HMC 1.1 cells. Cells were pre-t reated with DS70 10^{-5} M and 10^{-6} M. Mediators in supernantants of the co-coltures were measured at 22 hours of incubation; B Effect of PMA, calcimycin and DS70 10^{-5} M, 10^{-6}M e 10^{-7}M on β-hexosaminidase release by HMC 1.1 cells. Degranulation is expressed as percentage of the cells total mediator content obtained by lysis of cell with Triton X-100. Mean ± S.E.M.; n=5.*

6.2 IN VIVO RESULTS

6.2.1 Effect of DS70 in allergic conjunctivitis induced by ovalbumin in guinea pigs

Guinea pigs were actively immunized by intraperitoneal injection of ovalbumin and 4 weeks later were challenged with ovalbumin instilled into the conjunctival sac. In guinea pigs treated with the vehicle+ovalbumin, one and two hours after challenge, during the early phase ocular reaction, swelling of the eyelids and chemosis were more marked than in controls treated with the vehicle alone and not exposed to ovalbumin. During the early phase reaction, clinical observations also revealed typical symptoms of allergic conjunctivitis: tearing and discharge, conjunctival redness, chemosis and lid eversion. These symptoms persisted at least 6 hours showing a progressively decreasing reaction; 24 hours after challenge, the conjunctiva did not present any relevant clinical symptom.

Clinical scores were significantly increased from 1 hours up to 6 hours after ovalbumin challenge, with a maximum at 2 hours (Figure 6.11). DS70 induced a significant reduction of the clinical score compared with ovalbumin (OVA): in particular, it must to be noted that 6 hours after challenge, during the late phase of allergic conjunctivitis, the maximum reduction in the severity of conjunctival symptoms was observed. As shown in figure 6.11, 24 hours after ovalbumin challenge, clinical scores of guinea pigs treated with vehicle+OVA or OVA+DS70 0.1% were returned to the pre-challenge values and no significant eye alteration was noted.

6.2.2 Effect of DS70 0.1% eye drops on eosinophils peroxidase activity in ophthalmic lavage fluid (OLF) and conjunctiva

I chose to evaluate eosinophil peroxidase activity in ophthalmic lavage fluid (OLF) and in conjunctiva sections, taken as an indicator of eosinophil infiltration.

The adopted experimental model allows the evaluation of clinical and cytologic aspects of allergic conjunctivitis induced in the guinea pig. According to previous studies, it has been reported that allergen instillation into the conjunctival sac of sensitized guinea pigs induces an immediate hypersensitivity reaction that is biphasic. An early phase reaction (EPR), characterized by periorbital swelling, conjunctival edema and conjunctival redness, occurs within minutes of allergen challenge and reaches a maximum at 0,5 hours. The subsequent inflammatory response, termed the late phase reaction (LPR), occurs 6-10 h after a single antigen challenge. The LPR is sustained

locally by a cellular inflammatory reaction and is characterized by accumulation in the conjunctival substantia propria of eosinophils, neutrophils and lymphocytes up to 24 hours after the antigen challenge and when the clinical signals of allergic reaction are no longer present. Eosinophil peroxidase activity was measured in tears collected after ovalbumin exposure and treatment with compound under examination (Figure 6.12). DS70 0.1% was all effective in reducing tear eosinophil accumulation, evaluated indirectly by eosinophil peroxidase activity, with the same noticeable activity at 2, 4, 6 and 24 hours. I was able to confirm that eosinophil peroxidase activity increased proportionally after antigen challenge in ovalbumin-treated guinea pigs using conjunctiva sections, whereas there was a significant reduction in DS70-treated animals. Again, DS70 0.1%, administered 30 minutes in advance to ovalbumin, was effective in reducing eosinophil infiltration in the guinea pig conjunctiva.

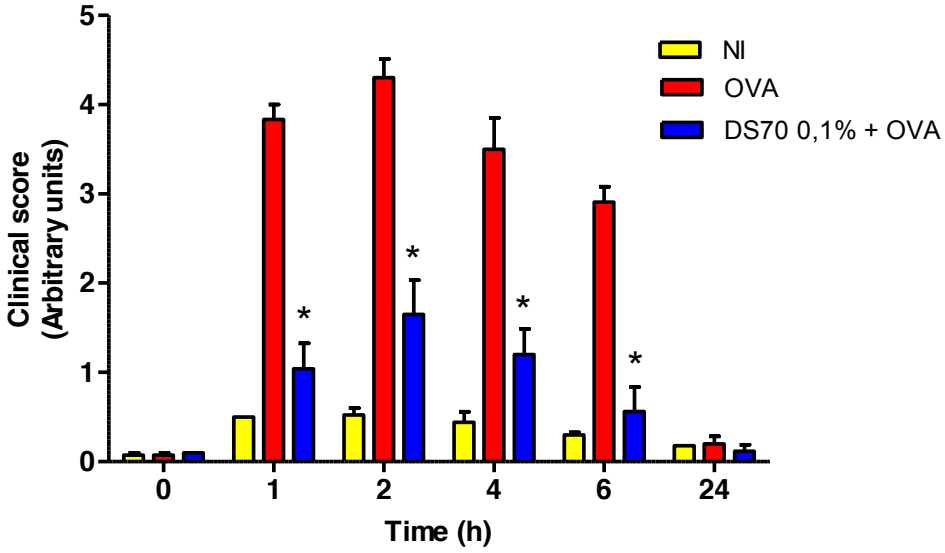


Figure 6.11 *Effects of DS70 on conjunctival symptoms induced by ovalbumin in guinea pigs. DS70 eye drops were administered to guinea pigs actively immunized by intraperitoneal injection of ovalbumin (OVA) and 4 weeks later challenged with OVA (30 µl of 2.5% solution) instilled into both eyes; 30 and 10 min before this challenge DS70 0,1% or the vehicle were instilled into both eyes (30 µl/eye). Controls received the vehicle alone and were not treated with OVA. Each group comprised five guinea pigs, and the score was based on changes before and 1, 2, 4, 6 and 24 h after challenge for the symptoms of itching, swelling, redness, and lid eversion as described under Methods. Data are presented as mean ± standard error of the mean, n=10 (both eyes were evaluated). *p<0.05 vs OVA. Abbreviations: No immunization represents controls; OVA represents ovalbumin.*

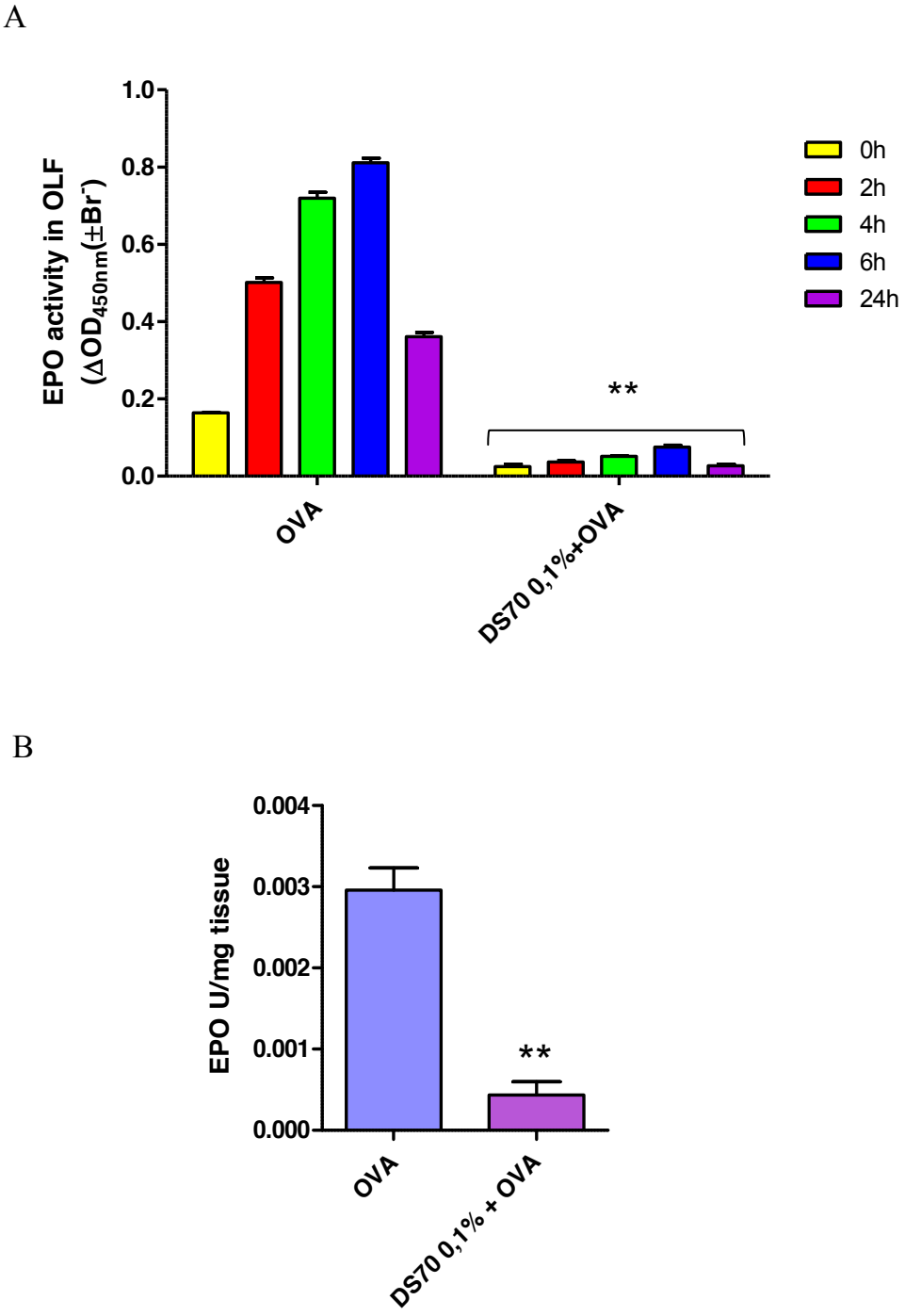


Figure 6.12 *Panel A, eosinophil peroxidase (EPO) activity in ophthalmic lavage fluid (OLF). Effect of DS70 eye drops suspension (0.1 %, 30 µL/eye) administered 30 minutes before antigen challenge on eosinophil peroxidase levels in ovalbumin-sensitised guinea pigs. Panel B, effect of DS70 on conjunctival eosinophil peroxidase levels 24 hours after topical challenge with OVA. Control guinea-pigs were not treated with ovalbumin. Values are the mean ± SEM of three values per each group. **p<0.001 vs ovalbumin.*

6.2.3 Effect of DS70 0.1% eye drops on conjunctival CCL5, CCL11, IL-1β, IL8 mRNA levels

Ovalbumin challenge induces, 24 hours later, a significant elevation of mRNA levels of CCL5, CCL11, IL-1β and IL8 in tarsal conjunctival specimens. DS70, administered 30 minutes in advance to ovalbumin, was effective in the same way in reducing mRNA levels of CCL5, CCL11 and IL-1β (Figure 6.13).

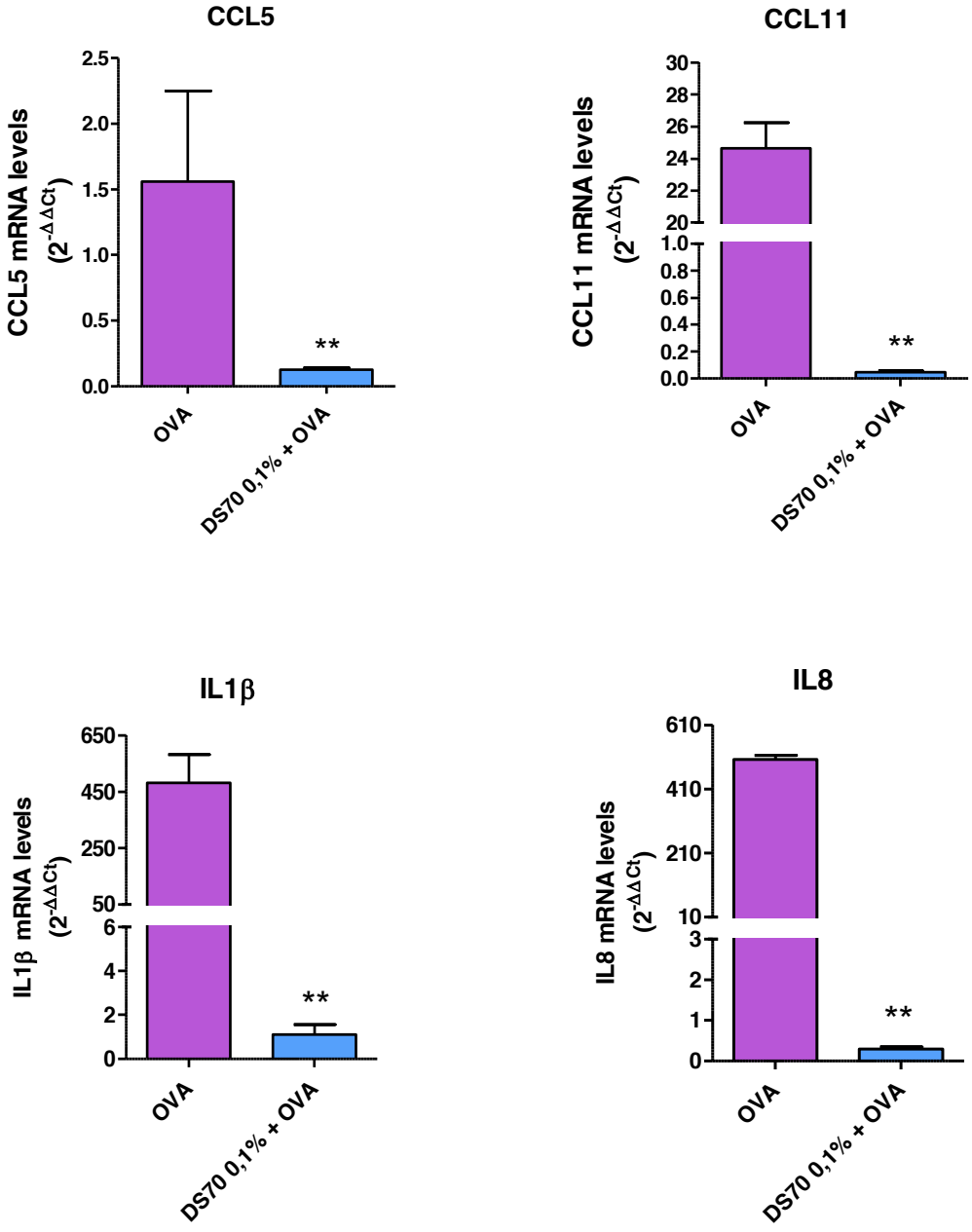


Figure 6.13 *Effect of DS70 0.1% eye drops suspension (30 µL/eye), administered 30 minutes before antigen challenge, on conjunctival cytokine/chemokine mRNA levels. Control guinea-pigs were not treated with ovalbumin. Values are the mean ± SEM of three values per each group. **p<0.001 vs vehicle+OVA.*

6.2.4 DS70 pre-treatment effects in histological sections: immunostaining for α_4 subunit and Giemsa staining for mast cells degranulation

To confirm any involvement of integrin α_4 in the allergic ocular response, I performed immunohistochemical analysis on conjunctival sections with aim to evaluate expression levels of integrin α_4 subunit. Histological analysis done in guinea pigs sacrificed 24 hours after ovalbumin challenge, showed elevated levels of this protein in correspondence of conjunctival outer edges, in comparison with basal levels observed in controls, not immunized animals. Black arrows indicated hot spot of expression levels after ovalbumin challenge. Finally, pre-treatment with DS70 0.1% decreased integrin expression: levels of α_4 integrin expression were comparable to untreated animals (Figure 6.14, panel A). Furthermore, it is also possible to observe reduction of mast cells degranulation with Giemsa staining In sections correspondent to treatment with compound, I could observe a poor number of degranulated mast cells (Figure 6.14, panel B).

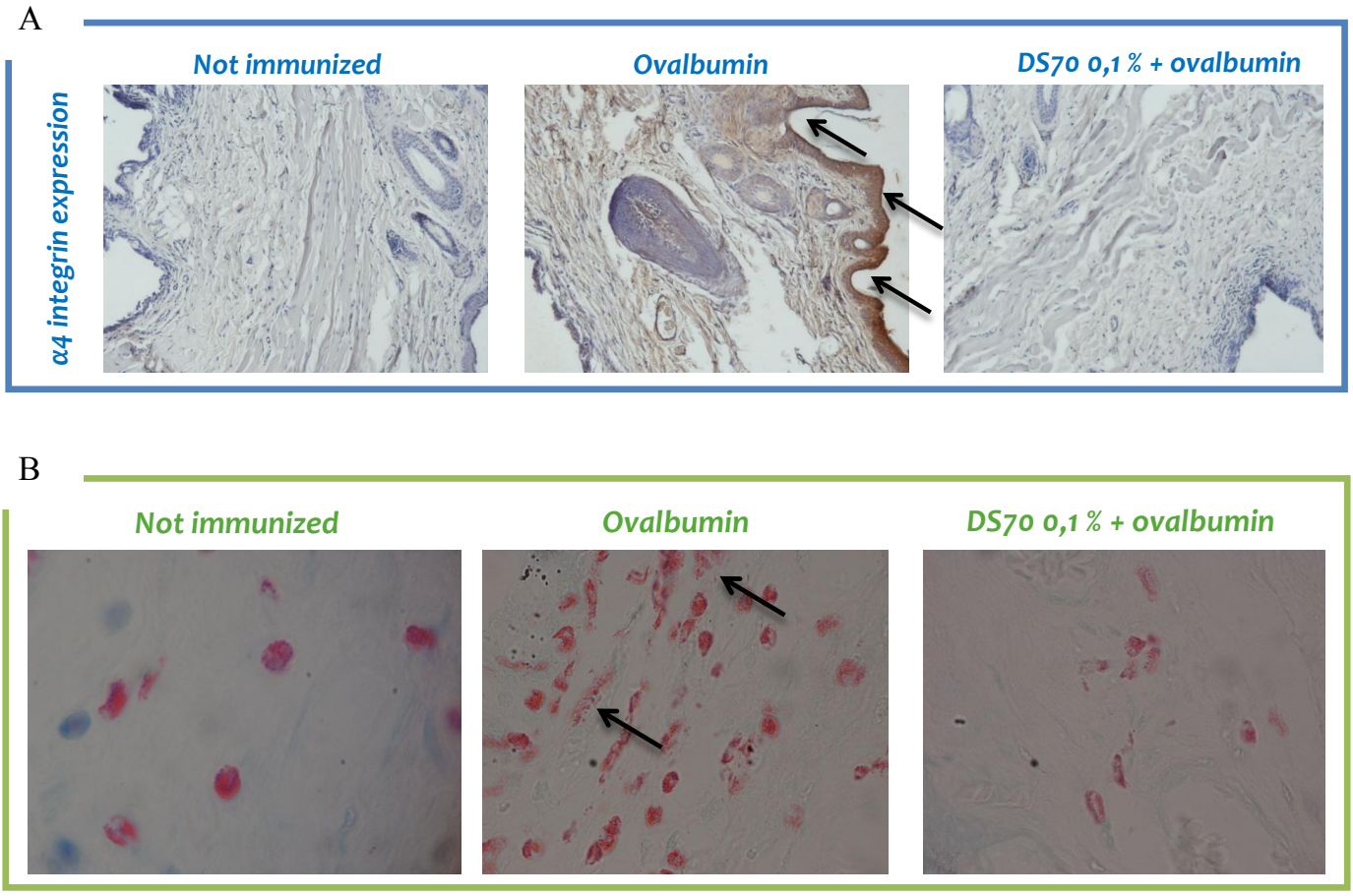


Figure 6.14 *Photomicrographs of the conjunctiva 24 hours after topical challenge with ovalbumin and pre-treatment with DS70. Panel A, effects of compound on conjunctival levels of α_4 integrin subunit 24 hours after topical challenge. Not immunized animals showed basal levels of expression, as expected. Black arrows in panel A indicated hot spot of expression. Panel B, effects of compound on mast cells degranulation 24 hours after topical challenge. Black arrows revealed presence of preformed granules, typical marker of happened degranulation. DS70 0.1% is able to prevent this release.*

7. DISCUSSION AND CONCLUSIONS

Inflammation is a complex response to a local injury or trauma involving various immunocytes and numerous mediators. The assembly of the inflammatory response would be impossible without the controlled migration of immune cells, of which cell adhesion molecules such as integrins are key components for migration and activation. Integrins are heterodimeric molecules and cell surface transmembrane glycoproteins that mediate cell-cell and cell-matrix interactions. Among these molecules, I focused my attention on integrin $\alpha_4\beta_1$. It requires the interaction with its ligands fibronectin, vascular cell adhesion molecule 1 (VCAM-1), and mucosal addressin cell adhesion molecule 1. Integrin $\alpha_4\beta_1$ is an important component in immune functions, playing a role in lymphocyte differentiation and homing, as well as in tissue-specific migration during inflammation [Alon *et al*., 1995]. α4 integrins, including $\alpha_4\beta_1$ and α4β7, have tightly regulated multi-step functions during rolling and arrest of leukocytes on the endothelium [Berlin *et al*., 2005].

Integrin $\alpha_4\beta_1$ is implicated in the pathogenesis of autoimmune diseases and chronic inflammation such as multiple sclerosis, Crohn's disease, asthma, stroke, rheumatoid arthritis, and inflammatory bowel disease [Hyun *et al*., 2010], but also in several allergic diseases [Pelaquini *et al*., 2011]. In allergic subjects a persistent or repetitive exposure to allergens, which are intrinsically innocuous substances frequently found in the environment, results in a chronic allergic inflammation. This in turn produces long-term changes in the structure of the affected organs and substantial abnormalities in their function. It is, therefore, important to understand the characteristics and consequences of acute and chronic allergic inflammation, and in particular to explore how integrin $\alpha_4\beta_1$ contributes to this event and how it can be modulated using small antagonist molecules.

Therefore, VLA-4 has been therapeutically targeted by antagonists such as blocking antibodies (Natalizumab). However, the inherent limitations of antibody therapy, high cost, potential immunogenicity, and the requirement for intravenous administration, strongly spurred efforts to develop ligand mimetic small molecules [Dattoli *et al*., 2013]. There are problems connected with the use of natural peptides, due to the low stability against proteolysis, resulting in a short duration of in vivo activity, and in a low bioavailability. One way to overcome these disadvantages is the employ modified peptides named peptidomimetics. This study focused on pharmacological characterization of a library of peptidomimetics synthetized by Professor Luca Gentilucci' group (Department of Chemistry, University of Bologna). The structures of the peptidomimetics were designed on the basis of the models developed by 3D-QSAR analysis of large libraries of compounds containing diphenylureas, or other substituted aromatic groups. Starting from the assumption that well determined pharmacophores are essential for the interaction with our target,

structures of this peptidomimetic inhibitors of integrin $\alpha_4\beta_1$ integrin are all based on a rigid central scaffold, β-amino acid residues, and partially modified retro-sequence.

First of all, inhibitory activity of all library of compounds was assessed *in vitro* through cell adhesion assays in Jurkat E6.1 cell line and a high-throughput screening (HTS) assay (scintillation proximity assay). This latter assay involves several steps to set up: purification of isolated integrin receptor from cultured cells, fibronectin labeling with ^{125}I (radioactive ligand), and optimization of different reagent concentrations and time incubation through establishment of a saturation curve.

Several of these compounds exhibit IC_{50} values extending from micromolar to nanomolar range. The best results have been obtained for compounds having the pharmacophores separated by 14 bonds, such as DS70. In fact, this compound shows has an IC_{50} value equal to 0,022 μM (± 0,003) in SPA assay and to 0,0020 (± 0,0005) μM in VCAM-1 mediated cell adhesion assay. This compound bears diphenylurea moiety to interacts with α subunit, a beta prolin, and a glycine carrying a carbossilic group.

DS70, as integrin $\alpha_4\beta_1$ antagonist, has been investigated to define its selectivity and specificity, in different cell lines expressing integrins, and in parallel, to analyze its effects on three principal component of inflammatory response: lymphocytes, mast cells and eosinophils.

Futher adhesion assays in Jurkat E6.1 cell line have allowed us to understand that the inhibition of cellular adhesion determined by DS70 is mediated only from integrin $\alpha_4\beta_1$ and not from integrin $\alpha_L\beta_2$, both expressed on such cells.

DS70 is able to inhibit cellular adhesion in an eosinophilic cell line, using as ligand both VCAM-1 and FN. DS70 results to be a dual antagonist of α_4 integrin family, because it also exerts inhibitory action towards integrin $\alpha_4\beta_7$, expressed in the cellular line RPMI8866. Albeit DS70 causes a significant reduction of cellular adhesion. It is less potent towards this integrin subtype. On the contrary, DS70 is a highly specific compound as it doesn't display any significant activity towards integrin $\alpha_5\beta_1$, integrin $\alpha_v\beta_3$ and integrin $\alpha_9\beta_1$, as shown in cellular adhesion assay in cell lines expressing these specific integrins.

After these experiments, I moved further to explore the ability of DS70 to modulate integrin signaling. Integrin receptors form structural and functional linkages between the extracellular matrix (ECM) and intracellular cytoskeletal linker proteins. Cell signaling mediated by integrin/ECM interactions is also integrated by cellular responses to growth factor signaling to regulate cellular proliferation, cytoskeletal reorganization, and other responses necessary for cell survival. Integrin receptors do not possess any kinase domains but they can activate a number of intracellular signaling pathways following extracellular adhesive interactions. Clusters of integrin/extracellular matrix interactions form focal adhesion points, concentrating together

cytoskeletal components and signaling molecules within the cell. The cytoplasmic tail of integrins serve as a binding site for α-actinin and talin which then recruit vinculin, a protein involved in anchoring F-actin to the membrane. In addition to actin polymerization/depolymerization, ligand binding to integrin receptors results in the talin-mediated oligomerization of FAK (Focal Adhesion Kinase). Focal adhesion kinase is best known for its role as an integrin-stimulated protein-tyrosine kinase. FAK is recruited to sites of integrin clustering via interactions of its C-terminal domain with integrin-associated proteins such as talin and paxillin. FAK contains a central kinase domain, C-terminal proline-rich regions that serve as binding sites for Src homology 3 (SH3) domain-containing proteins, and a protein 4.1, Ezrin, Radixin, Moesin domain (FERM) that acts to regulate FAK kinase activity through an auto-inhibitory mechanism [Schlaepfer *et al*., 2007]. The Tyr397 autophosphorylated FAK binds and activates Src and Fyn which in turn phosphorylate the FAK-associated proteins paxillin, tensin, and p130CAS. In addition, phosphorylated FAK has been shown to phosphorylate PI3K, PLCγ, and GRB7 leading to their activation. Activation of PI3K links integrin activation with the Akt signaling pathway for activation of cell survival mechanisms [Meng *et al*., 2009]. Phosphorylation of FAK at Tyr925 occurs by Src, thereby forming a complex with GRB2 and SOS, leading to the activation of Ras. Ras can function to activate numerous kinases including MEKKs, PAKs, MEKs, JNK, and SAPK. These kinases are key regulators of gene expression via the phosphorylation of multiple transcription factors including c-Myc, Elk1, Jun, and SRF (Serum Response Factor) [Harburger *et al*., 2009].

Therefore, I investigated any modulatory effect of compound DS70 on ligand-induced phosphorylation of FAK (Tyr197) in Jurkat E6.1 cells and of ERK 1/2 in all cell lines, expressing $\alpha_4\beta_1$ integrin. I set up an *in vitro* model, starting from preliminary experiments performed with other integrin receptor [Tolomelli *et al*., 2013]. First of all, I observed that VCAM-1-induced FAK phosphorylation in Jurkat cells after 30 minutes of incubation, and FN-induced FAK or ERK 1/2 phosphorylation in Eol-1 and TF-1 cell line after 60 minutes and 30 minutes, respectively; thus, this assay appeared to be quite robust. Then, I was able to confirm that pre-treatment with different concentrations of DS70 can prevent, in a dose dependent manner, FAK and ERK 1/2 phosphorylation. Optimal drug concentrations for each cell line were selected, given that integrin responsiveness strongly depends on cellular context. However, micromolar or submicromolar doses (10^{-5} or 10^{-6} M) revealed to be in general the most efficacious: these concentrations appeared to be interesting for a possible systemic administration of this compound *in vivo*.

Various integrins are expressed on mast cells and constitute an essential prerequisite for the accumulation of the cells at sites of inflammation. In order to clarify a potential contribution of

inflammatory cytokines to this process, I have studied the modulation of integrin expression and adhesion of immature human mast cells (HMC-1) to extracellular matrix proteins.

There is increasing evidence that adhesive interactions between mast cells and extracellular matrix (ECM) proteins play a major role in regulating anchorage, migration, differentiation, and function of these cells in various tissues. This concept is supported by the demonstration of the occurence of adhesion molecules on resting and/or activated mast cells [Metcalfe *et al*., 1997; Henz *et al*., 2001]. Furthermore, there is increasing evidence that exists a specific regulation of the expression and function of these molecules in normal tissue, under physiologic conditions or in various inflammatory states where the number of mast cells is increased.

Mast cells (MCs) are critical effector cells of both innate and adaptive immunity. In vertebrates, mast cells are widely distributed throughout vascularized tissues, particularly our body surfaces exposed to the external environment, including skin, airways, and gastrointestinal tract. Mast cells are considered to be one of the first cells of the immune system capable to interact with environmental antigens, toxins, or invading pathogens [Galli *et al*., 2010].

Like other myeloid cells, MCs derive from bone marrow–derived progenitors. These are mononuclear cells that lack the characteristic secretory granules of their mature counterparts. As assessed by cytofluorographic criteria, bone marrow–derived progenitors express high levels of α_4- and β_1-integrin. The high expression of $\alpha_4\beta_1$ subunits and PSGL-1 by these cells suggests that they may interact with VCAM-1 and the selectins, respectively. Immobilized soluble VCAM-1, as well as both recombinant P- and E-selectins, supported their attachment across a range of flow conditions in vitro. The adhesion to VCAM-1 was blocked nearly completely by anti–VCAM-1 and anti–α_4-integrin Abs, implying complete dependence on the $\alpha_4\beta_1$ and/or $\alpha_4\beta_7$ integrins for this event [Boyce *et al*., 2002]. Fully differentiated mast cells purified from human skin and uterus also express $\alpha_4\beta_1$ [Columbo *et al*., 1995]. Firstly, our data attest α_4 subunit presence in HMC 1.1 cells, a well established and widely used human cell line exhibiting many characteristics of mast cells. As reported in the literature, *in vitro* cellular adhesion of this cell line to VCAM-1 is mediated by integrin $\alpha_4\beta_1$ and is abrogated with pre-incubation of cell line with DS70, in a dose-dependent manner.

In 1994, Ra and colleagues demonstrated for the first time that engagement of $\alpha_4\beta_1$ and vitronectin receptor (β_3 integrin) promote mast cells degranulation induced by cross-linking of the high-affinity IgE receptor. Blocking these adhesion molecules by monoclonal antibodies remarkably reduces passive cutaneous anaphylaxis reaction *in vivo*.

β-hexosaminidase induced mast cells release is a marker for mast cell degranulation. It is triggered by high affinity IgE receptor (Fc epsilon RI)-mediated stimulation, and it enhances adhesion of

RBL-2H3 cells (rat mast cells) to either immobilized fibronectin, or monoclonal antibodies reactive with integrin chain alpha 4-1, alpha 5-1 or beta 3-1.

Fibronectin enhancement of β-hexosaminidase release is inhibited by specific antibodies as well as by GRGDSP and DELPQLVTLPHPNHLGPEILDVPST peptides that abrogate VLA-5/VNR (vitronectin) and $\alpha_4\beta_1$ binding to FN, respectively. In vivo, passive cutaneous anaphylaxis induced by IgE anti-DNP and DNP-BSA (2,4-dinitrophenylated BSA) was inhibited by concurrent injection of such antibodies. These results demonstrate that integrins expressed on rat mast cells bind to fibronectin and play an important role in regulating mast cell activation both in vitro and in vivo [Yasuda *et al*., 1995].

A number of studies reported that blocking integrin $\alpha_4\beta_1$ with specific antibodies could prevent both allergic early airway response (EAR) and late airway responses (LAR) and reduce the so-called nonspecific airway hyperresponsiveness in allergen challenged animals (rat, rabbit and sheep) [Rabb *et al*., 1994; Metzger *et al*., 1995; Abraham *et al*., 1997]. The inhibitory actions of anti-$\alpha_4\beta_1$ monoclonal antibodies (mAb) have been attributed mainly to preventing migration and/or activation of eosinophils and lymphocytes as well as mast cells in the sites of airway inflammation. Another study performed in Brown Norway rats, sensitized and challenged with ovalbumin, showed that treatment with a specific anti-$\alpha_4\beta_1$ mAb prevents allergen-induced airway responses, the release of mast cell mediators, and cys-LT synthesis in sensitized Brown Norway rats [Hojo *et al*., 1998].

In this frame, my aim was to investigate whether any reduction α_4-integrin dependent of early phase response is related to inhibition of mast cell degranulation and inflammatory mediator release, and to provide a possible explanation of this. Effectively, DS70 was able to reduce β-hexosaminidase levels in HMC 1.1 cells stimulated with both PMA and calcimycin and in PMA activated T-cells.

Recent in vitro studies indicate that mast cells may be triggered to degranulate and release cytokines upon heterotypic adhesion to activated, but not resting, T cells [Inamura *et al*., 1998; Baram *et al*.,2001; Salamon *et a*l., 2005]. Our results provide evidence that direct contact between cell-surface molecules on mast cells and on activated T cell membranes is sufficient to transduce the stimulatory signal necessary for mast cells degranulation and to promote cytokine release, independent of T cell intracellular function or production of cytokines or other mediators.

It was shown that direct contact of human mast cells with T lymphocyte membranes resulted in the phosphorylation of the MAPK family [Mor *et al*., 2010]. The MAPKs are known to play an important role in mediator release in human mast cells after IgE–cross-linking or on activation by inducers, such as SCF and TNF-α [Rivera *et al*., 2006; Wong *et al*., 2006]. In accordance with these findings, it has been reported that microparticle induced degranulation of LAD2 cells (a cell line of

mast cells) is regulated by MAPKs, as it is abrogated by the addition of a specific inhibitor (PD98059) to MAPK kinase/ERK, showing a significant reduction (~60%) in β-hexosaminidase levels. Given that the antagonist action of DS70 on $\alpha_4\beta_1$ integrin involves the modulation of this signaling pathway, it could be hypothesized a possible regulation at this step. My results, therefore, provide new insights on the activation of HMC-1.1 mast cell that requires a diversified and complex intracellular regulation. However, further investigations are required to provide a complete explanation of this mechanism.

With regards to *in vivo* assays, this study aimed to investigate the potential anti-allergic activity of DS70, specifically designed to be an $\alpha_4\beta_1$ integrin antagonist in a model of allergic conjunctivitis.
Ocular allergy represents one of the most common conditions encountered by immunolgists and ophthalmologists. Allergic conjunctivitis is often underdiagnosed and consequently undertreated. Allergic eye inflammation is a localized allergic condition that is frequently associated with rhinitis and occasionally with asthma but often observed as the only or prevalent allergic manifestation. The symptoms and signs can manifest alone in the eyelids as atopic blepharitis or contact blepharitis. Lid symptoms may occur together with conjunctival symptoms. Allergic conjunctivitis is the most common manifestation alone or together with lid symptoms. The eye is red and itchy, and there is lacrimation and slight discharge [Kari *et al*., 2012]. The possibility of curing allergic diseases is an essential issue for research because the medications currently used to treat these diseases, such as antihistamines, leukotriene receptor antagonists and glucocorticoids, only temporarily relieve symptoms by suppressing inflammation. Basic and clinical researches have provided a better understanding of cells, mediators, and immunologic events, which occur in ocular allergy.
The severity of allergic conjunctivitis (AC) correlates primarily with the degree of eosinophil infiltration into the conjunctiva, which is believed to be mediated by chemokines and adhesion molecules. Integrin $\alpha_4\beta_1$ and its ligand, vascular cell adhesion molecule (VCAM)-1, are known to play important roles in eosinophil infiltration, and in general in the recruitment of immune cells in inflammation sites.
The expression and function of integrin $\alpha_4\beta_1$ in allergic conjunctivitis have been investigated more in detail in recent years. Nowadays, adhesion molecules and their intracellular signals are validated targets to control allergic conjunctivitis [Baiula *et al*., 2012]. Advancement in understanding of the pathophysiology of ocular allergies has paved the way for the development of newer drug candidates, and the adhesion-based therapeutic strategies targeting α_4 integrin seem promising for ocular allergy. With regards to small molecules and similar, it is known by literature that levocabastine, in addition to its effects on H1 histamine receptors, binds to human integrin $\alpha_4\beta_1$ and

prevents eosinophil adhesion to VCAM-1, FN and human umbilical vascular endothelial cells *in vitro*. In a classical model of allergic conjunctivitis, levocabastine eye drops reduced the clinical symptomps of the late phase reaction and the conjunctival expression of $\alpha_4\beta_1$ integrin by reducing eosinophil infiltration [Qasem *et al*.,2008]. Authors hypothesized that blockade of integrin-mediated cell adhesion might be a target of the antiallergic action of levocabastine and may play a role in preventing eosinophil adhesion and infiltration in allergic conjunctivitis.

Ebihara and colleagues in 1998 have reported that in an actively sensitized allergic conjunctivitis model of guinea pig, treatment with an anti $\alpha_4\beta_1$-mAb was able to reduce almost completely eosinophil infiltration at least until 24 hours after the antigen challenge. Furthermore, treatment with both anti-$\alpha_4\beta_1$ and anti-VCAM-1 antibodies significantly suppressed the infiltration of eosinophils into the conjunctiva in mice that was induced by either active immunization with ragweed or or adoptive transfer of ragweed-primed splenocytes [Fukushima *et al*., 2006]. These results confirm that $\alpha_4\beta_1$-expressing cells infiltrate the conjunctiva and that the interaction between integrin $\alpha_4\beta_1$ and VCAM-1 is needed for the development of allergic conjunctivitis.

Effects on clinical score, eosinophils activity (in tears and in conjunctiva), levels of cytokines and chemochines mRNA, mast cells degranulation and α_4 integrin expression, were all assessed in this thesis. I focused on eosinophils and mast cells, two crucial effectors of both early and late phase of allergic response, since these cells mediate unique cytotoxic and inflammatory effects by the generation, storage, and release of their granule proteins and the production of cytokines, growth factors, reactive oxygen species, and proinflammatory lipid mediators. Their recruitment and activation are regarded as crucial to the development of allergic disorders, including conjunctivitis [Ono *et al*., 2005].

Here I report a significant reduction of clinical symptoms at a concentration of DS70 of 0,1% p/v. Eosinophil peroxidase levels, taken as an index of eosinophil accumulation, in tears and in tissues collected from guinea pigs pre-treated with DS70 and then with ovalbumin are equally decreased. The significant decrement of the chemokines and cytokines suggests that DS70 is capable to exert an influence in a relevant inflammatory response produced in the conjunctiva of sensitized guinea pigs by an allergen.

I found a lower expression of $\alpha_4\beta_1$ integrin in the conjunctiva of ovalbumin-sensitized guinea pigs treated with DS70: this might be a consequence of the reduction in recruitment and migration of $\alpha_4\beta_1$-expressing cells to the site of allergic inflammation. It is important to note that the same effect is also a typical mechanism of action of levocabastine.

I confirmed the positive effect of DS70 eye drops on early phase and late phase reactions inflammatory changes induced by allergen-specific conjunctival challenge. Histamine and

eicosanoids are responsible for the typical early phase reaction. 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 leads 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, sustaining a positive loop [La Rosa *et al*., 2013].

According to previous data *in vitro*, DS70's effect on early phase reaction seems to be mediated by the blockade of mast cells activation. I confirmed *in vivo* what observed before in *in vitro* studies: DS70 is able to prevent mast cells degranulation and, therefore, the consequent release of preformed granules in conjunctival tissue sections.

In ovalbumin-sensitized guinea pigs, this compound almost completely prevented conjunctival symptoms and this effect lasted up to 24 hours. Therefore, compound DS70 administrated topically may persist at an adequate conjunctival concentration for long periods of time, phenomenon that suggests an increased bioavailability in tissues than normal peptides.

Definitively, DS70 possesses potent and sustained antiallergic activity in preventing ocular inflammatory responses to ovalbumin exposure in sensitized guinea pigs. I propose that DS70 exerts a pleiotropic role as an antiallergic agent: it can interfere at different levels with accumulation and activation of immune cells (eosinophils, mast cells and leukocytes).

Late phase response is inhibited by DS70 pretreatment. Although it is possible that inhibition of the early phase which is often considered proemial to the late one accounts for the reduction of the latter (showing an inhibitory action on mast cells), an independent effect on the late phase could be also possible. Since eosinophils and lymphocytes are strikingly involved in the development of the late phase, the decrease in the magnitude of this response could result from the effect of DS70 on activation of these cells. A fine "tuning" of recruitment and activation of immune cells can be taken into account to explain DS70 mechanism of action. DS70 can represent without any doubt a lead compound in the field of anti-inflammatory drugs.

8. REFERENCES

- Abraham WM, Sielczak MW, Ahmed A, Cortes A, Lauredo IT, Kim J, Pepinsky B, Benjamin CD, Leone DR, Lobb RR, et al. Alpha 4-integrins mediate antigen-induced late bronchial responses and prolonged airway hyperresponsiveness in sheep. J Clin Invest. 1994 Feb;93(2):776-87.
- Alon R, Kassner PD, Carr MW, Finger EB, Hemler ME, Springer TA. The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. J Cell Biol. 1995. Mar;128(6):1243-53.
- Alon, R., H. Rossiter, X. Wang, T. A. Springer, and T. S. Kupper. Distinct cell surface ligands mediate T lymphocyte attachment and rolling on P and E selectin under physiological flow. J. Cell Biol. 1994. 127: 1485–1495.
- Baiula M, Bedini A, Carbonari G, Dattoli SD, Spampinato S. Therapeutic
- Banerjee ER, Jiang Y, Henderson WR Jr, Scott LM, Papayannopoulou T. Alpha4 and beta2 integrins have nonredundant roles for asthma development, but for optimal allergen sensitization only alpha4 is critical. Exp Hematol. 2007, Apr;35(4):605-17.
- Baram D, Vaday GG, Salamon P, Drucker I, Hershkoviz R, Mekori YA. Human mast cells release metalloproteinase-9 on contact with activated T cells: juxtacrineregulation by TNF-alpha. J Immunol. 2001 Oct 1;167(7):4008-16.
- Bartolomé RA, Sanz-Rodríguez F, Robledo MM, Hidalgo A, Teixidó J. Rapid up-regulation of alpha4 integrin-mediated leukocyte adhesion by transforming growth factor-beta1. Mol Biol Cell. 2003 Jan;14(1):54-66.
- Berlin C, Bargatze RF, Campbell JJ, von Andrian UH, Szabo MC, Hasslen SR, Nelson RD, Berg EL, Erlandsen SL, Butcher EC. alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. Cell. 1995 Feb 10;80(3):413-22.
- Bhattacharyya SP, Drucker I, Reshef T, Kirshenbaum AS, Metcalfe DD, Mekori YA. Activated T lymphocytes induce degranulation and cytokine production by human mast cells following cell-to-cell contact. J Leukoc Biol. 1998 Mar;63(3):337-41.
- Chigaev A, Sklar LA. Aspects of VLA-4 and LFA-1 regulation that may contribute to rolling and firm adhesion. Front Immunol. 2012 Aug 2;3:242.

- Chigaev A, Sklar LA. Aspects of VLA-4 and LFA-1 regulation that may contribute to rolling and firm adhesion. Front Immunol. 2012 Aug 2;3:242.
- Chigaev, A., Wu, Y., Williams, D.B., Smagley, Y., and Sklar, L.A.. Discovery of very late antigen-4 (VLA-4, α4β1 integrin) allosteric antagonists. J. Biol.Chem. 2011d. 286, 5455–5463.
- Columbo M, Bochner BS, Marone G. Human skin mast cells express functional beta1 integrins that mediate adhesion to extracellular matrix proteins. J Immunol. 1995 Jun 1;154(11):6058-64.
- Coulombel, L., Auffray, I., Gaugler, M.H., and Rosemblatt, M.. Expression and function of integrins on hematopoietic progenitor cells. Acta Haematol 1997. 97, 13–21.
- Dattoli SD, De Marco R, Baiula M, Spampinato S, Greco A, Tolomelli A,
- Ebihara N, Yokoyama T, Kimura T, Nakayasu K, Okumura K, Kanai A, Ra C. Anti VLA-4 monoclonal antibody inhibits eosinophil infiltration in allergic conjunctivitis model of guinea pig. Curr Eye Res. 1999 Jul;19(1):20-5.
- Engelhardt B, Kappos L. Natalizumab: targeting alpha4-integrins in multiple sclerosis. Neurodegener Dis. 2008;5(1):16-22.
- Friedlaender MH. Ocular allergy. Curr Opin Allergy Clin Immunol. 2011 Oct;11(5):477-82.
- Front Pharmacol. 2012 Dec 26;3:203.
- Fukushima A, Yamaguchi T, Ishida W, Fukata K, Ueno H. Role of VLA-4 in the development of allergic conjunctivitis in mice. Mol Vis. 2006 Apr 7;12:310-7.
- Gascoigne MH, Holland K, Page CP, Shock A, Robinson M, Foulkes R, Gozzard N. The effect of anti-integrin monoclonal antibodies on antigen-induced pulmonary inflammation in allergic rabbits. Pulm Pharmacol Ther. 2003;16(5):279-85. Review.
- Gazitt,Y. Homing and mobilization of hematopoietic stem cells and hematopoietic cancer cells are mirror image processes, utilizing similar signaling pathways and occurring concurrently: circulating cancer cells constitute an ideal target for concurrent treatment with chemotherapy and anti lineage- specific antibodies. Leukemia 2004. 18, 1–10.
- Gentilucci L. Synthesis and assay of retro-α4β1 integrin-targeting motifs. Eur J

- Ghandour, H., X. Cullere, A. Alvarez, F. W. Luscinskas, and T. N. Mayadas. Essential role for Rap1 GTPase and its guanine exchange factor CalDAG-GEFI in LFA-1 but not VLA-4 integrin mediated human T-cell adhesion. Blood 2007. 110: 3682–3690.
- Godot V, Arock M, Garcia G, Capel F, Flys C, Dy M, Emilie D, Humbert M. H4 histamine receptor mediates optimal migration of mast cell precursors to CXCL12. J Allergy Clin Immunol. 2007 Oct;120(4):827-34.
- Grimbaldeston MA, Chen CC, Piliponsky AM, Tsai M, Tam SY, Galli SJ. Mast cell-deficient W-sash c-kit mutant Kit W-sh/W-sh mice as a model for investigating mast cell biology in vivo. Am J Pathol. 2005 Sep;167(3):835-48.
- Guo CB, Kagey-Sobotka A, Lichtenstein LM, Bochner BS. Immunophenotyping and functional analysis of purified human uterine mast cells. Blood. 1992 Feb 1;79(3):708-12.
- Hu P, Luo BH. Integrin bi-directional signaling across the plasma membrane. J Cell Physiol. 2013 Feb;228(2):306-12.
- Imai, Y., Shimaoka, M., and Kurokawa, M. Essential roles of VLA-4 in the hematopoietic system. Int.J. Hematol 2010. 91, 569–575.
- Inamura N, Mekori YA, Bhattacharyya SP, Bianchine PJ, Metcalfe DD. Induction and enhancement of Fc(epsilon)RI-dependent mast cell degranulation following coculture with activated T cells: dependency on ICAM-1- and leukocyte function-associated antigen (LFA)-1-mediated heterotypic aggregation. J Immunol. 1998 Apr 15;160(8):4026-33.
- Izushi K, Nakahara H, Tai N, Mio M, Watanabe T, Kamei C. The role of histamine H(1) receptors in late-phase reaction of allergic conjunctivitis. Eur J Pharmacol. 2002 Apr 5;440(1):79-82.
- Johansson MW, Han ST, Gunderson KA, Busse WW, Jarjour NN, Mosher DF. Platelet activation, P-selectin, and eosinophil β1-integrin activation in asthma. Am J Respir Crit Care Med. 2012 Mar 1;185(5):498-507.
- Johansson MW, Kelly EA, Busse WW, Jarjour NN, Mosher DF. Up-regulation and activation of eosinophil integrins in blood and airway after segmental lung antigen challenge. J Immunol. 2008 Jun 1;180(11):7622-35.
- Johansson MW, Mosher DF. Integrin activation States and eosinophil recruitment in asthma. Front Pharmacol. 2013 Apr 1;4:33.

- Juliano RL, Carver K, Cao C, Ming X. Receptors, endocytosis, and trafficking: the biological basis of targeted delivery of antisense and siRNA oligonucleotides. J Drug Target. 2013 Jan;21(1):27-43.+
- Kari O, Saari KM. Diagnostics and new developments in the treatment of ocular allergies. Curr Allergy Asthma Rep. 2012 Jun;12(3):232-9.
- Kari O, Saari KM. Diagnostics and new developments in the treatment of ocular allergies. Curr Allergy Asthma Rep. 2012 Jun;12(3):232-9.
- Kashiwakura J, Yokoi H, Saito H, Okayama Y. T cell proliferation by direct cross-talk between OX40 ligand on human mast cells and OX40 on human T cells: comparison of gene expression profiles between human tonsillar and lung-cultured mast cells. J Immunol. 2004 Oct 15;173(8):5247-57.
- Khosravi E, Elena PP, Hariton C. Allergic conjunctivitis and uveitis models: reappraisal with some marketed drugs. Inflamm.Res 1995; 44:47-54.
- Kinashi T. Overview of integrin signaling in the immune system. Methods Mol Biol. 2012;757:261-78. doi: 10.1007/978-1-61779-166-6_17.
- Kita H. Eosinophils: multifaceted biological properties and roles in health and disease. Immunol Rev. 2011 Jul;242(1):161-77.
- Kubo S, Kobayashi M, Masunaga Y, Ishii H, Hirano Y, Takahashi K, Shimizu Y. Cytokine and chemokine expression in cigarette smoke-induced lung injury in guinea pigs. Eur Respir J. 2005 Dec;26(6):993-1001.
- Kuwano, Y., O. Spelten, H. Zhang, K. Ley, and A. Zarbock. Rolling on E- or P-selectin induces the extended but not high-affinity conformation of LFA-1 in neutrophils. Blood 2010. 116: 617–624.
- Lacy HM, Bowlin AK, Hennings L, Scurlock AM, Nagarajan UM, Rank RG. Essential role for neutrophils in pathogenesis and adaptive immunity in Chlamydia caviae ocular infections. Infect Immun. 2011 May;79(5):1889-97.
- Leonardi S, Miraglia del Giudice M, La Rosa M, Bellanti JA. Atopic disease, immune system, and the environment. Allergy Asthma Proc. 2007 Jul-Aug;28(4):410-7.
- levocabastine. Biochem Pharmacol. 2008 Sep 15;76(6):751-62.

- Lin TJ, Issekutz TB, Marshall JS. Human mast cells transmigrate through human umbilical vein endothelial monolayers and selectively produce IL-8 in response to stromal cell-derived factor-1 alpha. J Immunol. 2000 Jul 1;165(1):211-20.
- Luscinskas, F. W., G. S. Kansas, H. Ding, P. Pizcueta, B. E. Schleiffenbaum, T. F. Tedder, and M. A. Gimbrone, Jr. Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, β1-integrins, and β2-integrins. J. Cell Biol. 1994. 125: 1417–1427.
- McEver, R. P., and C. Zhu.. Rolling cell adhesion. Annu. Rev. Cell Dev. Biol. 2010. 26: 363–396. Med Chem. 2014 Feb 12;73:225-32.
- Mishra GP, Tamboli V, Jwala J, Mitra AK. Recent patents and emerging therapeutics in the treatment of allergic conjunctivitis. Recent Pat Inflamm Allergy Drug Discov. 2011 Jan;5(1):26-36.
- Mor A, Shefler I, Salamon P, Kloog Y, Mekori YA. Characterization of ERK activation in human mast cells stimulated by contact with T cells. Inflammation. 2010 Apr;33(2):119-25.
- Nakae S, Suto H, Iikura M, Kakurai M, Sedgwick JD, Tsai M, Galli SJ. Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. J Immunol. 2006 Feb 15;176(4):2238-48.
- Nandi, A., P. Estess, and M. Siegelman. Bimolecular complex between rolling and firm adhesion receptors required for cell arrest; CD44 association with VLA-4 in T cell extravasation. Immunity 2004. 20: 455–465.
- Njus BH, Chigaev A, Waller A, Wlodek D, Ostopovici-Halip L, Ursu O, Wang W, Oprea TI, Bologa CG, Sklar LA. Conformational mAb as a tool for integrin ligand discovery. Assay Drug Dev Technol. 2009 Oct;7(5):507-15.
- Oettgen H and Broide DH Introduction to mechanisms of allergic disease. Chapter 1. Allergy, 4th ed. 2012.
- Ono SJ, Abelson MB. Allergic conjunctivitis: update on pathophysiology and
- Oostendorp, R.A., and Dormer, P. VLA-4-mediated interactions between normal human hematopoietic progenitors and stromal cells. Leuk. Lymphoma 1997. 24, 423–435.
- Pelaquini EH, Guimarães Lde A, Benetti LR, Fernandes LG, Tamashiro WM, Conran N, Ferreira HH. Role of the Mac-1 and VLA-4 integrins, and concomitant Th2-cytokine

production, in nitric oxide modulated eosinophil migration from bone marrow to lungs in allergic mice. Int Immunopharmacol. 2011 Feb;11(2):204-11.

- Possa SS, Leick EA, Prado CM, Martins MA, Tibério IF. Eosinophilic inflammation in allergic asthma. Front Pharmacol. 2013 Apr 17;4:46.
- prospects for future treatment. J Allergy Clin Immunol. 2005 Jan;115(1):118-22.
- Qasem AR, Bucolo C, Baiula M, Spartà A, Govoni P, Bedini A, Fascì D,
- Reber, L. L.,Marichal, T., & Galli, S. J. Newmodels for analyzing mast cell functions in vivo. Trends Immunol 2012. 33, 613–625.
- Rigoli L, Briuglia S, Caimmi S, Ferraú V, Gallizzi R, Leonardi S, La Rosa M, Salpietro C. Gene-environment interaction in childhood asthma. Int J Immunopathol Pharmacol. 2011 Oct;24(4 Suppl):41-7.
- Rivera J, Gilfillan AM. Molecular regulation of mast cell activation. J. Allergy Clin Immunol. 2006 Jun;117(6):1214-25; quiz 1226. Review.
- Rodewald, H. R., & Feyerabend, T. B. Widespread immunological functions of mast cells: fact or fiction? Immunity 2012. 37, 13–24.
- Rosenberg HF, Dyer KD, Foster PS. Eosinophils: changing perspectives in health and disease. Nat Rev Immunol. 2013 Jan;13(1):9-22.
- Rothenberg ME, Hogan SP. The eosinophil. Annu Rev Immunol. 2006;24:147-74. Review.
- Salamon P, Shoham NG, Gavrieli R, Wolach B, Mekori YA. Human mast cells release Interleukin-8 and induce neutrophil chemotaxis on contact with activated T cells. Allergy. 2005 Oct;60(10): 1316-9.
- Sandborn WJ, Yednock TA. Novel approaches to treating inflammatory bowel disease: targeting alpha-4 integrin. Am J Gastroenterol. 2003 Nov;98(11):2372-82.
- Semko CM, Chen L, Dressen DB, Dreyer ML, Dunn W, Farouz FS, Freedman SB, Holsztynska EJ, Jefferies M, Konradi AW, Liao A, Lugar J, Mutter L, Pleiss MA, Quinn KP, Thompson T, Thorsett ED, Vandevert C, Xu YZ, Yednock TA. Discovery of a potent, orally bioavailable pyrimidine VLA-4 antagonist effective in a sheep asthma model. Bioorg Med Chem Lett. 2011 Mar 15;21(6):1741-3. doi:10.1016/j.bmcl.2011.01.075. Epub 2011 Jan 22.

- Springer TA, Dustin ML. Integrin inside-out signaling and the immunological synapse. Curr Opin Cell Biol. 2012 Feb;24(1):107-15.
- Tagari P, Black C, Marshall S, Ford-Hutchinson AW. A rapid biochemical method for measuring antigen-induced pulmonary eosinophil margination in allergic guinea pigs. J Immunol Methods. 1993 Jul 6;163(1):49-58.
- Targan SR, Feagan BG, Fedorak RN, Lashner BA, Panaccione R, Present DH, Spehlmann ME, Rutgeerts PJ, Tulassay Z, Volfova M, Wolf DC, Hernandez C, Bornstein J, Sandborn WJ; International Efficacy of Natalizumab in Crohn's Disease Response and Remission (ENCORE) Trial Group. Natalizumab for the treatment of active Crohn's disease: results of the ENCORE Trial. Gastroenterology. 2007 May;132(5):1672-83. Epub 2007 Mar 21.
- Tsai, M., Grimbaldeston, M. A., Yu, M., Tam, S. Y., & Galli, S. J. Using mast cell knock-in mice to analyze the roles of mast cells in allergic responses in vivo. Chem Immunol Allergy 2005. 87, 179–197.
- Valent P, Gleich GJ, Reiter A, Roufosse F, Weller PF, Hellmann A, Metzgeroth G, Leiferman KM, Arock M, Sotlar K, Butterfield JH, Cerny-Reiterer S, Mayerhofer M, Vandenberghe P, Haferlach T, Bochner BS, Gotlib J, Horny HP, Simon HU, Klion AD. Pathogenesis and classification of eosinophil disorders: a review of recent developments in the field. Expert Rev Hematol. 2012 Apr;5(2):157-76.
- Villablanca EJ, Cassani B, von Andrian UH, Mora JR. Blocking lymphocyte localization to the gastrointestinal mucosa as a therapeutic strategy for inflammatory bowel diseases. Gastroenterology. 2011 May;140(6):1776-84. doi: 10.1053/j.gastro.2011.02.015. Erratum in: Gastroenterology. 2011 Aug;141(2):778.
- Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. Nat Med. 2012 May 4;18(5):716-25.
- Williams, M. R., V. Azcutia, G. Newton, P. Alcaide, and F. W. Luscinskas.. Emerging mechanisms of neutrophil recruitment across endothelium. Trends Immunol. 2011. 32: 461–469.
- Wong CK, Tsang CM, Ip WK, Lam CW. Molecular mechanisms for the release of chemokines from human leukemic mast cell line (HMC)-1 cells activated by SCF and TNF-alpha: roles of ERK, p38 MAPK, and NF-kappaB. Allergy. 2006 Mar;61(3):289-97.

- Xu YZ, Konradi AW, Bard F, Dappen M, Dofiles L, Dreyer M, Gallager I, Garrido C, Krimm M, Liao Z, Messersmith E, Mutter L, Pleiss MA, Samant B, Semko CM, Smith J, Stappenbeck F, Stupi B, Vandervert C, Welch B, Wipke B, Yednock T. Arylsulfonamide pyrimidines as VLA-4 antagonists. Bioorg Med Chem Lett. 2013 May 15;23(10):3070-4. doi: 10.1016/j.bmcl.2013.03.010. Epub 2013 Mar 15.
- Yasuda M, Hasunuma Y, Adachi H, Sekine C, Sakanishi T, Hashimoto H, Ra C, Yagita H, Okumura K. Expression and function of fibronectin binding integrins on rat mast cells. Int Immunol. 1995 Feb;7(2):251-8.
- Zhang K, Chen J. The regulation of integrin function by divalent cations. Cell Adh Migr. 2012 Jan-Feb;6(1):20-9.
- Zhu J, Zhu J, Springer TA. Complete integrin headpiece opening in eight steps. J Cell Biol. 2013 Jun 24;201(7):1053-68.
- Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. J Cell Sci. 2006 Oct 1;119(Pt 19):3901-3.
- Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002 Sep 20;110(6):673-87.
- Byron A, Humphries JD, Askari JA, Craig SE, Mould AP, Humphries MJ. Anti-integrin monoclonal antibodies. J Cell Sci. 2009 Nov 15;122(Pt 22):4009-11.
- Garmy-Susini B, Varner JA. Roles of integrins in tumor angiogenesis and lymphangiogenesis. Lymphat Res Biol. 2008;6(3-4):155-63.
- von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. Nat Rev Immunol. 2003 Nov;3(11):867-78.
- Luo BH, Carman CV, Springer TA. Structural basis of integrin regulation and signaling. Annu Rev Immunol. 2007;25:619-47.
- Xie C, Zhu J, Chen X, Mi L, Nishida N, Springer TA. Structure of an integrin with an alphaI domain, complement receptor type 4. EMBO J. 2010 Feb 3;29(3):666-79.
- Xiong JP, Mahalingham B, Alonso JL, Borrelli LA, Rui X, Anand S, Hyman BT, Rysiok T, Müller-Pompalla D, Goodman SL, Arnaout MA. Crystal structure of the complete integrin alphaVbeta3 ectodomain plus an alpha/beta transmembrane fragment. J Cell Biol. 2009 Aug 24;186(4):589-600.

- Xiong JP, Stehle T, Diefenbach B, Zhang R, Dunker R, Scott DL, Joachimiak A, Goodman SL, Arnaout MA. Crystal structure of the extracellular segment of integrin alpha Vbeta3. Science. 2001 Oct 12;294(5541):339-45.
- Zhu J, Luo BH, Xiao T, Zhang C, Nishida N, Springer TA. Structure of a complete integrin ectodomain in a physiologic resting state and activation and deactivation by applied forces. Mol Cell. 2008 Dec 26;32(6):849-61.
- Takagi J, Springer TA. Integrin activation and structural rearrangement. Immunol Rev. 2002 Aug;186:141-63.
- Xiao T, Takagi J, Coller BS, Wang JH, Springer TA. Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature. 2004 Nov 4;432(7013):59-67.
- Kim C, Schmidt T, Cho EG, Ye F, Ulmer TS, Ginsberg MH. Basic amino-acid side chains regulate transmembrane integrin signalling. Nature. 2011 Dec 18;481(7380):209-13
- Margadant C, Monsuur HN, Norman JC, Sonnenberg A. Mechanisms of integrin activation and trafficking. Curr Opin Cell Biol. 2011 Oct;23(5):607-14.
- Calderwood DA, Yan B, de Pereda JM, Alvarez BG, Fujioka Y, Liddington RC, Ginsberg MH. The phosphotyrosine binding-like domain of talin activates integrins. J Biol Chem. 2002 Jun 14;277(24):21749-58.
- Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH, Campbell ID. Structural basis of integrin activation by talin. Cell. 2007 Jan 12;128(1):171-82.
- Anthis NJ, Wegener KL, Ye F, Kim C, Goult BT, Lowe ED, Vakonakis I, Bate N, Critchley DR, Ginsberg MH, Campbell ID. The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. EMBO J. 2009 Nov 18;28(22):3623-32.
- Tanentzapf G, Brown NH. An interaction between integrin and the talin FERM domain mediates integrin activation but not linkage to the cytoskeleton. Nat Cell Biol. 2006 Jun;8(6):601-6. Epub 2006 Apr 30.
- Ye F, Hu G, Taylor D, Ratnikov B, Bobkov AA, McLean MA, Sligar SG, Taylor KA, Ginsberg MH. Recreation of the terminal events in physiological integrin activation. J Cell Biol. 2010 Jan 11;188(1):157-73.

- Moser M, Nieswandt B, Ussar S, Pozgajova M, Fässler R. Kindlin-3 is essential for integrin activation and platelet aggregation. Nat Med. 2008 Mar;14(3):325-30.
- Moser M, Legate KR, Zent R, Fässler R. The tail of integrins, talin, and kindlins. Science. 2009 May 15;324(5929):895-9.
- Harburger DS, Calderwood DA. Integrin signalling at a glance. J Cell Sci. 2009 Jan 15;122(Pt 2):159-63.
- Juliano RL, Reddig P, Alahari S, Edin M, Howe A, Aplin A. Integrin regulation of cell signalling and motility. Biochem Soc Trans. 2004 Jun;32(Pt3):443-6.
- Aplin AE, Stewart SA, Assoian RK, Juliano RL. Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. J Cell Biol. 2001 Apr 16;153(2):273-82.
- Parsons JT. Focal adhesion kinase: the first ten years. J Cell Sci. 2003 Apr 15;116(Pt 8):1409-16.
- Schlaepfer DD, Mitra SK. Multiple connections link FAK to cell motility and invasion. Curr Opin Genet Dev. 2004 Feb;14(1):92-101.
- Cooper LA, Shen TL, Guan JL. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. Mol Cell Biol. 2003 Nov;23(22):8030-41.
- Lietha D, Cai X, Ceccarelli DF, Li Y, Schaller MD, Eck MJ. Structural basis for the autoinhibition of focal adhesion kinase. Cell. 2007 Jun 15;129(6):1177-87.