Attending persistent T cell activation in Alopecia areata: A therapeutic option

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"The ability to convert ideas to things is the secret to outward success."

To my loving parents and my family….

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Abbreviations

AA: Alopecia areata

Ab: Antibody

ADCC: Antibody dependent cell-mediated cytotoxicity

Ag: Antigen

AICD: Activation-induced cell death

Apc: Antigen presenting cell

APC: Allophycocyanin

ARG1: Arginase1

ATRA: All *trans* retinoic acid

BAD: Bcl_2- associated death promoter protein

BAX: Bcl_2- associated X protein

BAK: Bcl_2- homologous antagonist killer

BCL-XL: B-cell lymphoma-extra large protein

BID: BH3-interacting domain death agonist

BCL_2: B-cell lymphoma-2

BSA: Bovine serum albumin

Caspase: Cysteine aspartic proteases

CD: Cluster of differentiation

CD44s: CD44 standard

CD44v: CD44 variant

CFSE: Carboxyfluorescein succinimidyl ester

cm: centimeter

CMP: Common myeloid progenitor

Cys: Cysteine

DC: Dendritic cell

DCP: Diphenylcyclopropenone

DNCB: Dinitrochlorobenzene

DTH: Delayed type hypersensitivity reaction

ECM: Extra cellular matrix

ELAM: Endothelial leukocyte adhesion molecule

ERM: Ezrin, Radixin, Moesin

EAE: Experimental autoimmune encephalitis

FACS: Fluorescent activated cell sorting

FAK: Focal adhesion kinase

FCS: Fetal calf serum

FITC: Fluoresceinisothiocyanate

Flt-3: FMS-related tyrosine kinase 3

FN: Fibronectin

GEM: Glycolipid enriched membrane

GM-CSF: Granulocyte macrophages-colony stimulating factor

G-MDSC: Granulocytic- myeloid derived suppressor cells

HA: Hyaluronic acid

HF: Hair follicle

HLA: Human leukocyte antigen

HRP: Horseradish peroxidase

hrs: Hours

i.v.: Intravenous

IAP: Inhibitor of apoptosis

ICAM: intracellular adhesion moleclue

IFNγ: Interferon γ

Ig: Immunoglobulin

IL: Interleukin

IMCs Immature myeloid cells

IP: Immunoprecipitation

JNK: c-jun N-terminal kinase

Lck: Lymphocyte-specific protein tyrosine kinase

LNC: Lymph node cells

M: Molar

MAP Kinase: Mitogen activated protein kinase

M-CSF: macrophage -colony stimulating factor

MDSC: Myeloid derived suppressor cells

MHC: Major histocompatibility complex

ml: Millilitre

M-MDSC: Monocytic- myeloid derived suppressor cells

MMP: Matrix metalloproteinase

MT1-MMP: Membrane type 1- metalloprotease

ng: Nanogram

NK: Natural killer

NO: Nitric oxide

NF-κB: Nuclear factor-κB

o/n: over night

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PDGF: Platelet-derived growth factor

PE: Phycoerythrin

PI: Propidium iodide

PKC: Protein kinase C

PMA: Phorbol 12-myristate 13- acetate

PTK: Phospho tyrosine kinase

PUVA: Psoralen plus ultra violet A radiation

ROS: Reactive oxygen species

RPM: Revolution per minute

RT: Room temperature

RTK: Receptor tyrosine kinase

RT-PCR: Reverse transcriptase polymerase chain reaction

SADBE: Squaric acid dibutylester

SC: Spleen cells

SCF: Stem-cell factor

SCID: Severe combined immunodeficiency

SD: Standard deviation

SDF-1: Stromal cell-derived factor-1

Ser: Serine

SKIL: Skin infilterating leukocytes

SMAC: Second mitochondria-derived activator of caspase

STAT: Signal transducer and activator of transcription

TAMs: Tumor-associated macrophages

TCR: T cell receptor

TGF: Tumor growth factor

Thr: Threonine

TLR: Toll like receptor

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand

T_{reg} : Regulatory T cells

VCAM: Vascular cell adhesion molecule

VEGF: Vascular endothelial growth factor

VLA: Very late antigen

V: Volts

w/v: weight/volume

WB: Western blot

Zap-70: Zeta associated protein

%: Percentages

˚C: Degree Celcius

µg: Micro gram

µF: Micro farad

Zusammenfassung

Alopecia Areata (AA) ist eine organspezifische Autoimmunerkrankung, welche Haarfollikel in der anagenen Phase angreift und durch eine peri- und intrafollikuläre Infiltration mit $CD4^+$ und $CD8^+$ T Zellen charakterisiert ist.

Die wirkungsvollste Behandlungsmethode besteht in der Induktion eines milden chronischen Kontaktekzems durch die topikale Applikation eines Kontaktallergens wie Sqaricdibutylester, das über mehrere Monate appliziert wird. Die Mechanismen, welche dem therapeutischen Effekt eines Kontaktekzems bei AA zu Grunde liegen, sind weitgehend unbekannt. Es gibt jedoch Hinweise auf eine Expansion sogenannter myeloider Suppressorzellen, die Proliferation und Aktivierung von T-Zellen hemmen. Andererseits kann auch eine Blockade kostimulatorischer Moleküle der T-Zellaktivierung, wie z.B. CD44, therapeutisch wirksam sein.

Aktivierte T-Zellen sind durch eine Hochregulation der Expression von CD44 charakterisiert. Dies führt zu einer Steigerung der T-Zellproliferation und erhöht deren migratorisches Potenzial. Es wurde postuliert, dass diese Effekte auf eine Kooperation von CD44 mit CD49d zurückzuführen sind. Um diese Hypothese zu überprüfen und die zu Grunde liegenden Mechanismen zu klären, habe ich zwei Leukämiezelllinien, EL4 und Jurkat, die entweder CD44 (Jurkat) oder CD49d (EL4) exprimieren mit der CD44 bzw. CD49d cDNA transfiziert. Zusätzlich habe ich CD44 und CD49d Mutanten erstellt, die die Interaktion dieser beiden Moleküle miteinander oder die Interaktion mit dem Zytoskelett oder zytoplasmatischen Kinasen verhindern. Erste Befunde bestätigen die funktionelle Aktivität der transfizierten Moleküle und belegen, insbesondere im Kontext mit der Motilität der T-Zellen, das essentielle Zusammenspiel von CD44 und CD49d und ergaben Hinweise auf beteiligte Zytoskelett- und Signaltransduktionsmoleküle. Entsprechende Untersuchungen der transfizierten Leukämiezelllinien zum Einfluss von CD44 und CD49d auf die T-Zellproliferation belegten, dass das gewählte Modell zur Beantwortung der Frage geeignet ist. Untersuchungen zum molekularen Mechanismus sind noch nicht abgeschlossen.

Diese Befunde deuten auf eine Kooperation von CD44 und CD49d in der T-Zellmigration, -aktivierung und -proliferation hin. Eine Blockade von CD44 und CD49d könnte einen Ansatzpunkt für die Therapie von Autoimmunerkrankungen darstellen.

Um die Frage zu beantworten, ob myeloide Suppressorzellen maßgeblich für den therapeutischen Effekt eines chronischen Kontaktekzems verantwortlich sind, habe ich zerst gezeigt, dass eine Behandlung mit *trans*-Retinolsäure den therapeutischen Effekt eines chronischen Kontaktekzems aufhebt. Es ist bekannt, dass *trans*-Retinolsäure myeloide Suppressorzellen in die Differenzierung treibt und sie dabei ihre suppressive Eigenschaft verlieren. Somit belegt dieser Versuch, dass myeloide Suppressorzellen maßgeblich am Therapieeffekt eines Kontaktekzems bei AA beteiligt sind.

Um die Mechanismen zu klären, mittels derer ein chronisches Kontaktekzem bzw. myeloide Suppressorzellen mit den autoreaktiven T-Zellen der AA interferieren, wurden die Effekte einer Kontaktallergenbehandlung mit denen von *in vitro* Kokulturen von T-Zellen aus AA Mäusen mit myeloiden Suppressorzellen von Kontaktallergen-behandelten Mäusen verglichen. Kontaktallergenbehandlung und myeloide Suppressorzellen inhibieren die Proliferation der T-Zellen von AA Mäusen. Es wurde eine leichte Reduktion der ξ Kettenexpression und eine stark verminderte Aktivierung von Lck und Zap 70 beobachtet. und, weniger ausgeprägt, einer Reduktion der c-jun- und MAPK-Signalwege begleitet. Der stärkste Effekt wurde in Anwesenheit von AA-Hautlysaten, eines autoantigenen Stimulus, gesehen. Die Proliferation wurde aber auch bei T-Zellrezeptor-unabhängiger Stimulation durch PMA und Ionomycin gehemmt. Dieser Befund belegt, dass ein Kontaktallergen bzw. myeloide Suppressorzellen zumindest teilweise unabhängig vom T-Zellrezeptor-Komplex wirken. Tatsächlich steigern ein Kontaktallergen bzw. myeloide Suppressorzellen die Aktivierung verschiedener proapoptotischer Moleküle, die an der mitochondrialen Apoptose beteiligt sind, und hemmen den anti-apoptotischen PI3K/Akt Weg. Der letzt genannte Effekt korreliert mit der TNFα-Sekretion der myeloide Suppressorzellen und TNFRI-Expression in Kontaktallergen behandelten T-Zellen von AA Mäusen.

Zusammenfassend kann gesagt werden, dass die Kontaktallergenbehandlung myeloide Suppressorzellen induziert, welche T-Zellaktivierung inhibieren und dazu beitragen Apoptoseresistenz autoimmuner T-Zellen zu senken, indem sie die Aktivierung von pro-apoptotischen Proteinen induzieren. Kontaktallergen-induzierte myeloiden Suppressorzellen erscheinen daher für die Therapie organspezifischer Autoimmunerkrankungen in besonderer Weise geeignet.

1: Introduction

1.1 Skin and Hair

The skin is the largest organ of the integumentary system and it provides a protective barrier that keeps microbes out and essential body fluids in (1). Skin is composed of three primary layers: the epidermis, which provides water proofing and serves as a barrier to infection; the dermis, which serves as a location for the appendages of skin including hair follicles and the hypodermis (subcutaneous adipose layer) (2). Hair is a filamentous biomaterial, which grows from follicles found in the dermis of the skin and is primarily composed of protein, notably keratin. Hair is one of the defining characteristics of the mammalian class since it is exclusively found in mammals.

1.1.1 Hair Structure

A cross section of the hair may be divided roughly into three zones:

▪ Cuticle: it is the outermost part of the hair shaft. It is a hard shingle-like layer of overlapping cells. The hair cuticle is the first line of defense against all forms of damage; it acts as a protective barrier for the softer inner structure including the medulla and cortex and also controls the water content of the fiber (3).

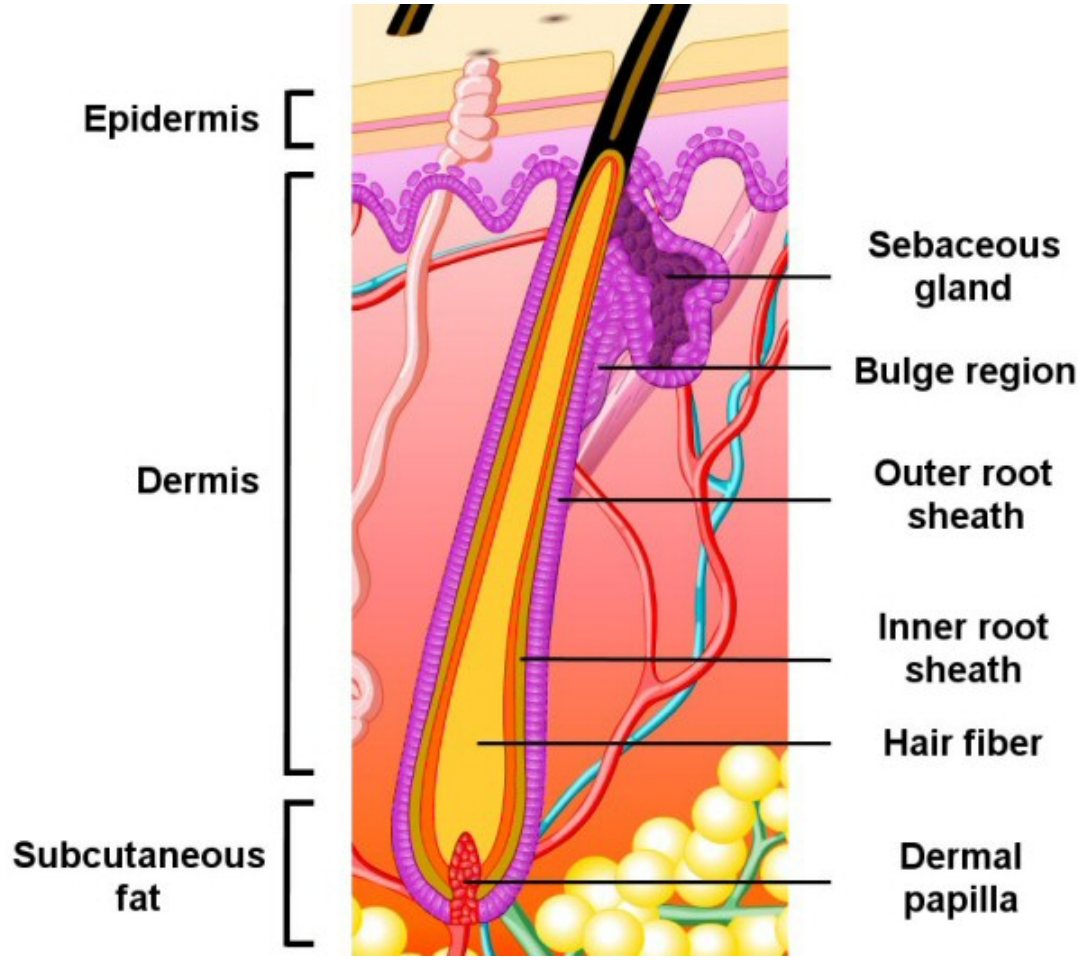
▪ Cortex: it contains the keratin bundles in cell structures and makes up the majority of the hair shaft. It is the cortex that gives hair its special qualities such as elasticity and curl.

▪ Medulla: it is also called the pith or marrow of the hair. It is composed of round cells, two to five rows across. Thick or coarse hair usually contains a medulla while fine hair lacks a medulla (4).

Hair grows from a follicular structure called hair follicle (HF) which serves as a hair root. The mature HF is a complex structure, composed of several concentric cylinders of epithelial cells, known as root sheaths which surrounds the hair shaft (5) The HF contains at its base a ball of specialized dermal cells, the dermal papilla which is fed by the blood stream and plays a very crucial role in the regulation of successive cycles of postnatal hair growth. Signals from the dermal papilla instruct epithelial stem cells

residing in the bulge region of the follicle to migrate to the base of the follicle, where they surround the dermal papilla, forming the hair matrix in response to further

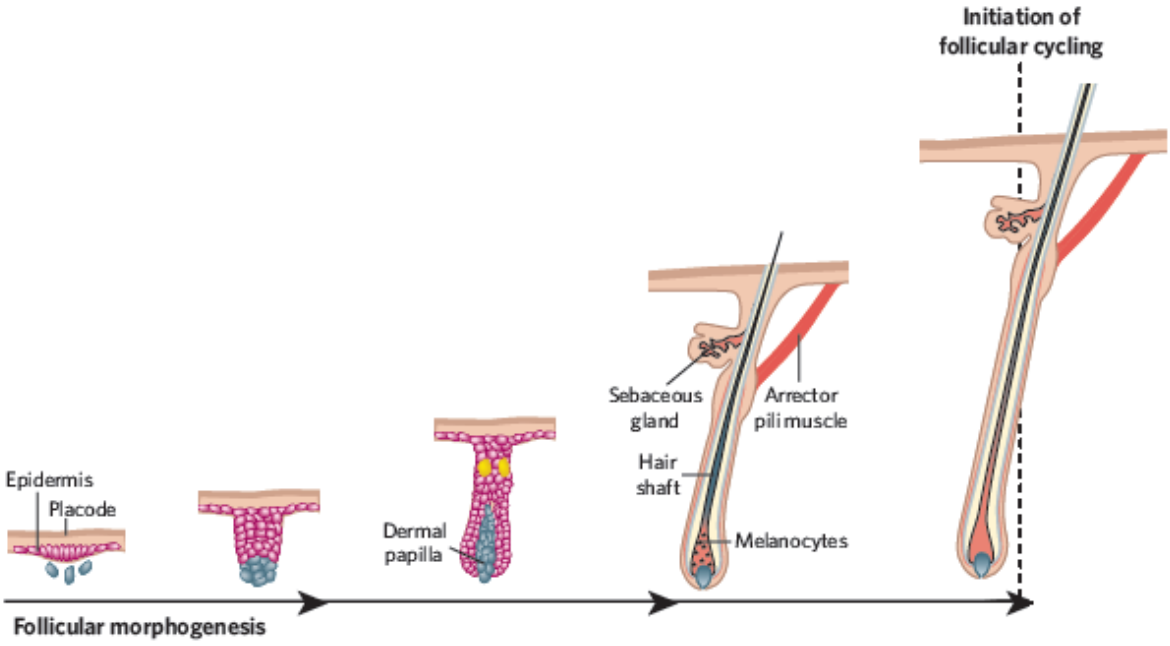
signals from dermal papilla matrix cells proliferate and differentiate to form hair shaft and inner root sheath (Scheme 1). Melanocytes also reside in HF and deposit pigment granules into the hair shaft as it forms (6-8). Keratins are a major structural component of the hair follicle. The hair shaft is a highly keratinized tissue formed within the hair follicle. When the specific epithelial cells in the hair shaft, known as trichocytes, pass through the keratinizing zone, keratinization actively occurs and the rigid hair shaft is generated (9). In humans, a total of 17 functional hair keratin genes (11 type I and 6 type II) have been identified. It has been shown that these hair keratins, except for one type II keratin K84 (Hb4), are abundantly and sequentially expressed in the hair shaft (10, 11, 12).



Scheme1. Structure of hair follicle.

1.1.2 Hair follicle morphogenesis

The formation of hair follicles occurs during embryogenesis through a series of signals between dermal cells and overlying surface epithelial cells resulting in differentiation of the hair shaft, root sheaths, and dermal papilla (13). The epithelial cells that receive this 'first dermal signal' form a thickening of columnar cells known as a placode. Subsequently, a signal from the placode leads to the formation of a mesenchymal (dermal) condensate just beneath the placode. In response to a 'second dermal signal' from the dermal condensate, the epithelial placode cells proliferate and invade the dermis, eventually surrounding the dermal condensate, which develops into dermal papilla. Further proliferation and differentiation of the epithelial cells results in the formation of the inner root sheath and hair shaft of the mature hair follicle. Finally, the epithelial cells undergo proliferation and eventual differentiation into several distinct layers (14, 15) (Scheme 2).



Scheme2. Follicular morphogenesis (17).

1.1.3 Hair Growth cycle

Each hair follicle undergoes a cycle of activity. The hair grows to a maximum length, then hair growth ceases and the hair is shed and replaced. At any one time we have only around 85% of our hair growing, the rest being in the resting stages. The hair

growth cycle has three distinctive phases (Scheme 3); Anagen: the period of active growth; Catagen: the period of breakdown and change; Telogen: the resting stage before resumption of growth

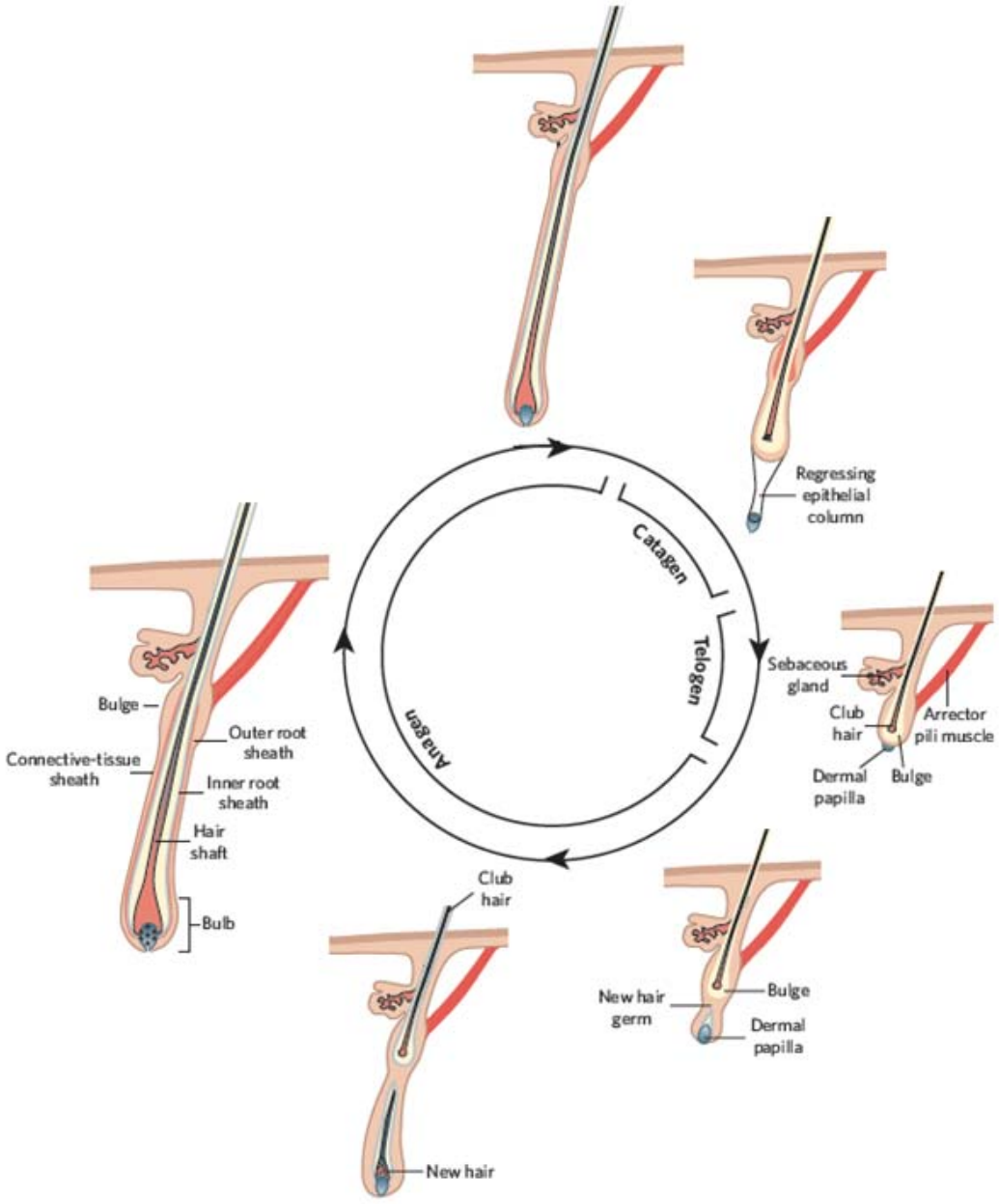
1.1.3.1 Anagen: The epidermal cells surrounding the dermal papilla form the germinal matrix or root of the hair. These cells are constantly dividing, and as new cells are formed they push the older ones upwards where they begin to change shape. By the time the cells are about one-third of the way up the follicle they are dead and fully keratinized (16). In the anagen phase, the HF grows downward and forms the matrix region at its base. These matrix cells actively proliferate and differentiate into several distinct cell types, giving rise to the companion layer, the inner root sheath, and the hair shaft (17). A scalp hair will grow actively for between one and a half and seven years (three years being an average growth period). The average growth rate is about half an inch per month. On average 85% of follicles are in the anagen stage.

1.1.3.1 Catagen: This is the end of the active growth period, and is marked by changes occurring in the follicle. The hair stops growing and becomes detached from the base of the follicle forming a club hair. The hair bulb begins to break down, resulting in the follicle becoming shorter. A small section of the outer root sheath remains in contact with the group of cells that formed the papilla. This period of breakdown or change lasts about three weeks. As the inner root sheath breaks down, the hair remains in the follicle due to its shape. On average, 1% of follicles are in the catagen stage. In the catagen phase, the lower portion of the HF undergoes apoptosis and regresses toward the permanent portion, before the HF returns to the telogen phase. Importantly, the mesenchyme-derived dermal papilla regresses and descends with the lower portion of the HF during catagen (17).

1.1.3.2 Telogen: The section of remaining root sheath still in contact with the papilla is known as the secondary or root germ. It is from this root germ that a new hair can grow. The shortened follicle rests for about three months. The hair may be brushed out at this time or at the onset of anagen. On average 14% of follicles are in the telogen stage.

After the telogen stage the cycle returns to anagen and the root germ begins to grow downwards and forms a new bulb around the dermal papilla. It is the lower end of the

germ that forms the new bulb, producing a new hair. The upper part of the germ forms the new cells that lengthen the follicle below the club hair. The new hair may push the old hair out; therefore sometimes one may see two hairs in the same follicle (18, 19).



Scheme3. The hair cycle: The stages of the hair cycle are depicted, starting from the first postnatal anagen, when the hair shaft is growing and protruding through the skin surface. Follicles progress synchronously to the destructive (catagen) phase, during which the lower two-thirds of the follicle undergo apoptosis and regress. The dermal papilla is brought to rest below the bulge-stem-cell compartment, and after the resting (telogen) phase, a critical threshold of activating factors is reached and the stem cells become activated to regrow the hair (17).

1.2 Diseases associated with hair

The loss of hair is known as Alopecia, this disease is not life threatening but the pressures of an image orientated society can make hair loss psychologically devastating for those affected, their families, and friends.

The loss of hair could be either permanent or temporary, based on these clinical observations; alopecia is further classified broadly as:

- Scarring (cicatricial) alopecia: hair loss associated with fibrosis and scar tissue that replaces and often permanently destroys the hair follicle.
- Non-Scarring alopecia: hair loss without permanent destruction of the hair follicle (20).

1.2.1 Scarring (cicatricial) Alopecia

This disorder destroys the hair follicle and replaces the follicle with a scar of fibrous tissue causing permanent hair loss. In some cases, the hair loss is gradual and no symptoms are evident for long periods. In other cases, scarring alopecia causes are associated with severe itching, burning and pain which are rapidly progressive. The inflammation destroys the follicle below the skin surface but above the skin surface generally not much inflammation is visible. Studies leads to a hypothesis that scarring alopecia involves the malfunctioning of one of the hair structure machineries, which includes the stem cell rich region in the hair follicle and the outer root sheath (21). Most kinds of the alopecia of the scarring type involve the inflammatory infiltration of different immune cells and affecting one of these structures.

On the basis of the predominant type of inflammatory cell component, the scarring alopecia is further classified into three groups: Lymphocytic scarring alopecia, Neutrophilic scarring alopecia and Mixed inflammatory scarring alopecia

1.2.1.1 Lymphocytic scarring alopecia: It is associated with infiltration of lymphocytes into structural component of hair. The most common example of this type of alopecia is Discoid Lupus Erythematosus (DLE) or Chronic Cutaneous Lupus Erythematosus. Clinically DLE is characterized by ill defined patches of alopecia, with decreased follicular orifices, scale erythema, follicular plugging, depigmentation and atrophy (22). Some studies suggest that exposure to ultraviolet light provokes or

incites keratinocyte apoptosis and a reactive T-cell- or immune-complex-mediated response.

1.2.1.2 Neutrophilic scarring alopecia: It involves intrafollicular and perifollicular neutrophil infiltration. One of the examples of this kind is Folliculitis Decalvans, characterized by inflamed hair follicles that discharge pus. This scarring alopecia is an outcome of the patient's susceptibility to infections due to systemic or local immune deficits and *Staphlococcus aureus* strain related properties. (Both acquired and inherited immune disturbances are associated with folliculitis decalvans) (23).

1.2.1.3 Mixed inflammatory scarring alopecia: It involves both neutrophil and lymphocytes infilteration in intrafollicular and interfollicular regions. Example includes, Folliculitis (Acne) Keloidalis, early in this disease there is follicular dialation with neutrophil and follicular rupture, late lesions shows perifollicular granulomas around naked hair shafts mixed with a lymphoplasmacellular cell infiltrate and hypertrophic scar with broad eosinophilic hyalinized keloidal collagen bundles. Sebaceous glands are absent in this disease (24-26).

1.2.2 Non- Scarring Alopecia:

Non scarring alopecia involves temporal hair loss. Hair follicles are intact and are not damaged. Clinically in non-scarring alopecia follicular orifices are grossly apparent on the scalp and histo-pathologically the follicular units are intact. Hence hair re-growth is possible in non-scarring alopecia. This kind of alopecia involves different kinds:

1.2.2.1 Androgenetic alopecia: It is the most common type of hair loss. It is a disorder of dominant inheritance with variable penetrance (27, 28) affecting approximately half of the population by the age of 50 years, of both the sexes (28, 29). Clinically it is a patterned alopecia, i.e. it is characterized by bitemporal recession and vertex balding in men (30) and in women (female pattern hair loss) by diffuse hair thinning of the crown with an intact frontal hair line (31). The total numbers of hair follicle remains unchanged but are genetically programmed, under the influence of androgens to undergo miniaturization (28, 32-34).

1.2.2.2 Telogen effluvium: it is a diffuse form of alopecia, in which the hair falling may be acute or chronic. Acute telogen effluvium can occur in both male and female and can be triggered by numerus factors like major surgery, injury, severe illness, childbirth, crash diet and numerous medications (33,35).

1.2.2.3 Traction alopecia: It is a non-inflammatory, non-scarring alopecia which occurs due to mechanical damage. Clinically, the hair loss is often seen at the margins of the scalp, involving the frontal, temporal and parietal regions. In early traction alopecia the hair loss is temporary; in late traction alopecia there is marked loss of the terminal follicles with preservation of the vellus hairs and sebaceous glands and can lead to scarring process and thus permanent hair loss (36).

1.2.2.4 Alopecia areata: Alopecia areata (AA) is an inflammatory hair loss disease that can affect men, women and children. Circumstantial evidence suggests alopecia areata is an autoimmune disease where cells of an individual's own immune system prevent hair follicles from producing hair fiber. Alopecia affects certain stages of hair growth cycle the most affected stage could be the active phase, anagen stage which is characterized by active DNA synthesis and melanogenesis.

Clinically, it is characterized by sudden onset of patches of nonscarring hair loss, with ‘exclamation-point’ hairs. It may involve the entire scalp (alopecia totalis) and body hair (alopecia universalis), nail changes (pitting, thickening and ridging) may be seen. The early active stage is characterized by a peribulbar lymphoid cell infiltrate (33, 37) affecting the terminal hair follicles (35) and may invade the follicular epithelium and the matrix, as well as extend above the hair bulb and into fibrous tracts (37). Initially, the terminal hairs are attacked, but subsequently also the vellus hairs become involved. Eosinophils (38) and plasma cells may be present (39, 37). There is a 70–90% ‘shift out of anagen’ of the hair follicles into catagen or telogen phase (33), but the number of hair follicles is unchanged. In longstanding (chronic) stages, with repeated episodes, the peribulbar lymphoid cell infiltrate also involves miniaturized hairs (35, 37). The majority of the hair follicles will be in catagen/telogen phase.

1.3 Alopecia areata as an autoimmune disease

Alopecia areata (AA) is a heterogeneous disease characterized by non scarring hair loss on the scalp or any hair-bearing surface. It is one of the most frequently occurring organ-restricted autoimmune diseases in humans targeted to hair follicle (40).

However, the antigenic targets, mechanisms, and consequences of autoimmune attack in AA have yet to be determined, but the evidence that autoimmunity may play a role in AA is dependant upon following factors:

i) Its association with other autoimmune diseases, like vitiligo and thyroid diseases including Hashimoto's Thyroiditis also, anti-thyroid antibodies and thyroid microsomal antibodies have been found in AA patients (41, 42).

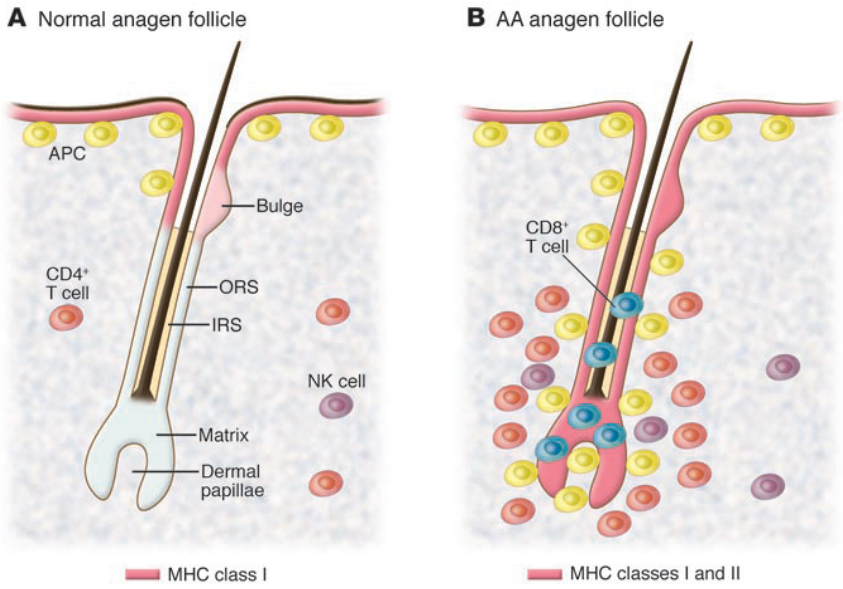
ii) Response of AA patient to immunosuppressive or immunomodulatory therapy.

iii) The characteristic infiltration of lymphocytes is present in and around the hair bulbs in active disease (43). In AA the infiltrate consists predominantly of activated T lymphocytes along with macrophages and Langerhans cells (44-46). Both $CD4^+$ and $CD8^+$ T lymphocytes are observed peri- and intra-follicularly. Studies involving injections of separated $CD4^+$ and $CD8^+$ or both cells together demonstrated that injection of purified single cell type alone did not result in hair loss, in contrast mixture of $CD4^+$ and $CD8^+$ T cells results in significant loss of hair. These results suggest that both $CD4^+$ and $CD8^+$ T cells have an important role in the pathogenesis of AA (47). An increased expression has been observed of class I MHC and class II MHC antigens in and around the lesions.

iv) Pro-inflammatory changes occur both in the skin and in immune system organs. Inflammatory markers include the upregulation of expression of intercellular cell adhesion molecule (ICAM) and endothelial leukocyte adhesion molecule (ELAM) on the endothelium of blood vessels closely associated with affected hair follicles (48-50). Changes in cytokine levels, particularly activating cytokines such as interleukin 2 (IL- 2) and interferon-γ (IFN-γ) have been noted in patients with AA compared to normal controls (51).

v) Associations between specific human leukocyte antigen (HLA genes) and AA lend further circumstantial evidence that autoimmune mechanisms are involved in the pathogenesis of disease. Normal hair follicle epithelium typically does not express the HLA class I antigen A, B and C. There is a concomitant increased expression of HLA class I (HLA-A, B, and C) and class II (HLA-DR) antigens in AA, (52, 53). In

particular, HLA class II alleles – DR4, DR5 (DR11), DR6 (54-61), and HLA-DQ3 (including both subtypes DQB1*0301 (DQ7) and DQB1*0302 (DQ8)) (54-56, 64, 65) have been most consistently associated with AA. Furthermore, specific HLA associations have been reported as markers of clinical sub-types of AA (64). In the more severe forms of the disease–alopecia totalis (AT) and alopecia universalis (AU) – DRB1*0401 (DR4) and DQB1*0301(DQ7) are expressed with increased frequency (64). Hair follicles in the anagen phase are recognized sites of immune privilege. HF immune privilege is characterized by down regulation of MHC class I expression and strong local expression of immunosuppressants. HF appear to maintain immune privilege by actively suppressing natural killer cells (66) and showed that the NK cell inhibitor, macrophage migration inhibitor factor, is strongly expressed by HF epithelium and that very few NK cells are observed in and around normal anagen HF compared to AA, in which they are prominently aggregated. A collapse of the immune privilege (IP) of the hair follicle results in the loss of hair, as seen in patients with AA (Scheme 4).



Scheme4. A model of immune privilege collapse in AA pathogenesis: Both a normal anagen (growing) hair follicle (A) and a hair follicle in AA (B) are shown. MHC class I molecules are expressed on the epidermis, and on the most superficial (distal) portion of the normal hair follicle epithelium. The inferior (proximal) portions of the hair follicle are immune privileged and deficient in expression of MHC classes I and II as well as APCs. By contrast, the AA anagen hair follicle expresses MHC class I and II molecules throughout the follicular epithelium, including the portion adjacent to the dermal papilla of the hair follicle. Active AA also exhibits a perifollicular infiltrate of $CD4^+$ T cells and an intrafollicular infiltrate of $CD8^+$ T cells. IRS, inner root sheath; ORS, outer root sheath (65).

Experimental studies also give stronger evidence that autoimmune phenomenon is involved in AA, for example lesional scalp skin from AA patients grafted onto nude (athymic) mice shows regrowth of hair, suggesting a loss of inhibition by T cells. These experiments strongly suggest that the key mediators of AA reside outside the follicle itself. Given that lesional skin grafts are able to regrow hair in an athymic host, functional T cell populations appear to be involved in the pathogenesis of this disease (67). The strongest evidence implicating autoimmune mechanisms in the pathophysiology of alopecia areata has been provided by studies involving mice with severe combined immunodeficiency (SCID) (68). AA was induced on human scalp explants transplanted onto SCID mice by injection of autologous T lymphocytes from lesional skin. In particular, only T lymphocytes cultured with hair follicle homogenate and antigen presenting cells were capable of inducing AA in scalp explants. Both the clinical and histopathologic features of AA were reproduced in this model, including hair loss, perifollicular T lymphocyte infiltration, and expression of HLA-DR and ICAM-1 in the follicular epithelium. The induction of these changes was not a nonspecific effect of T cell activation, as lesions could not be produced by injection of IL-2 activated T cells from peripheral blood or scalp. Moreover, the fact that these changes could not be induced by lesional scalp T cells that were not cultured with follicular homogenate strongly suggests that the T lymphocytes involved in AA are reacting to specific follicular antigens (68).

1.4 Treatment of AA

There are several potential therapeutic drugs for treatment of alopecia areata.

1.4.1 Immunosuppressive

▪ **Corticosteroids:** corticosteroids are known to exert a strong inhibitory effect on the activation of T Lymphocytes, and also these can result in Th-1 mediated immune attack on the hair follicle in AA. Topical, intralesional, and systemic corticosteroids have been used to treat AA, with different side-effects. But the rate of treatment success was not statistically significant (70-76). This treatment is only indicated in patchy AA with longstanding bald areas. It's a painful procedure and can lead to permanent atrophy after injection. Whereas, initially, oral corticosteroids were used daily or every other day for several months (30 and 150 mg daily) giving rise to

unacceptable side effects such as hypertension, diabetes, immunosuppression, osteoporosis and proneness to thrombosis. Taken together, corticosteroids treatment is reasonable in exceptional, selected cases of AA, but has potentially serious side-effects.

▪ **PUVA:** Several studies have examined treatment of AA with PUVA using either oral or topical application of 8-methoxypsoralen (8-MOP) with ultraviolet A radiation (UVA) on the scalp or the whole body (77-83). There are a large number of recurrences (between 30% and 50% of successfully treated patients).due to the fact that regrown hair inhibits UVA radiation from reaching the skin. Long treatment with PUVA can lead to high risk of skin malignancies

▪ **Tacrolimus:** FK506 is an immunosuppressive agent that can be applied topically to the skin. FK506 suppresses IL-2 production and release in activated T cells. Subsequently, activation and proliferation of T cells are inhibited (84). Therefore, FK506 is a promising candidate for the treatment of AA. In the C3H/HeJ mice model of AA, topically applied FK506 induces hair regrowth (85,86), accompanied by reduced peri- and intrafollicular infiltrates of $CD4^+$ and $CD8^+$ T cells and decreased expression of MHC class I, MHC class II and ICAM-1 on hair follicle epithelium. These encouraging results obtained in animal models suggested that topical application of FK506 could be effective in the treatment of human AA.

1.4.2 Immunomodulatory

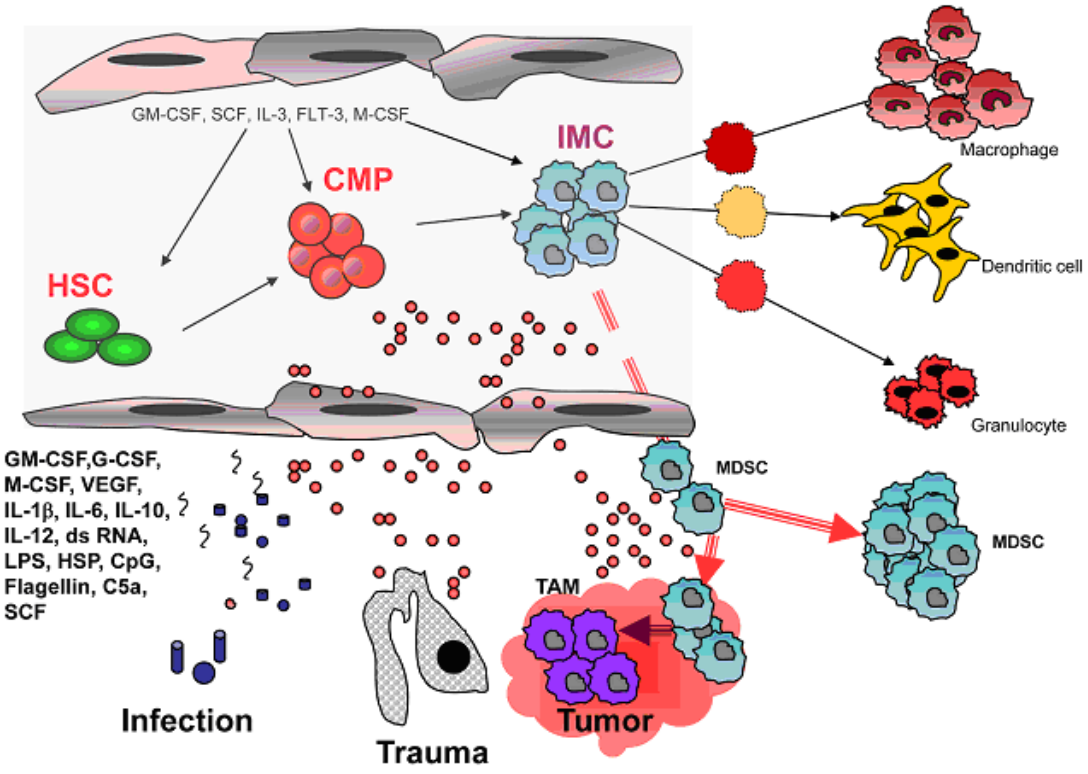
▪ **Squaric acid dibutylester (SADBE):** AA has been treated with contact sensitizers for more than 20 years. Dinitrochlorobenzene (DNCB) was the first to be used (87), but because it has been shown to be mutagenic in the Ames test, it can no longer be recommended (88, 89). Today diphenylcyclopropenone (DCP) or squaric acid dibutylester (SADBE), which are not mutagenic in the Ames test, are widely used. Treatment with contact sensitizers are preceded by sensitization of the patient with 2% SADBE solution on a small area of the scalp. Two weeks later, treatment is initiated by applying a 0.001% SADBE solution, followed by weekly applications of increasing concentrations until a mild eczematous reaction is obtained. In this way, an appropriate eliciting concentration of SADBE is identified for each patient. This concentration is then applied once a week to induce a mild eczematous reaction characterized by itching and erythema, without blistering or oozing. SADBE is used

in those patients who become tolerant to DCP. Initial hair regrowth is usually visible after 8 to 12 weeks. Treatment must be continued once weekly until complete hair regrowth is obtained. Treatment intervals are then decreased, and, eventually treatment may be discontinued. However, if relapse occurs, treatment can be restarted immediately to stop further progression of AA and to induce renewed hair growth. As applicable to many drug therapies, SADBE treatment also has some side effects like mild eczematous reactions and enlargement of retroauricular lymph nodes, these are usually well tolerated if patients are informed that they are desired for the therapeutic effect. Undesired side-effects are noted in 2 to 5% of patients (90). Dissemination of allergic contact dermatitis, urticarial or erythema multiforme-like reactions may also occur (91), but these can be treated successfully with topical corticosteroids. Pigmentary disturbances such as post-inflammatory hyperpigmentation with spotty hypopigmentation have been observed, especially in patients with dark skin, but these have resolved within 1 year after discontinuing treatment in most instances (92,93). Apart from these acute and subacute side-effects, no long-term side-effects have been reported after 21 years of SADBE (18 years of DCP) treatment worldwide of about 10000 patients, including children. However mechanism behind the curative effect is still unclear. Several studies point towards the decreased ratio of $CD4^+$:$CD8^+$ T lymphocytes with reduction in IFNγ expression elevated IL2, IL10 and TNFα expression and also an increase in apoptosis of autoreactive T cells. Recent studies have provided a strong evidence of hindrance of APC migration and a strong expansion of myeloid derived suppressor cells (MDSC) that suppress T cell activation. Till date, SDABE treatment has been proved to be the best treatment for AA in human with minimum of side effects.

1.5 Myeloid derived suppressor cells (MDSC)

In late 1970's an unknown population of cell with suppressive features were identified. These were named as natural suppressor cells and were first identified in bone marrow and spleens of tumor bearing mice which were able to suppress T cell responses *in vivo* and *in vitro*. Further characterization demonstrated their involvement in immune tolerance induction by inhibiting different activities of the immune system. These cells were later named as myeloid derived suppressor cells (MDSC) since they were involved in regulating myeloid cell differentiation. MDSC

represent a heterogenous population of immature myeloid cells that consists of myeloid progenitors and precursors of macrophages, granulocytes and dendritic cells and are characterized by a strong ability to suppressor various T cell functions (Scheme 5). This heterogenticity demonstrates the plasticity of this immune suppressive myeloid compartment and shows, how various tumors and infectious agent can have similar effects on myeloid cells despite the differences in the factors that they produce to influence the immune system (94).



Scheme5. The origin of MDSC: Immature myeloid cells (IMCs) are part of the normal process of myelopoiesis, which takes place in the bone marrow and is controlled by a complex network of soluble factors that include cytokines such as granulocyte/macrophage colony-stimulating factor (GM-CSF), stem-cell factor (SCF), interleukin-3 (IL-3), FMS-related tyrosine kinase 3 (FLT-3), macrophage colony-stimulating factor (M-CSF) and cell-expressed molecules including Notch (not shown). Haematopoietic stem cells (HSCs) differentiate into common myeloid progenitor (CMP) cells and then into IMCs. Normally, IMCs migrate to different peripheral organs, where they differentiate into dendritic cells, macrophages and/or granulocytes. However, factors produced in the tumour microenvironment and/or during acute or chronic infections, trauma or sepsis, promote the accumulation of IMCs at these sites, prevent their differentiation and induce their activation. These cells exhibit immunosuppressive functions and are therefore known as myeloid-derived suppressor cells (MDSC). MDSC can also differentiate into tumor-associated macrophages (TAMs) within the tumor environment, which are cells that have a phenotype and function that is distinct from MDSC (95).

1.5.1 MDSC surface markers and subsets

MDSC are identified as cells that simultaneously express the two markers CD11b and Gr-1. More recently MDSC were subdivided into two different subsets based on their expression of the two molecules Ly6C and Ly6G. The nuclear morphology and content of immunosuppressive substances have also been used to characterize mouse MDSC that are mononuclear, are considered "monocytic" and typically are $CD11b^{+}Ly6G^{+}Ly6C^{high}$, whereas those with multi-lobed nuclei are 'granulocytic/neutrophilic-like' and have $CD11b^{+}Ly6G^{+}Ly6C^{low}$ phenotype (96-98). Importantly, evidences indicate that these two subpopulations may have different functions in cancer, infectious and autoimmune diseases (99-101). The variations in MDSC phenotype are consistent with the concept that MDSC are a diverse family of cells that are in various intermediate stages of myeloid cell differentiation. Because the myeloid population contains many different cell types and myeloid cell differentiation is a continuum of processes, MDSC may display diverse phenotypic markers that reflect the spectrum of immature to mature myeloid cells (102). This diversity prompted the search for markers that could be used to identify such a population. Several potential candidates were suggested, such as CD115 (M-CSFR), CD124 (IL-4Rα), CD40 and CD80 (103-106), however further studies indicated that although these markers are undoubtedly expressed on MDSC, they do not define specific immune suppressive populations of MDSC (107).

In humans, the phenotype of these cells is less clearly defined, although recent studies have shown CD15 and CD66b as additional markers allowing for detection of G-MDSC and M-MDSC (108,109). These two major subsets of MDSC apparently have an important role in the antigen-specific versus non-specific nature of immune suppression. G-MDSC, which use reactive oxygen species (ROS) for their suppressive functions, require close cell - cell contact with T cells, which in turn is manifested by the strong reliance on antigen-specific interaction between MDSC and T cells (110). MDSC, which use up-regulation of NO and arginase production of immune suppressive cytokines and other mechanisms, effectively suppress antigen-dependent T cell responses without requiring direct cell-cell contact. Evidence from various reports suggests that on a per cell basis M-MDSC are more potent then G-MDSC (111-114). Experiments demonstrating that treatment with all trans-retinoid acid convert MDSC to DCs (115,116) support the concept that MDSC are normal

intermediaries. MDSC may play a role in normal homeostasis and maintenance of tolerance to self antigens.

1.5.2 MDSC and suppressive activity

MDSC suppress immunity, both innate and adaptive immune responses. Subsequent studies showed that the immunosuppressive functions of MDSCs require direct cell–cell contact and can be antigen-specific or non-specific, which suggests that they act either through cell-surface receptors and/or through the release of short-lived soluble mediators (95). Following are the mechanisms by which these cells suppress the immune response (Scheme 6):

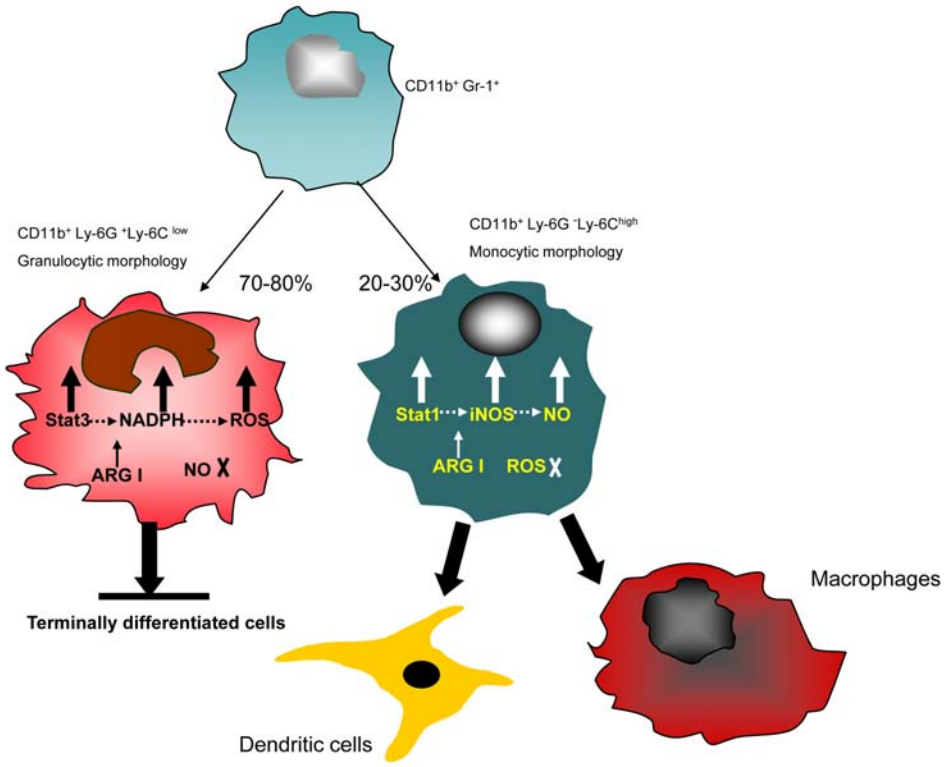
1.5.2.1 Arginase1 and iNOS: The suppressive activity of MDSCs has been associated with the metabolism of L-arginine. L-arginine serves as a substrate for two enzymes: iNOS, which generates NO, and arginase1 (ARG1), which converts L-arginine into urea and L-ornithine. MDSCs express high levels of both arginase and iNOS, and a direct role for both of these enzymes in the inhibition of T-cell function is well established (117,118). Recent data suggest that there is a close correlation between the availability of arginine and the regulation of T-cell proliferation (119,120). The increased activity of arginase in MDSC leads to enhanced L-arginine catabolism, which depletes this non-essential amino acid from the microenvironment. The shortage of L-arginine inhibits T-cell proliferation through several different mechanisms, including decreasing their CD3ζ expression (121). In absence of ζ chain $CD4^+$ and $CD8^+$ T cells are unable to transmit the required signals for activation and it also prevents the upregulation of the expression of the cell cycle regulators cyclin D3 and cyclin-dependent kinase 4 (CDK4) (122). Additionally inhibiting the activity of arginase and iNOS, which are expressed in malignant but not in normal prostate tissue and are key enzymes of L-arginine metabolism, led to decreased tyrosine nitration and restoration of T-cell responsiveness to tumor antigens (123). NO suppresses T-cell function through a variety of different mechanisms that involve the inhibition of JAK3 and STAT5 in T cells , the inhibition of MHC class II expression (124) and the induction of T-cell apoptosis (125).

1.5.2.2 Reactive oxygen species (ROS): Another important factor that contributes to the suppressive activity of MDSC is ROS. Increased production of ROS has emerged as one of the main characteristics of MDSC in both tumour-bearing mice and patients with cancer (126-130). Inhibition of ROS production by MDSC isolated from mice and patients with cancer completely abrogated the suppressive effect of these cells in vitro (126,127,129). Interestingly, ligation of integrins expressed on the surface of MDSC was shown to contribute to increased ROS production following the interaction of MDSC with T cells (126). In addition, several known tumour-derived factors, such as TGFβ, IL-10, IL-6, IL-3, platelet-derived growth factor (PDGF) and GM-CSF, can induce the production of ROS by MDSC (131).

1.5.2.3. Peroxynitrite: More recently, it has been described that peroxynitrite (ONOO-) is a crucial mediator of MDSC-mediated suppression of T-cell function. Peroxynitrite is a product of a chemical reaction between NO and superoxide anion (O^2 -) and is one of the most powerful oxidants produced in the body. It induces the nitration and nitrosylation of the amino acids cysteine, methionine, tryptophan and tyrosine (132). Increased levels of peroxynitrite are present at sites of MDSC and inflammatory-cell accumulation, including sites of ongoing immune reactions. In addition, high levels of peroxynitrite are associated with tumor progression in many types of cancer (132-135), which has been linked with T-cell unresponsiveness. High levels of nitrotyrosine were present in the T cells, which suggested the production of peroxynitrites in the tumor environment. In addition, it has been demonstrated that peroxynitrite production by MDSC during direct contact with T cells results in nitration of the T-cell receptor (TCR) and $CD8^+$ molecules, which alters the specific peptide binding of the T cells and renders them unresponsive to antigen-specific stimulation. However, the T cells maintained their responsiveness to nonspecific stimuli (136). This phenomenon of MDSC induced antigen-specific T-cell unresponsiveness was also observed *in vivo* in tumor-bearing mice (128).

1.5.2.4. Cysteine deprivation: Recent work demonstrates that MDSC also block T cell activation by depriving the environment of cysteine, an amino acid that is essential for T cell activation. T cells lack the enzyme to convert methionine to cysteine and the membrane transporter to import cysteine, which could be reduced intracellularly to cysteine, and therefore must obtain their cysteine from extracellular

sources. Under healthy conditions, APCs (i.e., DC and macrophages) synthesize cysteine from methionine and import extracellular cystine and convert it to cysteine. Surplus cysteine is then exported during antigen presentation and imported by T cells. MDSC are also unable to convert methionine to cysteine, so they are fully dependent on importing cystine for conversion to cysteine. When MDSC are present in high concentrations they import most of the available cystine, depriving DC and macrophages of cystine. Because MDSC do not export cysteine, their immediate environment is cysteine-deficient and T cells are unable to synthesize the necessary proteins for activation (102).



Scheme6. Suppressive mechanisms mediated by different subsets of MDSC: Myeloid-derived suppressor cells (MDSC) consist of two major subsets: granulocytic MDSC with a $CD11b^{+}Ly6G^{+}Ly6C^{low}$ phenotype and monocytic MDSCs with a $CD11b^{+}Ly6G^{-}Ly6C^{high}$ phenotype. In most tumour models, it is predominantly (70–80%) the granulocytic subset of MDSC that expands. It has been hypothesized that the granulocytic subset of MDSCs has increased activity of STAT3 (signal transducer and activator of transcription 3) and NADPH, which results in high levels of reactive oxygen species (ROS) but little nitric oxide (NO) production. ROS and, in particular, peroxynitrite (the product of a chemical reaction between superoxide and NO) induces post-translational modification of T-cell receptors and may cause antigen-specific T-cell unresponsiveness. The monocytic MDSC subset has upregulated expression of STAT1 and inducible nitric oxide synthase (iNOS) and increased levels NO but little ROS production. NO, which is produced by the metabolism of L-arginine by iNOS, suppresses T-cell function through a variety of different mechanisms that involve the inhibition of

Janus kinase 3 (JAK3) and STAT5, the inhibition of MHC class II expression and the induction of T-cell apoptosis. Both subsets have elevated level of arginase-1 (ARG-1) activity that causes T-cell suppression through depletion of arginine. Only monocytic MDSCs can differentiate into mature dendritic cells and macrophages *in vitro* (95).

1.5.2.5. Subset-specific suppressive mechanisms: Recent findings indicate that different subsets of MDSC might use different mechanisms to suppress T-cell proliferation. As already discussed, two main subsets of MDSC have been identified: a granulocytic subset and a monocytic subset. The granulocytic subset of MDSC was found to express high levels of ROS and low levels of NO, whereas the monocytic subset expressed low levels of ROS and high levels of NO and both subsets expressed ARG16. Interestingly, both populations suppressed antigen-specific T-cell proliferation to an equal extent, despite their different mechanisms of action. The suppressive activity of the granulocytic subset was ARG1-dependent, in contrast to the STAT1- and iNOS-dependent mechanism of the monocyte fraction (101).

1.5.2.6 Induction of regulatory T cells: MDSC can also promote the development of $FoxP3^+$ regulatory T (T_{Reg}) cells (137, 138). The induction of T_{Reg} cells by MDSC was found to require the activation of tumor-specific T cells and the presence of IFNγ and IL-10, but was independent of NO (138). In a mouse model of lymphoma, MDSC were shown to induce T_{Reg}-cell expansion through a mechanism that required arginase and the capture, processing and presentation of tumor-associated antigens by MDSC, but not TGFβ. In contrast, some other studies suggested that MDSCs were not involved in T_{Reg} cell expansion since it was found that the percentage of T_{Reg} cells was invariably high throughout tumor growth and did not relate to the kinetics of expansion of the MDSC population (101). Furthermore, in a rat model of kidney allograft tolerance that was induced with a CD28-specific antibody, MDSCs that were co-expressing CD80 and CD86 were found to have a limited effect on the expansion of the T_{Reg} cell population (139). Although further work is required to resolve these discrepancies it seems possible that MDSCs are involved in T_{Reg}-cell differentiation through the production of cytokines or direct cell–cell interactions. Furthermore, MDSC and T_{Reg} cells might be linked in a common immunoregulatory network.

1.5.3. Expansion and activation of MDSC

MDSC accumulation and expansion are driven by multiple factors which may be endogenous or exogenous and can be divided into two main groups. The first group includes factors that are produced mainly by tumor cells and promote the expansion of MDSC through stimulation of myelopoiesis and inhibiting the differentiation of mature myeloid cells. The second group of factors is produced mainly by activated T cells and tumor stroma, and is involved in directly activating MDSC (95).

1.5.3.1Granulocyte–macrophage-colony-stimulating factor: Granulocyte macrophage colony-stimulating factor (GM-CSF) acts at earlier stages of lineage commitment during the steady state regulating the expansion and maturation of early hematopoietic progenitors (113, 140). High concentrations of GM-CSF such as produced by activated T cells (141), NK-cells and DC (142) during immune responses may lead to expansion, redistribution and activation of Gr-1^{+}CD$11b^{+}$ cells. It has been demonstrated that GM-CSF acts differentially on the two MDSC subsets. It expanded both, CD$11b^{+}$Gr-1^{int} and CD$11b^{+}$Gr-1^{low} subsets in the spleen of tumor-bearing mice, but expanded only the CD$11b^{+}$Gr-1^{low} subset in the bone marrow (143).

1.5.3.2 Vascular endothelial growth factor (VEGF): VEGF and its receptors have significant effects in early developmental stages and differentiation of vascular and hematopoietic progenitors. Within tumor-associated diseases, VEGF reduces the amount of DC and interferes with their function *in vivo* and *in vitro*, whereas the number of immature precursors of DC increases (144-146). Blocking the activation of VEGF receptor was effective at controlling tumor growth and inhibiting the infiltration of suppressive immune cells like MDSC, regulatory T cells and macrophages, while increasing the mature DC fraction (147).

1.5.3.3 Prostaglandins: Prostaglandins, in particular PGE2, have been widely implicated in MDSC-mediated T cell inhibition. In an early study, signaling through the PGE2 receptor E-prostanoid (EP) 4 in MDSC was found to induce the expression and activity of arginase1. PGE2 promote tumor progression through non-immune mechanisms and by limiting anti-tumor immunity through the induction of higher levels and more suppressive MDSC (148).

1.5.3.4 Interferon-γ: IFNγ is secreted by many cells of the immune system and influences functions of T cell and MDSC (149-151). MDSC, in response to exogenous or autocrine production of IFNγ, generate ARG1 and iNOS together with an autocrine production of IL-13 and induce the expression of IL-4 and IL-13 receptors (149). It has been shown that MDSC inhibit the cytotoxicity and IFNγ, production by NK-cells. After incubation with MDSC, NK-cells could not be activated to produce IFNγ, (152). In addition to the cytokines already mentioned, the immunosuppressive cytokine transforming growth factor-β (TGF-β) (153-156) as well as stem cell factor (SCF) (157), IL-1α, IL-4, IL-6, IL-10, IL-12, IL-13, matrix metallo protease-9 (MMP-9), M-CSF and G-CSF seem to have influence on generation and function of MDSC (95, 158).

1.5.4 MDSC and signaling

STAT3 plays a central role in many molecular events governing tumor cell proliferation, survival and invasion. At the same time, STAT3 is involved in inhibition of anti-tumor immune responses. In myeloid cells, STAT3 signaling drives the expression of Bcl-XL, c-myc, cyclin D1 or survivin, which prevents cell apoptosis, promotes cell proliferation, and prevents differentiation to mature cell types (159). Earlier studies established a crucial role for STAT3 in MDSC expansion in mice (160,161). Recently, an association was demonstrated between upregulated STAT3 activation and MDSC accumulation in melanoma patients (162). Inhibition of STAT3 in vitro abolishes the suppressive activity of MDSC. Ablation of STAT3 expression in conditional knockout mice or selective STAT3 inhibitors markedly reduced the expansion of MDSC and increased T-cell responses in tumour-bearing mice (163,164). Abnormal and persistent activation of STAT3 in myeloid progenitors prevents their differentiation into mature myeloid cells and thereby promotes MDSC expansion. Several pathways downstream of STAT3 might be involved in the regulation of MDSC expansion and function. One such pathway involves the calcium-binding pro-inflammatory proteins S100A9 and S100A8 (165). In addition, it has been shown that MDSC also express receptors for these proteins on their cell surface. Activation of STAT3 in hematopoietic progenitor cells (HPC) upregulates S100A8 and S100A9. This in turn, inhibits DC differentiation and promotes MDSC

accumulation (166). The precise mechanism of this effect is not clear, but it was suggested that the S100A9 and S100A8 heterodimer participate in the formation of the NADPH oxidase (Nox2) complex that is responsible for production of ROS in myeloid cells. Increased production of ROS contributes to inhibition of myeloid cell differentiation. Upregulation of ROS in MDSC is dependent on increased expression of Nox2. STAT3 activation is directly responsible for upregulating transcription of the Nox2 components p47phox and gp91phox, increasing ROS production by MDSC in tumor-bearing mice (167). PKCbII is required for DC differentiation and is downregulated by activated STAT3. STAT3 could also play an indirect role in MDSC differentiation. It was shown recently that heat-shock protein 72 (Hsp72), which is associated with tumor-derived exosomes, induces suppressive activity of MDSC via activation of STAT3. Thus, STAT3 utilizes various molecular mechanisms to regulate MDSC expansion and function (166).

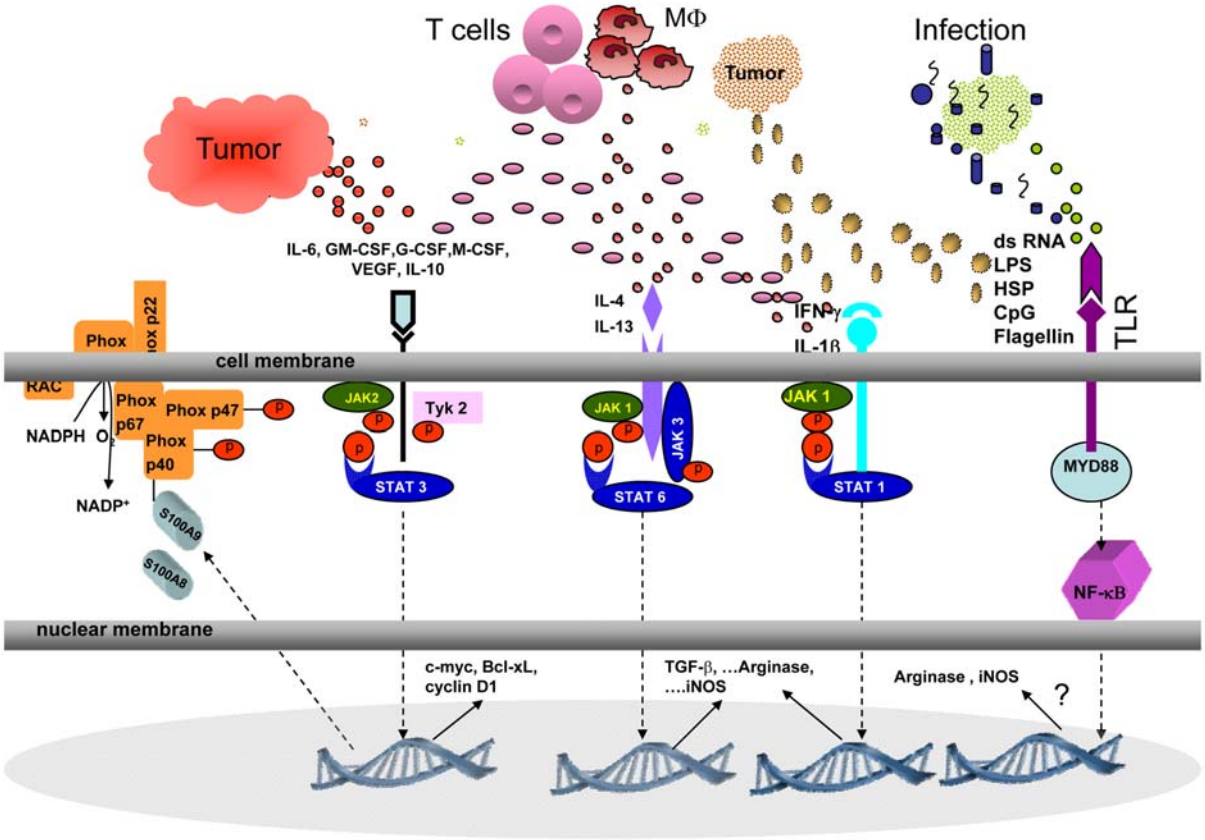
Evidences suggest an important role of STAT1 in regulation of MDSC, STAT1 is the main transcription factor activated by IFN-γ or IL-1β signaling and is implicated in the regulation of inducible nitric oxide synthase (iNOS) and arginase activity. MDSC from STAT1-/- mice failed to up regulate ARG1 and iNOS expression and therefore did not inhibit T cell responses (168). Blocking IFN-γ secretion by T cells also abrogates MDSC-mediated suppression, mainly via the block of iNOS upregulation (169,170). A recent study demonstrated that STAT1 is particularly important for the function of M-MDSCs (171).

STAT6 activation in MDSCs occurs in response to binding of IL-4 or IL-13 to the receptor CD124. This receptor is also described as a MDSC marker and is responsible for upregulation of arginase activity and increased TGF-β production by MDSC (172-175). Other experiments have shown that STAT6 deficiency prevents signalling downstream of the IL-4Rα and thereby blocks the production of ARG1 by MDSC (163) (Scheme 7).

1.5.4.1. MDSC and Toll like receptors

In myeloid cells, the toll-like receptor (TLR) family plays a prominent role in NF-kB activation, primarily via the myeloid differentiation primary response gene 88 (MyD88). TLR4 was shown to be involved directly in MDSC function (177), and LPS. In combination with IFN-γ, TLR4 could promote MDSC expansion, probably by inhibiting differentiation of DC (178). However, wild-type mice and mice lacking a

functional TLR4 protein had comparable expansion of the MDSC during polymicrobial sepsis, which suggests that signalling through TLR4 is not required for MDSC expansion and that MyD88-dependent signalling pathways that are triggered by other TLRs probably contribute to the expansion of MDSC in sepsis (179). This indicates that the activation of MDSC is a fundamental outcome of the host innate immune response to pathogens that express TLR ligands.



Scheme7. Signalling pathways involved in the expansion of MDSC populations:

The accumulation of myeloid-derived suppressor cells (MDSC) is regulated by several factors that are released by tumor cells, tumor stromal cells, activated T cells and macrophages, apoptotic tumor cells, bacterial and viral agents and by pathogen-infected cells. These factors trigger several different signalling pathways in MDSC that mainly involve the STAT (signal transducer and activator of transcription) family of transcription factors. STAT3 regulates the expansion of MDSC by stimulating myelopoiesis and inhibiting myeloid-cell differentiation. It also contributes to the increased production of reactive oxygen species (ROS) by MDSC. The activation of STAT6 and STAT1, as well as TLR-mediated activation of nuclear factor-κB (NF-κB), by these factors results in the activation of MDSC, which leads to the upregulation of iNOS and arginase and increased production of suppressive cytokines such as transforming growth factor-β (TGF-β). In combination with STAT3 they also contribute to upregulation of ROS production by these cells. S100A8 and S100A9 directly bind to p67phox and p47phox, which are crucial components of NADPH complex. This binding potentiates NADPH oxidase activation in MDSC, which causes increased production of ROS, leading to the

observed suppressive effects. It is likely that MDSC activation via TLR play especially important role during pathogenic infections (95).

It is important to note that an increase in the production and/or recruitment of IMCs in the context of acute infectious diseases or following vaccination does not necessarily represent an expansion of an immunosuppressive MDSC population. It is likely that under pathological conditions, the expansion of a suppressive MDSC population is regulated by two different groups of factors that have partially overlapping activity firstly, those that induce MDSC expansion and secondly, those that induce their activation (which leads to increased levels of ROS, arginase, or NO). This two-tiered system may allow for flexibility in the regulation of these cells under physiological and pathological conditions.

1.5.5. MDSC and Autoimmunity

Chronic inflammation and autoimmunity promote MDSC. In experimentally induced chronic inflammation of the skin (180), gut (181) and eye (182) an increase in MDSC populations is observed. The activation of TLR and the secretion of IL-1α, IL-10 and IL-12 are involved in this process (177, 183). In the murine model of multiple sclerosis, experimental autoimmune encephalitis (EAE), MDSC accumulate in the spleen of immunized mice. Moreover, this subset was capable of suppressing T-cell proliferation *in vitro* (184). There is also evidence suggesting a beneficial role of MDSC in autoimmune diseases. A significant increase in the number of MDSC was also detected in experimental autoimmune uveoretinitis, an animal model of human intraocular inflammatory disease (185) and in the skin and spleens (180) of mice that were repeatedly treated with a contact sensitizer to induce an inflammatory skin response and in inflammatory bowel diseases (186). MDSC were also found to infiltrate the spleen and suppress T-cell function in a model of traumatic stress (187). With the aim to find a possible therapy for alopecia areata, mice were persistently stimulated on the skin with a contact sensitizer in order to give rise to a chronic eczema that is accompanied by the generation and recruitment of MDSC, which, in turn, could control autoimmune T cells (180).

1.6 Alopecia areata and adhesion molecules

In AA, as discussed before there is an infiltration of leukocytes at the site of disease. Since, adhesion molecules like CD44 or integrins play an important role in leukocyte extravasation and homing, it becomes important to discuss about the significance of adhesion molecule with AA.

Adhesion molecules are important in inflammatory responses and may have roles in directing autoimmune processes through their expression on lymphoid and non-lymphoid tissues. Interaction of adhesion molecules with their ligands mediate adherence of leukocytes to other cells and also to extracellular matrix. Adhesion molecule regulates leukocytes circulation, lymphoid cell homing to tissue and inflammatory sites and transendothelial migration and also participates in lymphocyte co-stimulation, cytotoxicity, lympho-haemopoiesis and leukocyte apoptosis. Adhesion molecules relevant to leukocytes have been classified as;

- **Selectins**: selectins are expressed on leukocytes, platelets and endothelial cells. Their common structural component is an N terminal lectin binding domain. Selectins have been further classified as L-selectin (Lymphocytes), P-selectin (Platelets), E selectins (Endothelium). Selectin binding and rapid association and disassociation to glycosylated and sialyated ligands mediate leukocyte rolling along the endothelial cell wall and are involved in initial localization of leukocytes to inflammatory sites. Rolling of leukocyte at inflammatory sites exposes them to chemo-attractants and cytokines. This exposure leads to leukocytes activation, upregulation of additional adhesion molecules, chemotaxis and prolonged localization of cells to a site of inflammation (188).
- **Immunoglobulin super family (IGSF):** are named and classified together because each receptor has immunoglobulin like amino-acid domain. The IGSF includes several related cell surface proteins found on immunocompetent cells like CD4, CD8, T cell receptor/CD3 complex and MHC classI and Class II molecules. IGSF receptors functioning as adhesion molecule which include “lymphocytes function antigen” (LFA-2, CD2 and LFA-3), intracellular adhesion molecules (ICAM-1 and ICAM-2) and VCAM-1. ICAM-1 and VCAM-1 are principal receptors of LFA-1 and VLA-4 respectively. Cytokine stimulation of endothelial cells increased ICAM-1 expression, which promotes adherence of LFA-1 positive leukocytes to inflammatory sites. VLA-

4/VCAM-1 adhesion may be primarily responsible for prolonged cell adhesion at inflammatory sites (189).

- **Integrin:** Integrin adhesion molecules are heterodimeric proteins composed of non-covalently bound α and β subunits. Subunit combinations form functionally different receptors. Each contains a large extracellular domain, a transmembrane domain, and a generally short cytoplasmic tail. The extracellular domains of integrins bind ligands in the extracellular matrix (ECM) like fibronectin, vitronectin, laminin, collagen etc. or on the surface of other cell types to mediate either cell-substratum or cell-cell adhesion. In addition to forming these physical connections, integrins regulate cell signaling pathways through their cytoplasmic domains. These signaling pathways are important in coordinating cell migration. In particular, the α4β1 integrin (CD49d) mediates leukocyte migration essential for immune surveillance and inflammation. An ECM binding motif for many integrins is the amino acid sequence "arginine-lysine-aspartate" (RGD) domain. Fibronectin, vitronectin and other integrins binding extra-cellular proteins have this RGD domain (190).

  It has been shown that α4 integrin (CD49d) associates with CD44 and contributes to leukocyte extravasation. Lymph node cells of AA mice displayed increased motility, proliferative activity and apoptosis resistance, which were equally inhibited by both CD44- and CD49d-specific antibodies (191).

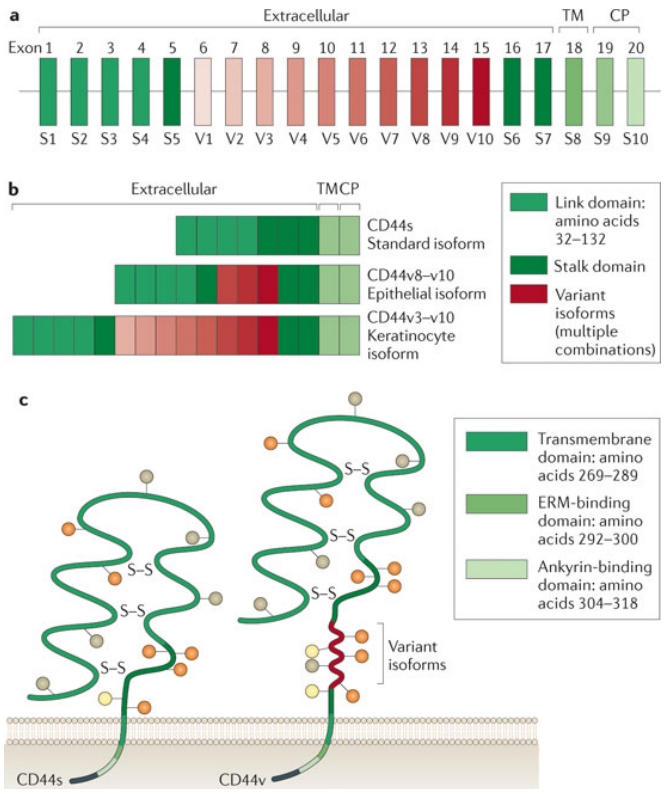
1.7 CD44

The CD44 proteins form a rather ubiquitously expressed family of cell surface adhesion molecules involved in cell-cell and cell-matrix interactions. The CD44 glycoproteins are well characterized members of the hyaluronate receptor family of cell adhesion molecules (192,198). CD44 has been described into a multitude of functions. The major physiological role is to maintain organ and tissue structure via cell-cell and cell-matrix adhesion. In leukocytes it has been characterized as an adhesion receptor engaged by migrating T cells including leukocyte extravasation. It also mediates T cell activation and thymic homing.

1.7.1 CD44 structure

1.7.1.1 Gene structure: The human CD44 gene has been mapped to the chromosomal locus 11p13 (192). CD44 glycoproteins are encoded by a single gene. They vary in

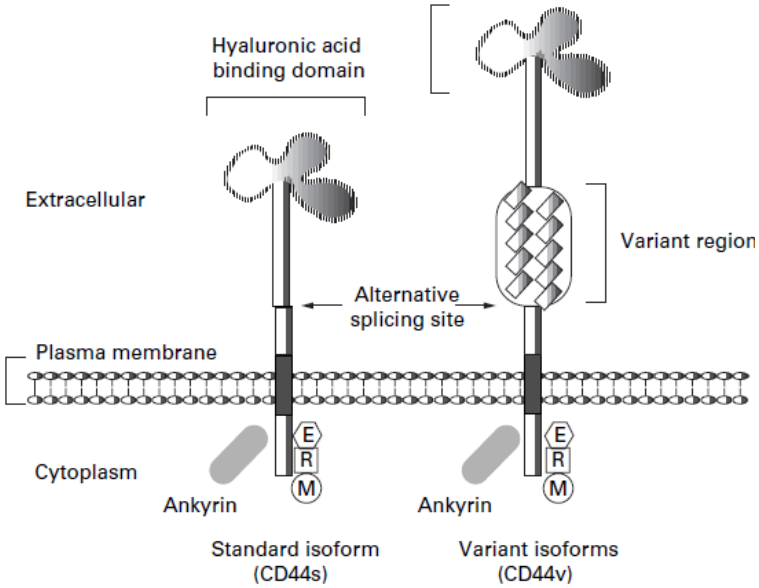
size owing to N-glycosylation and O-glycosylation and the insertion of alternatively spliced exon products in the extracellular domain of the molecule. The smallest standard or hematopoietic isoform (CD44s) is present on the membrane of most vertebrate cells. CD44 has seven extracellular domains, a transmembrane domain and a cytoplasmic domain (193). The standard CD44 consist of consists of an N-terminal signal sequence (exon 1), a Link-homology hyaluronan-binding module (exons 2 and 3), a stem region (exons 4, 5, 16 and 17), a single-pass transmembrane domain (exon 18) and a cytoplasmic domain (exon 20). Alternative splicing of CD44 predominantly involves variable insertion of different combinations of exons 6-15 (variant exons v1-v10) into the stem region. Nearly all CD44 cDNAs isolated have exon 19 spliced out, producing an open reading frame encoding a 73 amino acid cytoplasmic domain. The inclusion of exon 19 would generate a short 5 amino acid cytoplasmic tail terminating at Arg294 (194) (Scheme 8).



Scheme8. Gene structure of CD44 (195)

1.7.1.2 Protein structure: The most abundant standard isoform of human CD44 protein (CD44s) contains 363 amino acid (aa) and has a theoretical molecular mass of 37 kDa. The protein consists of three regions, 270 amino acid (aa) extracellular

domain, 21 aa transmembrane domain and 72 aa C-terminal cytoplasmic domain. The highly conserved cytoplasmic tail can exist as a short or a more prevalent long form by the inclusion of the C-terminal exon (196). The hydrophobic transmembrane domain is encoded by exon 18 and is 100% conserved between mammalian species. The extracellular domain can be subdivided further into conserved and non-conserved regions. The N-terminal ectodomain, encoded by exons 1 to 5 is highly conserved (~ 85%) between mammalian species and is thought to fold into a globular tertiary structure by the formation of disulphide bonds between three pairs of cysteine residues. Also present in this part of the molecule is a 100 aa region of homology with other hyaluronic acid (HA) binding proteins. This is termed the "link module" or CLP domain because of its resemblance to the HA binding domain of cartilage link protein (197). The variable region is the point at which up to 381 aa encoded by the 10 alternatively spliced variant exons are inserted at a site between exons 5 and 16 of the RNA transcript, corresponding to amino acid position 223. The membrane proximal region of the extracellular domain, encoded by exons 16 and 17 is less conserved (35%) between mammalian species and includes several carbohydrate modification sites (Scheme 9).



Scheme9. CD44 protein structure: The standard form binds its principal ligand, hyaluronic acid at the N-terminal, distal extracellular domain. The inclusion of combinations of the variant exons (v1-v10) within the extracellular domain can alter the binding affinity for hyaluronic acid and confer interaction with alternative ligands. The molecule interacts with the cytoskeleton through the binding of ankyrin and the ERM family to the cytoplasmic domain (192)

1.7.1.3 Post-translational modifications: The apparent molecular mass of the CD44s protein, as estimated by gel electrophoresis, is ~80 kDa and the largest possible protein, containing peptides from all variant exons (referred to as "epican") can be over 200 kDa which is much greater than the expected value as calculated from aa residues and is due to the extensive post-translational modification of CD44 isoforms (192). In cultured cells, CD44 is constitutively phosphorylated at Ser325 in the cytoplasmic tail (199-201). Ser325 phosphorylation is estimated to occur on ~25-40% of CD44 molecules and is mediated by Ca2+/calmodulin-dependent protein kinase II (CaMKII). Phosphorylation at Ser325 and Ser291 residues has been implicated in mediating cell migration on HA and in the interaction of CD44 with ezrin. Mutations at Ser325 site impair hyaluronan-mediated cell migration (202).

1.7.1.4 Palmitoylation: CD44 is reversibly palmitoylated (203,204), the prospective acylation sites being Cys286 and/or Cys295. In the case of CD44, acylation has been reported to impair anti-CD3 mediated signal transduction in lymphocytes (204) and enhance the association of CD44 with ankyrin. (203). Given the location of these cysteine residues in the CD44 sequence, palmitoylation might also play a role in partitioning CD44 into membrane subdomains and/or in regulating its association with ERM proteins.

1.7.1.5 Modification by proteolytic processing: It has been described that the extracellular domain of CD44 is subject to regulated proteolytic cleavage (205). Membrane type1 (MT1)-MMP and MT3-MMP has been shown to release soluble CD44 (206,207). CD44 cleavage can generate two cell associated CD44 species (~25 kDa and ~12 kDa) in addition to the secreted extracellular domain fragment (208-211). The ~25 kDa species corresponds to the residual membrane-bound C-terminal fragment (CTF), whereas the major product isolated from the ~12 kDa band is a CD44 intracellular domain (ICD) fragment resulting from a cleavage just inside the CD44 transmembrane domain.The released CD44-ICD fragment translocates to the nucleus and stimulates transcription, one of its target genes is the gene encoding CD44 itself (211).

1.7.2 CD44 Functions

CD44 proteins have essential functions in life and their dysfunction, absence or over expression can lead to pathogenic phenotype. It has been described that CD44 null mice were viable and had relatively mild phenotype. Interestingly activated T cells survived longer in null mice than wild type mice which led to a resistance to hepatitis that could be explained by the involvement of CD44 in proapoptotic signaling (213). The multiple functions of the CD44 family of proteins are centered around the binding of HA and, to a lesser extent, other extracellular molecules. A transmembrane domain and a minimal cytoplasmic domain are required for efficient ligand binding (214,215), probably stabilizing CD44 at the plasma membrane and promoting receptor clustering.

1.7.2.1 Cell adhesion and migration

Cell adhesion and migration are critical steps in cancer progression and inflammatory responses and cell migration is important for tissue remodeling, wound healing and leukocyte migration. The CD44 dependent adhesion mechanism is most important for mobilization of effector cells at sites of infection and inflammation. Adhesion of CD44 to its ligand(s) induces up-regulation of additional adhesion molecules, mostly integrins like CD49d that strengthen adhesion. It has been suggested that CD44 and CD49d comes into proximity during T cell activation. On activated T cells, CD44 can regulate tethering and rolling interactions with vascular endothelial cells that express HA (216). The affinity of CD44 for HA seems to be modulated from inside the cells, as its binding affinity is upregulated by mitogenic stimuli, influenced by glycosylation of the extracellular domains and also by the phoshphorylation of specific serine residues in the cytoplasmic domain of CD44 (217), deletion of the cytoplasmic tail of CD44 prevents firm adhesion of cells to endothelium, which follows rolling as the first step in leukocyte extravasaation (218). There has been evidence for the physiological importance of the involvement of CD44s in leukocyte extravasation. It has been described that the development of an oedema and leukocyte immigration into inflamed tissue as in delayed type hypersensitivity reactions can be strongly inhibited by anti-pan CD44 (219-221). In addition to the well defined role of CD44 in extravasation of T cells, CD44 also plays a crucial role in regulating cell motility within the tissue stroma. In tissue resident T cells CD44 and other adhesion receptors

localizes to the leading edges and lamellopodia (222). Intracellular signals not only guide CD44 to the leading edge but also support CD44 mediated cell motility by cleavage of extracellular domain by membrane type metalloproteinases 1 (MT1-MMP) (209,223).

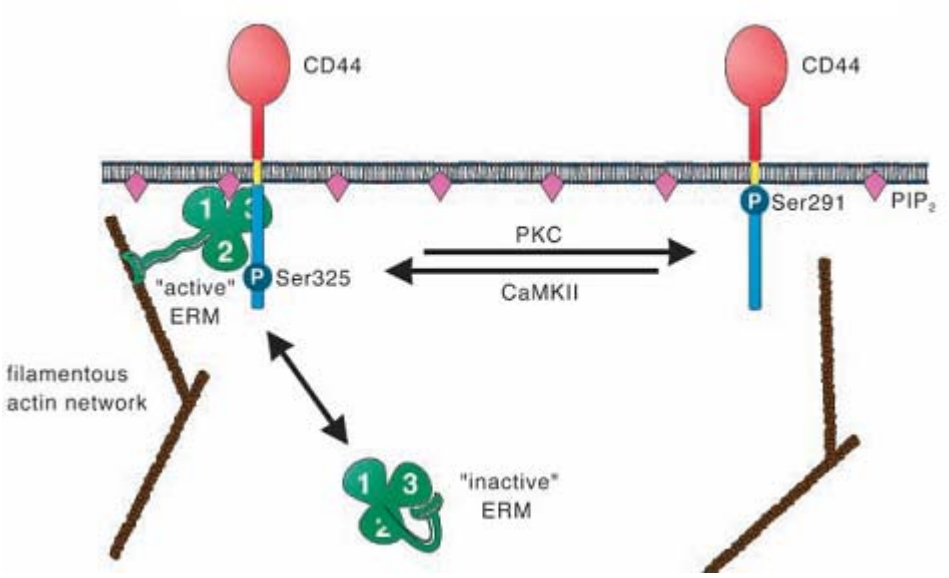
1.7.2.2 Interaction with the cytoskeleton

The cytoskeleton is a highly dynamic structure that reorganizes when cells respond to extracellular stimuli via actin polymerization and the rearrangement of the underlying cortical actin filaments (199-201).

CD44 cytoplasmic domain lacks a binding site for actin. Therefore it mediates the interaction with actin via adaptor proteins like ankyrin, ERM family of proteins (ezrin, radixin and moesin) and related protein merlin (202). An ankyrin binding domain has been identified (203) and binding is influenced by many factors including palmitoylation (204). PKC mediated phosphorylation and GTP binding. Ankyrin binding is important for HA binding and cell adhesion as deletion of the ankyrin binding domain results in nearly complete loss of HA binding (227).

ERM proteins act as key linkers between transmembrane proteins and cytoskeleton. The ERM proteins have a 300 amino acid domain at the N-terminus, α-helical central region and a C-terminal domain which has the F-actin binding site. ERM proteins are activated by phosphorylation and by binding to membrane phospholipids. It is the phoshorylated (active) form of ERM that binds to CD44 (202). ERM activation is regulated via Rho GTPase family. Binding of ROK, PKC and phosphatidyl inositol 4, 5 bisphosphate (PIP2) to ERM proteins results in phosphorylation of these proteins (228-230). Thr_{567} Ezrin, Thr_{564} Radixin and Thr_{558} Moesin phosphorylations result in rearrangement of the cytoskeleton (231). CD44 binding to the cytoskeleton is further regulated by its cytoplasmic domain. In resting cells CD44 is phosphorylated at Ser325. The switch from Ser325 phosphorylation to Ser291 is triggered via PKC and leads to a break in the association between ezrin and CD44 (Scheme 10). In addition, Ser291 phosphorylation is also involved in directional migration of cells. Merlin protein has 65% homology to ERM proteins. Its activity is also regulated through phosphorylation and dephosphorylation (232). Merlin does not have an actin binding site and merlin-CD44 complex cannot bind to cytoskeleton (233). The ability of ERM/merlin proteins to switch between phosphorylated and dephosphorylated forms along with the competition between ERM and merlin to bind to CD44 leads to making

and breaking of CD44 interactions with the cytoskeleton [234]. Merlin is inactivated after its phosphorylation and prohibits its binding to CD44 When cells are in the growth phase, phosphorylated ERM binds to CD44 mediating cytoskeletal interactions. During this time merlin gets phosphorylated by PAK2 (p21-active kinase -2) and is now unable to bind to CD44. During growth arrest the opposite mechanism is under rule and MAPK activation is blocked (235, 236).



Scheme10. Model for the regulation of the CD44-ERM complex by dynamic phosphorylation of the CD44 cytoplasmic tail: The ERM (ezrin, radixin, moesin)-family proteins can function to crosslink transmembrane receptors, including CD44, to the cytoskeleton. Their basic structure consists of the three-lobed N-terminal FERM domain followed by a coiled-coil region and a C-terminal domain that contains an F-actin binding site. In their 'inactive' conformation, the C-terminal domain binds to the FERM domain, masking both transmembrane receptors and F-actin interaction sites. Conformational regulation between the 'inactive' and 'active' forms involves complex mechanisms including phosphorylation and binding to the membrane phospholipids phosphatidylinositol 4,5-bisphosphate (PIP2). The cytoplasmic tail of CD44 is phosphorylated at Ser325 by CaMKII and this form of the receptor binds to an 'active' ERM protein that links CD44 to the actin cytoskeleton. PKC activation results in a concomitant dephosphorylation of Ser325 and phosphorylation of Ser291, resulting in disengagement of the ERM proteins and loss of cytoskeletal association (212).

1.7.2.3 Role in lymphocytic function

Several lymphocyte functions appear to be dependent upon CD44 expression. Increased surface levels of CD44 proteins are characteristic of T cell activation after encounter with its cognate antigen (238). Cell surface CD44 on lymphocytes can mediate the adhesion of lymphocytes to vascular endothelial cells via binding of HA,

and this interaction is used for activated T cell extravasation into sites of inflammation in mice (239) and in humans (240). This targeting of lymphocytes to effector sites by CD44–HA binding is enhanced by the induction of HA synthesis in vascular endothelium by the proinflammatory cytokines, TNF-α and IL-1α (241). Accordingly, the presence of CD44 splice variants appears to be obligatory for the migration and function of Langerhans cells and dendritic cells from peripheral organs to lymph nodes for antigen presentation (242). CD44 has a role in mediating the functions of regulatory T cell as well, which are essential for the prevention of autoimmunity. CD44 deficient Tregs fail to persist after transfer *in vivo*. The *in vitro* ligation of CD44 on activated wild type Tregs promotes persistent expression of the transcription factor FoxP3, which is essential for regulatory activities. These functions of CD44 are shown to be dependent upon the interaction with high molecular weight forms of HA that are found in the absence of inflammatory responses.

1.7.2.4 CD44 signaling

The location of CD44 in GEMs is of particular importance with respect to CD44 mediated signal transduction, as the inner side of these microdomains is known to harbor signal transducing molecules. CD44 signaling can result in opposing effects depending on the cellular context, the expressed variant isoform and the associated signaling partner, for example, CD44 engagement can lead to proliferation or inhibition of proliferation, apoptosis or inhibition of apoptosis, resulting in up-regulation and down-regulation of several signaling pathways involved in cell activation.

However CD44 lacks intrinsic catalytic activity and its signaling depends upon its association with receptor tyrosine kinases and the Src family of phosphotyrosine kinases (PTKs). Src kinases act as molecular switches on the cell membranes linking extracellular events to intracellular signaling. They are activated through engagement with many receptors such as TCR/CD3 complex, CD4, CD8, B cell receptor, Fc receptors, integrins, GPI anchored receptors and growth factor receptors (193). When activated they mediate several signaling events including activation of additional PTKs such as ZAP-70 or Syk, phospholipases, cytoskeletal proteins and adaptor proteins. CD44 and the associated lck are recruited into membrane microdomains where they interact with the CD3/TCR complex. Association of CD44 with lck and fyn and in turn their co-localisation with the TCR allow for recruitment of several

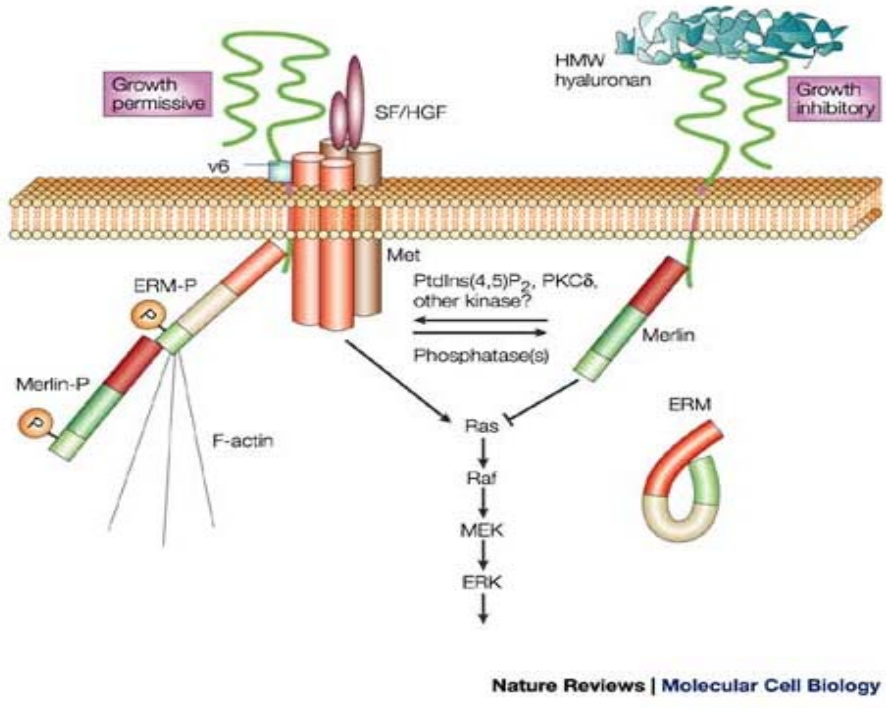
other kinases necessary for T cell activation (243, 244). The co-stimulatory function of CD44 is demonstrated by its ability to induce cell proliferation on freshly isolated lymph node cells in the presence of sub-threshold levels of anti-CD3. This is mediated by activation of several tyrosine kinases and is accompanied by strong activation of ERK and c-jun and involvement of MAPK (Scheme 11). Consequently, IL-2 production and CD69 and CD25 expression are up-regulated in T cells. The situation differs in a T helper line (IP-12) where it was observed that CD44-cross-linking with CD3 leads to upregulation of CD95 and CD95L expression resulting in apoptosis induction (or activation induced cell death AICD). This argues for differential effects of CD44 on different cell types. It is important to note that, in both peripheral T cells and a T helper line, CD44 cross linking alone did not exert any effect on signaling by itself. This is in line with two signals one from the TCR and the other from a co-stimulatory molecule. In antigen-specific T cell activation, CD44 has been described to deliver co-stimulatory signals for T cell activation (243).

CD44 mediated cytoskeletal reorganization appear to be dependent on Rac1 activation. Small GTPase Rac1 activation is required for this process. Rac1 is also known to co-localise with ezrin (245). These events along with phosphotyrosine kinase activations are responsible for cytoskeletal re-organizations in the cell. In T lymphocytes cross-linking anti-CD44 Ab leads to CD44-dependent spreading through F-actin polymerization, accompanied by T cell adhesion, flattening and spreading (244). Vav1 protein encoded by vav proto-oncogene is a 95 kD protein that is an upstream regulator of Rac1. Vav catalyses the GDP to GTP exchange on Rac1. Vav1 activation involves phosphoinositides binding and tyrosine phosphorylation that is dependent on Src family kinases (lck and fyn) which associate with CD44. Thus, this could be a possible explanation for CD44 mediated effects on the cytoskeleton (244, 246,247). Another mediator of CD44 signalling is RhoA and its downstream effector Rho-Kinase (ROK). ROK phosphorylates the cytoplasmic domain of CD44v3 which strengthen the interaction between these and ankyrin and supports tumor cell migration. In endothelial cells, migration is stimulated after CD44v10–HA binding by interaction between ROK and CD44v10 (248).

Some studies have shown that CD44 is capable of activating human resting T cells and mouse cytotoxic T cells by itself and can promote proliferation of T cells that is dependent on IL-2 provision. In this study, anti-CD44 cross-linking does not phosphorylate the ξ chain of the TCR complex indicating that the signals generated in

this case are independent of TCR engagement. Indeed, it was demonstrated that this process is mediated by tyrosine kinases associated with CD44 (249).

Using a mouse model for the autoimmune disease alopecia areata Marhaba et al., have shown an association between CD44 and CD49d. This association resulted in the formation of a signaling complex between CD44, CD49d and the underlying signaling machinery allowing for each surface molecule (CD44 or CD49d) to avail from the associated signaling molecules of the other. In this case, cross-linking of CD44 resulted in the activation of the focal adhesion kinase (FAK) associated with CD49d, and CD49d cross-linking allowed the activation of Ezrin and Lck associated with CD44. Thus signaling pathways initiated through both the molecules CD44 and CD49d are triggered which could well be important in lymphocyte activation and function (191).



Scheme11. CD44 and signaling (250).

1.7.2.5 CD44 and Alopecia areata

The involvement of CD44 in autoimmune diseases is well known. As it has been already discussed above, AA is most effectively cured by the induction of DTH via chronic contact eczema treatment by SADBE. One of the mechanisms behind is expansion and activation of MDSCs and another aspect which is important in the cure of AA by chronic contact eczema is that there should be an impaired T cell activation

as a result of hindrance in APC migration towards draining lymph node. CD44 is described as lymphocyte homing receptor and plays a major role in leukocyte extravasation. In autoimmune diseases and allergic reactions, there are several reports suggesting alterations in the intensity of CD44 expression as well as changes in the pattern of CD44 and variant isoforms in autoimmune diseases. It has been shown that both CD44 and isoforms are supposed to function as costimulatory molecules (251-253). Induction of AA in mice can be prevented by repeated injections of CD44v10. The blockade of CD44 on endothelial cell can contribute to the inhibition of leukocyte extravasation, but the different efficacy of an anti-CD44 blockade in diseased as compared with healthy mice depends upon leukocytes. The anti-CD44 mediated inhibition of T cell extravasation preferentially affected activated ($CD69^{+}CD154^{+}$) T cells. T cell migration is more strongly inhibited with anti-panCD44 than anti-CD44v10. Instead macrophage recruitment was more affected by anti-CD44v10 (254). Another important role of CD44 in leukocyte rolling and firm adhesion to vessel endothelium is due to its association with CD49d. Since autoimmune diseases are reflected by an increased percentage of peripheral blood leukocytes expressing activated CD44, it became relevant to look for the CD44 and CD49d association. This wide range of CD44 activities leads to a speculation that association of CD44 with CD49d could play an important role in leukocyte activation and /or apotosis resistance. In additionan antibody blockade of different CD44 isoforms may provide therapeutics in T cell mediated autoimmune disease like Alopecia areata.

1.8 Aims of the study

Alopecia areata (AA) is an organ related autoimmune disease that is cured most efficiently by chronic contact eczema. However, the underlying mechanism is unknown. To unravel the mechanism I aimed to shed some more light on the mode of T cell activation in Alopecia areata and the mechanisms promoting the therapeutic efficacy of chronic contact eczema, I approached to the following questions:

1. **Do adhesion molecules, particularly CD44 and CD49d account for T cell expansion and hyperactivity in Alopecia areata:** This work builds on previous studies in our lab which showed that in T cells from Alopecia areata, but not of healthy mice, CD44 associates with CD49d. This associatrion support T cell migration, proliferation and apoptosis resistance. To prove that the CD44-CD49d association is essential for the observed effects, I generated T cell lines expressing either mutated CD44 or mutated CD49d. The T cell lines differed, in addition, by CD3/TCR expression. This allowed to concomitantly attend the question of a TCR-dependant *versus* TCR-independent modulation of T cell activity *via* adhesion molecules.

2. **Are myeloid derived suppressor cells (MDSC), the driving force in the SADBE-induced cure of Alopecia areata:** Myeloid derived suppressor cells have been found to be expanded in SADBE-treated Alopecia areata (AA) mice. However, studies were still missing to demonstrate their essential contribution to the therapy of Alopecia areata. I approached this question by a direct comparison of *ex-vivo* analyzed leukocytes from AA and SADBE-treated AA mice with co-cultures of AA T cells with separated MDSC. In addition, I started to unravel the molecular pathways, whereby MDSC derived from SADBE-treated AA mice affect autoreactive T cells.

2: Materials and methods

2.1 Materials

2.1.1 Animals

Mice	**Origin**
C3H/HeJ	Jackson Laboratories, Bar Harbour, USA

2.1.2 Bacterial strain

E.coli DH5α	Genotype: F^-, Φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk^-,mk^+), *pho*A, *sup*E44, *thi*-1, *gyr*A96, *rel* A1, λ^- (Invitrogen, Darmstadt, Germany)

2.1.3 Cell Lines

Cell Line	**Origin**
EL-4	Mouse Thymoma, American Type Culture Collection (ATCC) number: TIB- 39
EL4+wtCD49d	EL4 cells transfected with mouse wild type CD49d
EL4+mutCD49d	EL4 cells transfected with mouse CD49d muataed at Ser988 to alanine
Jurkat	Human T lymphocyte ATCC TIB-152
Jurkat+wtCD44	Jurkat cells transfected with mouse wild type CD44
Jurkat+mutCD44	Jurkat cells transfected with mouse CD44 mutated at Ser291 and Ser325 to alanine
Jurkat+truncCD44	Jurkat cells transfected with mouse CD44 excluding cytoplasmic tail

2.1.4 Primers

mouseCD44wt	**F** 5'-GCAGTGAATTCCCACCATGGACAAGGTTTGGTGGCAC -3'
	R 5'-CGACGCTCGAGCACCCCAATCTTCATATCCAC -3'
mouseCD44mut ser291	**F** 5'-CATTTCCTGAGACTTGGCGGCCTCCCCGTTGGGGTACCCC -3'
	R 5'-GGGTACCCCAACGGGGAGGCCGCCAAGTCTCAGGAAATG-3'
mouseCD44mut ser325	**F** 5'-GTCTGCATCGCGGTCAATGCTAGGAGAAAGG -3'
	R 5'-CTGCCCACACCTTCTCCTAGCATTGACGGCGATCCACAG -3'
mouseCD44-truncated	**F** 5'- GCAGTGAATTCCCACCATGGACAAGGTTTGGTGGCAC -3'
	R 5'- TAGTCTAGAACTATTGACCGCGATGCA-3'
mouseCD49d	**F** 5'-GAGAATTCATGGCTGCGGAAGCGAG-3'
	R 5'-CGCTCGAGTCAGTCATCATTGCTTTTGC-3'
mouseCD49d mut ser298	**F** 5'-CGACGCGTATTATTACCATCGCCTTGCTACTTGG-3'
	R 5'-CGACGCGTCCAAGTAGCAAGGCGATGGTAATAAT-3'

2.1.5 Primary Antibodies

Antibody	**Company**
Anti-Actin	Becton Dickinson, Heidelberg, Germany
Anti-akt	Becton Dickinson, Heidelberg, Germany
Anti-BAD	Becton Dickinson, Heidelberg, Germany
Anti-Bax	Becton Dickinson, Heidelberg, Germany
Anti-Bcl2	Becton Dickinson, Heidelberg, Germany
Anti-Caspase 3	Becton Dickinson, Heidelberg, Germany
Anti-Caspase 8	Becton Dickinson, Heidelberg, Germany
Anti-Caspase 9	Becton Dickinson, Heidelberg, Germany
Anti-Caspase 9 cleaved	Cell Signalling, Frankfurt , Germany
Anti-CD1d	Becton Dickinson, Heidelberg, Germany
Anti-CD3ζ	Abcam Cambridge UK
Anti-CD4 (YTA)	European Animal Cell Culture collection, UK
Anti-CD8 (YTS169)	European Animal Cell Culture collection, UK
Anti-CD11b (YBM)	European Animal Cell Culture collection, UK

Anti-CD11c	Becton Dickinson, Heidelberg, Germany
Anti-CD16/32	Becton Dickinson, Heidelberg, Germany
Anti-CD18	Becton Dickinson, Heidelberg, Germany
Anti-CD25	Becton Dickinson, Heidelberg, Germany
Anti-CD28	Becton Dickinson, Heidelberg, Germany
Anti-CD40	Becton Dickinson, Heidelberg, Germany
Anti-CD44(IM7)	American type culture collection
Anti-CD44	Becton Dickinson, Heidelberg, Germany
Anti-CD49a	Becton Dickinson, Heidelberg, Germany
Anti-CD49b	Becton Dickinson, Heidelberg, Germany
Anti-CD49c	Becton Dickinson, Heidelberg, Germany
Anti-CD49d	Becton Dickinson, Heidelberg, Germany
Anti-CD49d (PS/2)	American type culture collection
Anti-CD49f	Becton Dickinson, Heidelberg, Germany
Anti-CD50	Becton Dickinson, Heidelberg, Germany
Anti-CD54 (YN1/1.7.4)	American type culture collection
Anti-CD54	Becton Dickinson, Heidelberg, Germany
Anti-CD62E	Becton Dickinson, Heidelberg, Germany
Anti-CD62L	Immunotools, , Friesoythe; Germany
Anti-CD62L-lig	Becton Dickinson, Heidelberg, Germany
Anti-CD62P	Becton Dickinson, Heidelberg, Germany
Anti-CD69	Becton Dickinson, Heidelberg, Germany
Anti-CD80	Becton Dickinson, Heidelberg, Germany
Anti-CD86	Becton Dickinson, Heidelberg, Germany
Anti-CD95	Becton Dickinson, Heidelberg, Germany
Anti-CD95l	Becton Dickinson, Heidelberg, Germany
Anti-CD102	Becton Dickinson, Heidelberg, Germany
Anti-CD106	Becton Dickinson, Heidelberg, Germany
Anti-CD120a	Becton Dickinson, Heidelberg, Germany
Anti-CD120b	Becton Dickinson, Heidelberg, Germany
Anti-CD152	Becton Dickinson, Heidelberg, Germany
Anti-CD154	Becton Dickinson, Heidelberg, Germany

Anti-CD253	Biozol Eching, Germany
Anti-CD254	Becton Dickinson, Heidelberg, Germany
Anti-CD265	Becton Dickinson, Heidelberg, Germany
Anti-CD284	Biolegend, Uithoorn, Netherlands
Anti-c-jun	Santa Cruz, Heidelberg, Germany
Anti-cytochrome c	Becton Dickinson, Heidelberg, Germany
Anti-ERK1/2	Becton Dickinson, Heidelberg, Germany
Anti-Ezrin	Becton Dickinson, Heidelberg, Germany
Anti-FAK	Becton Dickinson, Heidelberg, Germany
Anti-FoxP3	Becton Dickinson, Heidelberg, Germany
Anti-Gr1	Becton Dickinson, Heidelberg, Germany
Anti- IFNγ	Becton Dickinson, Heidelberg, Germany
Anti-IL-1α	Becton Dickinson, Heidelberg, Germany
Anti-IL-10	Becton Dickinson, Heidelberg, Germany
Anti-IL-12	Becton Dickinson, Heidelberg, Germany
Anti-IL-2	Becton Dickinson, Heidelberg, Germany
Anti-IL-4	Becton Dickinson, Heidelberg, Germany
Anti-IL-6	Becton Dickinson, Heidelberg, Germany
Anti-iNOS	Becton Dickinson, Heidelberg, Germany
Anti-JNK	Becton Dickinson, Heidelberg, Germany
Anti-LAT	Santa Cruz, Heidelberg, Germany
Anti-Lck	Santa Cruz, Heidelberg, Germany
Anti-Ly6C	Becton Dickinson, Heidelberg, Germany
Anti-LY6G	Becton Dickinson, Heidelberg, Germany
Anti-NFkB	Santa Cruz, Heidelberg, Germany
Anti-pAkt	Becton Dickinson, Heidelberg, Germany
Anti-Paxillin	Becton Dickinson, Heidelberg, Germany
Anti-pBAD	Cell Signalling, Frankfurt , Germany
Anti-p-c-jun	Santa Cruz, Heidelberg, Germany
Anti-pERK1/2	Santa cruz, Heidelberg, Germany
Anti-pI3K	Santa Cruz, Heidelberg, Germany
Anti-pJNK	Becton Dickinson, Heidelberg, Germany

Anti-pLck	Santa Cruz, Heidelberg, Germany
Anti-pZAP70	Becton Dickinson, Heidelberg, Germany
Anti-Smac	Becton Dickinson, Heidelberg, Germany
Anti-Stat3	Becton Dickinson, Heidelberg, Germany
Anti-Stat6	Becton Dickinson, Heidelberg, Germany
Anti-TNFRI	Becton Dickinson, Heidelberg, Germany
Anti-TNFRII	Becton Dickinson, Heidelberg, Germany
Anti-TNFα	Becton Dickinson, Heidelberg, Germany
Anti-Trail	Becton Dickinson, Heidelberg, Germany
Anti-ZAP70	Becton Dickinson, Heidelberg, Germany

2.1.6 Secondary antibodies

Name	**Company**
Anti-mouse IgG HRP	Amersham, Freiburg, Germany
Anti-rabbit IgG HRP	Amersham, Freiburg, Germany
Anti-rat IgG HRP	Amersham, Freiburg, Germany
Anti-mouse IgG PE	Jackson Laboratories, Bar Harbor,USA
Anti-hamster IgG FITC	Jackson Laboratories, Bar Harbor,USA
Anti-mouse IgG FITC	Jackson Laboratories, Bar Harbor,USA
Anti-mouse IgG APC	Jackson Laboratories, Bar Harbor,USA
Anti-rat IgG FITC	Jackson Laboratories, Bar Harbor,USA
Anti-rat IgG PE	Jackson Laboratories, Bar Harbor,USA
Anti-rat IgG APC	Becton Dickinson Heidelberg, Germany
Streptavidin FITC	Jackson Laboratories, Bar Harbor,USA
Streptavidin PE	Jackson Laboratories, Bar Harbor,USA
Streptavidin APC	Jackson Laboratories, Bar Harbor,USA
Streptavidin HRP	Rockland, PA USA

2.1.7 Enzymes

Restriction enzymes	MBI Fermentas, St. Leon-Rot, Germany
Taq polymerase	MBI Fermentas, St. Leon-Rot, Germany

2.1.8 Instruments

Name	Company
Agitator for bacterial cultures	Edmund Buehler GmbH, Hechingen, Germany
Camera system Spot CCD	Diagnostic Instruments, Sterling Heights, USA
Cell chamber neubauer improved	Brand, Wertheim, Germany
Centrifuge Sorvall RC5B Plus	Kendro, USA
Centrifuge Biofuge fresco	Heraeus, Hanau, Germany
DNA-agarose gel electrophoresis chamber	Bio-Rad, Munich, Germany
Eagle eye (Mididoc)	Herolab, Wiesloch, Germany
ELISA plate reader	Anthos labtec, Wals, Austria
FACS Calibur	Becton-Dickinson, Heidelberg, Germany
Hyper processor (for processing films)	Amersham, Freiburg, Germany
Incubator for bacteria	Melag, Berlin, Germany
Incubator for cell culture	Labotec, Goettingen, Germany
Invert microscope DM-IL	Leica, Bensheim, Germany
LSM710 (laser scanning microscope)	Zeiss, Goettingen, Germany
Master cycler (PCR cycler)	Eppendorf, Hamburg, Germany
Magnetic stirrer 3000	Heidolph, Keilheim, Germany
Microscope DMBRE	Leica, Bensheim, Germany
Microwave	Phillips, Wiesbaden, Germany
Photocassette	Amersham, Freiburg, Germany
Ph-Meter-761 Calimatic	Knick, Berlin, Germany
Photometer Ultraspec III	Amersham, Freiburg, Germany
Pipettus-Akku	Hirschmann, Eberstadt, Germany
Pipettes	Eppendorf, Hamburg, Germany
Powersupply PS 9009	GIBCO, Darmstadt, Germany
Rotor GSA	Kendro, USA
Rotor SW34	Kendro, USA
Rotor SW41 Ti	Beckman Coulter, Krefeld, Germany
Sterile bench	Heraeus, Hanau, Germany

Sonicator Sonoplus	Bandelin, Berlin, Germany
Tabletop centrifuge	Heraeus, Hanau, Germany
Transferapparatus Mini Trans-Blot®	Bio-Rad, Munich, Germany
Thermo-mixer	Eppendorf, Hamburg, Germany
Homogenizer	Bandelin Electronik, Germany
UV-transilluminator	Biotec Fischer, Germany
Water-bath	Julabo, Seelbach, Germany
Weighing scale RC210 D	Sartorius, Goettingen
Whirlmixer Vortex Genie	Si Inc., New York, USA

2.1.9 Miscellaneous Materials

Cell culture flasks 25cm^2, 75cm^2	Greiner, Frickenhausen, Germany
Cell culture 96-well, 24-well, 6-well plates	Greiner, Frickenhausen, Germany
Centrifugal concentrators Vivaspin 6ml, 20ml	Vivascience, Hannover, Germany
Cryovials	Greiner, Frickenhausen, Germany
Coverglass	R. Langenbrinck, Emmendingen, Germany
Falcon tubes 15ml, 50ml	Greiner, Frickenhausen, Germany
Needles	BD Biosciences, Heidelberg, Germany
Nitrocellulose membrane Hybond ECL	Amersham, Freiburg, Germany
Parafilm	American Nat. Can., Greenwich, Great Britain
Petriplates	Greiner, Frickenhausen, Germany
Pipette tips	Sarstedt, Numbrecht, Germany
Sterile filter 0,2µm	Renner, Darmstadt, Germany
Syringes	BD Biosciences, Heidelberg, Germany
Trans-well migration (Boyden) chambers 48 well	Neuroprobe, Gaithusberg, USA
WhatmanTM 3MM paper	Scleicher & Schull, Dassel

2.1.10 Chemicals:

Acetic acid	Riedel-de Haen, Seelze
Acetone	Fluka, Buchs, Switzerland
Agarose	Sigma, Steinheim
Ammonium persulphate (APS)	GIBCO, Darmstadt
Ampicillin sulphate	Calbiochem, Darmstadt
Annexin FITC V	Becton Dickinson,Heidelberg
Bactoagar	Fluka, Buchs, Switzerland
Bio-Rad, Munich Bradford reagent	Bio-Rad, Munich
Biotin-X-NHS	Calbiochem, Darmstadt
Bovine Serum Albumin (BSA)	PAA, Pasching, Austia
Brij 96	Fluka, Buchs, Switzerland
Bromo phenol blue	Merck, Darmstadt
Calcium chloride	Merck, Darmstadt
CFSE	Invitrogen, Darmstadt
Chloroform	Riedel-de Haen, Seelze
Coomassie R-250	Merck, Darmstadt
Crystal violet	Sigma, Steinheim
Dimethyl formamide	Merck, Darmstadt
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Ethanol	Riedel-de Haen, Seelze
Ethidium bromide	Merck, Darmstadt
Ethylenediamine tetraacitic acid (EDTA)	Sigma, Steinheim
Fibronectin	Sigma, Steinheim
Foetal Calf Serum (FCS)	PAA, Pasching, Austria
Formaldehyde (37%)	Merck, Darmstadt
G418 sulphate	PAA, Pasching, Austria
Gelatine (cold water fish skin)	Merck, Darmstadt
Glucose	Merck, Darmstadt
Ladder Gene ruler DNA	MBI Fermentas, St. Leon-Rot
Ladder Prestained Protein	MBI Fermentas, St. Leon-Rot
L-Glutamine	AppliChem, Darmstadt

Glycerine	Roth, Karlsruhe
Glycine	GERBU, Gaiberg
HEPES	GERBU, Gaiberg
HiPerfect-Reagent for transfection	Quiagen, Hilden
Hyaluronan	SIGMA, Steinheim, Germany
Hydrochloric acid (HCl)	Riedel-de Haen, Seelze
Hygromycin	PAA, Pasching, Austria
Hyperfilm ECL	Amersham, Freiburg, Germany
Isopropanol	Fluka, Buchs, Switzerland
Magnesium carbonate	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Magnesium sulphate	Merck, Darmstadt
Magnetic Beads	Miltenyl Biotec, Bergisch Gladbach, Germany
Milk powder	Roth, Karlsruhe
Methanol	Riedel-de Haen, Seelze
N,N,N'N'-Tetramethylenediamine (TEMED)	Sigma, Steinheim
Paraformaldehyde	Sigma, Steinheim
Penicillin	Sigma, Steinheim
Phenylmethylsulphonylfluoride (PMSF)	Sigma, Steinheim
Phorbolmyristateacetate (PMA)	Sigma, Steinheim
Propidium Iodide	Sigma, Steinheim
Potassium acetate	Sigma, Steinheim
Potassium carbonate	Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Potassium dihydrogenphosphate	Merck, Darmstadt
Potassium tetrathionate	Merck, Darmstadt
Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim
Protein G Sepharose 4 Fast Flow	Amersham Biosciences, Freiburg
Rotipherose Gel 30 (Acrylamide-mix)	Roth, Karlsruhe
RPMI 1640	GIBCO, Darmstadt cell culture

Silver nitrate	Roth, Karlsruhe
Sodium acetate	Merck, Darmstadt
Sodium azide	AppliChem, Darmstadt
Sodium carbonate	AppliChem, Darmstadt
Sodium chloride	Fluka, Buchs, Switzerland
Sodium hydrogen phosphate	Merck, Darmstadt
Sodium dodecyl sulphate (SDS)	GERBU, Gaiberg
Sodium hydrogen carbonate	AppliChem, Darmstadt
Sodium hydroxide	Riedel-de Haen, Seelze
Sodium pyruvate	Merck, Darmstadt
Sodium thiosulphate	Merck, Darmstadt
SP-Dio$_{18}$(3) dye for exosome labelling	Invitrogen, Darmstadt
Tris	Roth, Karlsruhe
Triton-X-100	Sigma, Steinheim
Trypan bue	Serva, Heidelberg
Trypsin	Sigma, Steinheim
Trypton	AppliChem, Darmstadt
Tween 20	Serva, Heidelberg
Yeast Extract	GIBCO, Darmstadt

2.1.11 Buffers and solutions

Name	**Composition**
Annexin FITC/PI Binding buffer	10mM HEPES pH7.4, 140nM NaCl, 25mM $CaCl_2$
Bicarbonate buffer pH 9.6	15mM Na_2CO_3, 35mM $NaHCO_3$. Fill to 900ml with distilled water. Adjust pH to 9.6 and make it upto 1l with water.
Blot buffer (5x)	10g SDS, 142g Glycine, 30.3g Tris base and make upto 1l with distilled water. 1X buffer was made fresh by taking 5x Blot buffer, methanol and distilled water in the ratio 1:1:3
Cell culture complete medium	RPMI 1640 medium, 10% FCS, 1% Glutamine, 1% Penicillin/ Streptomycin antibiotic solution, additional antibiotic if needed.
DEPC water	200µl DEPC reagent in 1l distilled water and autoclave

Ethidium Bromide	0.2g Ethidium bromide, distilled water 20ml. Stored in dark.
Freezing medium	90%FCS, 10%DMSO
Glycine solution	0.2M glycine in PBS
HEPES buffer	25mM HEPES pH7.2, 150mM NaCl, 5mM $MgCl_2$, 1mM PMSF, 1x Protease inhibitor, 1mM $NaVO_4$, detergent as indicated in experiments
LB medium	10g Peptone, 5g Yeast extract, 10g NaCl, fill upto 1l with Distilled water. For LB plates 15g agar was added.
Laemmli buffer	62.5mM Tris HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue
PBS (pH7.2)	137mM NaCl, 2.7KCl, 4.3mM Na_2HPO_4 in distilled water
Running buffer for protein gels (10x)	10g SDS, 144g Glycine, 30g Tris, filled upto 1l with distilled water
TAE Buffer	242g Tris base, 57.1ml Glacial acetic acid, 100ml 0.5m EDTA pH 8.0, Add distilled water 1l and adjust final pH to 8.5.
TNES buffer	50mM Tris, 0.4M NaCl, 100mM EDTA, 1% SDS.

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Chemically competent cells

From a 50 ml DH5α overnight culture, 1 litre of LB medium (without antibiotic) was cultivated till the OD 600nm reaches 0.5-0.6. Cells were then centrifuged in the Sorvall GSA rotor (250 ml centrifuge bottle) at 5,000 RPM for 10 minutes at 4^{o}C, bacteria pellet was gently resuspended in 1/4 volume of ice cold sterile $MgCl_{2.}$ Cells were again centrifuged at 4000 RPM in the Sorvall GSA rotor for 10 minutes and bacterial pellet was now re-suspended in 1/20 volume of ice cold $CaCl_{2.}$ Cells were centrifuged at 4,000 RPM in the GSA rotor for 10 minutes and the cell pellet was re-suspended in 1/50 volume of ice cold, sterile 85 mM $CaCl_2$ in 15% glycerol w/v. Cells were aliquoted in 50 µl each and stored at -80^{o}C.

2.2.1.2 Transformation

Competent cells were thawed on ice and 1-10ng of plasmid was added and incubated on ice for 30 mins. Competent cell plus plasmid was given a heat shock at 42°C for 2 mins and kept on ice for another 5 mins. To this 1 ml of LB medium (no antibiotics) was added and kept at shaker for 1 hr at 37°C. 10 to 100 µl was then plated on LB agar plate containing selection drug (ampicilline at 100µg/ml). Plates were incubated overnight at 37°C.

2.2.1.3 Mini-Prep

Single colonies were recovered from LB agar plates in 2 ml LB medium containing selection agent (ampicilline at 100µg/ml) on a shaker set at 200 rpm at 37°C overnight.1 ml bacterial suspension was used for miniprep following producer's recommendations (Miniprep kit, Qiagen). Positive clones were obtained by doing enzymatic digestion.

2.2.1.4 Enzymatic restriction digestion

For double digestion 1 µg purified plasmid with insert was mixed with 0.5µl (5U) primary restriction enzyme and appropriate digestion buffer (1X). Total volume was made up with distilled water to 10 µl and digestion was performed for 2 hrs in a water bath at 37°C. After digestion, DNA was purified over Qiagen mini-column and a

second enzymatic digestion was performed following the same procedure. Samples containing right sized fragments were further proceeded *via* midi-prep for transfection.

2.2.1.5 Midi-PreP

To obtain sufficient amount of vector DNA necessary for transfection, overnight cultures of positive clones were incubated overnight on a shaker set at 200 rpm at 37°C in 100ml of LB medium containing appropriate selection antibiotic (ampicillin at 100µg/ml). Whole bacterial culture was used for midi-prepration following producer's recommadations (Midi-prep Kit, Qiagen).

2.2.1.6 Isolation of DNA fragment from agarose gel

The DNA fragments were separated on agarose gel and were illuminated with UV-light. The desired band was excised using a clean scalpel and transferred into 1.5ml tube. The DNA was purified from the excised agarose gel using Qiagen gel extraction kit following manufacturer's protocol.

2.2.1.7 Quantification of DNA

DNA was quantified spectroscopically using a Spectronic-Unicam spectrophotometer. Concentration was determined by measuring the absorbance at 260nm and 280nm. Absorbance at 260nm should be higher than 0.1 but less than 0.6 for reliable determinations. A ratio of A260/A280 between 1.8 and 2 indicated a sufficient purity of the DNA preparation.

2.2.1.8 RNA preparation, cDNA synthesis and amplification

Total RNA isolation was done from 10^7 cells with Tri reagent following manufacturer's instructions (Applichem, Darmstadt, Germany). Quality of RNA preparation was checked by running RNA sample diluted in RNA sample buffer on 1% agarose/formaldehyde gel. cDNA synthesis and amplification was performed by RT–PCR (reverse transcriptase polymerase reaction). 1-2 µg template RNA was mixed with 0.5µg oligo dT primer and heated at 70°C, 5 min; chilled on ice, then mixed with 1µl Im Prom II reverse transcriptase (10U), 1-2mM dNTP, 1x Im Prom II buffer 5x, 6mM $MgCl_2$ and made upto a total of 20µl with nuclease free water. The program continues as 25°C-5min, 42°C-60min and 70°C-15min.

PCR was performed in 25 µl volume containing template, 1-2 mM dNTP, 1.5µl Red Taq polymerase, 2.5 µl 10x Taq buffer and 0.2 µM primer forward and reverse. Total volume was completed to 25 µl with autoclaved distilled water.

PCR Program:

wtCD44 : 94°C-5min, 94°C-30secs, 55°C-30secs, 72°C-1min -32cycles, 72°C-10min

mutCD44 : 94°C-5min, 94°C-30secs, 50°C-30secs, 72°C-1min, 22cycles, 72°C-10min

truncCD44 :94°C-5min, 94°C-30secs, 53°C-30secs, 72°C-1min -22 cycles, 72°C-10min

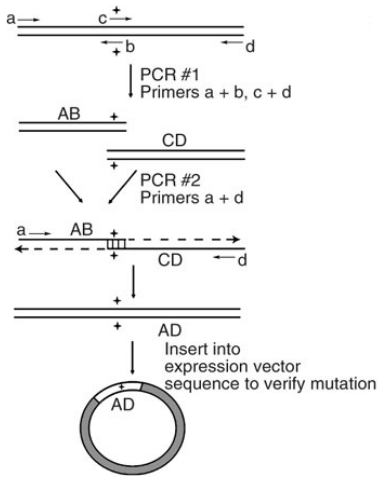
wtCD49d : 94°C-5min, 94°C-30secs, 55°C-30secs, 72°C-3.5min -32 cycles, 72°C-10min

mutCD49d : 94°C-5min, 94°C-30secs, 54°C-30secs, 72°C-3.5min, 22cycles, 72°C-10min

GAPDH : 94°C-5min, 94°C-30secs, 55°C-30secs, 72°C-1min -32 cycles, 72°C-10min

2.2.1.9 Site directed Mutagenesis

Site-directed mutagenesis is performed by using mutagenic primers (b and c) and flanking primers (a and d) to generate intermediate PCR products AB and CD that are overlapping fragments of the entire product AD. Products AB and CD are denatured when used as template DNA for the second PCR; strands of each product hybridize at their overlapping, complementary regions that also contain the desired mutation (indicated by the cross). Amplification of product AD in PCR #2 is driven by primers a and d. Final product AD is inserted into pcDNA3 vector (gray circle) to generate larger quantities of DNA.



Restriction sites were introduced into the mutated primers (CD49dmut MluI) to confirm the efficacy of mutants. Care should be taken that these sites should be unique (should not be present in insert and vector) and should not make any change in the protein structure,

2.2.1.10 DNA gel electrophoresis

PCR products or samples containing DNA of interest were checked by running an agarose gel of 1 to 2%, depending on the size of the product. When Red Taq polymerase was not used then DNA sample was mixed with DNA loading dye (6x) to locate the running front. Markers (1kb or 100bp) were run in parallel. The gel was run at 100 volts in a migration tank containing 1x TAE buffer. Bands were visualized on a U.V. transilluminator.

2.2.2 Protein Chemistry

2.2.2.1 Antibody purification

Antibody purification was done by affinity chromatography using a sepharose protein G-4B column. To purify IgG fractions, sterile filtered hybridomas supernatants were passed over a sepharose protein G-4B column. The column was washed with 0.1M phosphate buffer, pH 7.5. Bound IgG was eluted from the column with 0.1M Glycine buffer pH 2.7. Protein containing fractions were dialyzed against PBS, concentrated and filter-sterilized. The protein amounts obtained were photometrically analysed for protein concentration by Biorad assay.

2.2.2.2 Biorad assay

This test is based on the affinity of the coomassie dye G-250 for proteins. In microassay it allows detections of BSA amounts under 25µg/ml. To proceed with detection of the soluble proteins, 1µl of each sample to test were diluted in a flat micotiter well with 99µl Biorad reagent prediluted (1 :10 in distilled water). In parallel, a 10mg/ml BSA solution in water was serially diluted to allow drawing of a standard curve. After 5min incubations, optical densities were read at 595nm on an ELISA reader. Optical densities were reported to the standard curve for concentration calculation.

2.2.2.3 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

Protein components were separated using SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS, an anionic detergent denatures and imparts negative charge to all proteins in the sample. Proteins can be separated according to their size in an electrical field. Two gels were used for this purpose. At the bottom resolving gel was poured to separate the proteins and at the top a stacking gel. The stacking gel concentrates all proteins in one band and allows them to enter the resolving gel at the same time; it can be mounted with a comb to load samples in the wells. Before loading the gel, samples were boiled for 5 minutes at 95° C for proteins to allow complete protein denaturation. Around 25-30 µl of sample was loaded into wells. The inner and outer chambers were filled with running buffer (1x).

2.2.2.4 Immunoprecipitaion

Freshly harvested cells from lymph node, spleen or cultured cells (10^7) were taken, washed 3 times in cold PBS. Cells were lysed in 1ml HEPES buffer containing protease inhibitor and phosphate inhibitors as well as detergent (1% Lubrol) for 1 hour at 4°C on a rocking plate-form. Lysates were then centrifuged 10min at 13,000 rpm, 4°C. Lysate supernatants were collected. Immunoprecipitation was done using 1 to 5 µg specific monoclonal or polyclonal antibodies at 4°C on a rocking plate-form for over night. Following over night incubation at 4°C, 5% of protein G-sepharose was added to the precleared lysates for 1 hour. Protein G coupled antibody was washed 3 times with lysis buffer completed with detergent. Sepharose pellet was resuspended in 80µl Laemmli buffer and boiled 5min at 95°C. After 1min

centrifugation at maximum speed, 20µl of the supernatant were taken and loaded on acrylamide gel to be resolved by SDS-PAGE. If indicated, cells were biotinylated before lysis and immunoprecipitation. Washed cells were resuspended in HEPES buffer containing 0.1mg/ml NHS-water soluble biotin. Biotinylation was performed 30min under agitation at 4°C. The reaction was terminated by 3 washes with 0.2M glycine PBS solution. Cells were lysed 1 hour at 4°C in 1ml HEPES buffer containing protease and phosphatases inhibitors as well as detergent of choice. Following steps were similar as described above.

2.2.2.5 Western Blotting

Following gel electrophoresis proteins were electroblotted onto a nitrocellulose membrane (Amersham Biosciences) overnight at 30 volts and analysed by immunoblotting using specific primary and secondary antibodies. The gel, foam pads and 3MM Whattmann papers were equilibrated in blotting buffer; the gel was placed on the membrane which in turn was placed on Whattmann paper followed by foam pads on either side. The whole set was placed in a cassette holder, followed by a tank blotting apparatus such that the membrane was placed towards the anode side. After overnight transfer the blots were blocked with PBS/5% milk powder or BSA (for phosphospecific antibodies) for 1 hour, followed by primary antibody for 1 hour. Blots were washed with PBS/0.1%Tween thrice and 5 minutes each wash. Then the blots were incubated in secondary antibody for an hour, followed by washing again. The blots were developed with Enhanced Chemiluminescence system (ECL, Amersham Biosciences) and exposed to X-ray film (Amersham Biosciences) for desired time points and developed.

2.2.3 Cell biology

2.2.3.1 Cell culture

Cells were grown in a humidified incubator at 37°C, 5% CO_2. Cells were maintained in RPMI 1640 medium in 10% FCS as per the requirements. Cells were usually passaged at a ratio of 1:4. For long term cell storage, cells were washed once with medium and frozen in cryovials in FCS, 10% DMSO. The vials were placed for 1 hour at -80°C before transferring into liquid nitrogen. Cells were thawed from the cryovials by placing the vials from liquid nitrogen first on ice, followed by 37°C

water bath and immediately placed in falcon containing medium and centrifuged at 1600 rpm for 5 minutes. The medium was sucked off and new medium was added and transferred to flasks at high density to maximise recovery.

Cell viability was determined using hemacytometer and trypan blue staining.

Tryptan blue: 0.4% Trypan blue (4vol)

4.5% NaCl (1vol).

2.2.3.2 Transfection of suspension cells

10^7 Jurkat cells or/and El4 cells were washed in cold serum-free RPMI. Cells were then resuspended in 200 µl serum-free RPMI, kept on ice (Jurkat cells) or at room temperature (EL4 cells) in 0.4 cm electroporation cuvette for 10 mins. 10-15 ug of sterile DNA is added to the cells in the cuvette and mixed. Electroporation was done at 250 V, 960 µF. After the pulse cuvette is kept on ice (Jurkat cells) or at room temperature (EL4 cells) for 10 min. Later cells were plated in 1 ml of RPMI+10%FCS without antibiotic mix for 12 hrs and then were plated in 1ml of complete medium with 750µg/ml (Jurkat cells) and 250µg/ml (El4 cells) G418. Cells were tested within 48 hrs for the transfection efficiency via flow cytometry. Once positive for the desired protein, cells were kept in culture for another 2 weeks and can be subjected to single cell cloning.

2.3.3 Single cell cloning

Single cell cloning can be done by single cell per well seeding or by serial dilution using 96 well plate in RPMI+10%FCS+G418 medium. For serial dilution $5x10^4$ cells were plated in a single well of 96 well plates and were serially diluted down the lane. After 4 weeks clones were checked by flow cytometry, positive clones were expanded.

2.2.3.4 Magnetic Beads separation

Spleen cells and lymph node cells were prepared as described below. $CD11b^+/Gr1^+$ spleen cells were enriched by magnetic bead isolation. T cells were enriched in LNC by magnetic bead depletion of $CD11b^+$, $CD19^+$, and $CD16/CD32^+$ cells.

Viability of the separated populations was >95%.

2.2.3.5 Flow cytometry

Fluorescent activated cell sorting (FACS) allows cell segregation based on size and granulation and also allows detecting the expression levels of proteins in cells. This method is based on diffraction of light and measure of fluorescence, which reflects cell size and amount of fluorescent antibody labelled cells.

Cells were washed in PBS/0.5% BSA. About $5x10^5$ cells were added to round bottomed 96 well plates. After centrifugation cells were suspended in 50 µl of primary antibody diluted in PBS/0.5% BSA and incubated for half an hour on ice. This was followed by washing the cells thrice with PBS/0.5%BSA, 5min each wash. Secondary antibody (50µl) coupled to fluorochrome was added to each well and incubated for half an hour on ice in the dark. The cells were again washed. After the last wash cells were suspended in 200µl PBS/0.5%BSA for immediate measuring. Staining was evaluated using FACS-Calibur (Becton Dickinson, Heidelberg, Germany). In case of double or triple fluorescence the same procedure was repeated with adequate antibodies and blocking steps wherever necessary (e.g. different antibodies from the same species).

For cytokines, intracellular FACS was performed. The cells were first incubated with formalin 1% to fix them for 10 minutes on ice. The cells were washed with PBS 1% BSA 3X as mentioned above. Thereafter the cells were incubated with 0.1 % Tween for 15 minutes on ice to create pores on the cell membrane and facilitate the entry of antibodies against the cytokines into the cells. The cells were again washed and antibodies were added following the usual protocol.

2.2.3.6 Immunohistochemistry

Immunoassay was performed on 5µm cross-section for skin tisues. The section was fixed with choloroform/acetone (1:1) for 4 mins. The endogenous peroxidase reactivity was blocked by washing with levamisole solution. Sections were fixed with 4% PFA, after washing with PBS, non-specific binding was blocked with 2% normal serum derived from the same species as the secondary antibodies. The sections were washed with PBS followed by incubating in secondary biotinylated antibody for 30 min. After 3 washing of 15 mins each section was incubated with avidin-biotin complex. After washing with PBS, the sections were detected by staining with AEC reagent. The reaction was terminated by adding water. Sections were counterstained with Mayer's hematoxylin and mounted afterwards.

2.2.3.7 Migration assay

Cell lines and LNC were freshly prepared. The cells were counted and $5x10^4$ cells were incubated with or without anti-CD44, anti-CD49d and PMA for 30 mins on ice and then were seeded onto the upper well of a boyden chamber in 30 µl of RPMI without FCS. The lower part of the chamber is separated by a 5µm (LNC) and 8µm (cell lines) pore sized polycarbonate membrane. The lower part of chamber contained chemotaxis buffer with or without HA (5µg/ml), FN (250µg/ml). The chamber was incubated in 5% CO_2 at 37°C for 4 hrs and cells in the lower chamber were counted by hemacytometer and trypan blue staining.

2.2.3.8 Coating of plates for crosslinking

HA (10µg/ml) and FN (50µg/ml) in bicarbonate buffer was used to coat 6-well plate (1ml) or 96-well plate (100µl). Plates were kept at 4°C o/n or at 37°C for 1 hr, plates were than washed 2X with PBS+0.1%BSA and blockd with PBS+1%BSA for another 1 hr at 37°C. Washed 2X with PBS+0.1%BSA and can be stored in the same washing buffer untill use, at 4°C.

2.2.3.9 Apoptosis assay

Apoptosis assay was performed using Annexin V-FITC and propidium iodide (PI) (R & D systems, Wiesbaden-Nordenstadt, Germany) double staining. Early apoptotic cells bind to Annexin V because of the exposed phosphatidylserine on the outer cell membrane. Late apoptotic cells are positive for Annexin V and PI. Necrotic cells bind only PI. LNC ($1x10^5$, triplicates) were co-cultured with and without MDSC. Cultures contained AA skin lysates (100µg/ml) or PMA (10^{-8}M)/ionomycin (10^{-6}M). Cell lines were activated over crosslinked HA, FN and with PMA and were treated with or without anti-CD44 and anti-CD49d. Apoptosis was determined after 48h by AnnexinV/PI staining, 96-well plates were centrifuged at 1600 rpm for 5 minutes and washed with PBS/1% BSA. Cell labelling was performed according to manufacturer's instructions. Cell were incubated in the dark at RT for 15 min and detected by FACS using the FL1 channel for Annexin FITC and FL-3 channel for PI.

2.2.3.10 Proliferation assay

LNC ($1x10^5$, triplicates) were co-cultured with and without MDSC. Cultures contained AA skin lysates (100µg/ml) or PMA (10^{-8}M)/ionomycin (10^{-6}M). Cell lines

were activated over crosslinked HA (5µg/ml) and FN (250µg /ml) and with PMA (10^{-8}M)/Ionomycin (10^{-6}M) in solution and were treated with or without anti-CD44 and anti-CD49d to check for the proliferative activity, cells were evaluated for 72 hour in the presence or absence of anti-CD44 or anti-CD49d by ^{3}H-thymidine incorporation.

2.2.3.11 Cytosol, nuclei and mitochondria preparation

Cells were incubated in hypotonic buffer, homogenized and centrifuged at 800rpm for pelleting the nuclei. For separating the cytosolic from the mitochondrial fraction, $2.5x10^{6}$ cells were lysed in 0.5ml lysis buffer. After adding Nonidet-P40 (0.5%), vortexing, and centrifugation (1600rpm, 5min), cytosolic proteins were recovered from the supernatant. The pellet (mitochondria) and the pelleted nuclei were washed, resuspended in lysis buffer (1% TritonX-100, 1% SDS) and sonicated (7sec, 9cycles).

2.2.4 Animal experiments

2.2.4.1 Skin Transplantation

Donor mouse was sacrificed and recipient mice were anesthesized by injecting 0.12-0.15ml ketamine i.p. Antero-posterior midline of the graft recipients was shaved. Disinfection of mice before grafting was done with the ethanol pad. For grafting 1 cm pieces in diameter of alopecia areata affected skin from the donor were cut and collected in PBS (till donor mice are ready). The recipient mice were cleaned with iodine solution at the area shaved before making any surgery. Round piece of skin from the graft recipient were removed from antero-posterior midline (shaved area). Graft was put onto the gap, stiches were made on four sides (with 3 knots each) and the gaps were sealed with histoacryl-glue. Bandages were applied once glue is dry. Mice drinking water was supplied with antibiotic Sulfadimidin (1g in 1L water) from day 0 (grafting day) to day 4 and from day 7 to day 11 after grafting.

To look for effect of MDSC on hair re-growth, mice received MDSC ($1x10^{7}$, i.v.) or an ATRA depot pellet (sub cutaneous). Both applications were repeated after 3 weeks.

2.2.4.2 Lymphatic organ preparation

Lymph nodes and/or spleen were collected in the medium (RPMI) or PBS +1%FCS from 16-20 weeks old mice. Organs were meshed on the sterile gauze and washed the gauze 2-3 times with sterile medium. Cells were collected in 50 ml of RPMI medium

supplemented with 1% FCS and cells were centrifuged at 1600rpm for 5 minutes. Now these cells can be used for further experiment.

2.2.4.3 Skin infiltrating leukocytes (Skil) preparation

Skin is taken from whole of the back and is made free from fat, tissue and hair. Skin is first treated with trypsin (1mg/ml) for 30 min at 37°C and then pressed (epidermis) onto the sterile gauge (very harshly), cells were collected and washed with medium (RPMI) two times. This procedure was repeated 3 times and then cells were pooled and kept at 37°C for 2 hrs or o/n in RPMI 1640 medium supplemented with 10% FCS, antibiotic mix to rejuvenate them.

2.2.4.4 Skin lysate preparation

Skin is taken from whole of the back and is made free from fat, tissue and hair. Skin was then cut into very small pieces with scalpel and was homogenized with help of an ultra turrax in 5 ml of ice cold PBS. Lysates were centrifuged at 2000rpm for 15 mins to settle down the debris and were frozen at -20^0C until use.

2.2.4.5 Delayed type hypersensitivity (DTH) reaction

Whole back of the mice was shaved and sensitized by applying 1% SADBE (squaric acid dibutylester) in acetone on the dorsal side (back) 3-4 times with the help of cotton bud. Mice were then weekly sensitized by topical applications of 0.5% SADBE in acetone on the back and the abdominal wall to induce a moderately severe contact dermatitis lasting for 2–3 days. Mice were challenged 3-4 times. The skin becomes red, swollen, itchy, or blistered. This kind of skin reaction is a sign that the contact sensitizer has worked. Mice were sacrificed by cervical dislocation 3 days after the last challenge.

2.2.5 Statistical analysis

Significance of differences was calculated according to the Student's T test (*in vitro* studies). Functional assays were repeated at least 3 times. Mean±SD of *in vitro* studies are based on 3-4 replicates.

3. Results

Autoimmune diseases are frequently characterized by persisting activation and expansion of autoreactive T cells as well as resistance towards activation induced cell death. This also accounts for alopecia areata, an autoimmune disease affecting anagen stage hair follicles, where the topical application of contact sensitizers presents the most efficient therapy in human as well as in a mouse model (257-259). I tried to answer particularly two questions, first what are the molecular mechanisms that allows for persisting T cell activation and second, how can the induction of an immune disorder, chronic contact eczema, be a therapeutic for another immune disorder, an organ related autoimmune disease.

3.1 Persisting T cell activation

I started with the first question, the persisting activation and expansion of T cells. In the healthy organism antigen-specific T cells become activated via TCR/CD3 complex and co-stimulatory signals. Activated T cells become regulated by transient TCR internalization, inhibitory T cells such as regulatory T cells ($T_{reg)}$ and a mechanism called activation induced cell death. Alternatively to T cell activation *via* the TCR/CD3 complex, though not mutually exclusive, there is some evidence that T cells could become activated circumventing TCR engagement. Such an alternative mechanism could explain the escape from regulatory mechanisms and provides a means for persisting T cell activation and expansion as frequently seen in autoimmune diseases. I explored this question with T cell lines, which has the advantage of a uniform population of T cells and offers the opportunity to modulate individual molecules. In addition, I selected a T cell leukaemia, which expresses the TCR/CD3 complex and a thymoma, which does not express the TCR/CD3 complex. This allowed me to distinguish between TCR-dependant versus TCR-independent effects.
CD44 and CD49d can act as accessory molecules supporting TCR-initiated activation signals.Yet, they have also been suggested to contribute to TCR-independent T cell activation. In fact, previous work in our lab provided evidence that these two molecules might, in addition, co-operate.

3.1.1 Selection of an appropriate model system

To explore the activity of CD44 as well as the suggested joint activities of CD44 and CD49d, I selected EL4 (Thymoma cell line) cells which are $CD44^+$, $CD49d^-$ and $TCR/CD3^-$ and Jurkat cells (Leukemia T cell line) which are $CD44^-$, $CD49d^+$ and $TCR/CD3^+$ and for comparison naïve and activated LNC (Fig.1). Jurkat cells were transfected with CD44, CD44 mutated at position Ser325 and Ser291 (mutCD44) or CD44 without cytoplasmic tail (truncCD44). The mutCD44 will prohibit ezrin binding, since binding of ezrin requires an exchange of Ser325 versus Ser291 phosphorylation (202), truncCD44 will prohibit binding of all cytosolic linker and signal transduction molecules. This will also provide evidence, whether the HA binding form of CD44 (activated form) is essential for the association with CD49d. EL4 cells were transfected with CD49d or CD49d mutated at position Ser988 (mutCD49d). The latter will interfere with CD49d phosphorylation and prevents paxillin and FAK binding (260,261).

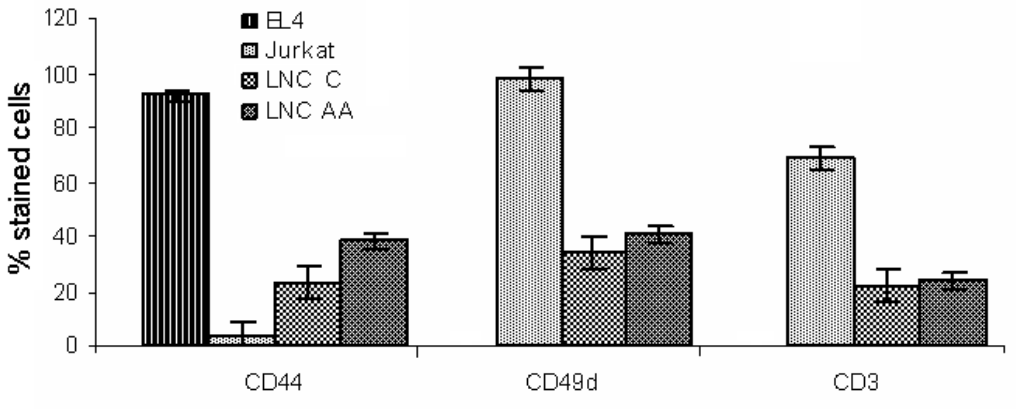


Figure1. CD44, CD49d and CD3 expression in EL4 and Jurkat cells: EL4, Jurkat and for comparison LNC from naïve (control) and AA mice were stained with the indicated antibodies and expression was checked by flow cytometry**.**

3.1.2 Cloning of CD44 and CD49d mutants

Cloning of CD44 without cytoplasmic domain (truncCD44):

As the first step wild type murine CD44 was re-cloned in pcDNA3 vector. Restriction digestion with EcoRI and XhoI confirmed correct insertion of wild type CD44 (1.2

Kb) (Fig.2A). This plasmid was used for constructing the CD44 mutants- without cytoplasmic tail (truncCD44) and CD44 mutated at Ser291 and Ser325 to alanine (mutCD44).

For the construction of CD44 tailless mutant, a gradient PCR was performed for an appropriate annealing temperature. A good amplification was obtained at all the tested temperatures (Fig.2B). The amplified products were ligated to pcDNA3 at restriction sites EcoRI and XhoI and ligation was confirmed by restriction digestion with the same set of enzymes (Fig.2C), positive clones were confirmed by sequencing.

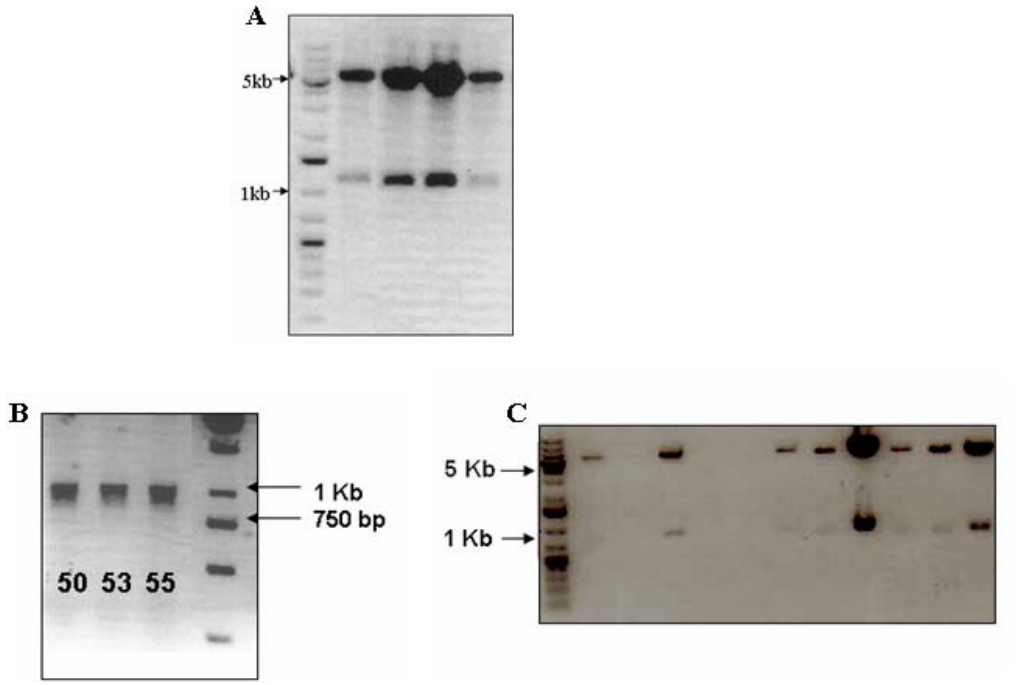
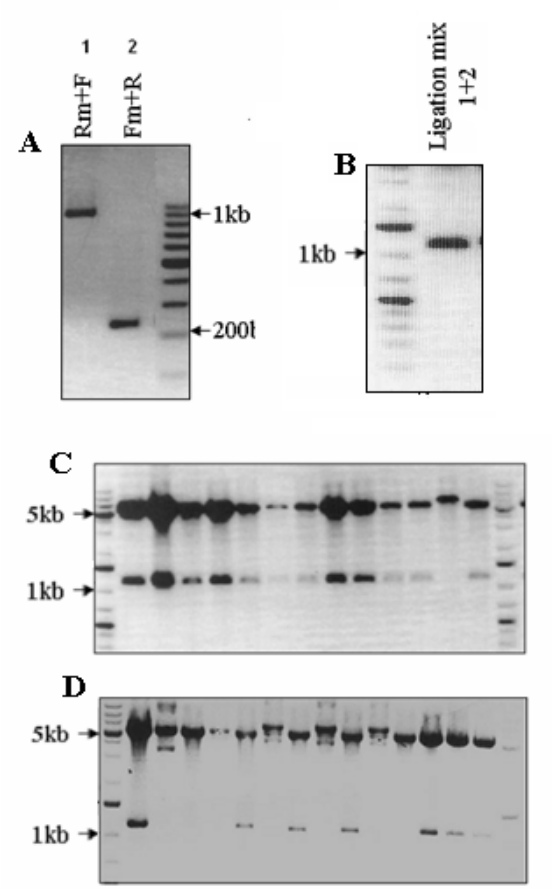


Figure2. Amplification of CD44 excluding the cytoplasmic domain (truncCD44): (A) Confirmation of wild type CD44 in pcDNA3 by restriction digestion with EcoRI and XhoI at 37° C for 2 hrs. The products were run on 1% agrose gel. The desired fragment was obtained at 1.2 kb. (B) Gradient PCR for truncated CD44. CD44 plasmid from (A) was used as a template for gradient PCR at 50°C, 53°C and 55°C. Amplified products were run on 1% agrose gel. The desired product was obtained at 1kb. (C) Amplified products were cloned in pcDNA3 and clones were confirmed by restriction digestion with EcoRI and XhoI at 37° C for 2 hrs. The products were run on 1% agrose gel. The desired fragment was obtained at 1 kb.

Cloning of mutated CD44 at Ser291 and Ser325 to alanine

The CD44 wild type plasmid described above was used as a template for amplification with the primers with the desired base pair changed from AGT (Serine)

to GCT (Alanine). It was done in two parts; first mutation at Ser325 was introduced followed by the mutation at Ser291. Amplification using forward primer (F) and reverse primer with the mutation (Rm); reverse primer (R) and forward primer with the mutation (Fm) was performed, desired DNA amplification was obtained at 1.0 kb and 210 bp, respectively (Fig.3A). These amplified products were ligated with each other and were used as template for the second amplification using forward and reverse primer. The desired DNA fragments were obtained at 1.2 Kb (Fig.3B). The amplified products were ligated to pcDNA3 at restriction sites EcoRI and XhoI. Ligation was confirmed by restriction digestion with EcoRI and XhoI (Fig.3C). Positive clones were confirmed by sequencing. A positive clone from the first mutation was used as template for the second mutation at Ser291 and was cloned in pcDNA3. Restriction digestion with EcoRI and XhoI was performed to confirm the positive clone (Fig.3D). Positive clones were confirmed by sequencing (Fig.3E).



E

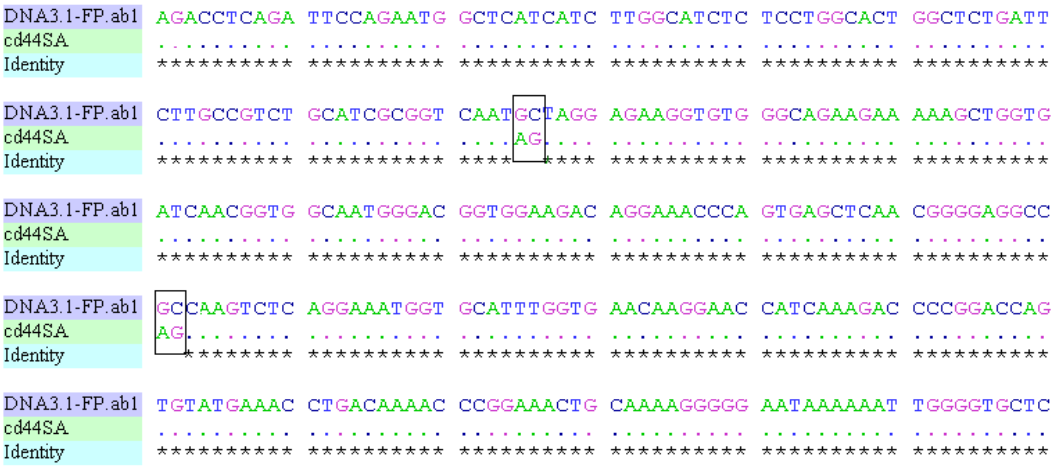
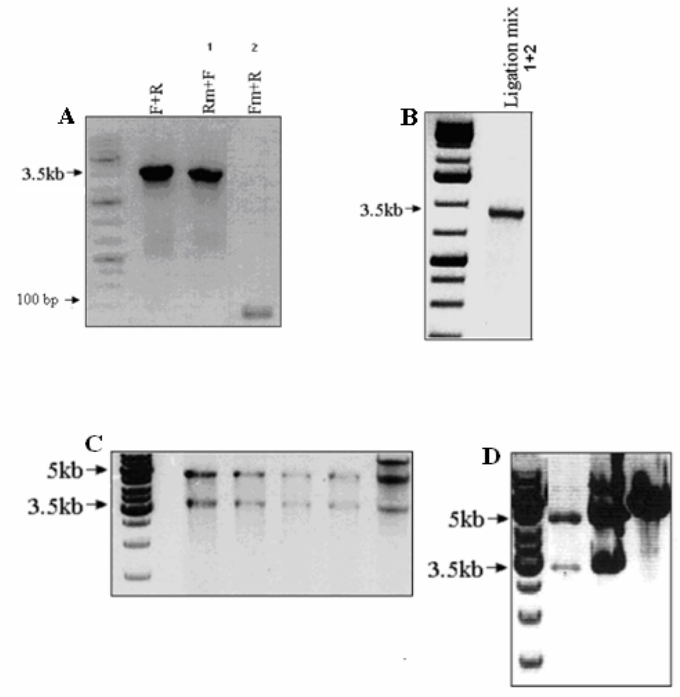


Figure3. Cloning of CD44 mutated at Ser291 and Ser325 positions: (A) Amplification for the first mutation (Ser325) was done at annealing temperature 50° C using wt CD44 as template with forward primer F+ reverse primer with mutated base pair Rm (1kb) and reverse primer R+ forward primer with mutated base pair Fm (210). Products were run on 1% agrose gel. (B) Second amplification was done using template from (A) with forward and reverse primer. (C) The amplified product was cloned into pcDNA3. Restriction digestion was done with EcoR1 and Xho1 at 37° C for 2 hrs. The desired fragment was obtained at 1.2 kb. (D) The second mutation (Ser291) was done using a positive clone from (C), amplification was done using primers as in (A), amplified products were cloned in pcDNA3 and restriction digestion was done with EcoR1 and Xho1 at 37° C for 2 hrs, products were run on 1% agrose gel. The desired fragment was obtained at 1.2 kb. (E) Sequence analysis of the positive clone, desired mutations are shown with boxes.

Cloning of mutated CD49d at Ser988 to alanine

For inducing mutation in CD49d (3.5 kb) at Ser988 to alanine, wild type CD49d was used as template and amplification with the primers with the desired base pair changed from TCT (serine) to GCT (alanine) was performed. First, by using forward primer (F) and reverse primer with the mutation (Rm), second, using reverse primer (R) and forward primer with the mutation (Fm). The desired DNA amplification was obtained at 3.4 kb and 75 bp, respectively (Fig.4A). These amplified products were ligated to each other and used as template for the second amplification reaction.The desired DNA fragments were obtained at 3.5 Kb (Fig.4B). Amplified products were ligated to pcDNA3 at restriction sites EcoRI and XhoI. Ligation was confirmed by restriction digestion with EcoRI and XhoI (Fig.4C) and with MluI and EcoR1 (Fig.4D), which was introduced into the mutated primer. Positive clones were confirmed by sequencing (Fig.4E).



E

```
NA3.1-RP1.ab1  CTAGATGCAT GCTCGAGTCA GTCATCATTG CTTTTGCTGT TGACATAACT CCAGCTGTCT
cd49d..        ---------- -------... .......... .......... .......... ..........
Identity                  *** ********** ********** ********** **********

NA3.1-RP1.ab1  CTCCTGTTTG CTTCTTGTAG GATAGATTTG TACTGTCTTT TAAAGAATCC AGCCTTCCAC
cd49d..        .........T .......... .......... .......... .......... ..........
Identity       *********  ********** ********** ********** ********** **********

NA3.1-RP1.ab1  ATAACACATG AAATTAATAA AAGTACAATA AGTCCAAGTA GCAAGCTGAT GGTAATAATA
cd49d..        .......... .......... .......... .......... .......... ..........
Identity       ********** ********** ********** ********** ********** **********
```

Figure4. Cloning of CD49d mutated at Ser988: (A) Amplification was done with forward primer F+ reverse primer with mutated base pair Rm (3.4 kb) and reverse primer R+ forward primer with mutated base pair Fm (75 bp). Products were run on 1% agrose gel. (B) The second amplification was done using template from (A) with forward and reverse primer. (C, D) The amplified product from (B) was cloned into pcDNA3 and restriction digestion with (C) EcoR1 and Xho1and (D) MluI at 37^{o}C for 2 hrs was done. Samples were run on 1% agrose gel. The desired fragment was obtained at 3.5 kb. (E) Sequence analysis of positive clone, the desired mutation is shown with box.

3.1.3 Efficacy of transfectants

EL4 and Jurkat cells were stably transfected with non-mutated and mutated CD49d and CD44, respectively. Both lines showed high and comparable expression of CD44 and CD49d. Furthermore, transfection-induced expression was well in the range of native expression in activated leukocytes (Fig.5 A, B).

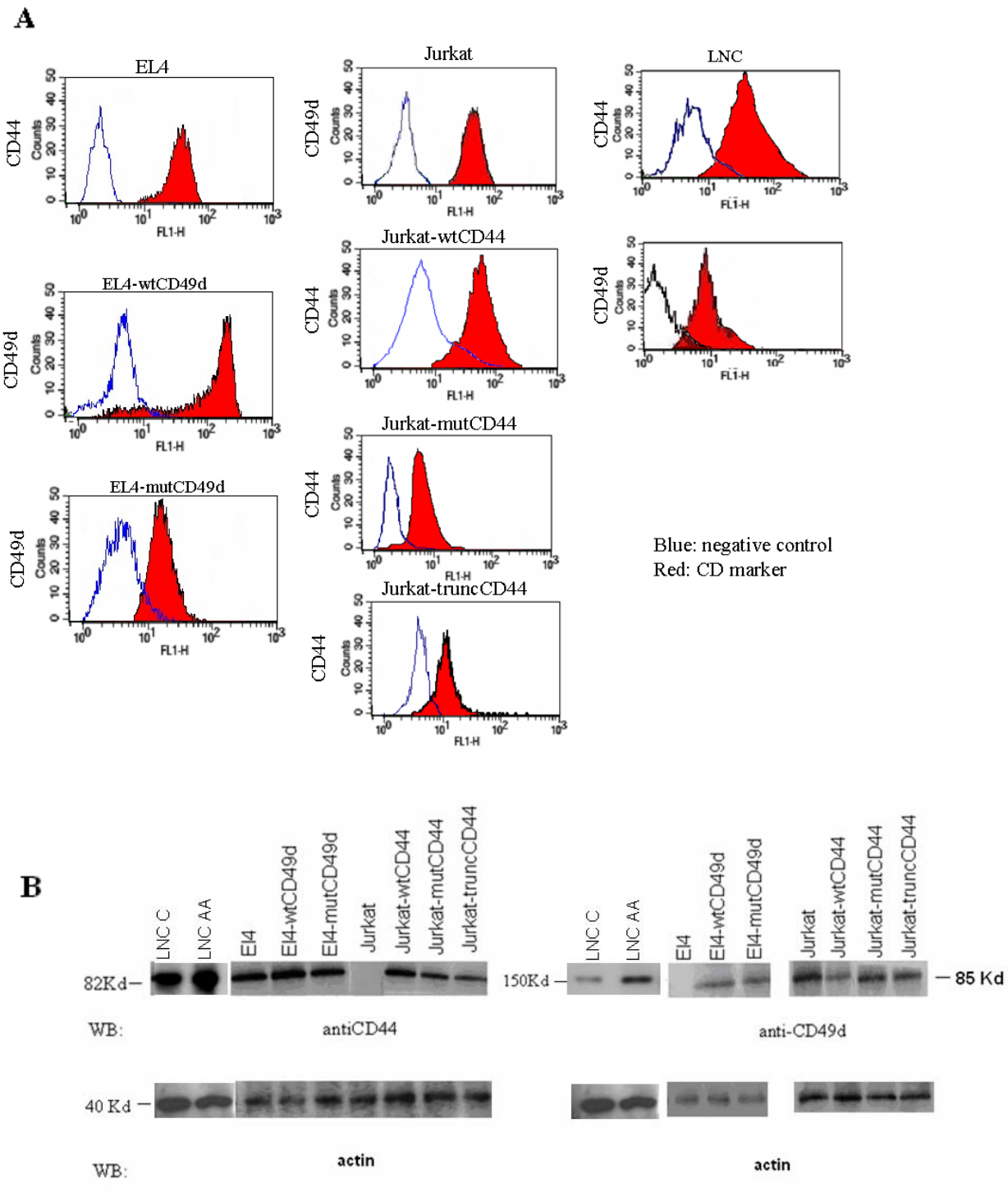


Figure5. Efficacy of transfection: (A) Transfection efficiency was checked by flow cytometry. EL4, EL4-CD49d, EL4-mutCD49d, Jurkat, Jurkat-CD44, Jurkat-mutCD44, Jurkat-truncCD44 cells were stained with the indicated antibodies. (B) Cells were lysed and were run on SDS-PAGE, proteins were transferred to nitrocellulose membrane and blotted with the indicated antibodies.

3.1.4 Characterization of transfected lines

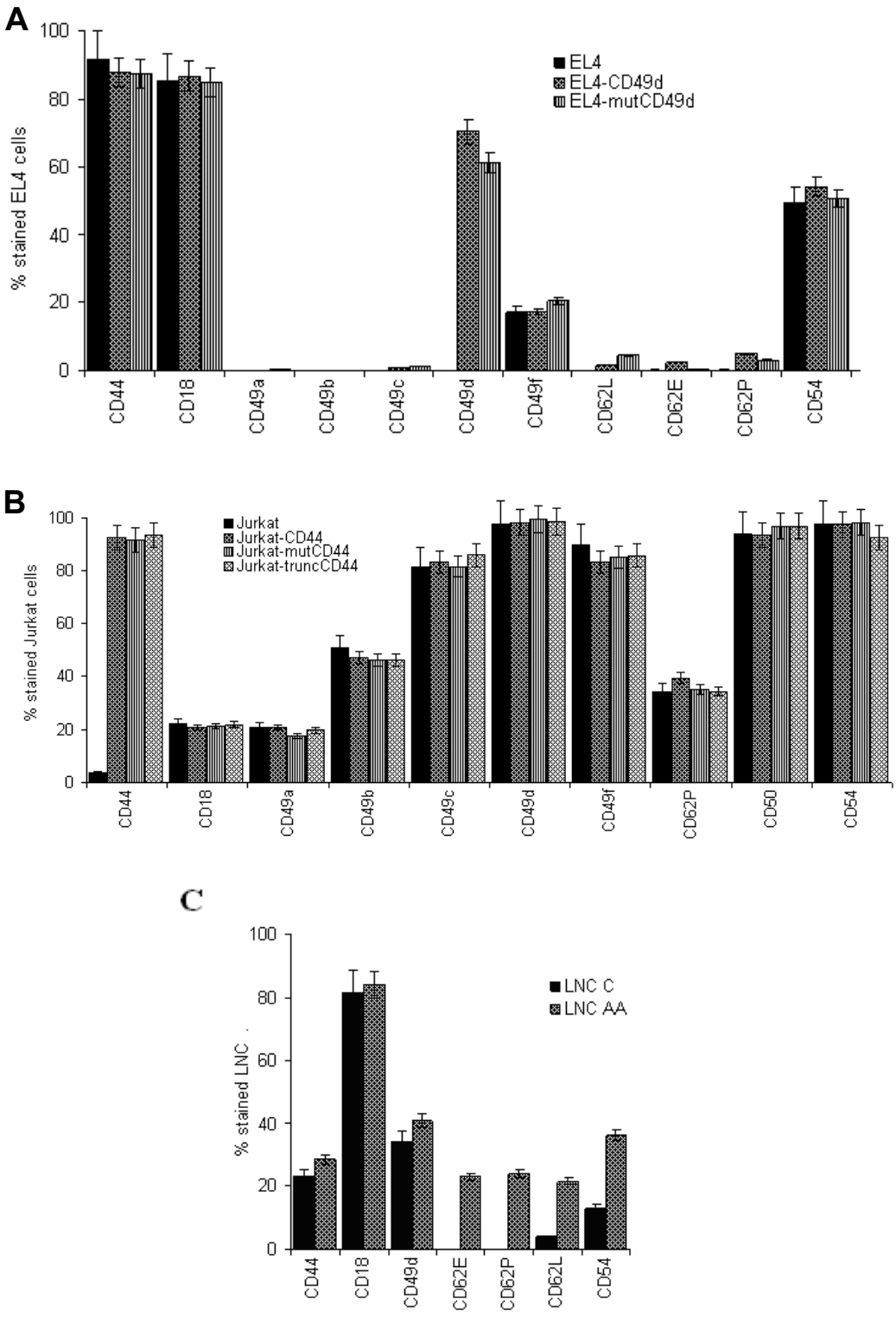
As a prerequisite to define the impact of CD44-CD49d cooperativity on leukocyte / leukemia migration, activation and apoptosis resistance, it was mandatory to define the expression profile of adhesion molecules, T cell and T cell activation markers in EL4 and Jurkat cells and to control for possible alterations in expression by transfection of CD49d and CD44, respectively. EL4 cells along with CD44, express other adhesion molecule CD18 (β_2), but do not express selectins and express CD54 (ICAM1) at medium level. EL4 cells hardly express β1 integrins like CD49a (α1β1), CD49b (α2β1), CD49c (α3β1), CD49d (α4β1), except for low level expression of CD49f (α6β1). Expression of none of these molecules is altered by CD49d transfection. Jurkat cells do not express CD44. They express CD18 (β_2) at a low level as well as β1 integrins CD49a, CD49b, CD49c, CD49d and CD49f, strongest expression being seen for CD49c and CD49d. Jurkat cells weakly express selectin CD62P (P-selectin), but not CD62L (L-selectin) and CD62E (E-selectin). They express CD50 (ICAM3) and CD54 (ICAM1) at a high level. Expression is not altered by CD44 cDNA transfection (Fig.6A-C).

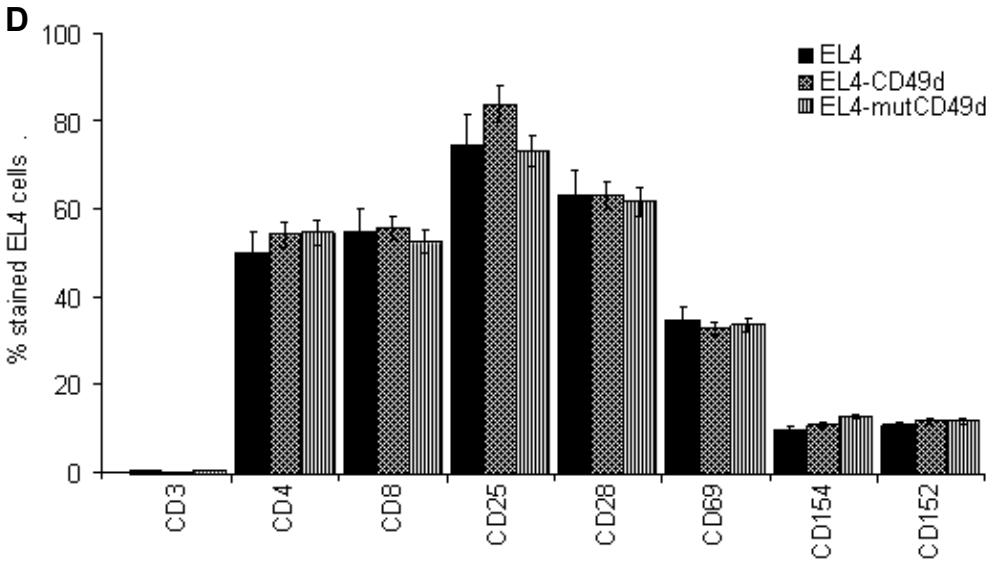
EL4 cells do not express the T cell receptors TCRαβ or TCRγδ chains or CD3, but express CD4 (helper T cell specific for MHC classII) and CD8 (cytotoxic T cell specific for MHC class I) at high levels. This is in line with their origin from thymus, where early pre-T cells express CD4 and CD8 shortly before TCR and CD3 complex expression. Jurkat cells express CD3 and CD4.

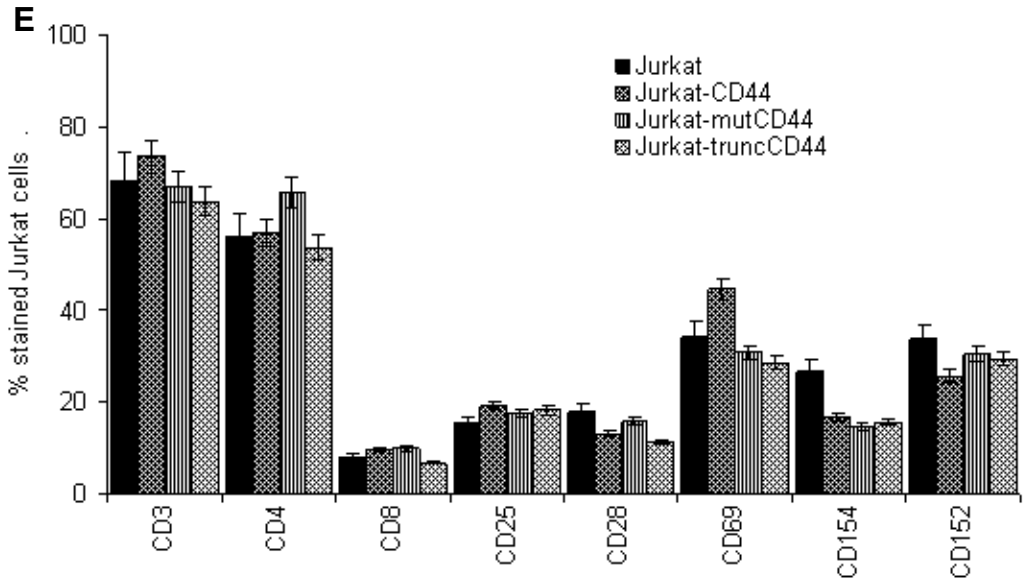
Besides adhesion molecules, it was also important to know the expression of so called accessory molecules, which support the TCR complex. EL4 cells express CD25 at a high level and CD28, CD69 at medium level, and CD154 and CD152 at a low level. Non-transfected Jurkat cells express CD69, CD154 at high level and CD152, CD25 and CD28 at medium level. Expression of these molecules is unaltered after CD44 cDNA transfection (Fig.6D, E).

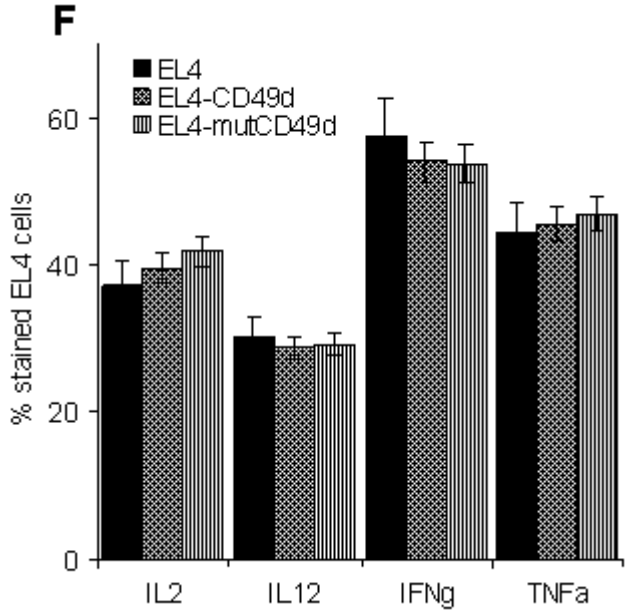
Both EL4 and Jurkat cells express the cytokines IL2 and IL12 which are involved in T cell activation. Only EL4 cells express IFNγ and TNFα at a high level. Expression of these cytokines is low in naïve lymphocytes, but increased in AA LNC (Fig.6F-H). Finally and in view of the question on apoptosis induced cell death, it should be noted that Jurkat cells express the apoptosis receptor CD95 (death receptor), and its ligand CD95L (death receptor ligand), CD120a (TNF receptor) which binds to TNF and

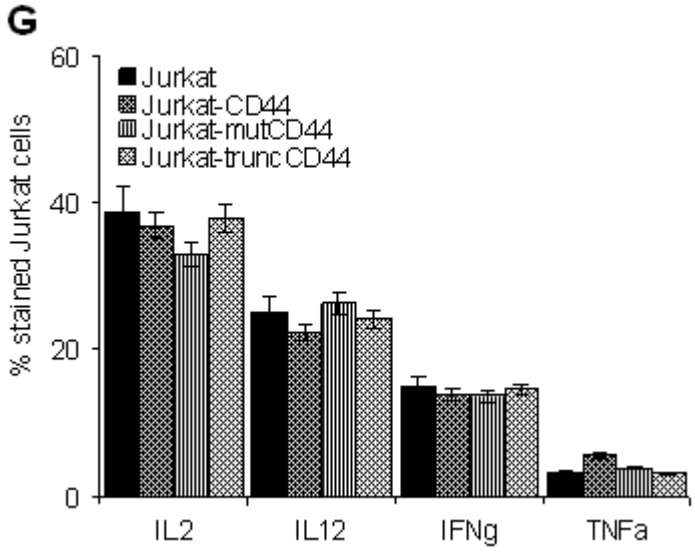
mediates apoptosis) and CD284 (Toll like Receptor-4). EL4 cells only express CD254 (TNFR ligand expressed by helper T cells). Naïve lymphocytes express CD95 and CD254 at a medium high level. Expression of CD254 is decreased in AA LNC, CD95L and CD284 expression is increased in AA LNC (Fig.6I-K).

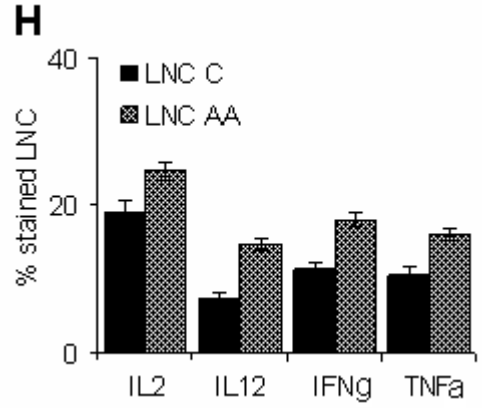



D
% stained EL4 cells
100
80
60
40
20
0
EL4
EL4-CD49d
EL4-mutCD49d
CD3
CD4
CD8
CD25
CD28
CD69
CD154
CD152


E
% stained Jurkat cells
100
80
60
40
20
0
Jurkat
Jurkat-CD44
Jurkat-mutCD44
Jurkat-truncCD44
CD3
CD4
CD8
CD25
CD28
CD69
CD154
CD152


F
EL4
EL4-CD49d
EL4-mutCD49d
% stained EL4 cells
0
20
40
60
IL2
IL12
IFNg
TNFa


G
Jurkat
Jurkat-CD44
Jurkat-mutCD44
Jurkat-trunc CD44
% stained Jurkat cells
0
20
40
60
IL2
IL12
IFNg
TNFa


H
LNC C
LNC AA
% stained LNC
0
20
40
IL2
IL12
IFNg
TNFa

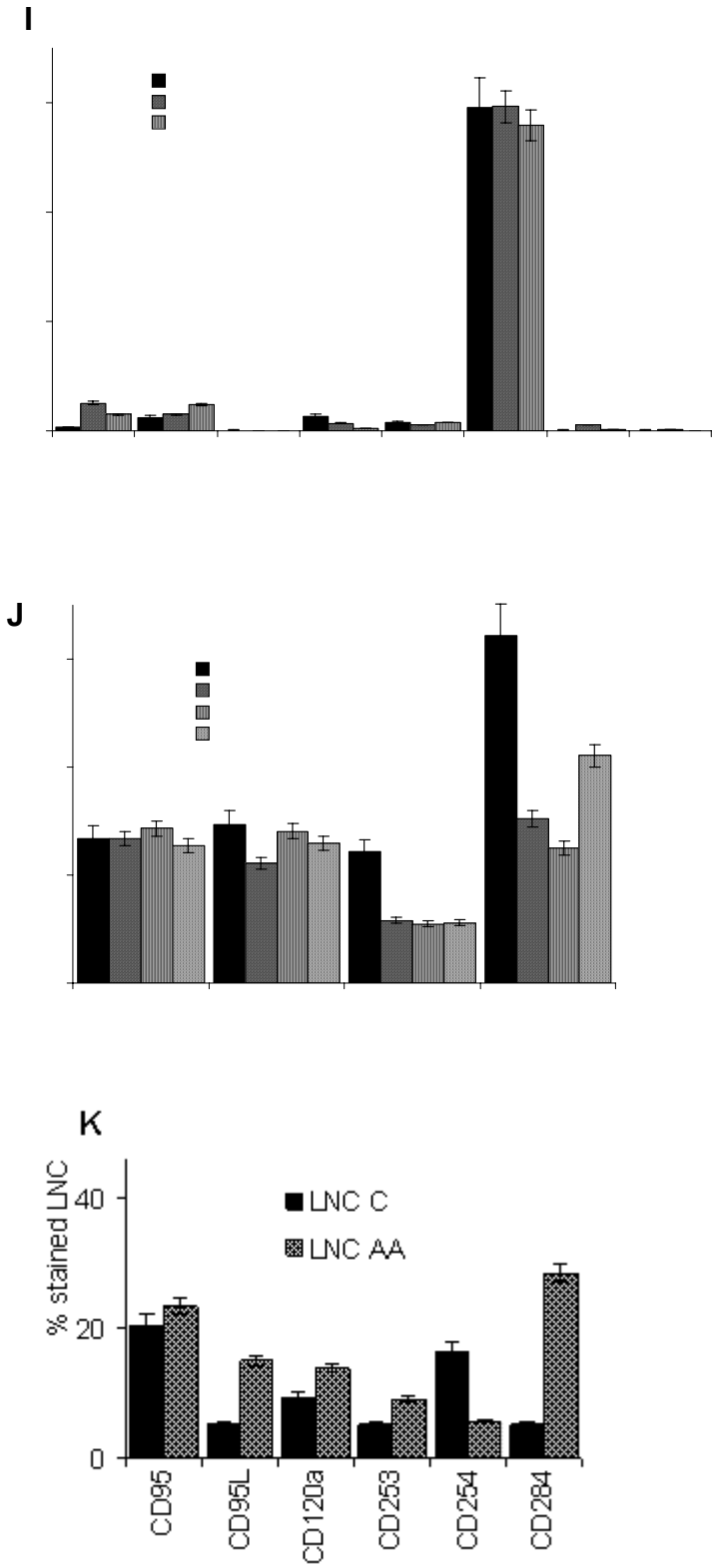


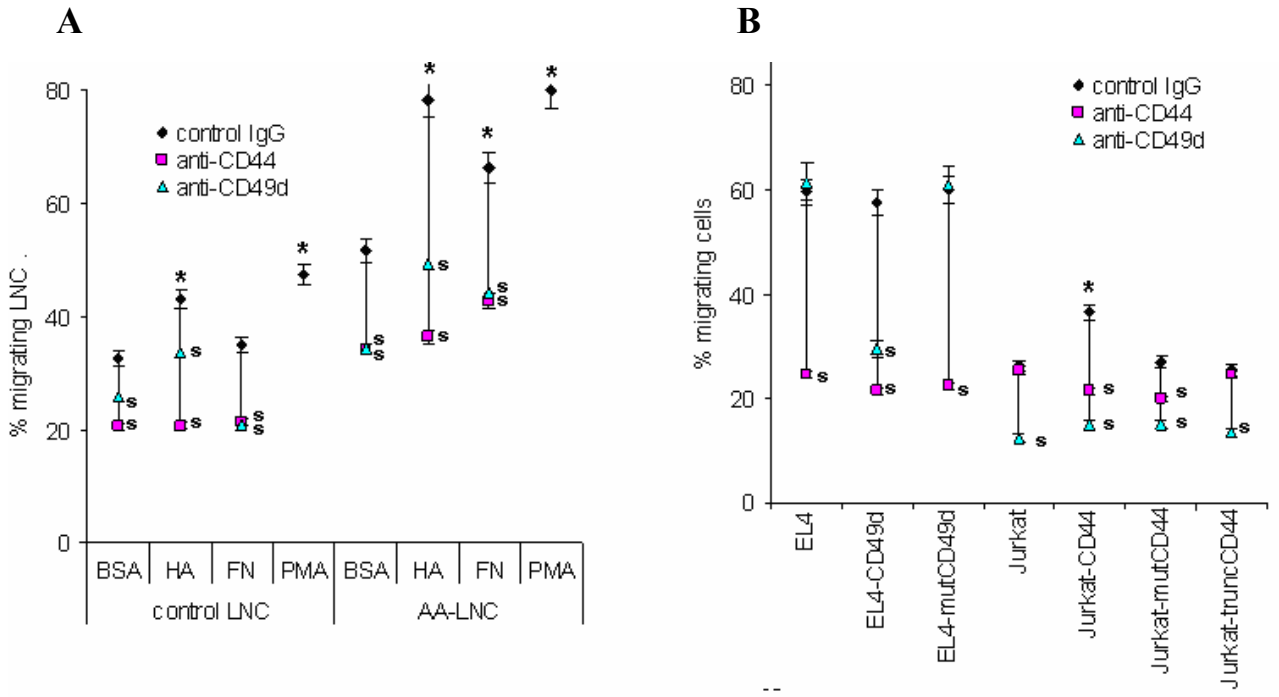
Figure6. Characterization of the transfected El4 and Jurkat cell lines: LNC from naïve (control) and AA mice and El4, El4-wtCD49d, EL4-mutCD49d, Jurkat, Jurkat-wtCD44, Jurkat-mutCD44, Jurkat-truncCD44 cells were stained with the indicated antibodies and expression was checked by flow cytometry.

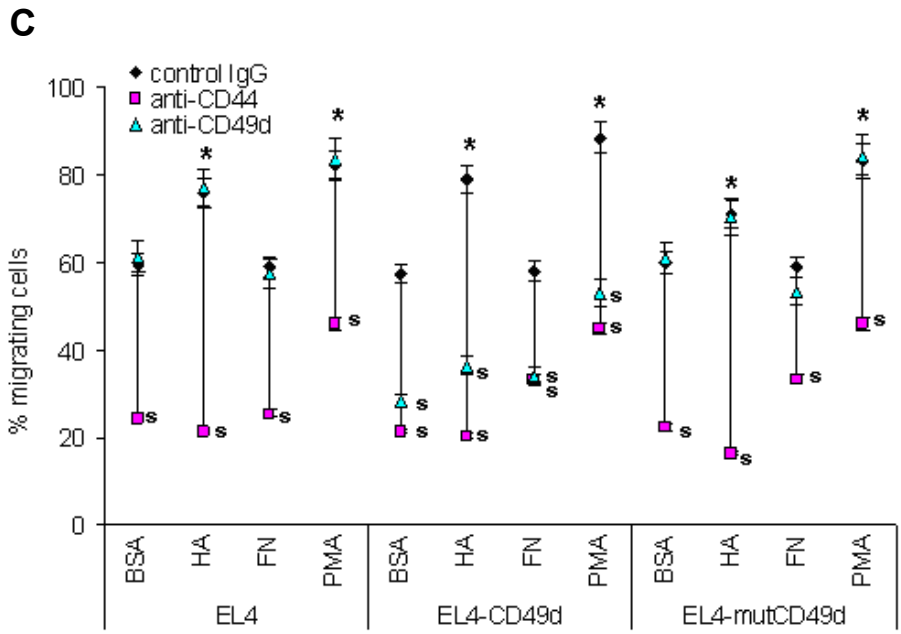
Taken together, transfected EL4 and Jurkat cells express native and mutated CD49d, CD44, respectively at a comparably high level. With the exception of CD120a (TNFRI), which is downregulated in CD44 cDNA transfected Jurkat cells, adhesion molecule, T cell and T cell activation marker expression as well as death receptor expression were not affected by transfection. Thus, activation induced changes in transfected EL4 and Jurkat cells can be considered to rely on de novo expression of CD44 and CD49d, respectively.

3.1.5 CD44 and CD49d are engaged in leukocyte migration

CD44 and CD49d both can contribute to leukocyte migration and cooperative activity of CD44 and CD49d have been described to strengthen migratory activity. To control for the relevance of CD44-CD49d co-operativity in leukocyte migration, the impact of anti-CD44 and anti-CD49d on EL4 and Jurkat cells and as control on naïve and AA LNC, was evaluated. Migration was tested in Boyden chambers using hyaluronic acid (HA), fibronectin (FN) or PMA as stimulus.

Control LNC displayed a lower migratory activity than AA LNC, which was more strongly inhibited by anti-CD44 than anti-CD49d, though both antibodies exerted a significant effect. The inhibitory effect was stronger in response to HA and FN than BSA. This accounted particularly for anti-CD49d in response to FN (Fig.7A). Transfection of EL4 with CD49d exerted no measurable impact on the migratory activity towards BSA. However, transfection of Jurkat cells with CD44 increased the migratory activity. Migration of non-transfected EL4 and mutCD49d transfected EL4 cells was only inhibited by anti-CD44. Similarly, only migration of CD44 wt transfected Jurkat cells was inhibited by anti-CD44 (Fig.7B). Irrespective of the stimulus, the migratory activity of EL4 cells was not influenced by CD49d cDNA transfection. However, EL4-CD49d, but not EL4 and EL4-mutCD49d cell migration was significantly inhibited by anti-CD44 and anti-CD49d, anti-CD49d being most efficient in response to FN (Fig.7C). Independent of the stimulus, migration of untransfected and transfected Jurkat cells was inhibited by anti-CD49d. Distinct to EL4-mutCD49dcells, migration of Jurkat-mutCD44 and Jurkat-truncCD44 cells was partly inhibited by anti-CD44 (Fig.7D).


A
% migrating LNC
control IgG
anti-CD44
anti-CD49d
BSA
HA
FN
PMA
control LNC
AA-LNC
B
% migrating cells
EL4
EL4-CD49d
EL4-mutCD49d
Jurkat
Jurkat-CD44
Jurkat-mutCD44
Jurkat-truncCD44


C
% migrating cells
control IgG
anti-CD44
anti-CD49d
BSA
HA
FN
PMA
EL4
EL4-CD49d
EL4-mutCD49d

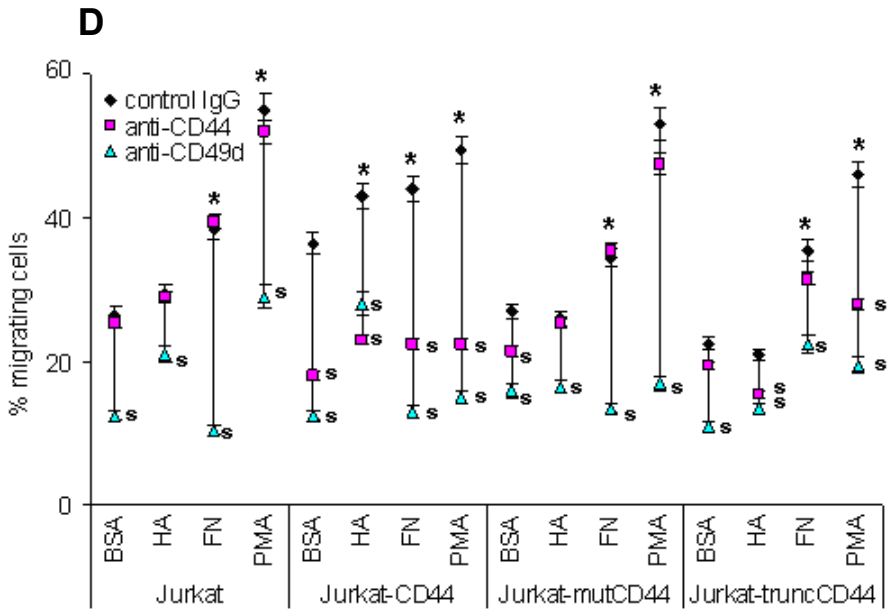
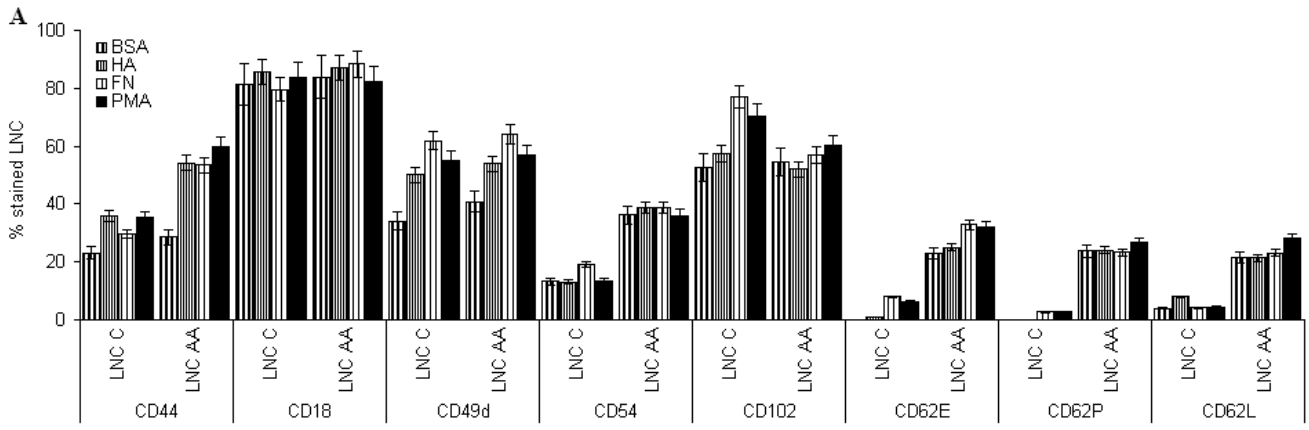
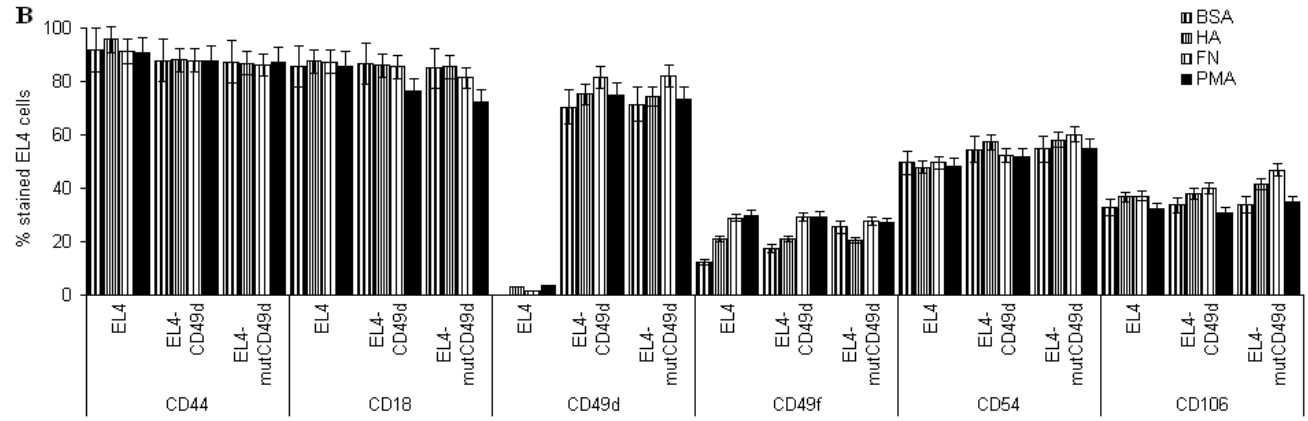


Figure7. Migration of the transfected EL4 and Jurkat cell lines: EL4, EL4-CD49d, El4-mutCD49d, Jurkat, Jurkat-CD44, Jurkat-mutCD44, Jurkat-truncCD44 cells and LNC ($5X10^4$) were incubated with anti-CD44 or anti-CD49d, washed and seeded in triplicate in the upper well of a Boyden chamber, the lower well contained BSA, Hyluronic acid (HA), Fibronectin (FN) or PMA. After incubation for 4hrs at 37^o C, 5% CO2, cells in the lower chamber were counted. The percentage of migrating cells is shown (mean SD of three separate experiments).

To control, whether migration and inhibition of migration may be influenced by altered adhesion molecules expression due to stimulation with matrix proteins and PMA, adhesion molecule expression was evaluated in naïve and AA LNC, non-transfected and transfected EL4 and Jurkat cells after stimulation on HA, FN or by PMA/Ionomycin. Except of a downregulation of CD49c in PMA-stimulated Jurkat cells, adhesion molecule expression in EL4 and Jurkat cells remained unaltered. In naive and AA LNC, CD44 and CD49d expression increased in HA, FN and PMA stimulated LNC. Increased expression of CD102 was only seen in naïve LNC (Fig.8A-D).


A
% stained LNC
BSA
HA
FN
PMA
LNC C
LNC AA
CD44
CD18
CD49d
CD54
CD102
CD62E
CD62P
CD62L


B
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BSA
HA
FN
PMA
EL4
EL4-CD49d
EL4-mutCD49d
CD44
CD18
CD49d
CD49f
CD54
CD106

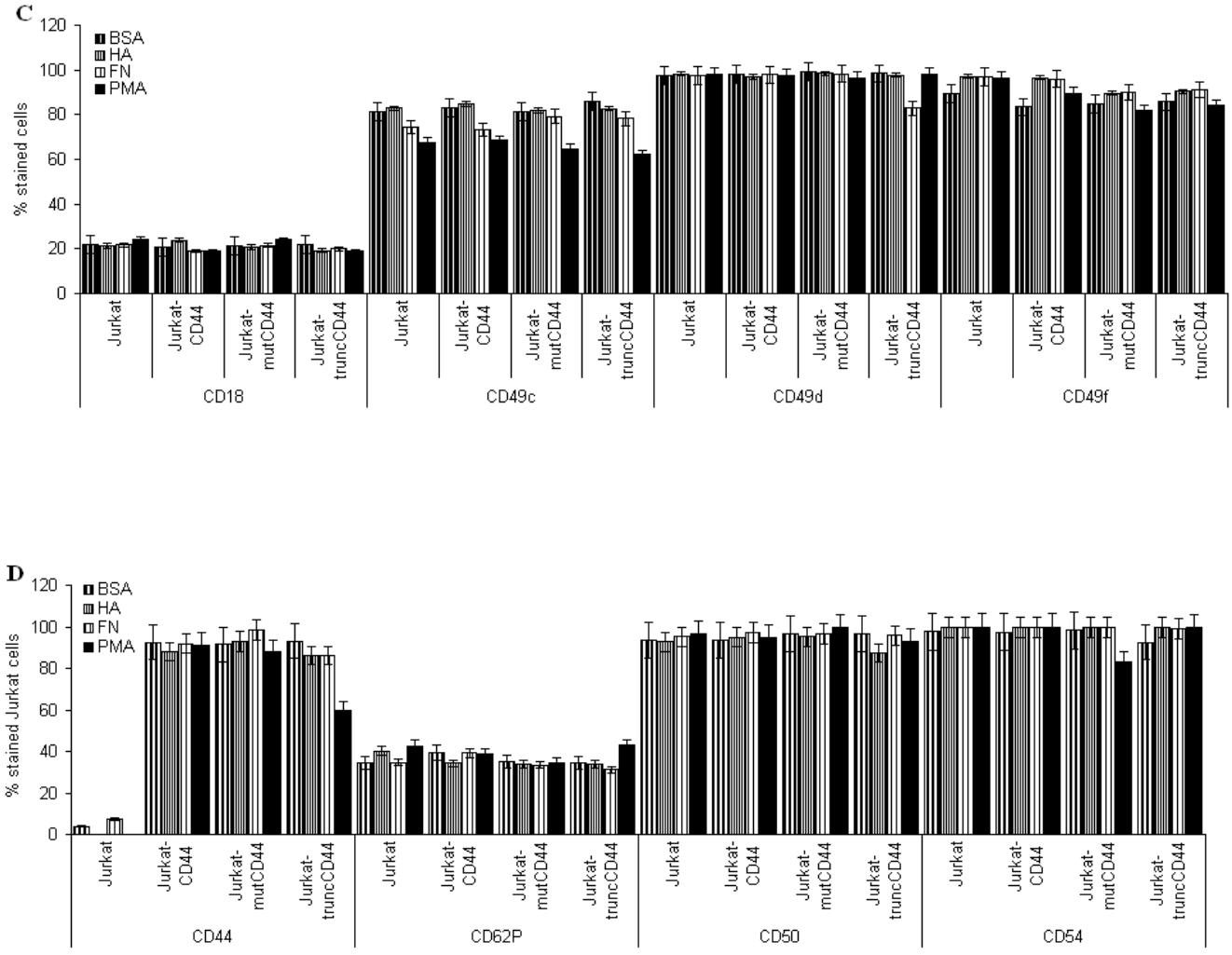


Figure8. Expression profile of adhesion molecules in LNC and transfected EL4 and Jurkat cells after stimulation: LNC from naïve (control) and AA mice and El4, El4-wtCD49d, EL4-mutCD49d, Jurkat, Jurkat-wtCD44, Jurkat-mutCD44, Jurkat-truncCD44 cells were activated on HA or FN coated plates and by PMA for 24 hrs follwed by staining with the indicated antibodies. Expression was checked by flow cytometry.

Taken together, migration of naive and AA LNC is inhibited by anti-CD44 and anti-CD49d, though stronger by anti-CD44. In line with this, HA promotes a stronger migratory stimulus than FN. Migration of EL4 and Jurkat cells is only inhibited by anti-CD44 and anti-CD49d respectively. Furthermore, anti-CD49d inhibits EL4-CD49d, but not EL4 migration. Jurkat-mutCD44, Jurkat-truncCD44 becomes only weakly inhibited by anti-CD44 compared to Jurkat–CD44. From there we conclude that in hematopoietic cells CD44 is the major contributor to motility, yet can be supported by CD49d. The inefficacy of the extracellular domains of both molecules

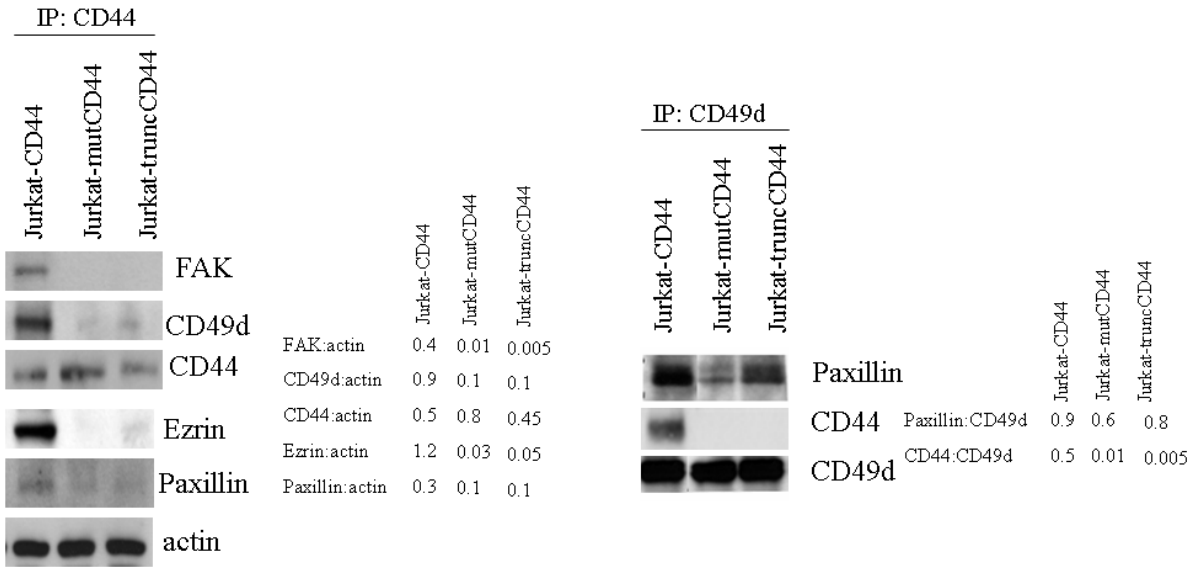
point towards joint signal transduction rather than towards adhesion strengthening by an increase in the docking sites.

3.1.6 Induction of the migratory phenotype is supported by CD44-CD49d co-operativity

To control for the hypothesis that possibly CD44 and CD49d initiated joint signal transduction accounts for strengthened motility, co-immunoprecipitation studies were done to get an insight into the CD44 and CD49d association with each other and downstream signalling molecules that might affect T cells migration.

Co-immunoprecipitation confirmed the association of CD44 with CD49d, ezrin, FAK and paxillin in Jurkat-CD44 cells. However, co-immunoprecipitation of CD49d with CD44 was not observed in Jurkat-mutCD44 and Jurkat-truncCD44 cell lysates, similarly co-immunoprecipitation with CD49d showed association with CD44 in Jurkat-CD44 and not with Jurkat-mutCD44 and Jurkat-truncCD44 (Fig.9A). CD44 crosslinking via HA and PMA stimulation strengthend the association of CD44 with CD49d, Ezrin and FAK in Jurkat-CD44 cells, but did not suffice mutCD44, truncCD44 to associate with CD49d, Ezrine and FAK (Fig.9B).

A



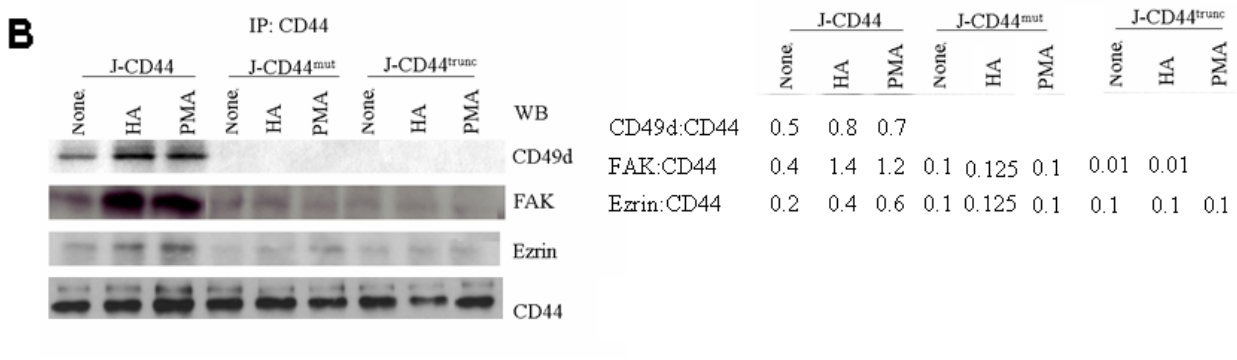


Figure9. CD44 and CD49d association: (A) Jurkat, Jurkat-CD44, Jurakt-mutCD44, Jurkat-truncCD44 cell lysates were immunoprecipitated with anti-CD44and anti-CD49d. Precipitates were dissolved by SDS-PAGE. After transfer, associated proteins were detected with the indicated antibodies. (B) Jurkat, Jurkat-CD44, Jurakt-mutCD44, Jurkat-truncCD44 cells were activated for 24 hrs with PMA or by crosslinking *via* HA. Cells were lysed and immunoprecipitated with anti-CD44, precipitates were dissolved by SDS-PAGE. After transfer associated proteins were detected by western blot with the indicated antibodies.

These data provide clear evidence that only CD44 and CD49d co-operativity supports T cell and leukemic T cell migration as truncCD44 and mutCD44 which do not promote migration, also do not allow CD44 to associate with Ezrin or with CD49d-associated FAK.

3.1.7 Effect of CD44-CD49d co-operativity on cell proliferation

Besides its impact on leukocyte migration, CD44 functions as accessory molecule in TCR complex-mediated signal transduction and can promote T cell proliferation and survival. AA LNC are in activated state and display a significantly increased proliferation rate in comparison to naïve LNC. AA LNC responded to HA, but not to FN and showed a stronger response to PMA than naïve LNC (Fig10A). When comparing the proliferation rate of EL4-CD49d, EL4-mutCD49d and Jurkat-CD44, Jurakt-mutCD44, Jurkat-truncCD44, in the absence of a stimulus, no increase in the proliferation rate was observed. Also CD44- and /or CD49d-crosslinking on HA or FN-coated plates did not suffice for increasing the proliferation rate, irrespective of whether only CD44 or CD49d or both, non-mutated molecules were expressed. Instead, PMA-treatment led to a sufficient reduction in the proliferation rate, which was independent of the transfection with mutated/non-mutated CD44 or CD49d. A

similar effect of PMA has been described by Han *et al* (156). I could only evaluate antibody inhibition as a possible indicator of CD44-CD49d cooperativity. Anti-CD44 effectively reduced the proliferation of EL4-CD49d, but had no or only marginal effect on EL4-mutCD49d (Fig10B). Similar observations accounted for Jurkat and transfected Jurkat cells (data not shown).

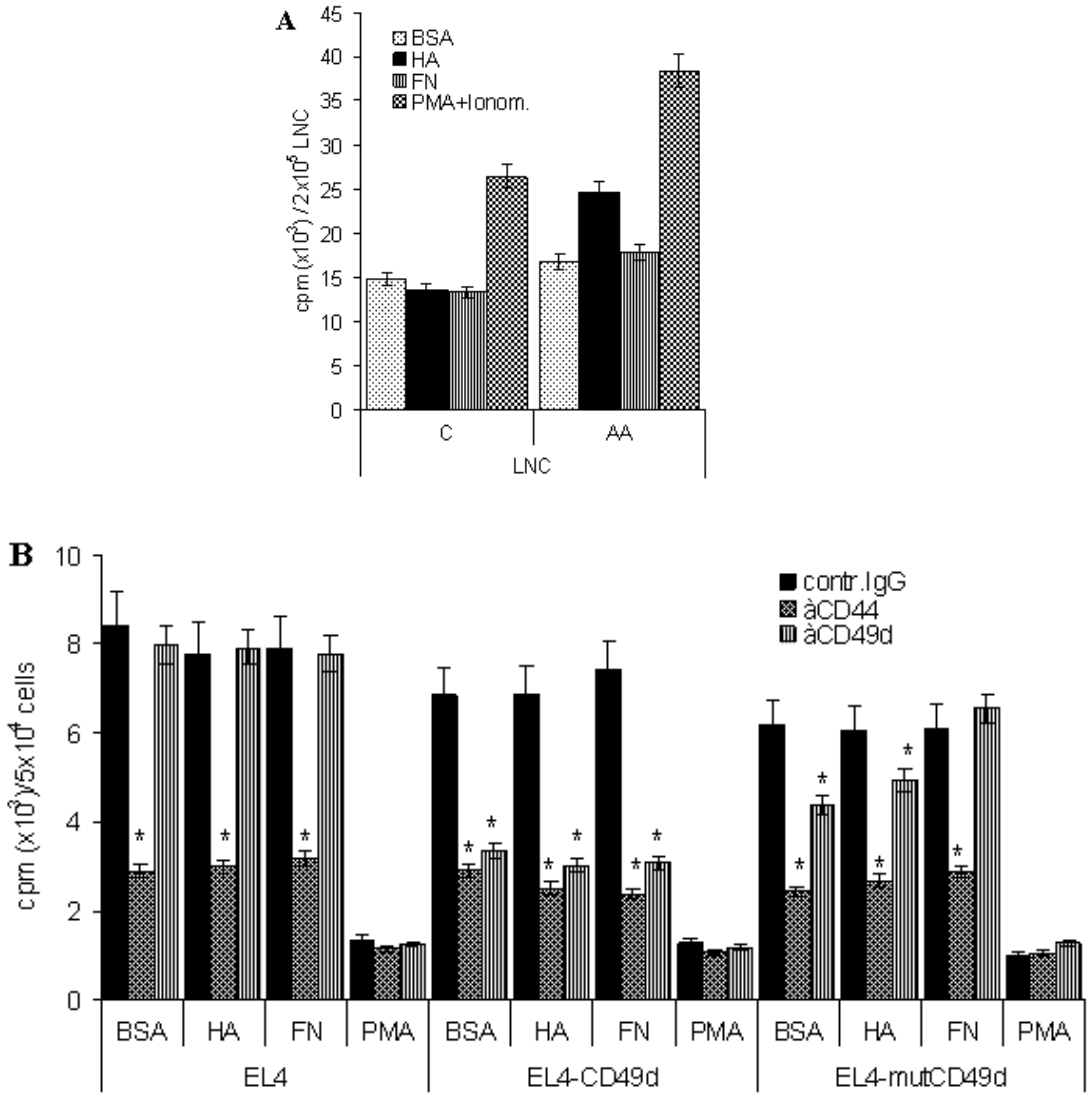


Figure10. Impact of CD44 and CD49d on T cell proliferation: (A) LNC from naïve (control) and AA mice and (B) El4, El4-wtCD49d, EL4-mutCD49d cells were activated on HA, FN and by PMA for 24 hrs and proliferation was checked by ^{3}H-thymidine incorporation. Significant differences in proliferation rate after antibody treatment are indicated by *.

Our present state of knowledge relies exclusively on antibody inhibition. Nonetheless, these data would be in line with cooperativity between CD44 and CD49d also having an impact on non-transformed and Leukemic T cell proliferation. I want to mention that preliminary evidence points towards CD44-CD49d interaction not to play a major role in protecting leukemic T cells from apoptosis as it has been observed in T cells (data not shown). This work is ongoing to define signaling pathways that become jointly activated via CD44-CD49d ligand binding.

Additional experiments are required to conclusively answer the question on the pathway whereby CD44-CD49d cooperativity promotes T cell proliferation and whether this cooperativity suffices for T cell receptor independent T cell proliferation. With the tool of EL4 and Jurkat mutated lines, I created, this should be possible and is currently pursuate in the lab.

3.2 Myeloid derived suppressor cells as the driving force in the DTH-induced cure of AA

Persisting T cell activation and expansion in autoimmune disease, including AA may partly be due to pronounced activity of accessory molecules like CD44 or CD49d, but also is promoted by a failure of downregulation of response. As SADBE treatment is curative in AA, the comparison of leukocytes of AA and SADBE treated AA mice offered itself to see, whether AA leukocytes escape the inherit control mechanisms of the immune system including immunosuppression and how a chronic contact eczema cope up with these suggested escape mechanisms.

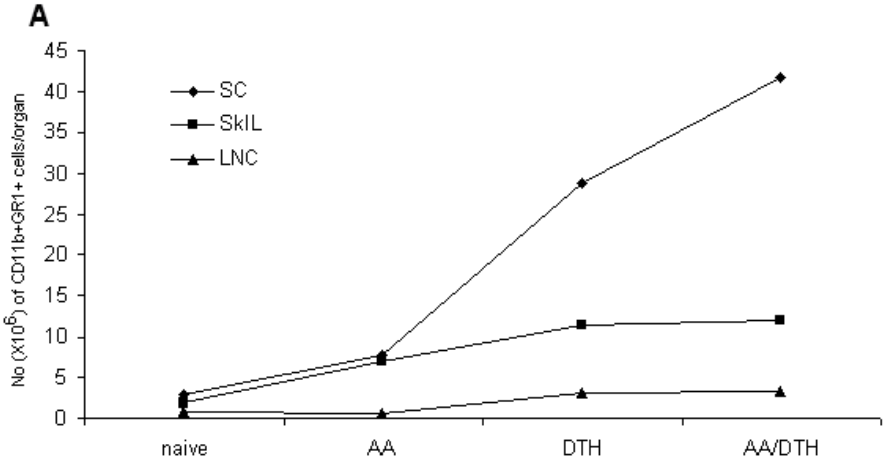
3.2.1 Myeloid derived suppressor cells in AA and chronic contact eczema

Immune responses occur following the initial step of specific antigen recognition and the transmission of activating signals. However, upon chronic stimulation, like persisting inflammation and /or cancer, immunoregulatory cells become activated. It is known that there is a defect in immunoregulatory cells in autoimmune diseases, including AA. Thus, it can be speculated that activation of immune regulatory cells by a chronic eczema may correct the deficit in AA. Two types of cells are most important for regulating T cell responses, T_{reg} and myeloid derived suppressor cells (Gr-1^{+},

$CD11b^+$ MDSC). The latter have been suggested to become particularly expanded and activated during chronic inflammation. Thus, I first evaluated the expansion of myeloid derived suppressor cells, which negatively affect T cell expansion and activation, and are engaged in AA in the therapeutic efficacy of a DTH in AA mice (SADBE treated AA mice). I started with the evaluation of MDSC in different lymphoid organs of untreated and SADBE treated mice. $Gr\text{-}1^+$ $CD11b^+$ cells were strongly increased in the spleen of DTH (naive mice treated with SADBE) and AA-DTH (AA mice treated with SADBE). MDSC also are increased in lymph node cells (LNC) and skin infilterating leukocytes (SkIL) in both DTH and AA-DTH mice in comparison to the spleen and SkiL of control and AA mice (Fig. 11A-B).

There are subgroups in MDSC which differ in their activity, therefore it also became important to look for the difference between AA and AA/DTH MDSC, since differences in the activation status of MDSC in these two groups may play an important role in functional activities.

AA/DTH MDSC express Ly6C at a higher percentage than AA MDSC. In addition, a higher percentage of AA/DTH than AA MDSC expresses IL1α and IL6. High TNFα, STAT1, STAT6 and NF-kB expression in AA MDSC is further increased in AA/DTH MDSC (Fig.11C).



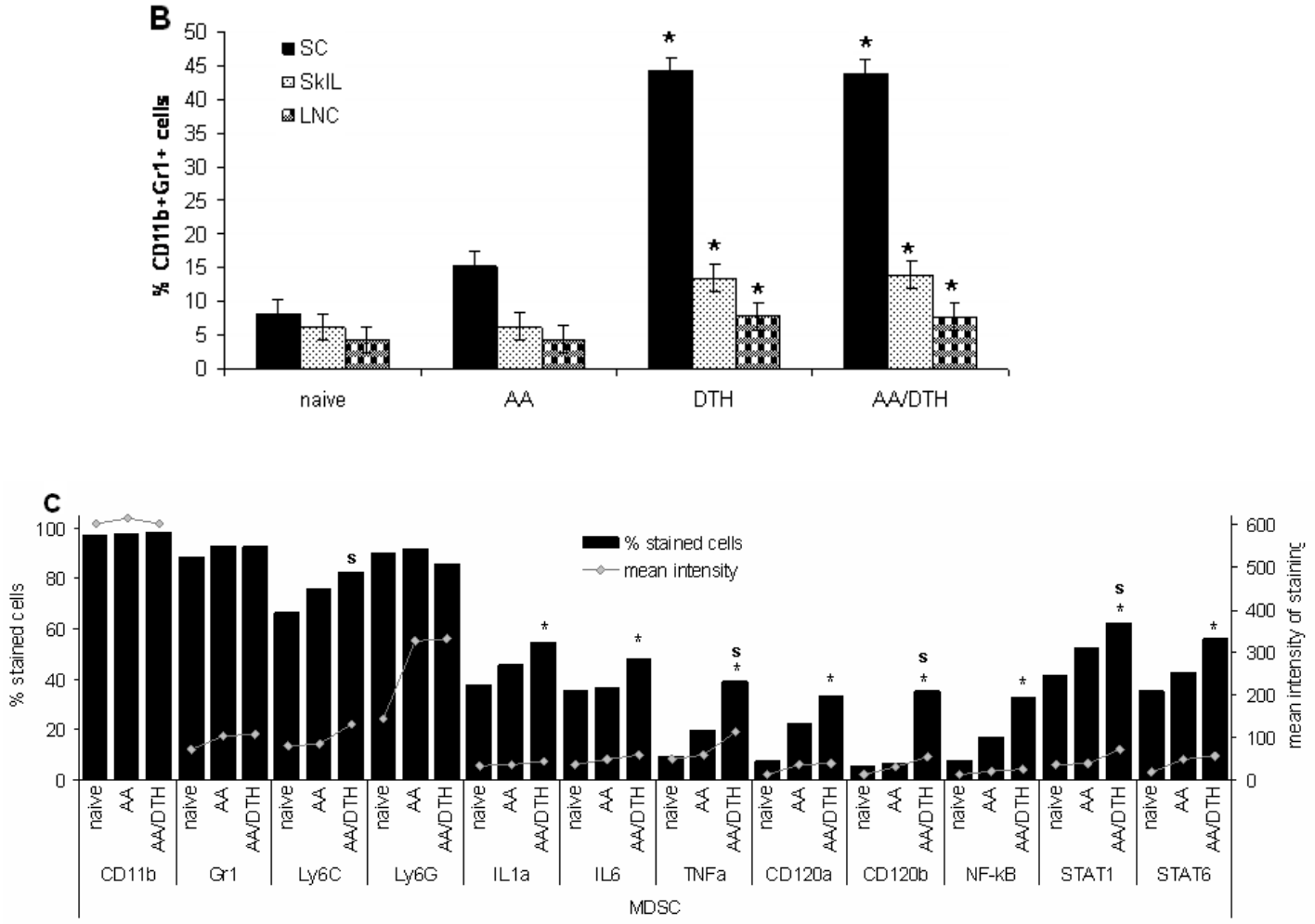


Figure11. Expression and characterization of MDSC: (A,B) The percentage of MDSC (Gr-1$^+$ CD11b$^+$) were double stained and checked via flow cytometry in SC, LNC and SkIL of naïve, AA, DTH and AA/DTH mice. The percentage (mean±SD) and the total number of Gr-1$^+$ CD11b$^+$ cells are shown. Significant differences in the percentage of spleen cells, SkIL and skin-draining LNC between DTH/AA-DTH mice and normal mice are indicated by *. (C) Expression of the indicated markers was evaluated by flow cytometry in naïve-, AA-, AA/DTH-MDSC, which were separated according to CD11b and Gr1 expression by magnetic beads. Significant differences between AA- and AA/DTH-MDSC in the percentage of marker+ cells are indicated by *, significant differences in intensity of expression are indicated by **s.**

Expansion of MDSC in DTH and AA/DTH mice points towards a putative role of MDSC in the curative action of contact sensitizers. In addition, higher expression of STAT1, STAT6 and NFκB expression, which are elevated in AA/DTH-MDSC compared to AA-MDSC, is known to be associated with the activated state of MDSC. These findings point towards a more activated status of AA/DTH-MDSC.

3.2.2 DTH- induced MDSC account for hair re-growth in AA

SADBE treatment induces hair re-growth in AA mice and it has been shown that there is an expansion of MDSC in DTH and AA-DTH mice (Fig11A). To confirm that the hair growth likely is a consequence of MDSC expansion and activation, mice received ATRA (all *trans* retinoic acid), together with SADBE. ATRA is known for driving MDSC into differentiation and therefore MDSC no longer behave as suppressor cells. The application of SADBE was repeated weekly and ATRA application was repeated once in 3 weeks. ATRA treatment itself exerted no effect. However, when applied concomitantly with SADBE only 1 out of 10 mice showed partial hair re-growth, whereas 9 out of 10 mice showed partial or complete hair growth during SADBE treatment. Also confirming the importance of MDSC, 7 out of 10 AA mice that received intravenous (i.v.) injections of MDSC derived from SADBE treated AA mice also showed partial hair re-growth, which was prevented by concomitant ATRA treatment (Fig.12A).

Immunohistological studies on skin sections showed that there was a reduction in $CD4^+$ and $CD8^+$ T cells in SADBE- and MDSC-treated mice, while no reduction was seen in SADBE or MDSC plus ATRA-treated mice (Fig.12B). Instead fewer $CD11b^+$ and $GR1^+$cells were detected in ATRA-treated mice (data not shown).

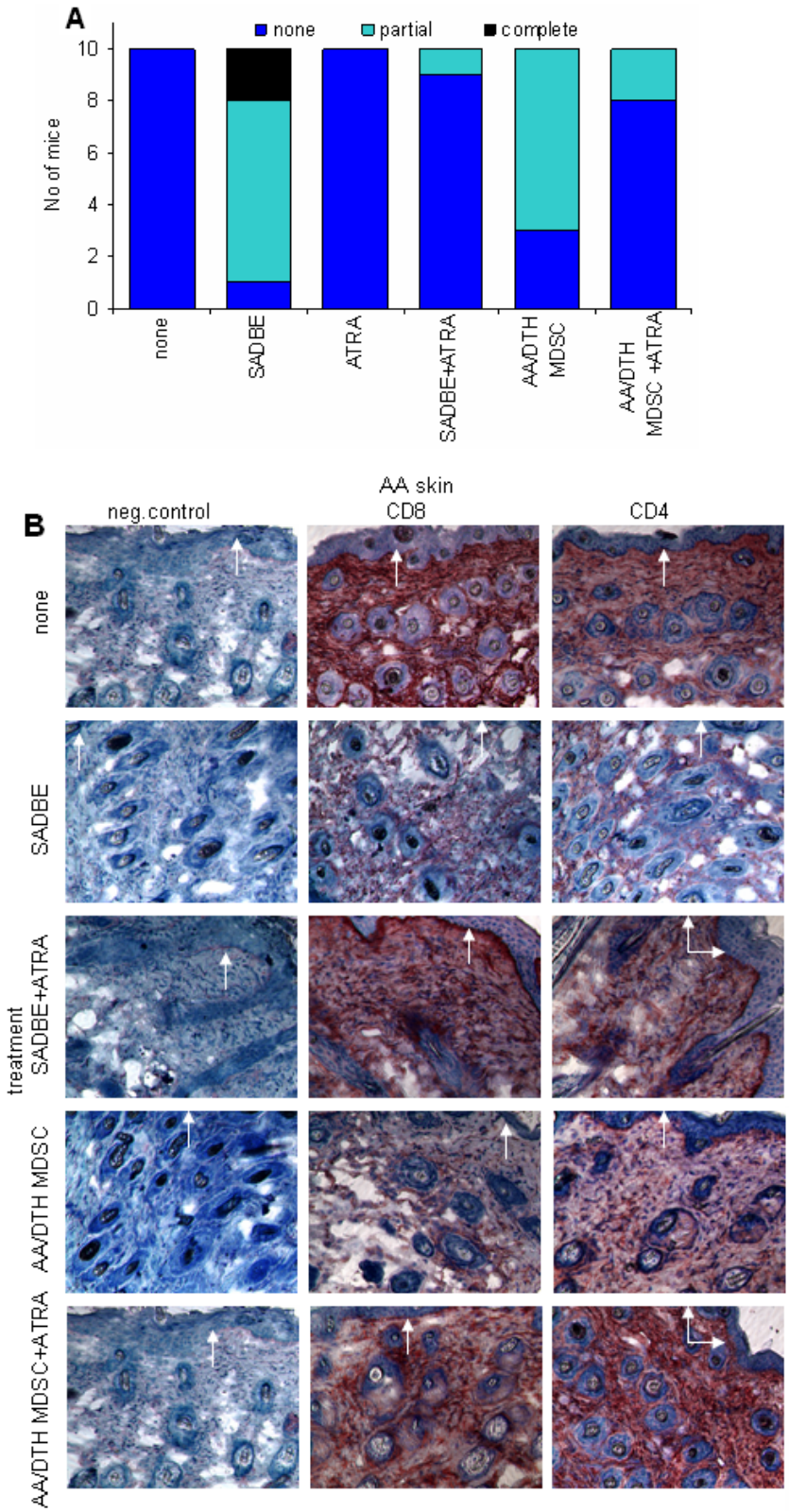


Figure12. MDSC and hair re-growth in AA mice: (A) C3H/HeJ mice with total hair loss 10 week after transplantation of AA-affected skin were treated with SADBE, ATRA, SADBE+ATRA, AA/DTH MDSC or AA/DTH MDSC+ATRA. The number of mice with partial or total hair re-growth is shown. (B) Immunohistology (CD4, CD8) of shock frozen skin section as indicated (White arrows: orientation towards the epidermis).

Hair re-growth in AA mice after SADBE treatment or after transfer of MDSC and suppression of hair re-growth in mice concomitantly receiving ATRA provides a strong hint towards a central role of MDSC in the SADBE therapy for AA. To sustain this hypothesis, I made a direct comparison of the immune status of lymphocytes recovered from AA/DTH mice with the immune status of LNC co-cultured with MDSC.

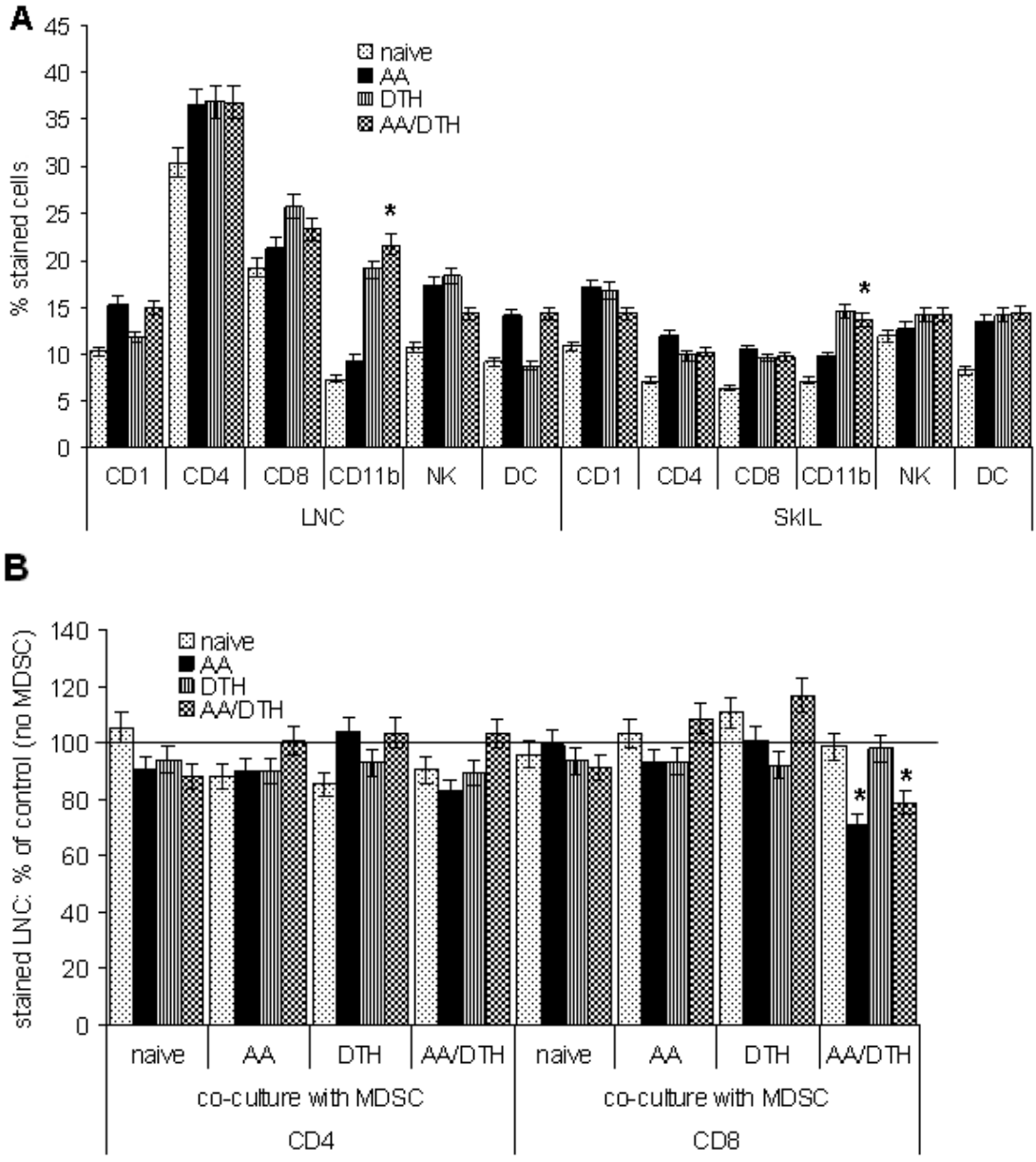
3.2.3 Activation status of T cells in AA and AA/DTH mice and impact of MDSC

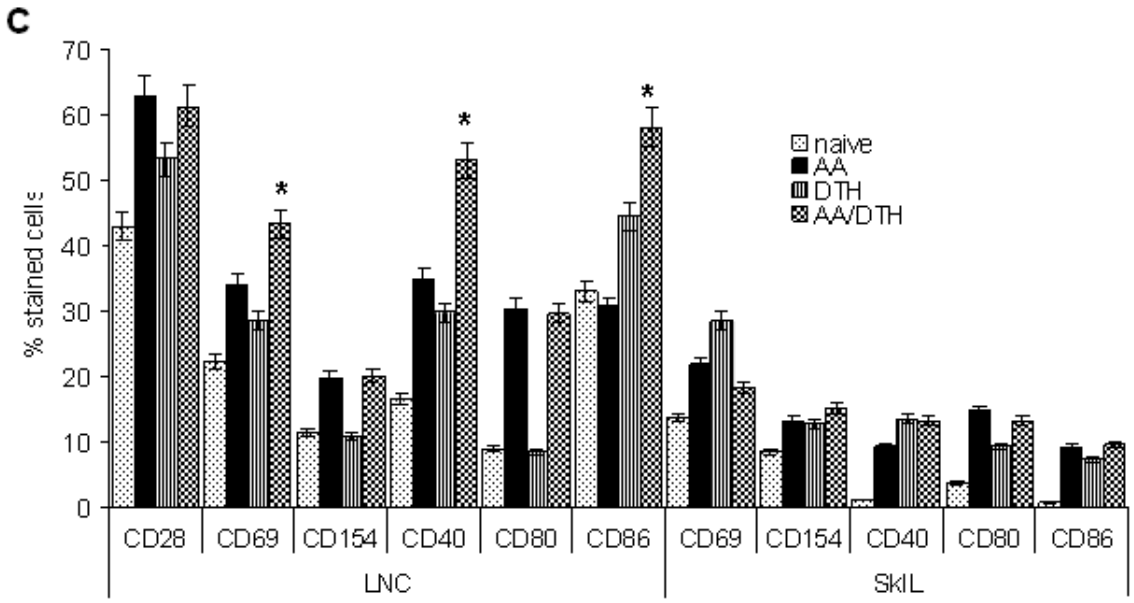
It has been stated before that T cells in AA are in an activated state and therefore it was important to see the effect of SADBE and MDSC on the activation status of these cells. It was observed that SADBE treatment exerted no major effect on the distribution of leukocyte subpopulations in LNC and SkIL of AA mice. Only $CD11b^+$ cells were recovered at a higher level in AA/DTH than AA mice, however the level was comparable to that in DTH mice (Fig.13A). To see the impact of MDSC on the activation status of T cells, a co-culture system was established, where T cells from naïve, AA, DTH and AA/DTH were co-cultured with MDSC derived from naïve, AA, DTH and AA/DTH. No significant changes in the percentage of $CD4^+$ and $CD8^+$ LNC were observed after co-culture, except for a slight reduction of $CD8^+$ AA and AA/DTH LNC after co-culture with AA/DTH- derived MDSC (Fig. 13B).

Expression of the T cell activation markers CD28, CD69 and CD154 were high in AA and AA/DTH LNC and SkIL. Only expression of CD69 was further increased in AA/DTH mice compared to AA LNC. Expression of the co-stimulatory molecules CD40, CD80 and CD86 was increased in AA LNC and, particularly, SkIL and remained high or increased further in AA/DTH LNC (Fig.13C). After co-culture with DTH- or AA/DTH-MDSC, the expression of CD28 was slightly, while expression of CD69 was strongly reduced in AA (Fig13D).

Except for an unexpected reduction in IL-4 and a strong increase in IL-6, there were no major DTH-induced changes in cytokine expression in AA LNC and SkIL (Fig.13E) and co-culture with MDSC exerted no significant effect. Instead CD152 and FoxP3 are low in AA LNC and SkIL but are strongly upregulated in AA/DTH LNC and SkIL. The latter also accounts for $CD25^+$ cells in LNC and SkIL (Fig.13F).

The percentage of CD152^{+} LNC also increased in co-cultures with DTH- or AA/DTH-MDSC, but not with naïve- or AA- MDSC (Fig. 13G).

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% stained cells
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50
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30
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10
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AA
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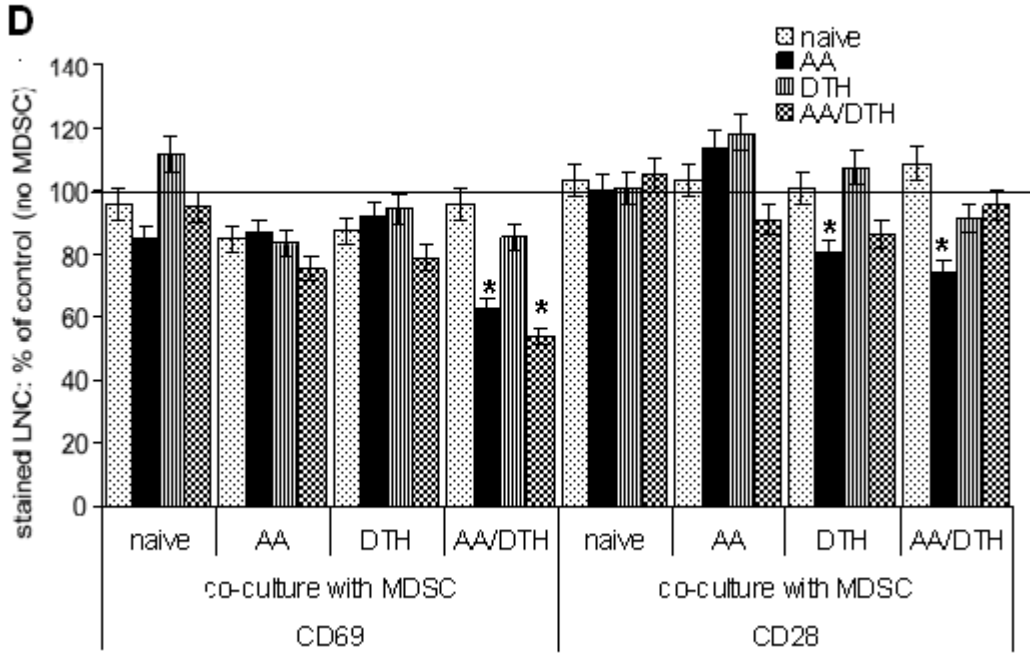
D
stained LNC: % of control (no MDSC)
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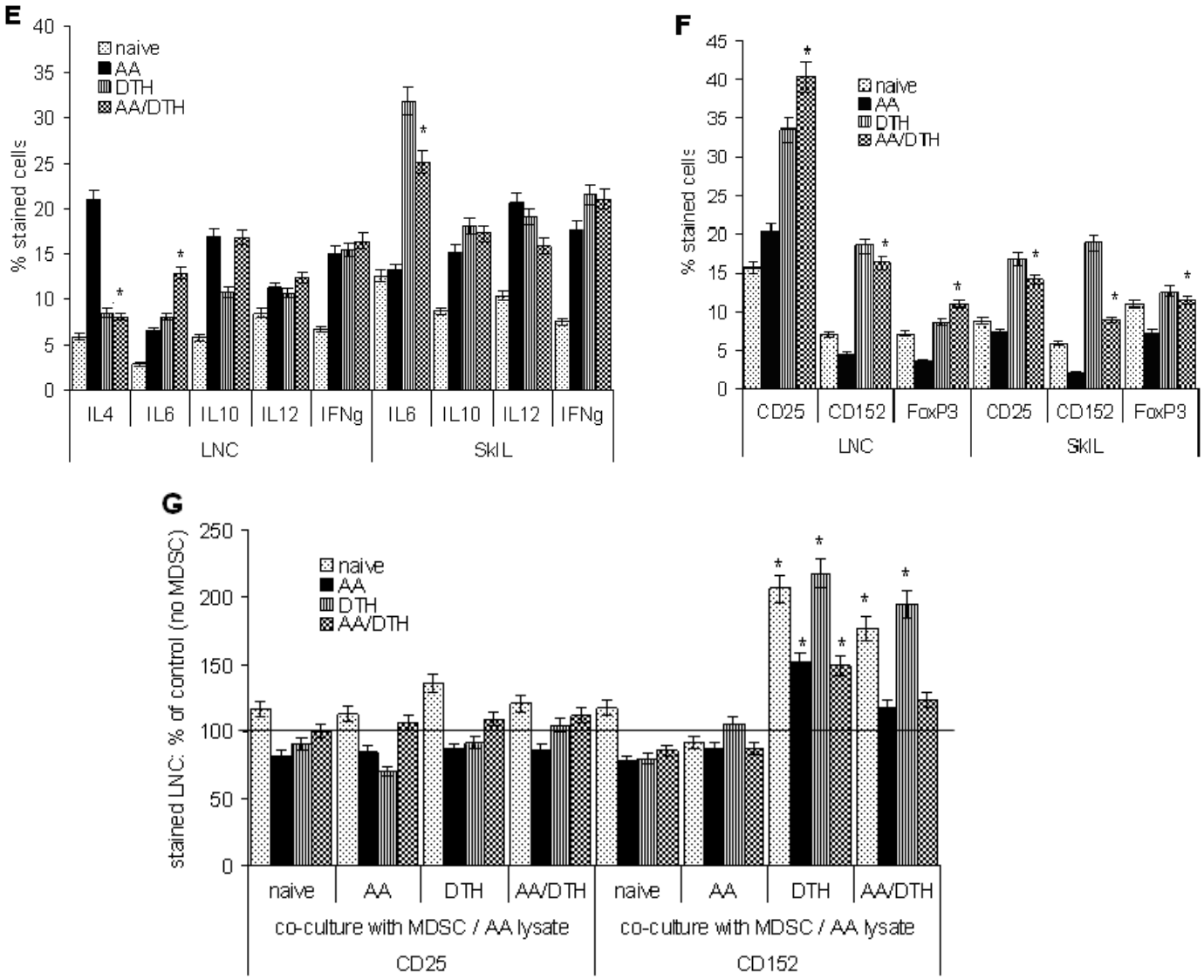


Figure13. Activation status of autoreactive T cells in AA, AA/DTH and impact of MDSC: (A,C,E,F) LNC and SkIL of naive, AA, DTH and AA/DTH mice were stained with the indicated antibodies. Expression was evaluated by flow cytometry .The percentage of stained cells (mean±SD) is shown. (B,D,G) Draining LNC of naïve, AA, DTH and AA/DTH mice were co cultured for 48 hrs with MDSC from naïve, AA, DTH, AA/DTH mice and were stained with the indicated antibodies. Staining of LNC cultured in the absence of MDSC was taken as 100%. A significant decrease or increase in marker$^+$ cells as compared to draining LNC and in co-culture with MDSCis indicated by *.

Though SADBE treatment of AA mice does not affect the distribution of leukocyte subpopulations, it promotes T cell activation as revealed by up-regulation of activation marker CD69 and co-stimulatory molecules CD40 and CD86. While after co-culture with DTH- or AA/DTH-MDSC the percentage of the activation marker CD69 was significantly reduced. Instead expression of CD152, which is a negative

regulator of T cell activation, was upregulated. Thus, MDSC derived from AA/DTH appear potent to control activation of autoreactive T cells.

3.2.4 Regulatory T cells in AA and AA/DTH mice and impact of MDSC

Regulatory $CD4^+CD25^+FoxP3^+$ T cells (T_{reg}) play an important role in maintaining homeostasis. Accordingly they are frequently reduced in autoimmune diseases. In fact, these are also recovered at a reduced level in AA LNC. T_{reg} were slightly increased in AA/DTH LNC, but did not reach the level seen in naïve LNC (Fig.14A). Instead, when LNC were co-cultured with DTH- or AA/DTH-MDSC, normal or even elevated levels of T_{reg} could be seen (Fig.14B).

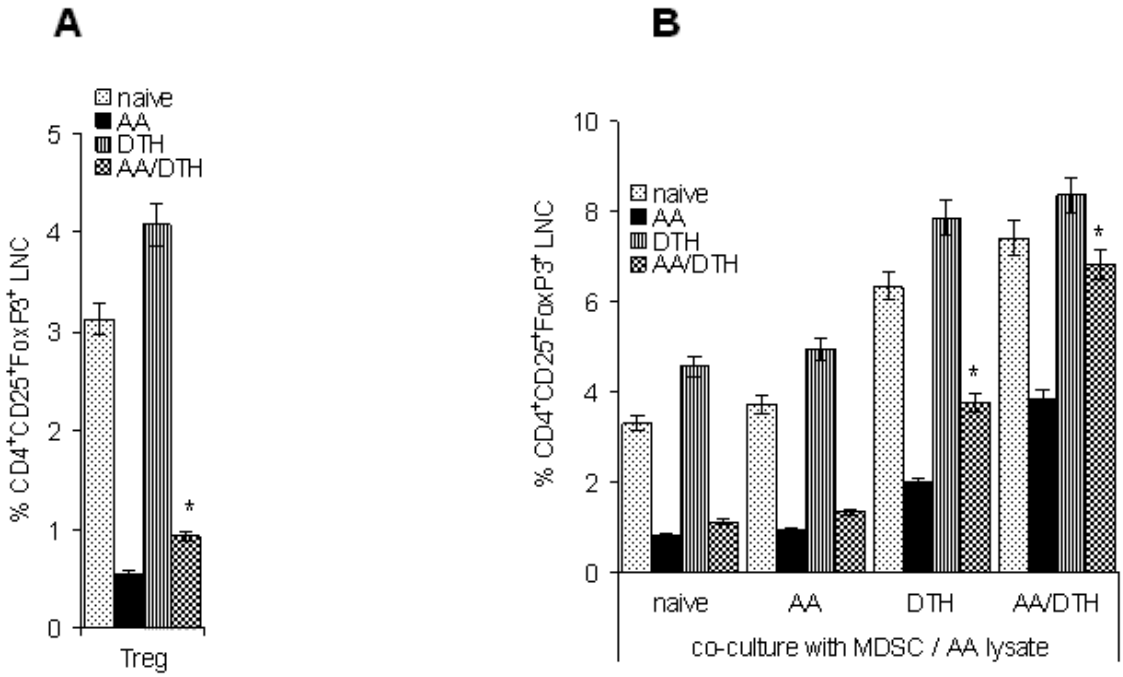


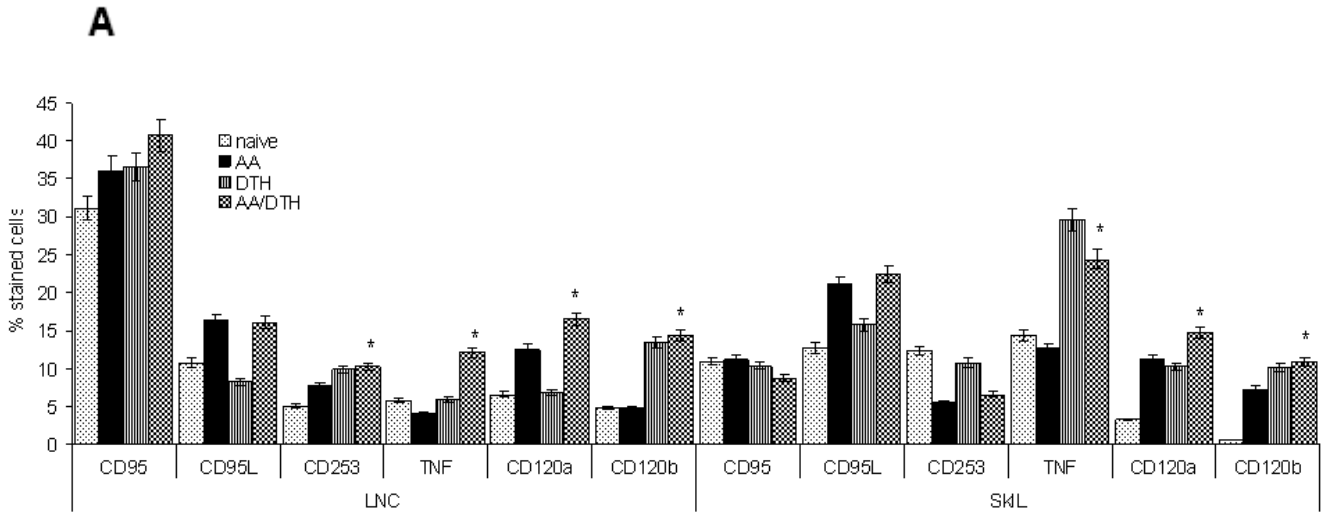
Figure 14.The impact of MDSC on regulatory T cells: (A) LNC were triple stained for CD4, CD25 and FoxP3. The percentage of triple-positive cells is shown. Significant changes between AA and AA/DTH LNC are indicated by *. (B) LNCs were co-cultured with MDSC for 48 hrs and triple stained for CD4, CD25 and FoxP3. The percentage of triple positive cells is shown. Significant changes in T_{reg} by co-culture with MDSC are indicated by *.

Co-culture of LNC with MDSC derived from DTH and AA/DTH mice allowed for rescuing T_{reg} to a significant level indicating a strong impact of MDSC on T_{reg}

expansion and their importance in controlling disease by activation of immunosuppression mechanisms, as T_{reg} are important regulatory cells of the immune system and that mostly affect activated T cells.

3.2.5 Impact of MDSC on death related marker expression in AA effector T cells

Besides the reduction in T_{reg}, autoimmune disease T cells are frequently characterized by resistance against activation induced cell death. Nonetheless, in AA, apoptosis resistance is unexpected as SkIL and LNC express death receptors CD95L (FAS-L) and TNFRI (CD120a) at a high level. However, SADBE treatment further strengthens the expression of death receptors like Trail (CD253), TNFα, TNFRI and TNFRII (CD120b) (Fig. 15A).After co-culture with AA/DTH MDSC, high CD95L expression is further increased (Fig.15B). Both AA- and AA/DTH-MDSC support Trail and TNFα expression but only AA/DTH-MDSC support TNFRI and TNFRII expression (Fig.15C). $TNFRI^+$ and $TNFRII^+$ LNC were mostly $CD4^+$. Trail was expressed at a comparable percentage of $CD4^+$ and $CD8^+$ cells. This distribution was not strikingly altered by co-culture with AA/DTH-MDSC (Fig.15D).



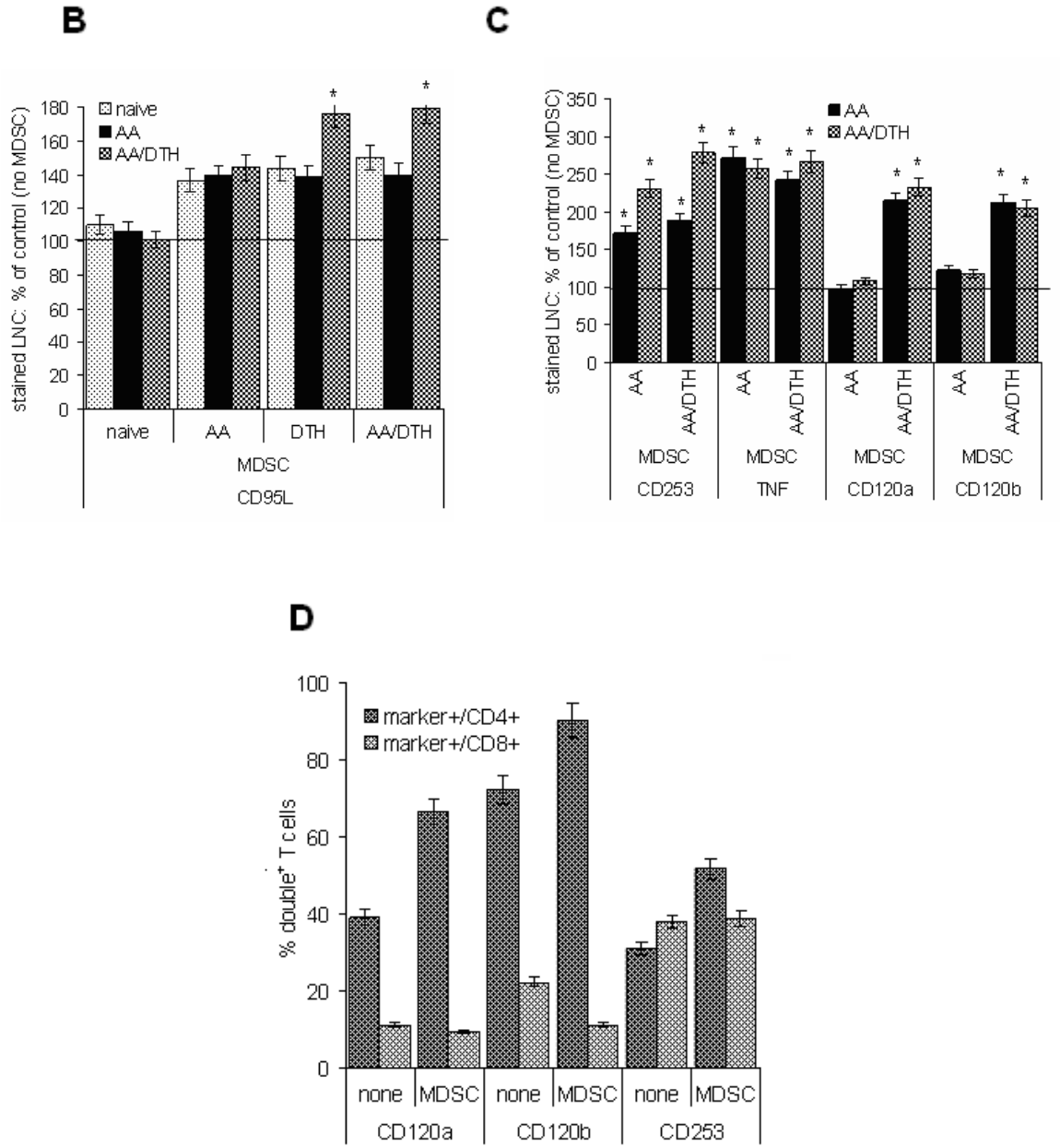


Figure15. The Impact of MDSC on death related marker expression in draining LNC and SkIL: (A) LNC and SkIL were stained with the indicated antibodies. The percentage of stained cells is shown. Significant changes in marker expression in AA/DTH compared to AA LNC or SkIL is indicated by *. (B,C) Draining LNC, as indicated, were co-cultured with MDSC for 48 hrs and stained for the indicated markers. Staining of LNC cultured in the absence of MDSC was taken as 100%. A significant decrease or increase in marker$^+$ cells as compared to draining LNC cultured in the absence of MDSC is indicated by *. (D) LNC were double stained with anti-CD4 or anti-CD8 and anti-CD120a, -CD120b or -CD253. The percentage of double positive cells per CD4$^+$ and CD8$^+$ cells are shown.

In the presence of DTH or AA/DTH MDSC the percentage of death receptors $CD95L^+$, $TRAIL^+$, $TNF\alpha^+$, $TNFRI^+$ and $TNFRII^+$ LNC was strongly increased, indicating a possible impact of MDSC on apoptosis induction.

3.2.6 MDSC suppresses AA effector cell proliferation and promotes apoptosis

In the absence of an antigen-specific stimulus (10% FCS) as well as in the presence of a polyclonal, but TCR-independent stimulus (PMA/Ionomycin), the proliferative activity of AA LNC exceeded not only that of naïve and DTH LNC, but also that of AA/DTH LNC. This difference became more striking in the presence of AA skin lysate that served as surrogate auto-antigen (since alopecia is an autoimmune disease of skin affecting hair follicles, autoantigens are expected to be in the hair follicles) (Fig.16A), which indicates that SADBE treatment may contribute to an antigen unspecific downmodulation of activated T cell proliferation, but possibly is most effective in interfering with an antigen specific response. Similarly, in the presence of 10% FCS, DTH- and AA/DTH-MDSC inhibited AA, DTH and AA/DTH LNC (activated LNC) proliferation more efficiently than that of naïve LNC (Fig.16B). To support the suggested antigen-specific component of MDSC activity, LNC were co-cultured with AA/DTH-MDSC in the presence of AA skin lysate or PMA/Ionomycin, where the latter circumvents the requirement of TCR engagement. It was observed that AA/ DTH-MDSC more efficiently suppressed the response of AA and AA/DTH than DTH LNC towards AA skin lysate. However, circumventing the requirement of TCR engagement (PMA/Ionomycin) AA/DTH-MDSC suppressed proliferation of AA, DTH and AA/DTH LNC with comparable efficacy (Fig.16C).

Expectedly, AA/DTH-MDSC most efficiently affected the “antigen-specific” response of AA and AA/DTH LNC, but additionally exerted TCR-independent activity. The activity of MDSC appeared to be contact dependent. Since supernatant of MDSC culture for 48hrs under the same conditions did not suppress LNC proliferation (data not shown).

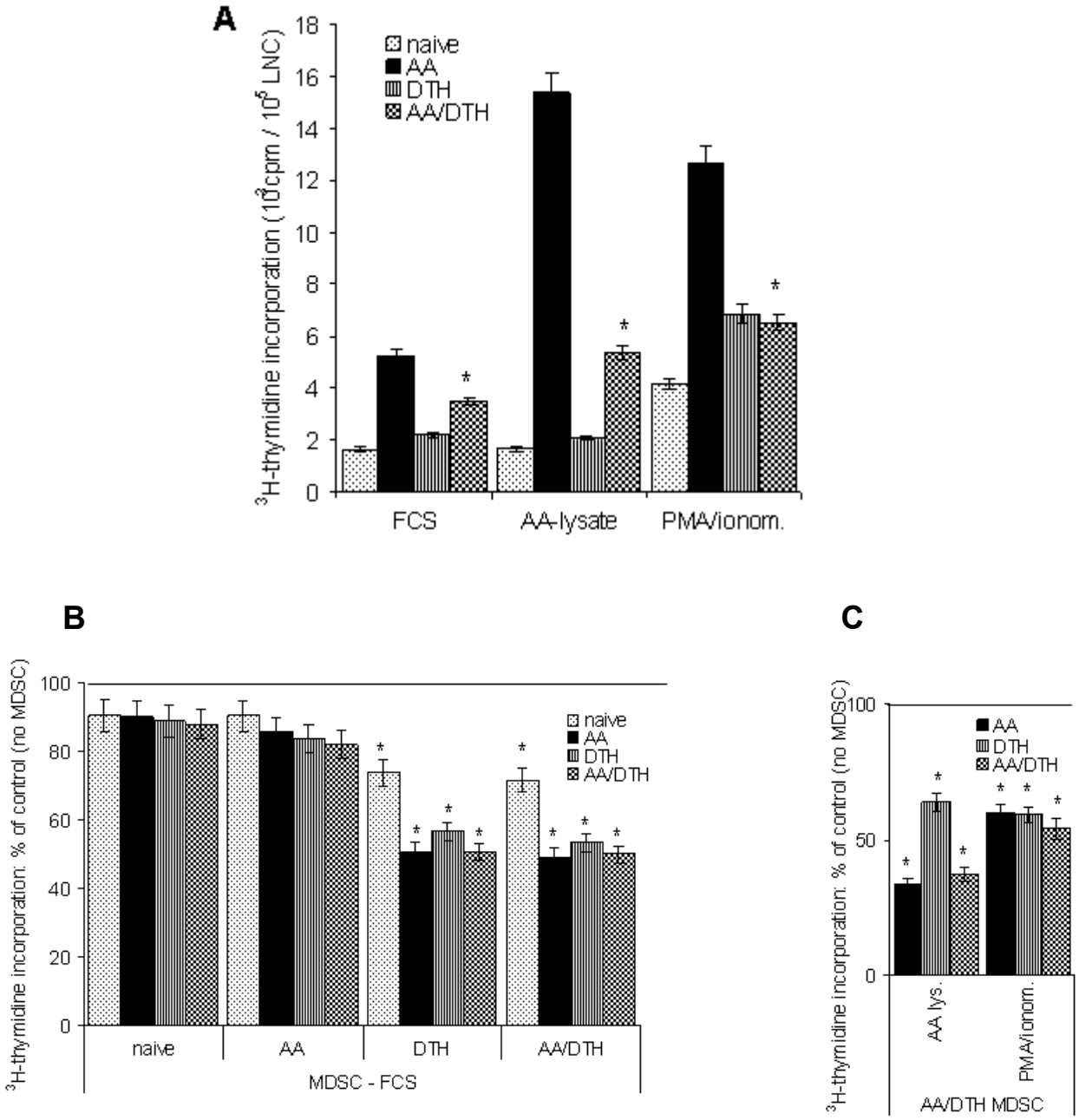


Figure16. AA and AA/DTH draining LNC distinctly respond to nominal antigen and PMA/Ionomycin: (A) ^{3}H-thymidine incorporation of naïve, AA, DTH and AA/DTH LNC in response to 10% FCS, AA-skin lysate and PMA/Ionomycin. Significant differences in the response of AA/DTH compared to AA LNC are indicated by *. (B, C) Relative ^{3}H-thymidine incorporation of draining LNC T cells after 48 hrs of co-culture with (B) MDSC/10%FCS (C) AA/DTH-MDSC/ AA skin lysate or AA/DTH-MDSC/ PMA/Ionomycin compared to cultures without MDSC. Values are shown for a ratio of T cells: MDSC=10:1. A significant reduction in proliferative activity by MDSC is indicated by *.

The proliferative response of AA/DTH LNC is significantly reduced compared to AA LNC and MDSC derived from DTH and AA/DTH LNC display stronger suppressive activity than MDSC from naïve and AA mice. In addition, there is evidence for antigen specificity of response suppression by MDSC (10% FCS versus AA lysate). MDSC also suppresses proliferation in response to PMA/Ionomycin. Taken together AA/DTH-MDSC most efficiently affected the "antigen-specific" response of AA and AA/DTH LNC, but also exerted TCR-independent activity.

To have an insight of apoptosis in AA and AA/DTH mice, LNC from naïve, AA, DTH and AA/DTH mice were cultured in RPMI/10% FCS for 48hrs. It was observed that the apoptosis rate of AA LNC was significantly lower than that of naïve and DTH LNC. The percentage of apoptotic AA/DTH LNC was higher than that of AA LNC, but did not reach the level of naïve LNC (Fig.17A). When LNC from naïve, AA, DTH and AA/DTH mice were co-cultured with naïve- and AA-MDSC in RPMI/10%FCS, no significant increase in the apoptosis rate was seen,but in co-culture with AA/DTH-MDSC, a slight increase was observed. Instead in the presence of PMA/Ionomycin, DTH and AA/DTH MDSC sufficed to strongly mitigate apoptosis resistance of AA and AA/DTH LNC. The same tendency, though, less pronounced was seen when AA or AA/DTH LNC were co cultured with DTH- or AA/DTH-MDSC in the presence of AA skin lysate (Fig.17B, C). To explore whether apoptosis resistance relies on $CD4^+$ or $CD8^+$ LNC and in which of these two subpopulations MDSC support apoptosis induction, so, it was observed that both $CD4^+$ and $CD8^+$ LNC from AA and AA/DTH showed comparable apoptosis resistance in presence of 10% FCS. No difference was seen in these two subpopulations when co-cultured with AA/DTH MDSC containing AA skin lysate or 10%FCS, but with PMA/Ionomycin mostly $CD8^+$ cells become apoptotic (Fig.17D).
Thus MDSC from naïve and AA mice do not promote apoptosis. However, DTH- and AA/DTH-MDSC interferes with apoptosis resistance of AA and AA/DTH LNC, particularly when cells become stimulated circumventing TCR engagement (PMA/Ionomycin).
These distinct features of AA versus AA/DTH MDSC likely rely on the differences between these two populations of myeloid derived cells. As described before, AA/DTH-MDSC showed a more activated state similar to that of M-MDSC (Monocytic-MDSC), which shows a higher expression of Ly6C. Thus, the highly

activated state of AA/DTH MDSC could well provide the fundaments for attacking autoreactive T cells.

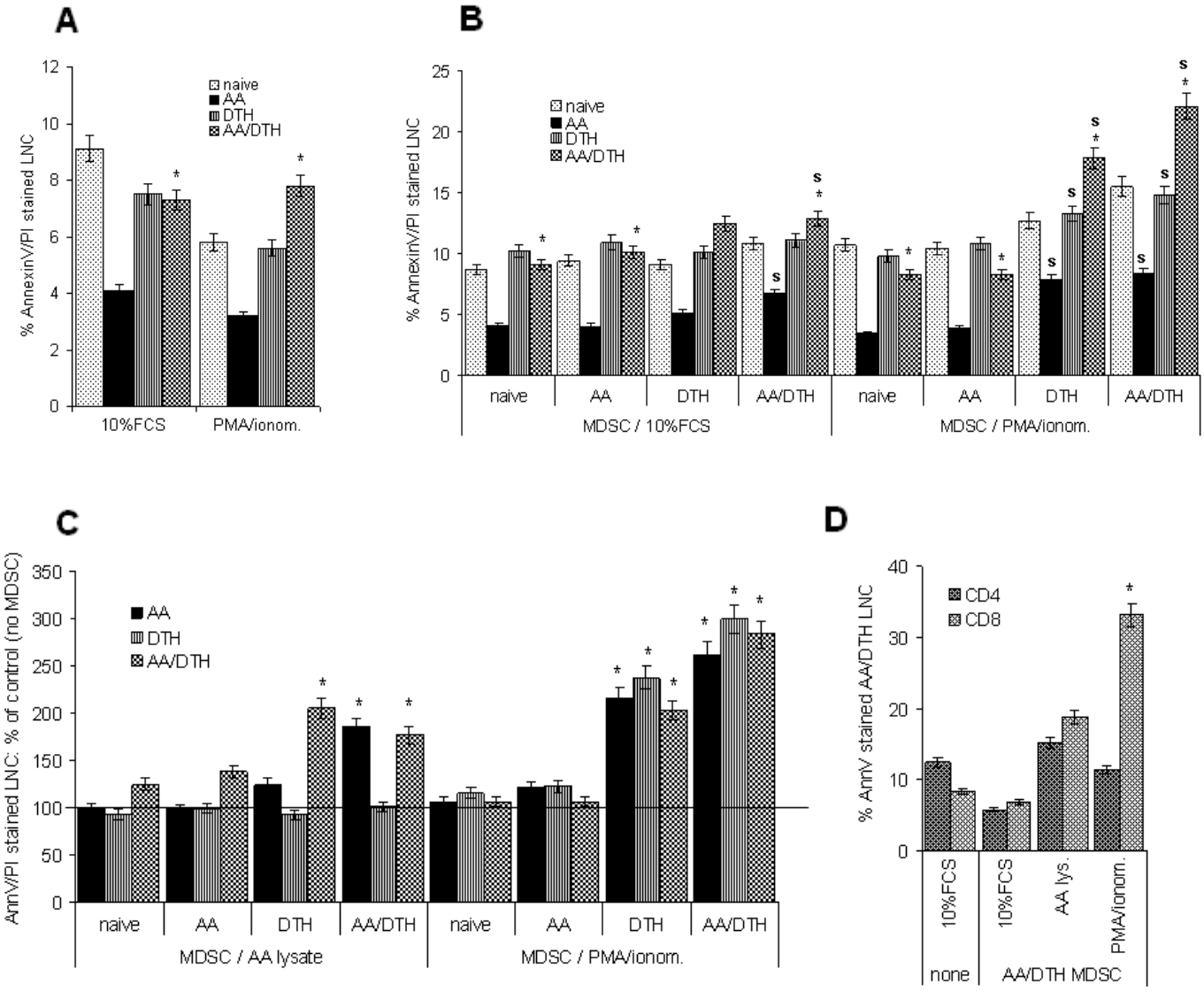


Figure17. The impact of MDSC on apoptosis resistance of AA LNC: (A) LNC were cultured for 48hrs in the presence of 10%FCS or PMA/Ionomycin and apoptosis assay was performed using Annexin V-FITC and propidium iodide (PI). Early apoptotic cells bind Annexin V and necrotic cells take up PI. Cells were incubated in the dark at RT for 15 min and analyzed by flow cytometry using the FL1 channel for AnnexinV FITC and FL-3 channel for PI. The percentage of stained LNC is shown. A significant increase in apoptosis of AA/DTH as compared to AA LNC is indicated by *. (B) LNC were cultured for 48 hrs in the presence of MDSC (LNC: MDSC= 10:1) and 10% FCS or PMA/Ionomycin and were stained with AnnexinV-FITC/PI. The percentage of stained LNC is shown. A significant increase in the apoptosis of AA/DTH as compared to AA LNC is indicated by *. A significant increase in apoptosis in the presence of naïve versus AA/DTH MDSC is indicated by **s**. (C) LNC were cultured for 48 hrs in the presence of MDSC

(LNC:MDSC= 10:1) and AA skin lysate or PMA/Ionomycin and were stained with AnnexinV/PI. The relative percentage of stained LNC in comparison to cultures without MDSC is shown. A significant increase in apoptosis in the presence of MDSC is indicated by *. (D) AA/DTH LNC were cultured for 48 hrs in the absence or presence of MDSC and 10%FCS or AA skin lysate or PMA/Ionomycin. The percentage of $CD4^+$/$AnnexinV^+$ and $CD8^+$/$AnnexinV^+$ LNC is shown. Significant differences in apoptosis susceptibility of $CD4^+$ versus $CD8^+$ LNC are indicated by *.

DTH- and AA/DTH-MDSC display stronger suppressive activity than MDSC derived from naive and AA mice and also AA LNC are more severely affected than naive or DTH LNC. There is evidence for antigen-specificity of response suppression by MDSC but MDSC also suppress proliferation in response to PMA/Ionomycin. The latter particularly accounts for the impact of AA/DTH MDSC on apoptosis resistance.

To sustain these hypotheses, I proceeded to evaluate T cell receptor (TCR) ligation initiated signal transduction as well as activation of the extrinsic or the intrinsic pathways of apoptosis.

3.2.7 SADBE and MDSC interfere with TCR-initiated signal transduction

ξ chain, a component of the T cell receptor (TCR/CD3) complex essential in T cell activation and there is evidence that MDSC can interfere with T cell activation via ξ chain downregulation. In freshly harvested AA/DTH LNC a minor reduction in ξ $chain^+$ cells was observed (data not shown). In co-culture, naïve- and AA-MDSC exerted no effect and DTH-MDSC only had a slight effect on ξ chain expression. The expression of ξ chain, except in naïve mice, was significantly reduced in AA, DTH and AA/DTH LNC after co-culture with AA/DTH-MDSC in the presence of AA skin lysate.

To support the interpretation that MDSC accounts for down-regulation of ξ chain expression, $CD4^+$ and $CD8^+$ LNC were co-cultured with MDSC. It was observed that DTH- and AA/DTH-MDSC more strongly affected $CD4^+$ than $CD8^+$ ξ chain expression (Fig. 18A-C).

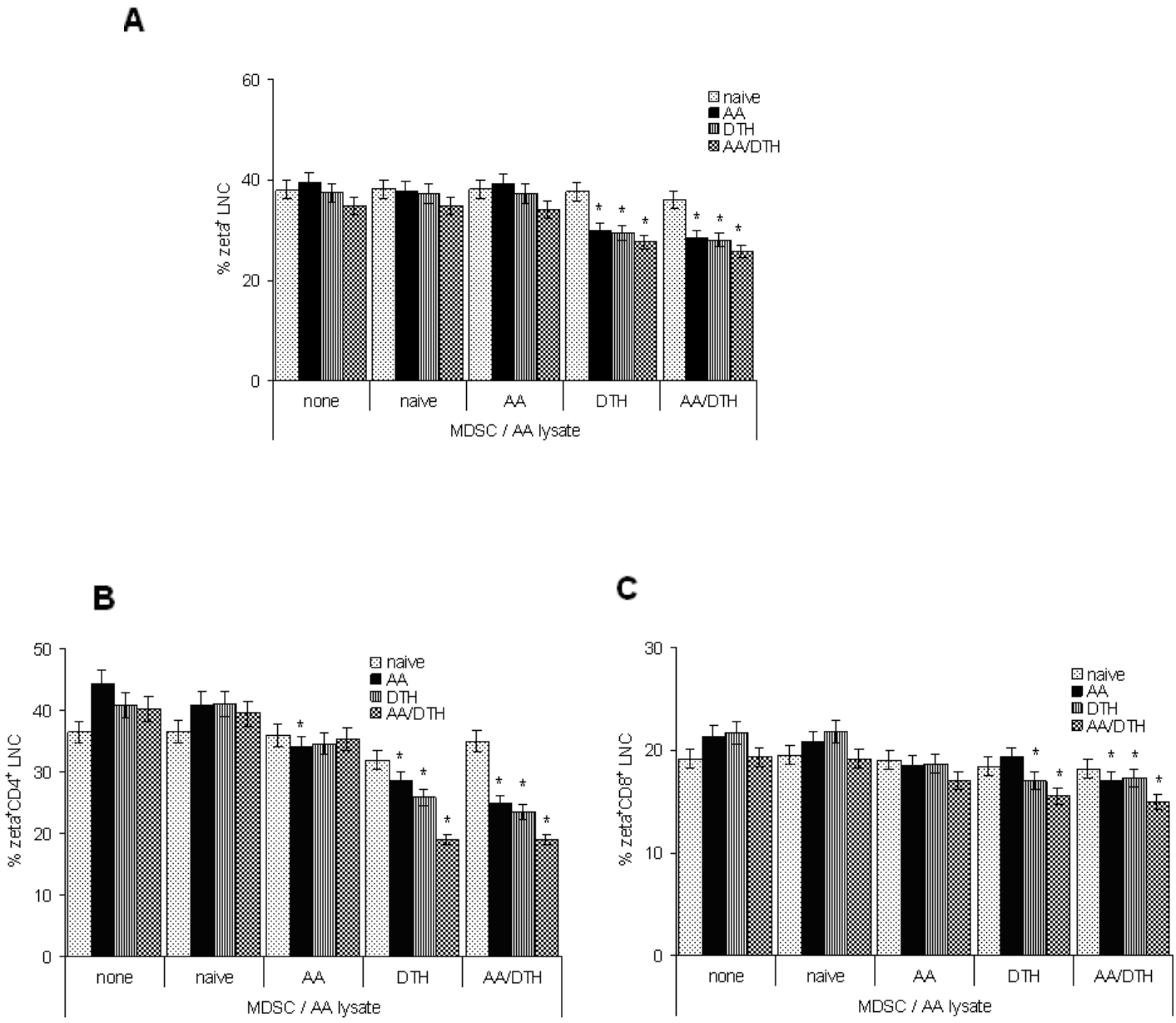
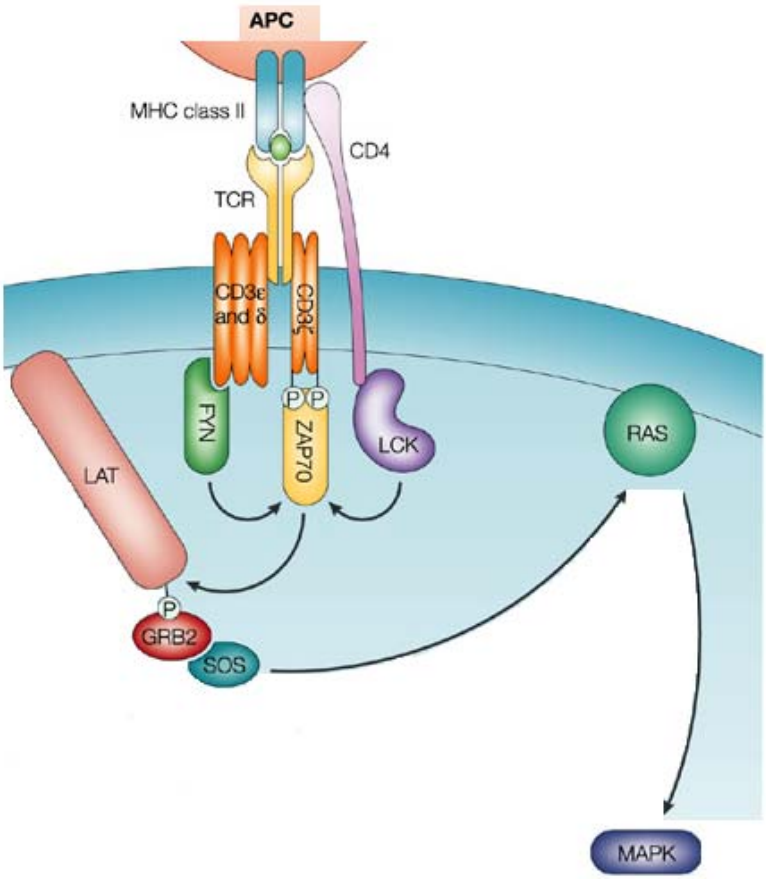


Figure18. The impact of AA/DTH MDSC on ξ chain expression: (A) LNC were cultured for 48 hrs in the absence or presence of MDSC (LNC: MDSC= 10:1) and AA skin lysate. The percentage of ξ chain$^+$ LNC was evaluated by flow cytometry. (B, C) LNC were cultured as described in A, cells were double stained for CD4 or CD8 and ξ chain. The percentage of $CD4^+$ ξ^+ and $CD8^+$ ξ^+ LNC is shown. (A-C) A significant decrease in the percentage of ξ^+cells by co-culture with MDSC is indicated by *.

In the presence of surrogate autoantigen (AA skin lysate) DTH and AA/DTH MDSC promote more efficiently $CD4^+$ than $CD8^+$ ξ chain reduction.

There was no strong effect on ξ chain expression by SADBE or AA/DTH-MDSC. However, high level of the T cell signaling molecules (Scheme 12) lck, ZAP-70, c-jun and ERK1,2 phoshphorylation in AA LNC were significantly reduced in AA/DTH LNC as revealed by flow cytometry and western blot analysis (Fig. 19A-D).



Scheme12. T cell signaling components: T cell receptors are clustered by binding to peptide-MHC complexes on an antigen-presenting cell, This brings the Src-like cytoplasmic tyrosine kinase lck/fyn into the signaling complex and activates it. Activated, lck phosphorylates tyrosines on the ζ and ε chains of the CD3 complex, which now serve as docking sites for another cytoplasmic tyrosine kinase called ZAP-70. lck phosphorylates, and thereby activates, ZAP-70. ZAP-70 then phosphorylates tyrosines on the tail of another transmembrane protein, which then serve as docking sites for a variety of adaptor proteins and enzymes. These proteins then help relay the signal to the nucleus and other parts of the cell by activating the MAP kinase signaling pathways

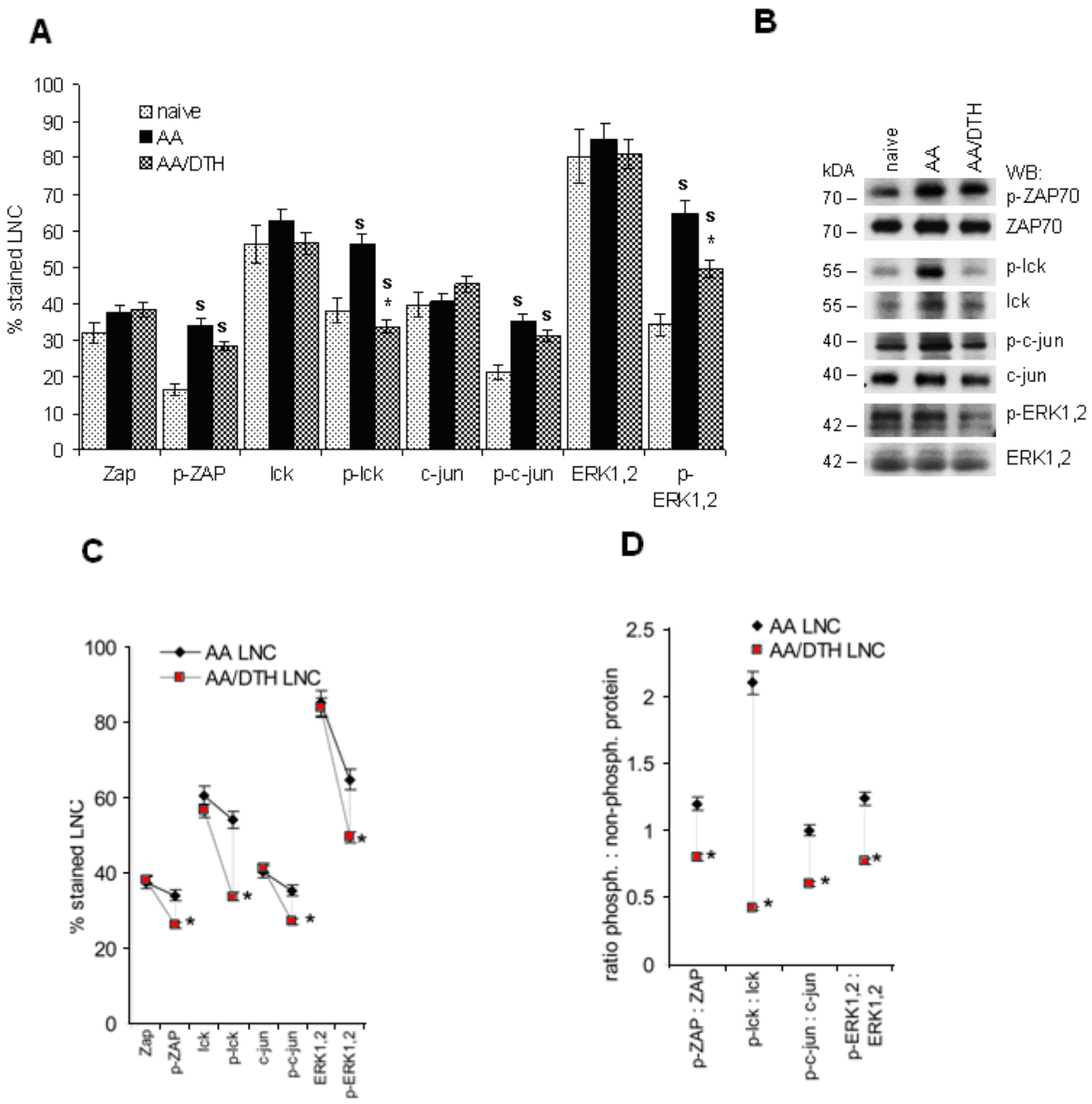
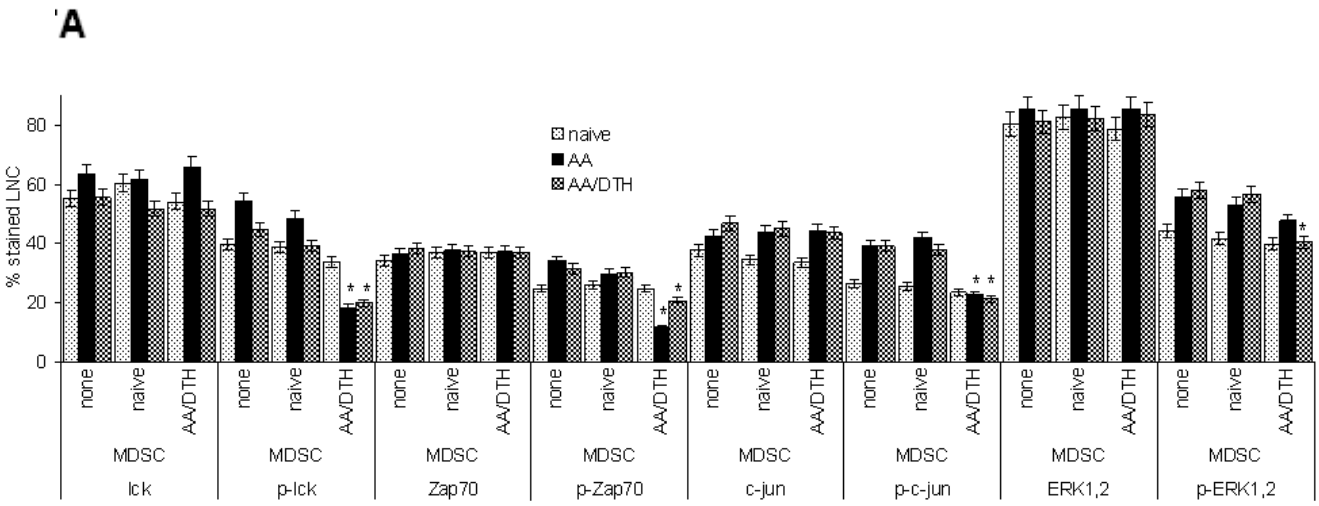
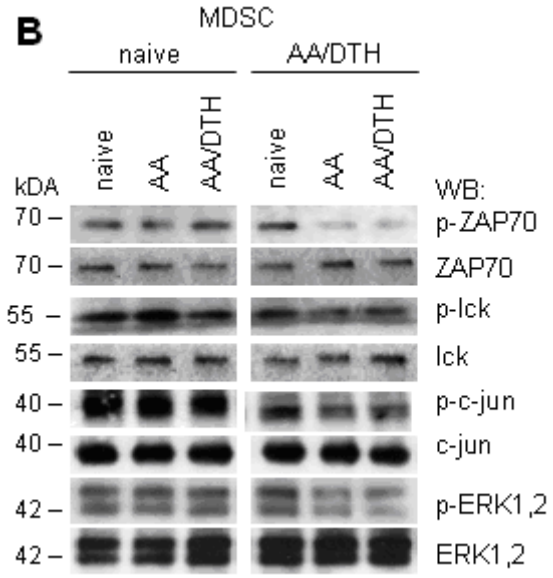


Figure19. TCR associated signal transduction in AA/DTH LNC: (A,B) Phosphorylation of Zap-70, lck, c-jun and ERK1,2 was evaluated in freshly harvested LNC from naïve, AA, DTH and AA/DTH mice by flow cytometry and western blot. (A) Significant differences in the percentage of phosphorylated protein expression in AA and AA/DTH versus naïve LNC are indicated by **s**; significant differences between LNC from AA versus AA/DTH are indicated by *. (C, D) Ratio of phosphorylated to non-phosphorylated molecules in AA and AA/DTH LNC as revealed by Flow cytometry (C) and western blot (D). Significant differences between AA/DTH versus AA LNC(C) and proteins (D) are indicated by *.

Activation of lck, Zap-70, c-jun and ERK1, 2 is impaired in AA/DTH LNC compared to AA LNC.

A similar picture emerged when LNC were co cultured with MDSC. Phosphorylation of ZAP-70, lck, c-jun and less pronounced, ERK1,2 was impaired in AA and AA/DTH, but not naïve LNC, when cultured in the presence of AA/DTH-MDSC. On the other hand, naïve-MDSC hardly exerted any effect (Fig.20A, B). Calculating the ratio of LNC expressing phosphorylated ZAP70, lck, c-jun and ERK1,2 as well as calculating the relative protein amount confirmed the strong effect of AA/DTH-MDSC on TCR signaling pathway activation, where AA and AA/DTH LNC were most and naïve LNC least sensitive to AA/DTH-MDSC (Fig 20C,D).





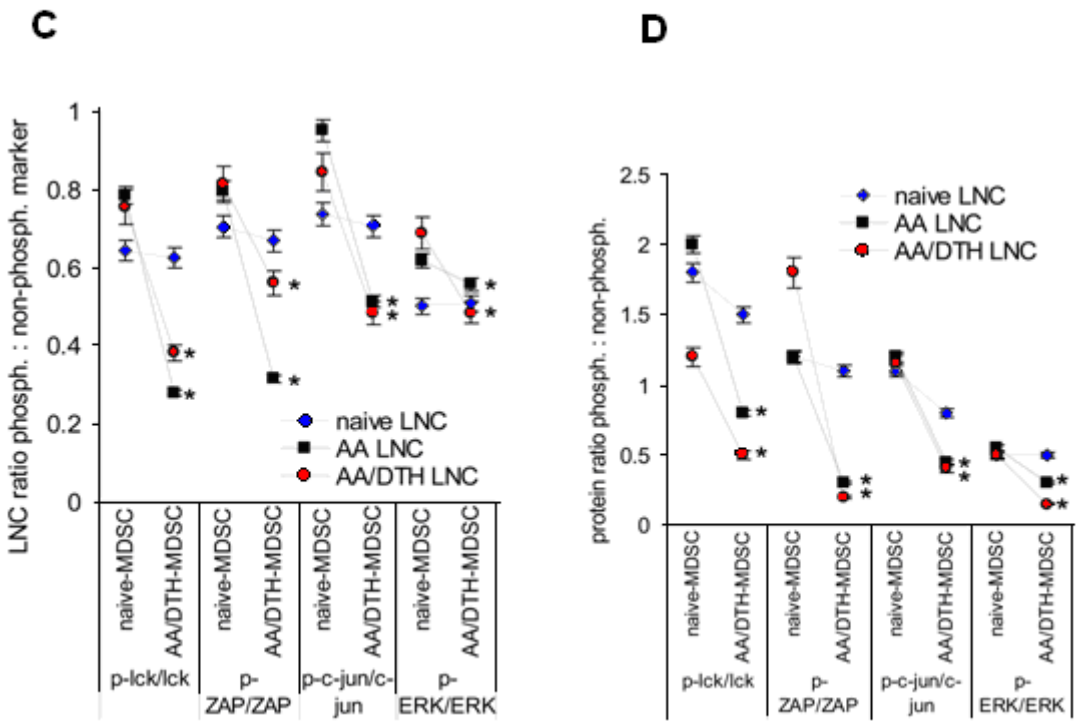


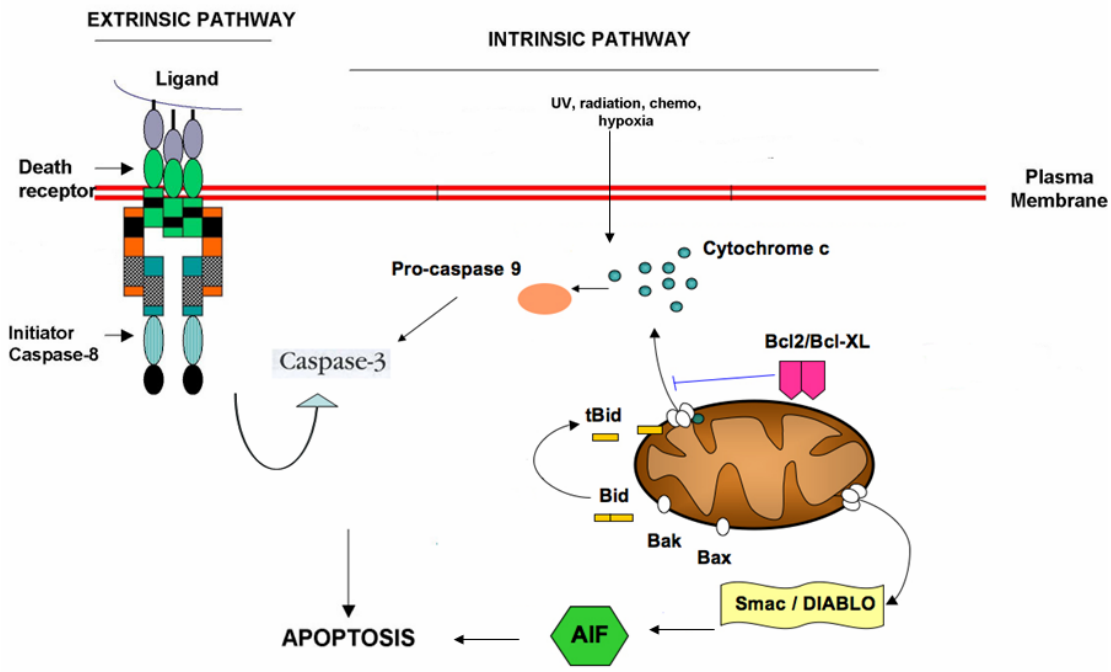
Figure20. Impact of AA/DTH MDSC on TCR-associated signal transduction: (A,B) LNC were co-cultured with MDSC for 48hrs (LNC: MDSC = 10:1). Expressions of non-phosphorylated and phosphorylated proteins were evaluated by (A) flow-cytometry and (B) western blot. (A) Significant differences in the ratio of phosphorylated to non-phosphorylated proteins in dependence on the presence of MDSC are indicated by *. (C, D) The ratio of phosphorylated to non-phosphorylated proteins was evaluated by flow cytometry (C) for LNC (Flow cytometry) and (D) proteins (WB) in cultures containing naive or AA/DTH-MDSC. Significant differences between AA/DTH versus AA LNC (C) and proteins (D) are indicated by *.

Taken together activation of lck, Zap70, c-jun and ERK1, 2 is impaired in AA/DTH LNC compared to AA LNC. AA/DTH MDSC promote ξ-chain reduction, but more efficiently mitigate lck, ZAP70, c-jun and, less pronounced ERK1, 2 activation.

Thus, SADBE and AA/DTH-MDSC strongly inhibit sustained activation of signaling cascades promoting T cell proliferation. ζ-chain internalization which is initiated by AA/DTH-MDSC only, is likely contributing to impaired ZAP70 phosphorylation. However, the more striking reduction in Lck phosphorylation which is upstream of ξ-chain did not essentially require TCR engagement.

3.2.8 MDSC participates in breaking apoptosis resistance

AA effector cells are strongly resistant to apoptosis despite of high level expression of death ligands and CD120a. In addition, AA- and AA/DTH-MDSC promote TNFα and Trail, only AA/DTH-MDSC promotes CD120a (TNFRI) and CD120b (TNFRII) expression. This may correspond to higher apoptosis susceptibility of AA and AA/DTH LNC when co-cultured with AA/DTH MDSC, particularly in the presence of PMA/Ionomycin. However, PMA/Ionomycin should rather promote T cell proliferation. Therefore it became important to unravel the mechanism of this TCR-independent break of apoptosis resistance supported by MDSC. Evaluation of expression of major anti-apoptotic protein (Scheme 13) was done.



Scheme13. Apoptotic pathways

It was observed that in freshly harvested LNC from naïve, AA and AA/DTH mice, revealed upregulation of p-Akt, p-Bad, Bcl_2 and Bcl-Xl in AA compared to naïve LNC. As demonstrated by flow cytometry and western blots, Akt and Bad phosphorylation was reduced in AA/DTH LNC, but Bcl_2 and Bcl-Xl expression was not reduced (Fig.21A-D).

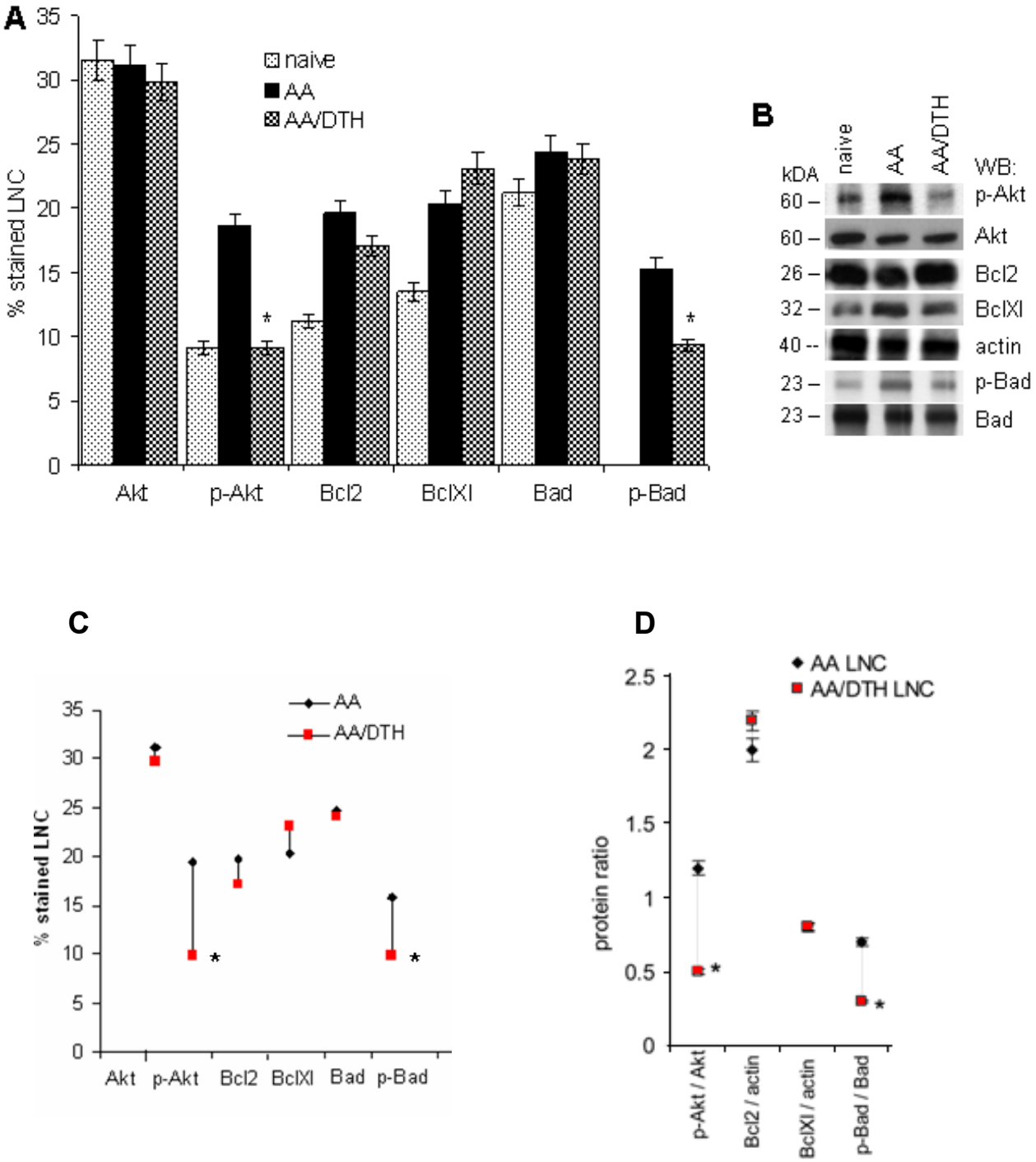
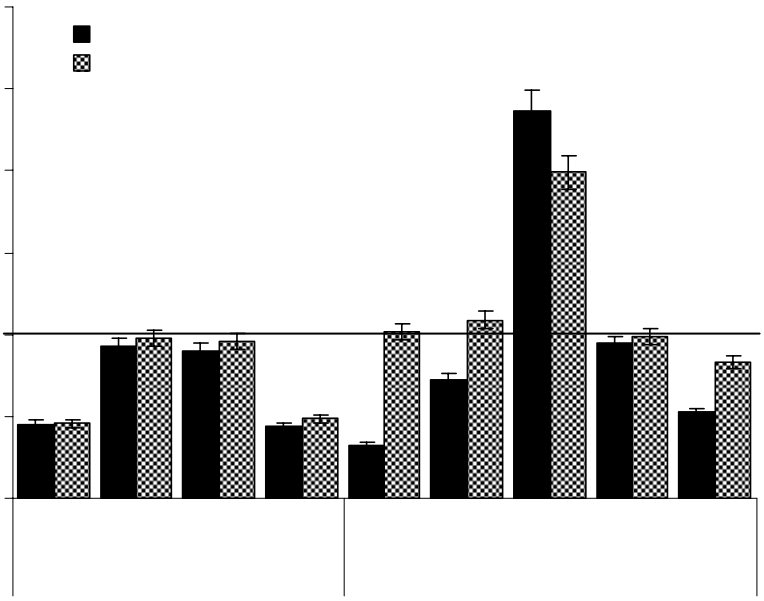


Figure21. Impact of SADBE on anti-apoptotic protein expression: (A, B) Anti-apoptotic protein expression evaluated in draining LNC by (A) flow cytometry and (B) WB. Significant differences in the percentage of stained AA/DTH compared to AA LNC are indicated by *. (C, D) The ratio of the anti-apoptotic proteins in AA/DTH compared to AA LNC as revealed by flow cytometry (C) and western blot (D). Significant differences between AA/DTH versus AA LNC (C) and proteins (D) are indicated by *.

Similarly, in the presence of AA skin lyaste AA/DTH MDSC interfered with Akt and Bad phosphorylation, but had no effect on Bcl_2 and Bcl-Xl. In the presence of PMA/Ionomycin, AA/DTH MDSC rather promoted than suppressed Bcl_2 and Bcl-Xl expression (Fig.22A, B). The ratios of cells expressing phosphorylated versus non-phosphorylated Akt or Bad and the ratio of Bcl_2 and Bcl-Xl versus actin as well as the corresponding relative protein band intensities are summarized for AA LNC cultured in the presence or absence of AA/DTH MDSC, supplemented with AA skin lysate or PMA/Ionomycin (Fig.22C).

A



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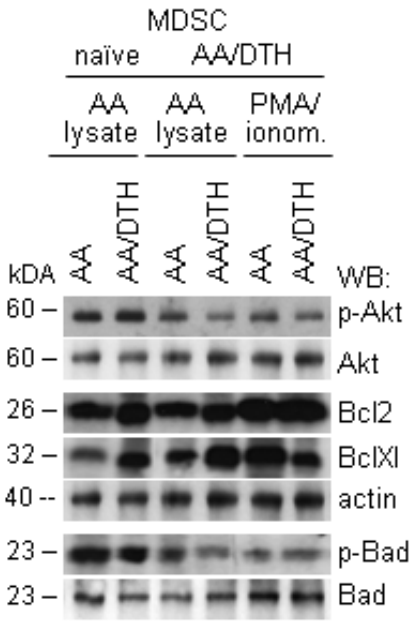

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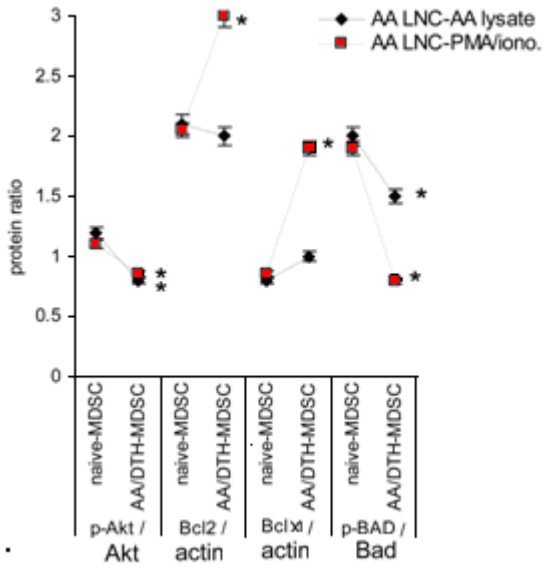


Figure22. Impact of MDSC on anti-apoptotic protein expression: (A, B) Expression of anti-apoptotic proteins in LNC after co-culture with MDSC (LNC: MDSC= 10:1) in the presence of AA-skin lysate or PMA/Ionomycin as revealed by (A) flow-cytometry and (B) WB. Significant differences in anti-apoptotic protein expression in dependence on the presence of MDSC are indicated by *. (C) The ratios of phosphorylated versus non-phosphorylated marker proteins or marker proteins versus actin were evaluated in AA LNC, for co-cultures with naïve- versus AA/DTH-MDSC in the presence of AA lysate and PMA/Ionomycin. Significant differences in the relative expression intensity of anti-apoptotic proteins in cultures containing AA/DTH- versus naïve- MDSC are indicated by *..

SADBE treatment is accompanied by a significant reduction in anti-apoptotic proteins. The impact of MDSC varies depending on the donor and the stimulus. AA/DTH-MDSC more efficiently than naïve-MDSC, suppresses Akt and Bad phosphorylation in the presence of AA skin lysate. In the presence of PMA/Ionomycin suppression becomes weaker and expression of Bcl_2 and BclXl is strengthened.

SADBE and AA/DTH-MDSC efficiently interfere with Akt activation and Bad phosphorylation, but do not cope with high level Bcl_2 and BclXl expression. Thus, downregulation of anti-apoptotic proteins may not contribute to a major degree to AA/DTH-MDSC-induced apoptosis susceptibility of AA LNC. Alternatively, SADBE and /or AA/DTH-MDSC may promote pro-apoptotic molecule expression and activation.

Expression of caspase8, the direct target in receptor-mediated apoptosis, was unaltered in AA and AA/DTH. Instead, Bid, Bax, Smac, cytochrome C, cleaved caspase9 and caspase3 expression were significantly increased in AA/DTH compared to AA LNC (Fig.23A-D).

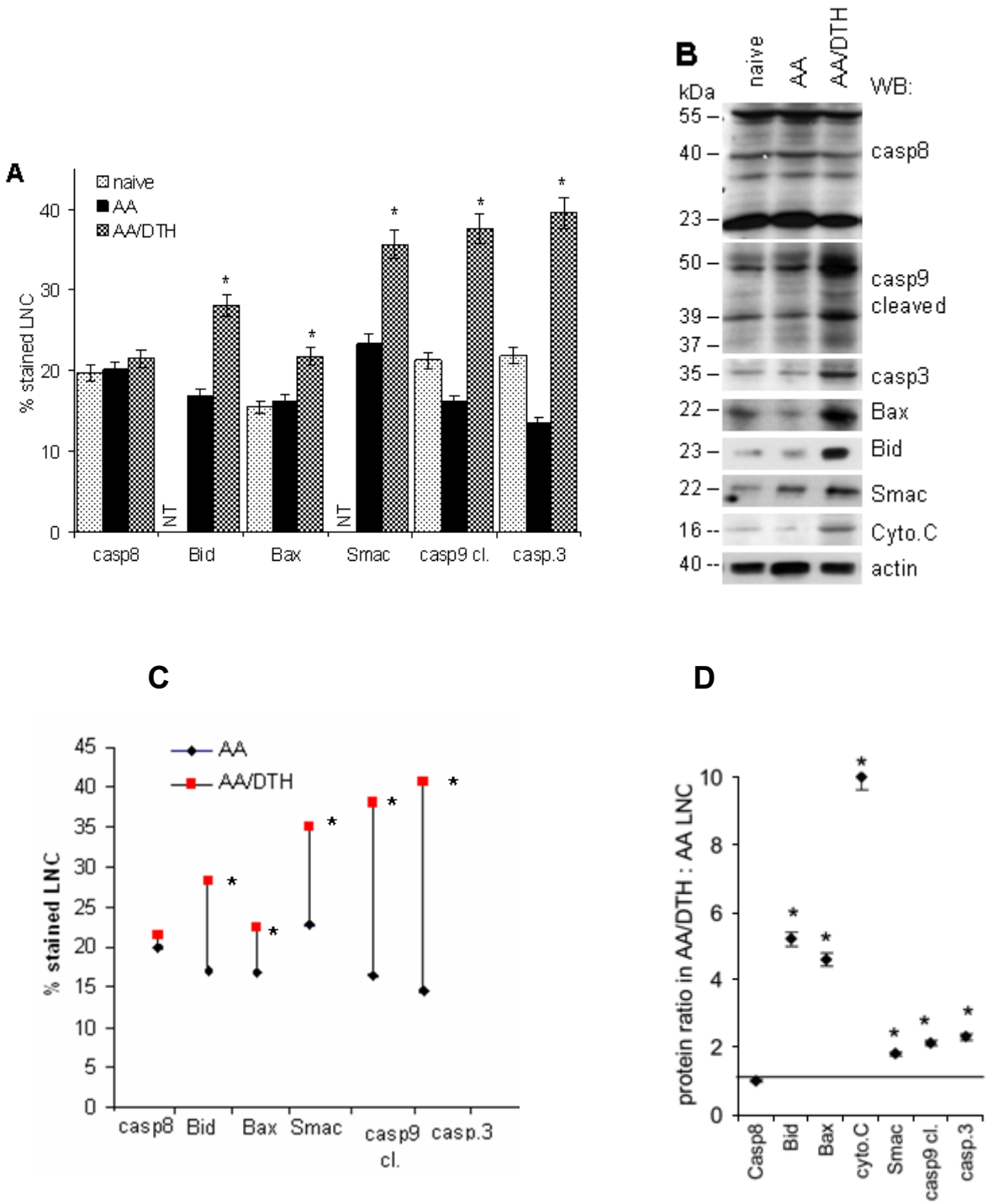
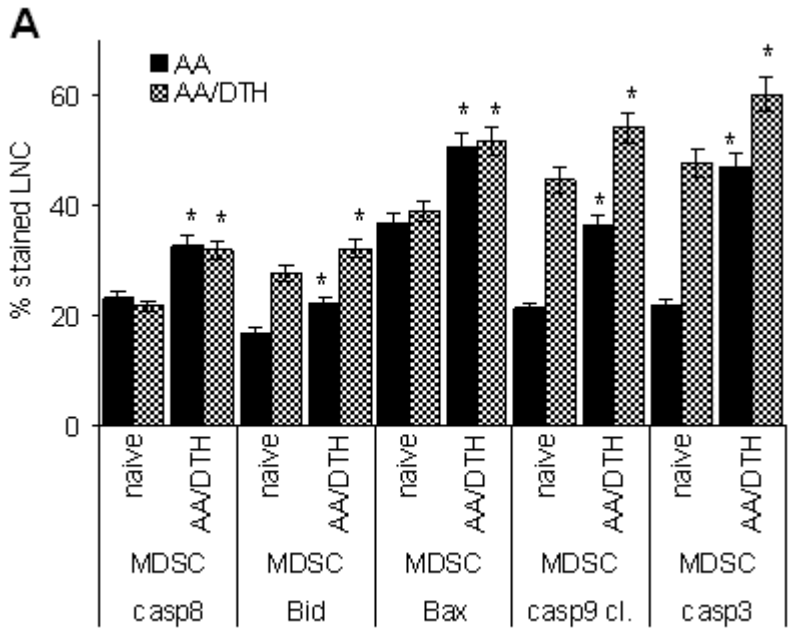


Figure23. Impact of SADBE on pro-apoptotic protein expression: (A, B) Pro-apoptotic protein expression was evaluated by flow cytometry (A) and WB (B). (A) Significant differences in pro-apoptotic protein expression in AA/DTH compared to AA LNC are indicated by *. (C, D) The cell and

protein ratio in AA/DTH versus AA LNC lysates as revealed by flow cytometry (C) and Western Blot (D). Significant differences between AA/DTH versus AA LNC (C) and proteins (D) are indicated by *.

Upregulation of pro-apoptotic molecules engaged in the mitochondrial apoptosis pathway in AA/DTH LNC pointed towards AA/DTH-MDSC possibly activating pro-apoptotic molecules in AA LNC. Bid, Bax, cleaved caspase9, caspase3 were expressed a higher percentage of AA and AA/DTH LNC, when co-cultured in the presence of PMA/Ionomycin with AA/DTH- than with naïve-MDSC (Fig.24A). This also accounted for Bax, cytochrome C, Smac, cleaved casp9 and casp3 protein levels (Fig.24B). Calculating the protein ratios for AA LNC confirmed upregulated expression of molecules engaged in the mitochondrial apoptosis pathway in co-cultures with AA/DTH-, but not naive-MDSC (Fig.24C). Similar, but less pronounced effects were seen in co-cultures containing AA skin lysate (data not shown).



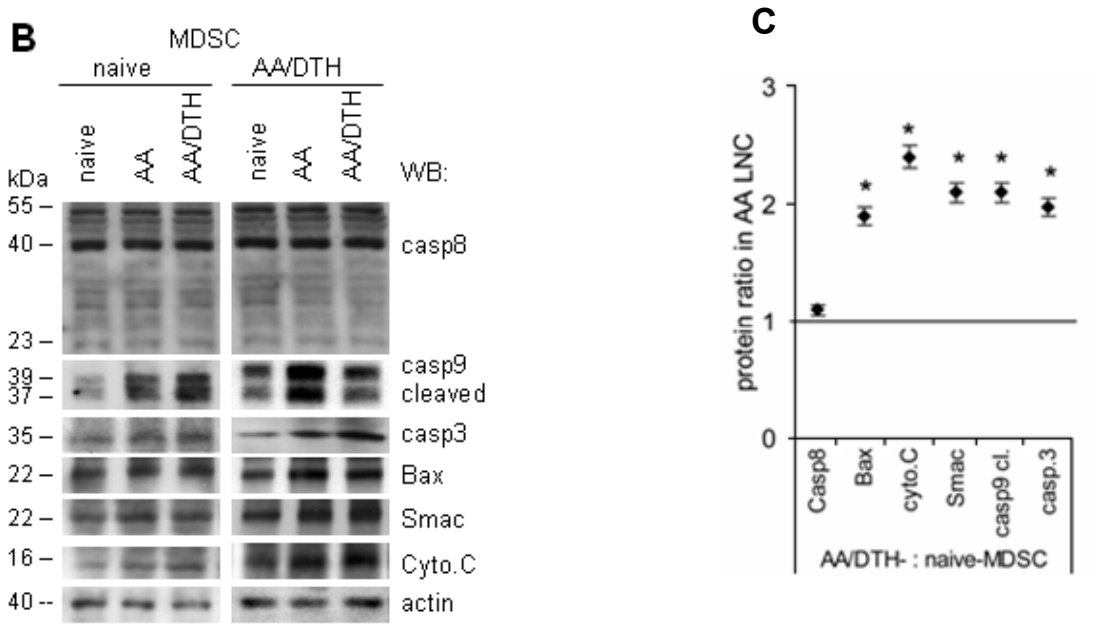


Figure24. Impact of MDSC on pro-apoptotic protein expression: (A, B) Expression of pro-apoptotic proteins in LNC after co-cultured with MDSC (LNC: MDSC =10:1) in the presence of PMA/Ionomycin as revealed by flow cytometry (A) and WB (B) NT: not tested. (C) The ratios of pro-apoptotic marker expressing AA LNC and proteins were evaluated for co-cultures with AA/DTH versus naïve MDSC in the presence of PMA/Ionomycin. Significant differences in protein expression between AA/DTH- versus naive MDSC are indicated by *.

Taken together, SADBE and AA/DTH-MDSC are not very effective in counter-regulating high level anti-apoptotic protein expression in AA LNC, but most efficiently promote activation of the mitochondrial apoptosis pathway

A chronic dermal DTH reaction supports induction and recruitment of highly efficient myeloid derived suppressor cells. A co-culture system provided evidence that reduced responsiveness of T cells relates to impaired activation of TCR-associated signaling cascades that only partly can be explained by ξ-chain downregulation. More importantly SADBE and AA/DTH-MDSC efficiently break autoimmune T cell apoptosis resistance by downregulation of anti-apoptotic proteins. They also support expression and stimulation of pro-apoptotic proteins.

4: Discussion

Alopecia areata is a T cell-mediated autoimmune disease of the skin that affects anagen stage hair follicle, and does not become life-threatening (257). The human disease is closely mimicked by C3H/HeJ mice that develop AA spontaneously or after the transfer of full thickness skin grafts from AA affected mice. The mild disease state as well as the availability of an animal model makes this autoimmune disease unique inasmuch as these features permit to elaborate the complex immune interactions promoting the persistence/exacerbation of an autoimmune disease without an iatrogenic or late stage autoimmune disease-associated collapse of the immune system. Taking in account the classical autoimmune disease features of AA, expansion and activation of autoreactive T cells, reduction in T_{reg}, apoptosis resistance, this autoimmune disease model is well suited to define the molecular pathways of disease maintenance/progression and also should allow elaborating therapeutics interventions that could be of general validity in organ-related autoimmune disease.

I used this model to elaborate the molecular mechanisms, whereby a chronic eczema acts as the most efficient therapeutics in AA. Yet, before attending this, I wanted to shed more light on another feature of autoimmune T cells, their persistent activation and expansion.

4.1 Undue T cell activation and expansion in autoimmune disease

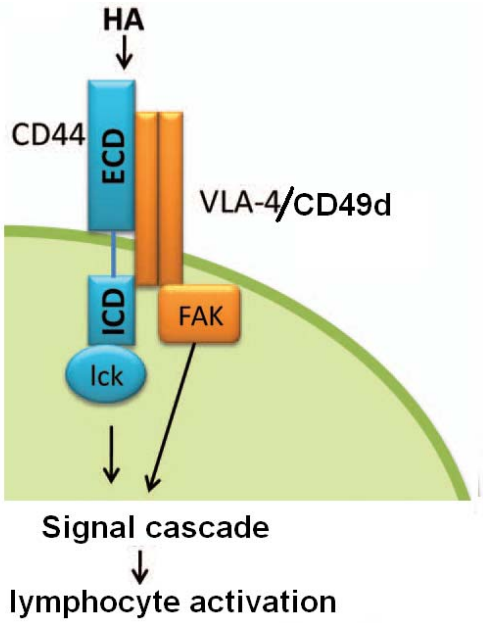
T cells respond to an antigenic stimulus that is mediated by TCR-complex. After antigen elimination, responding T cells can be driven into activation induced cell death (AICD) or return to the resting state due to antigenic deficiency or may became shut-off by transient internalization of the entire TCR/CD3 complex. All of these mechanisms can be disturbed in autoimmune disease. In addition, T cells may become activated independent of the TCR/CD3 complex. This phenomenon has been described to be mostly mediated by so called accessory molecules, the most important one being CD18. We described that CD44 can fulfill similar activities. Interestingly, previous work in our lab indicated that this kind of TCR/CD3 complex-independent T cell activation may also play a role in AA. This work also revealed that CD44 can co-operate with CD49d (integrin) in undue T cell activation. In the first part I have tried to approach this question at the molecular level.

4.1.1 Involvement of CD44-CD49d association in AA

The involvement of CD44 in autoimmune disease is well known. CD44 plays an important role in leukocyte extravasation in autoimmune disease and allergic reaction and has been implicated in the processes associated with changes in T cell morphology. Originally CD44 has been described as adhesion molecule mediating lymphocyte homing. CD44 is also important for the adhesion of leukocytes to endothelial cells as an initial step before extravasation. Adhesion of CD44 to its ligand(s) induces up-regulation of additional adhesion molecules, mostly integrins that strengthen adhesion. In line with this, the CD44-CD49d association supports leukocytes' firm adhesion to vessel endothelium. CD44 associates with CD49d in the membrane of activated T cells through its cytoplasmic tail and *in vivo* this receptor combination can regulate T cell extravasation into the peritoneum after induced inflammation (258,259).

It has been described that CD44 associates with src kinases like lck in glycolipid enriched membrane (GEM). Furthermore, CD44 and CD49d associate with each other *via* adaptor or signal transducing molecules. In fact CD44 crosslinking can induce FAK (Focal adhesion kinase) phosphorylation (Scheme 14) (260) and CD49d can promote c-src activation independent of FAK (261).

The safest way to prove the relevance of the CD44-CD49d interaction in T cell activation is by using T cell lines or leukemic lines that differ in absence or presence of one of these two molecules, and to transfect these lines with the missing partner molecule with or without mutation. In the latter case mutation should be selected such that the two molecules can no longer interact with each other. This has been my starting point, Jurkat leukaemic line was transfected with CD44, CD44 mutated at Ser325 and Ser291 which prohibit ezrin binding, and CD44 without the cytoplasmic domain, which prohibit cytosolic signalling. EL4 T cell line was transfected with CD49d and CD49d mutated at Ser988 which prohibit paxillin binding.



Scheme14. CD44-CD49d association: Ligation of CD44 (e.g., through HA) can facilitate aggregation of CD44-integrin-kinase signaling components in lipid rafts. Src family kinases, such as Lck, associate with the cytoplasmic tail of CD44 and activate signaling pathway which can strongly influences lymphocytes activation and function.

4.1.2 The CD44-CD49d complex promotes T cell motility

Activated T cells display a migratory phenotype, where migration can be inhibited by both anti-CD44 and anti-CD49d. The importance of this co-operation was confirmed by increased motility of CD44 transfected Jurkat ($CD49d^+$) and CD49d transfectred El4 ($CD44^+$) cells. T cell motility requires the cytoplasmic tail of CD44 to form an association with ezrin, which acts as a linker and links CD44 to F-actin (262), Furthermore; ezrin binding requires an exchange of Ser325 versus Ser291 phosphorylation. Therefore, as expected, Jurkat-truncCD44 (without cytoplasmic tail) and Jurkat-mutCD44 (Ser291and Ser325) had a lower migration rate. Similarly in EL4-mutCD49d less migration was observed since phosphorylation of S988 is required for CD49d mediated migration and affects paxillin binding, which in turn affects FAK binding.

Thus, from the functional point of view, the two cell lines transfected with non-mutated or mutated CD44 and CD49d fully support the engagement of CD44 and CD49d in T cell motility as well as the additional requirement of ezrin, paxillin and FAK. Co-immunoprecipitation with CD44 and CD49d studies revealed that no association between CD44 and CD49d was observed in the cell lines transfected with

truncCD44, mutCD44 and mutCD49d. Immunoprecipitations with CD44 on Jurkat-CD44, Jurkat-mutCD44, Jurkat-truncCD44 revealed that association with ezrin, FAK and paxillin was observed only in the Jurkat-CD44 but fails to show any association in mutated or truncated CD44 which was cross confirmed by immunoprecipitation with CD49d. Thus, the association of CD44 and CD49d is essential for downstream with ERM proteins as well as paxillin and FAK which allows for the migration
Taken together only the activated form of CD44 can associate with CD49d. Both, the cytoplasmic tail and ezrin binding are essential to promote the shift towards a mobile phenotype.

4.1.3 The CD44-CD49d association promotes leukocyte proliferation

Particularly CD44 also has been described to act as an accessory molecule in TCR complex mediated signal transduction (243). Thus, the question arose whether CD44 in combination with CD49d also suffice to initiate downstream of the TCR/CD3 complex activation cascade independent of the TCR/CD3 complex engagement. Particularly the EL4 thymoma line, which does not express the TCR and CD3 complex, was well suited to answer this question. El4-CD49d showed higher rate of proliferation which was inhibited by both anti-CD44 and anti-CD49d, while EL4-mutCD49d showed lesser proliferation and was inhibited only in presence of anti-CD44 (TCR independent). In Jurkat-$CD44^{mut}$, Jurkat-$CD44^{trunc}$ showed less proliferation, which was inhibited by anti-CD49d only instead Jurkat-CD49d showed a higher proliferation rate which was equally inhibited by both anti-CD49d and anti-CD44. Thus, CD44 and CD49d association is important for the proliferation of the T cells and at this point I clould define this only the basis of antibody blockade but this work is ongoing to look for the signal cascade.
From above data it can be speculated that the CD44-CD49d association supports TCR/CD3 initiated activation, but can also promotes TCR/CD3 independent activation and proliferation. The molecular pathway of signal transduction underlying the co-operativity of TCR with CD44-CD49d remains to be explored. In addition the impact of CD44-CD49d association on activation induced cell death (AICD) is yet to be defined. Further work is needed on this aspect such that it can provide an answer to the mechanism underlying the persisting activation and proliferation of autoreactive T

cells in autoimmune disease. Blockade of these bystander pathways could be a therapeutic approach in autoimmune disease including alopecia.

4.2 Autoimmunity: T cell regulation and a chronic DTH as therapy

Autoimmunity is characterized, besides undue persisting activation, by resistance against apoptosis induced cell death and a deficit in immunoregulatory elements, particularly regulatory T cells (T_{reg}) and myeloid derived suppressor cells (MDSC). Furthermore, it has been suggested that chronic eczema may be a therapeutic due to its impact on the activation and expansion of MDSC.

4.2.1 Characterization of MDSC

Treatment of AA mice with SADBE results in an expansion and activation of myeloid derived $CD11b^{+}Gr1^{+}$ suppressor cells (MDSC). MDSC are characterized by their myeloid origin, immature state and most importantly by their potent ability to suppress different aspects of immune responses especially T cell proliferation and activation. MDSC were enriched in dermis and spleen of AA mice treated with SADBE (AA/DTH). Spleen of SADBE treated mice also contained an increase number of activated $CD11b^{+}$ macrophages. In line with other reports (263), these activated macrophages were not suppressive and could even weaken the efficacy of MDSC suppression (180). Characterization of MDSC in AA and AA/DTH mice showed that AA/DTH-MDSC expressed Ly6C at a higher level which is a characteristic of M-MDSC (Monocytic-MDSC $Ly6^{high}$). Evidence from various laboratories suggests that on a per cell basis M-MDSC has more potent suppressive behavior than the G-MDSC (Granulocytic-MDSC$Ly6C^{low}$). Previous work in our lab showed that AA/DTH-MDSC also expressed TGFβ (180), which can be involved in NO production (150,175) and can be induced by IFNγ (103), which supports MDSC activation (264). AA/DTH-MDSC showed an increased expression of STAT1, STAT6 and NFkB in comparison to AA-MDSC. STAT1 is the main transcription factor activated by IFNγ or IL1β signaling and is implicated in the regulation of NO and arginase activity on the other hand STAT 6 is also described as a MDSC marker and is responsible for upregulation of arginase activity (172-174) and increased TGFβ production by MDSC (175). According to several studies it appears that STAT 1 and STAT 6 play an important role in MDSC activation. NFkB is also involved in

expansion of MDSC, and plays an important role in signal activation of MDSC and acquisition of immune suppression function. M-MDSC is the main population of MDSC where the NFkB pathway has been shown to be active (265). AA/DTH also showed a higher expression of IL1α and IL6. IL1 α induces accumulation of MDSC and enhances their suppressive activity against $CD4^+$ and $CD8^+$ T cells (266), IL6 is also involved in expansion and activation of MDSC. Finally, TNFα expression and secretion as well as TNFRI (CD120a) and TNFRII (CD120b) expression are up regulated in MDSC from AA/DTH mice.

Taken together MDSC d from AA/DTH mice are in a highly activated and suppressive state and thus could well be important in mitigating T cell activation.

4.2.2 The relevance of MDSC in mitigating AA

SADBE treatment cures alopecia and can be replaced (though slightly less efficient) by the transfer of AA/DTH-MDSC (180). It is known that ATRA, a member of retinoid family of molecules structurally related to vitamin A, exerts profound effects on cell proliferation, induction of differentiation and apoptosis in normal cells and cancer cells (115). Previously, it has been demonstrated that all *trans* retinoic acid (ATRA) had a potent activity in eliminating MDSC and drives these into differentiation towards mature myeloid cells. Treatment of cancer patients and tumor bearing mice with ATRA resulted in substantial decrease of these cells and improvement of immune responses (116,267). In our studies it has been shown that ATRA treatment prevents hair regrowth when applied along with SADBE or MDSC. In line with this the immunohistological studies demonstrate that dermal infiltrates of $CD4^+$ and $CD8^+$ cells ,which are a hallmark of AA disease were dramatically reduced after MDSC transfer and by SADBE. But, the infiltration remains unaltered upon concomitant treatment. Thus, all these factors points towards an active contribution of MDSC to hair re-growth.

4.2.3 Comparing the impact of SADBE and MDSC on leukocyte subsets and activation status of AA T cells

It has been reported previously that AA is characterized by an increase in $CD1^+$ (expressed on antigen presenting cells), $CD4^+$ and $CD8^+$ cells in the skin and also show an upregulation of T cell activation markers CD28, CD69 and CD154 as well as

of co-stimulatory molecules CD40, CD80 and CD86 in SkIL and draining LNC (268). Besides an expected increase in $CD11b^+$ cells, SADBE treatment did not induce any significant change in the leukocyte subpopulation composition. SADBE treatment showed a reduction in IL-4 and a strong increase in IL-6, and also strengthened CD69, CD40 and CD86 expression only in lymph node and not in skin. In co-cultures with AA/DTH-MDSC, a slight decrease in $CD8^+$ LNC was observed. Unlike SADBE treatment, AA/DTH-MDSC resulted in a decrease in the expression of activation markers CD28, CD69 and an increase in CD152 (CTLA 4) which transmits an inhibitory signal to T cells. CD152 expression was only slightly affected by SADBE treatment. Thus, MDSC do not actively eliminate T cell subsets and have no impact on co-stimulatory molecule expression by antigen prrsenting cell. However, they mitigate expression of accessory molecule on T cells

Regulatory T cells (T_{reg}) are a specialized subpopulation of T cells that act to suppress activation of immune cells and thereby maintain homeostasis of the immune system and tolerance to self antigens.The importance of the immunesuppressive potential of these cells become obvious in autoimmune disease, where T_{reg} are frequently recovered at a reduced level (175). This holds true for AA also (268). Thus expression of T_{reg} in AA become of special interest. T_{reg} are defined as $CD25^+CD4^+Foxp3^+$ cells. AA lymph node cells and SkIL displayed a reduced level of CD152 and FoxP3. In LNC of AA mice treated with SADBE, the percentage of T_{reg} cells was increased significantly, but did not reach the level of naïve LNC. Impact of MDSC on T_{reg} has been described (103). In line with this, after co-culture with AA/DTH-MDSC a further increase in T_{reg} was observed in AA and AA/DTH lymph nodes which were even higher than that of naïve mice lymph nodes. IFNγ, IL10 and CD152 have been suggested to be required for T_{reg} induction by MDSC (103). The impact of SADBE treatment as well as of MDSC on T_{reg} expansion could provide a first explanation for the therapeutic efficacy of a chronic eczema /MDSC in AA.

Apoptosis resistance of autoimmune T cells is an additional important feature of sustaining an autoimmune disease state. Autoimmune T cells were reportedly found to be resistant to apoptosis (268). In AA, apoptosis resistance is unexpected as SkIL and draining LNC express the death receptor CD95L at an elevated level. Death receptors are characterized by a unique intracellular death domain (DD), which is crucial for death ligand-induced apoptosis. SADBE and co-culture of LNC with MDSC had only slight effect on the expression of CD95L, but expression of other death molecules like

TNF (can induce apoptotic cell death) and Trail (TNF-related apoptosis-inducing ligand) expression become upregulated by both SADBE treatment and also in co-culture with AA/DTH-MDSC. Furthermore, expression of TNFRI and TNFRII (without a death domain) was also increased after SADBE treatment as well as in co-culture with AA/DTH MDSC, but not by co-culture of AA-MDSC. These distinct features of death receptor expression in AA and AA/DTH LNC rely on the difference in the activation status of MDSC in these mice. As described before, MDSC derived from AA/DTH mice represents a more activated and are more suppressive sub population than that of MDSC derived from AA mice

4.2.4 The impact of MDSC on the activation state of T cells is reflected by the potency in suppressing T cell proliferation

SADBE treatment, as well as, isolated MDSC from SADBE-treated AA mice efficiently suppresses AA effector T cell proliferation. Suppression was weakest in the absence of a nominal antigen and strongest in the presence of AA skin lysate which serves as a surrogate auto-antigen. Two major subsets of MDSC apparently have an important role in the antigen-specific versus non-specific nature of immune suppression. G-MDSC relies on antigen-specific interaction between MDSC and T cells, M-MDSC, which use upregulation of NO and arginase, production of immunosuppressive cytokines, and other mechanisms, effectively suppress antigen specific as well as antigen-independent T-cell responses. The idea that MDSC-mediated T-cell suppression occurs in an antigen-specific manner is based on findings that antigen-specific interactions between antigen-presenting cells and T cells result in much more stable and more prolonged cell–cell contact than nonspecific interactions (269-271). Such stable contacts are necessary for MDSC derived ROS and peroxynitrite to mediate effects on the molecules on the surface of T cells that render the T cells unresponsive to specific antigen. However suppression was also observed in presence of PMA/Ionomycin, which argues against an exclusive reliance on T cell receptor (TCR)-initiated signaling. There are many ways by which MDSC can suppress the activation of T cells. One of the pathways relies on down regulating the TCR-associated ξ chain (180,272-274). In absence of ξ chain, $CD4^+$ and $CD8^+$ T cells are unable to transmit the required signals for activation.bIt was noticed that SADBE treatment did not show any significant down regulation of ξ chain expression in

freshly harvested LNC, but in co-cultures with DTH- or AA/DTH-MDSC there was a partial down regulation of the ξ chain predominantly in $CD4^+$ cells. It has been observed previously in our lab that upon cross linking of the TCR with anti-ε, a stronger down regulation of the ξ chain in $CD8^+$ than $CD4^+$ LNC was observed (180). Thus, suggest that the AA skin lysate contains antigenic entities more efficiently recognized by $CD4^+$ than $CD8^+$ LNC. However, though ξ chain expression was not strongly affected by SADBE, SADBE sufficed for a significant change in the T cell signaling components. Activation of T cells brings the Src-like cytoplasmic tyrosine kinase lck into the signaling complex and activates it. Once activated, lck phosphorylates tyrosines on the ζ and ε chains of the CD3 complex, which now serve as docking sites for another cytoplasmic tyrosine kinase called ZAP-70. lck phosphorylates, and thereby activates, ZAP-70, ZAP-70 then phosphorylates tyrosines on the tail of another transmembrane proteins, which then serve as docking sites for a variety of adaptor proteins and enzymes. These proteins then help relay the signal to the nucleus and other parts of the cell by activating the MAP kinase, the JNK and the NFkB signaling pathways. SADBE, cause a reduction in lck, Zap70, c-jun and less pronounced ERK1, 2 phosphorylation. In co-culture of LNC with AA/DTH-MDSC there was also a strong reduction in the phosphorylation of lck and thereby control the phosphorylation of ZAP- 70 followed by c-jun and ERK1, 2 phosphorylation. Because lck activation is upstream of the ξ chain phosphorylation, these findings imply that ξ chain down regulation is not the dominating feature in SADBE- and AA/DTH MDSC initiated suppression of LNC proliferation. It also should be mentioned that one of the major pathways of ξ chain down regulation proceeds *via* Toll like receptor-4 (TLR4) (274), which is defect in C3H/HeJ mice (275). However TLR4 may not be essentially required, as in absence of TLR4, known to be important in controlling the susceptibility to contact hypersensitivity, contact hypersensitivity is not significantly impaired (276). As apparent by the therapeutic efficacy of SADBE treatment in AA mice, contact hypersensitivity is also not impaired in C3H/HeJ mice. Taken together, the mild down regulation of ξ chain and the strong reduction in lck phosphorylation argue for additional inhibitory signals that could have been transmitted *via* up regulated CD152 or *via* T_{reg} or via not yet defined signals. The efficacy of MDSC to suppress LNC proliferation in the presence of PMA/Ionomycin also points towards additional immunosuppressive mechanisms.

It can be concluded from these studies that, SADBE treatment as well as co–culture with MDSC is accompanied by a striking reduction in the activation of signaling cascades promoting T cell proliferation. ξ chain down regulation can contribute to impaired responsiveness, but does not exclusively account for the observed effects.

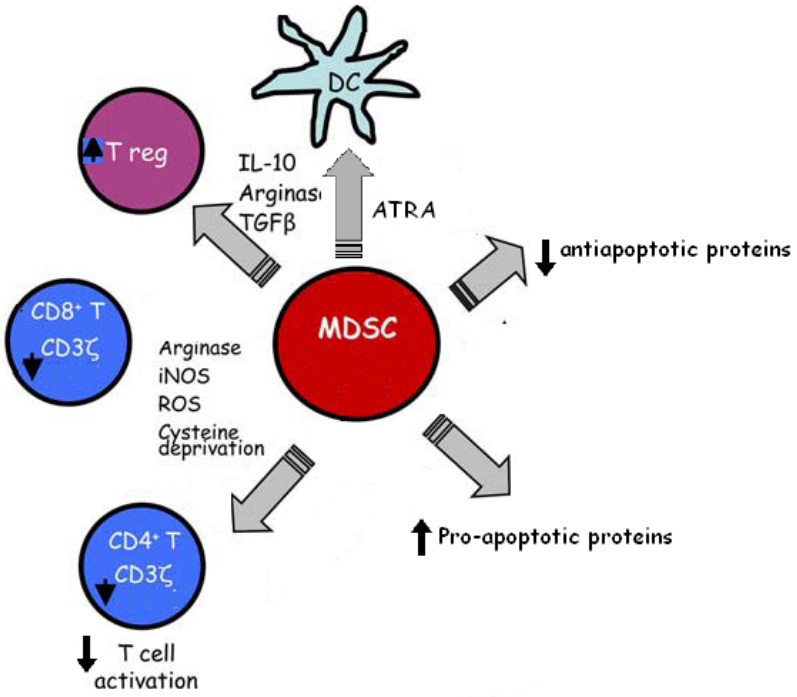
4.2.5 SADBE, MDSC and apoptosis resistance of AA effector cells

As described above autoimmune T cells frequently escape apoptosis i.e. are apoptosis resistant. Proliferation stimulating signaling cascades like the Akt pathway has been shown to inhibit apoptosis by phosphorylating Bad. AA is characterized by high level Akt and Bad phosphorylation and also shows high expression of anti-apoptotic proteins Bcl_2 and BclXl.

The phosphorylation of Akt and Bad is downregulated when AA mice were treated with SADBE and the same effect was observed in co-cultures with AA/DTH–MDSC, both in the presence of nominal antigen i.e. AA skin lysate and in presence of PMA/Ionomycin which circumvents the TCR, but there was no change in the expression of Bcl_2 and expression of BclXl even increased in the presence of PMA/Ionomycin. High apoptosis resistance of AA LNC may be supported by high expression of anti-apoptotic protein expression and this, to some extent is negatively affected by SADBE and MDSC. But counteregulation is not very effective and seemingly does not effect Bcl_2 and BclXl expression. A possible explanation for this could be the high level TNFα secreation by MDSC and upregulated TNFR expression in AA and AA/DTH LNC initiating JNK and NFkB activation (277), where the latter promotes, besides others, transcription of BclXl (277). On the other hand, TNFR engagement can transduce both survival and apoptotic signals (278), where the elements accounting for the dominance of one or the other are not yet completely unraveled (274). Although there seems to be a balance between survival and apoptotic signals also in SADBE treated AA mice, I interpret the findings as indicating a slight dominance of pro-apoptotic signaling as most elements of the mitochondrial pro-apoptotic pathway have been strongly upregulated. SADBE as well by AA/DTH-MDSC promoted Bid expression (279), which activates Bak and Bax that translocates to mitochondrial membrane (280). Bax has been shown to have pore-forming capabilities. Following a conformational change, they could form channels or even holes in the outer mitochondrial membrane (281) and results in the release of

cytochrome c from the mitochondrial intermembrane space. Mitochondrial membrane permebilization also results in release of Smac/Diablo (282) a mitochondrial factor that can facilitate caspase activation through neutralizing endogenous inhibitors of caspases, the inhibitor of apoptosis proteins (IAPs). Cytochrome c is considered to be the primary mitochondrial factor in caspase-mediated apoptosis. Together with Apaf-1 and procaspase-9, cytochrome c forms the apoptosome, which is a potent activator of caspase-3. Consecutively, the executioner caspase-3 is recruited to the apoptosome, where it is activated by the resident caspase-9. Caspase-3 then cleaves key substrates in the cell to produce many of the cellular and biochemical events of apoptosis.
Taken together, I interpret my findings in the sense that the dominance of SADBE and AA/DTH-MDSC initiated pro-apototic signals could well rely on the provision of TNFα and on upregulated TNFR-expression.

To summarize, the curative effect of persisting contact eczema predominantly relies on induction and recruitment of highly efficient M-MDSC characterized by upregulated TNFα expression. MDSC derived from AA/DTH contributes to ξ-chain downregulation, but exert additional immunosuppressive features that could proceed *via* T_{reg}, upregulated CD152 or activation of an inhibitor upstream of Lck. Besides inhibiting T cell activation, AA/DTH-MDSC also break autoimmune T cell apoptosis resistance. Downregulation of anti-apoptotic molecules supports increased apoptosis susceptibility; the major contribution relies on the activation of the mitochondrial apoptosis pathway, where TNFRI might provide the initial trigger (Scheme 15). In view of the strong effects on autoimmune T cell activation and survival, DTH-MDSC should be considered a potent therapeutics in organ-related autoimmunediseases.



Scheme15. MDSC: Mode of action: T cell activation is suppressed by the production of arginase and ROS, the nitration of the TCR, cysteine deprivation, and the induction of Tregs. MDSC also act by downregulation of anti-apoptotic proteins and upregulation of pro-apoptotic protein.

5: Summary

Alopecia Areata (AA) is an organ related autoimmune disease affecting anagen stage hair follicles and is characterized by a peri- and intrafollicular infiltrate of $CD4^+$ and $CD8^+$ T cells. The most effective way of treating AA is the application of contact sensitizers like diphencyprone or squaric acid dibutyl ester (SADBE). This topical application is refreshed for several months so that a mild form of a chronic eczema is persistently maintained. The molecular mechanisms underlying therapeutic efficacy of DTH in AA are not known, but evidences suggest a strong expansion of myeloid derived suppressor cells (MDSC) that hamper T cell proliferation and activation. Alternatively, a blockade of accessory molecules in T cell activation could possibly be of therapeutic benefit. CD44 and CD49d being candidate accessory molecules.

In T cells, CD44 became upregulated during activation. Upregulated expression promotes leukocyte migration and proliferation. It has been suggested that these activities are jointly performed by CD44 and CD49d, which come into proximity during T cell activation. To define the underlying mechanism the CD49d-negative EL4 lymphoma line was transfected with CD49d or point mutated CD49d, which prohibits phosphorylation and FAK binding. In addition, a CD44-negative Jurkat leukaemia line was transfected with murine CD44, point mutated CD44 in the ezrin binding site and with a cytoplasmic tail truncated CD44. Untransfected and transfected EL4 and Jurkat cells and for comparison native and activated leukocytes from alopecia areata mice were evaluated for migratory and proliferative activity. Migration of leukocytes, EL4-CD49d and Jurakt-CD44 cells was more and equally inhibited by anti-CD44 and anti-CD49d, migration of EL4-$CD49d^{mut}$ and Jurkat-$CD44^{mut}$, Jurkat-$CD44^{trunc}$ cells was less affected and only inhibited by anti-CD44 and anti-CD49d respectively. Similar phenomena were observed with respect to proliferation, clarifying molecular pathway(s) are still being in progress

It can be suggested from these findings that CD44 and CD49d works in cooperation to promote T cell migration, activation and proliferation. The blockade of these bystander pathways could be a therapeutic approach in autoimmune disease.

Another important aspect underlying the curative effect of SADBE treatment in AA relies on the expansion and activation of myeloid derived suppressor cells (MDSC). A therapeutic effect of the latter was abolished by all *trans* retinoic acid which drives MDSC into differentiation. This finding suggested a central role of MDSC in the

SADBE therapy of AA. To prove whether the curative effect of SADBE treatment, indeed, relies on MDSC induction, the effect of *in vivo* SADBE treatment was compared with the effect of *in vitro* co-cultures of AA lymph node cells with MDSC derived from SADBE treated AA mice. SADBE as well as MDSC strongly interfered with AA LNC proliferation accompanied by weak down regulation of ξ chain, and strongly impaired activation of Lck and Zap 70, and less pronounced the c-jun and MAPK pathway. The strongest effect was seen in presence of AA skin lysate used as surrogate autoantigen. Proliferation was also impaired in the presence of PMA plus Ionomycin indicating that SADBE / MDSC act at least partly independent of the TCR complex. In fact SADBE /MDSC promoted activation of several proapoptotic molecules engaged in the mitochondrial apoptotic pathway and interfered with the anti-apoptotic PI3K/Akt pathway. The latter effects strongly correlated with TNFα secretion by MDSC and TNFRI expression in AA/DTH lymphocytes.

Taken together, SADBE treatment results in the expansion of MDSC which impair T cell activation and contribute to breaking autoimmune T cell apoptosis resistance via promoting activation of pro apoptotic proteins. These activities qualify DTH-MDSC as a promising therapeutics in organ-related autoimmune diseases.

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Publications

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2. **Vibhuti Singh,** Pia Freyschmidt-Paul and Margot Zöller. Distinct impact of CD44 and CD49d on T cells and leukemic T cell migration and apoptosis resistance (in preparation).

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Abstracts

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