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*Wenn man zuversichtlich seinen Träumen folgt,
wird man unerwartet von Erfolg gekrönt.
(Henry David Thoreau)*

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ZUSAMMENFASSUNG

Mesenchymale Stamm- bzw. Stroma Zellen (MSZ) konnten bislang in einer Vielzahl fötaler und adulter Gewebe nachgewiesen werden, wobei MSZ aus dem Knochenmark und dem Nabelschnurblut besonders gut charakterisiert sind. Sie verfügen über eine enorme Plastizität indem sie sich in verschiedene gewebespezifische Zellen differenzieren können und darüber hinaus immunregulatorische Eigenschaften besitzen. Ob sich MSZ tatsächlich in der Haut befinden und welche Rolle sie vor Ort spielen, ist nach wie vor ein umstrittenes Thema. Da die Haut aufgrund ihrer einfachen Zugänglichkeit und des hohen Regenerationsvermögens eine vielversprechende Alternative im Bereich der autologen Geweberekonstruktion und für Einsätze bei zellbasierten Therapien darstellen könnte, sind detaillierte Untersuchungen notwendig um dermale MSZ näher zu charakterisieren. Mithilfe diverser zellbiologischer und molekularbiologischer Methoden ist es uns kürzlich gelungen, unterschiedliche Zellpopulationen in der humanen Dermis zu identifizieren und zu isolieren, deren Oberflächenmarkerprofil und das mesenchymale Differenzierungspotential den MSZ aus dem Knochenmark nahezu ident sind.

Das Ziel dieser Arbeit war es zu untersuchen ob adhärente dermale MSZ auch immunmodulatorische Fähigkeiten aufweisen. Unsere *in vitro* Daten haben gezeigt, dass MSZ Populationen aus der Dermis tatsächlich in der Lage waren, eine mit Kontrollzellen (MSZ aus dem Knochenmark und der Nabelschnur) vergleichbare Inhibition der Mitogen- oder Alloantigen-stimulierten T Zellproliferation zu erwirken. Insbesondere adhärente CD271⁺ dermale MSZ wiesen im Vergleich zur Gesamtfraktion adhärenter dermaler Zellen einen starken inhibitorischen Effekt auf. Um zu klären ob lösliche Faktoren für den suppressiven Effekt auf T Zellen verantwortlich sind, wurden dermale MSZ mit T Zellen, Mitogen und dem Prostaglandin E2 Inhibitor Indomethacin kultiviert. Damit wurde der inhibitorische Effekt dermaler Zellen auf die T Zellproliferation tatsächlich partiell aufgehoben, was den Schluss zulässt, dass Prostaglandin E2 an dem suppressiven Effekt beteiligt ist.

Zusammenfassend konnten wir zeigen, dass MSZ aus der Haut ein mit MSZ aus dem Knochenmark und aus der Nabelschnur vergleichbares immunregulatorisches Potential aufweisen. Diese Daten lassen vermuten, dass dermale MSZ eine wichtige regulatorische Funktion in der Haut auszuüben in der Lage sind.

ABSTRACT

Mesenchymal stem or stromal cells (MSC) mainly reside in the bone marrow but can be found in minute numbers also in a variety of fetal and adult tissues. They possess an immense plasticity by differentiating into multiple cells of the mesodermal lineage and have potent immunosuppressive effects on immune cell activation and proliferation. At the moment it is still a matter of debate whether MSC are also present in human skin and if so, which role they are playing. Due to the relatively easy accessibility and the high regeneration capability of the skin, autologous MSC could represent a promising alternative in the field of tissue reconstruction and for applications in cell-based therapies. Therefore, detailed analyses about dermal MSC are desired. Using different cellular and molecular methods we found that the human dermis contains diverse cell populations expressing mesenchymal stem cell markers. Comparative analysis of plastic-adherent dermal cells and bone marrow-derived cells displayed notable similarities concerning their marker profile and their differentiation capacity.

In this study we aimed to investigate whether plastic-adherent dermal MSC also exhibit immunosuppressive functions. Our *in vitro* results indicated that the proliferation of mitogen- or alloantigen-stimulated T cells was indeed inhibited by dermal MSC and was comparable to our positive controls (MSC from bone marrow and umbilical cord). In particular, adherent CD271⁺ dermal MSC showed a high suppressive effect compared to total plastic-adherent dermal cells. To unravel whether soluble factors are responsible for the immunosuppressive effect, dermal MSC were cultured with T cells, mitogen and the prostaglandin E2 inhibitor indomethacin. Indeed, we found that the T cell proliferation could be partially abolished suggesting that besides multiple other mechanisms, prostaglandin E2 is involved in the suppressive effect of dermal MSC.

In summary, we showed that dermal MSC possess immunomodulatory capacities similar to that described for bone marrow as well as umbilical cord MSC and, thus, may play important regulatory functions in the skin.

TABLE OF CONTENTS

Danksagung	5
Zusammenfassung	7
Abstract	9
Table of contents	11
1 Abbreviations	13
2 Introduction	15
2.1 Stem cells (SC).....	15
2.1.1 Hematopoietic stem cells (HSC)	15
2.1.2 MSC.....	15
2.1.3 Immunological features of MSC.....	16
2.1.3.1 Various cell types of the immune system can be affected by MSC	17
2.1.3.2 MSC produce numerous soluble factors.....	18
2.2 UC(B)-MSC and UC-endothelial colony forming cells (ECFC)	21
2.3 Clinical applications of MSC.....	23
2.4 Skin	24
2.4.1 Functions of the skin.....	24
2.4.2 Structure of the skin.....	24
2.4.3 Epidermis.....	25
2.4.4 Dermis	26
2.4.5 Hypodermis.....	27
2.4.6 Progenitor cells and MSC in the skin.....	27
3 Objective of the thesis	29
4 Materials & Methods	31
4.1 Isolation of human T cells.....	31
4.2 Human skin preparation and cell isolation	32
4.2.1 Isolation of CD271 ⁺ dermal cells.....	32
4.3 Cultivation of MSC and ECFC.....	33
4.4 Evaluation of optimal mitogen concentrations for T cell proliferation.....	33
4.5 Proliferation assays.....	35
4.6 Flow cytometry	35
5 Results and Discussion.....	37
5.1 Plastic-adherent dermal cells suppress T cell Proliferation in response to allogeneic stimuli.....	37
5.2 BM-MSC suppress T cell proliferation in response to mitogenic stimuli.....	37
5.3 UC-MSC but not UC-ECFC suppress T cell proliferation in response to mitogenic stimuli	39
5.4 Plastic-adherent dermal cells suppress T cell proliferation in response to mitogenic stimuli	45
6 Reference List	51
7 Appendix.....	61
7.1 Apparatuses and instruments	61
7.2 Plastic material	61
7.3 Reagents.....	62
7.4 Media, buffers, cell isolation kit	62
8 Curriculum vitae	64

1 ABBREVIATIONS

³ H-TdR	³ H-thymidine
7-AAD	7-aminoactinomycin D
Ab	Antibody(ies)
APC	Antigen-presenting cell(s)
APC	Allophycocyanin
ASC	Adult stem cell(s)
Bq	Becquerel
BM	Bone marrow
CCR	Chemokine receptor
CD	Cluster of differentiation
Ci	Curie
cpm	Counts per minute
CPT	Cell preparation tube(s)
DC	Dendritic cell(s)
DMSO	Dimethylsulfoxide
ECFC	Endothelial colony forming cell(s)
EDTA	Ethylenediaminetetraacetic acid
EGM	Endothelial cell growth medium
ESC	Embryonic stem cell(s)
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GvHD	Graft versus host disease
Gy	Gray
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HGF	Hepatic growth factor
HLA	Human leukocyte antigen
HO	Heme oxygenase
HSC	Hematopoietic stem cell(s)
IFN	Interferon(s)
Ig	Immunoglobulin(s)
Indo	Indomethacin
IDO	Indolamine-2, 3-dioxygenase
IL	Interleukin(s)
iNOS	Inducible nitric oxide synthases
mAb	Monoclonal antibody(ies)
MACS	Magnetic-activating cell sorting
MEM	Maintenance medium
MHC	Major histocompatibility complex
min	Minute(s)
ml	Millilitre
MLR	Mixed leukocyte reaction

mm	Millimetre
MSC	Mesenchymal stem cell(s)
NCM	Normal culture medium
NK	Natural killer
NO	Nitric oxide
NOS	NO synthases
PBMC	Peripheral blood mononuclear cell(s)
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PGE	Prostaglandin E
PHA	Phytohaemagglutinin
RPMI	Roswell Park Memorial Institute 1640
SC	Stem cell(s)
SD	Standard deviation
SSEA-4	Stage-specific embryonic antigen-4
TGF	Transforming growth factor
T _h	T helper
TLR	Toll-like receptor(s)
TNF	Tumor necrosis factor
T _{reg}	Regulatory T
UC	Umbilical cord
UCB	Umbilical cord blood
VEGFR	Vascular endothelial growth factor receptor

2 INTRODUCTION

2.1 STEM CELLS (SC)

SC are pluripotent and can be subdivided into embryonic stem cells (ESC) and adult stem cells (ASC)¹. ESC originate from the inner cell mass of the embryo, while ASC are present in multiple tissues. Mesenchymal stem cells also named mesenchymal stromal cells (MSC) are one major subgroup of ASC^{1,2}.

2.1.1 Hematopoietic stem cells (HSC)

HSC, the best described somatic SC so far³, have the ability to self-renew and give rise to all blood cells, including cells of the immune system in the bone marrow (BM)^{4,5}. HSC are responsible for the balance of homeostasis and immune response⁶. Besides hematopoiesis, a stable pool of functional HSC must be continuously preserved. To fulfil these tasks, quiescent and active HSC are co-existing in specific microenvironments or niches⁷ that supply them with regulatory signals necessary for their proliferation, conservation and differentiation⁵. In 1978, Schofield⁸ described the existence of segregated zones which he referred to as the endosteal and the vascular niche⁶. The endosteal niche consists of osteoblasts that are responsible for deposition, synthesis and mineralization of extracellular matrix of bone, thus, fringing the bone surface⁹. Moreover, the proliferation of primitive CD34⁺ hematopoietic progenitor cells is supported by osteoblasts *in vitro*, suggesting that hematopoiesis is promoted by osteoblasts¹⁰. Only a few resting HSC are resident in the endosteal niche^{7,9}. In contrast, many activated HSC are associated with sinusoidal endothelium, supposing that endothelial cells generate a niche for HSC (= vascular niche)^{7,9,11}.

2.1.2 MSC

Friedenstein and his colleagues identified MSC as spindle-shaped cells originating from BM which developed colonies of fibroblasts, named colony-forming unit fibroblasts (CFU-F) and were plastic-adherent¹². Today, MSC can be expanded *in vitro* while preserving their differentiation potential¹³. They are characterized as a heterogeneous group of multipotent, mostly non-immunogenic, clonogenic, non-hematopoietic progenitor cells¹³⁻¹⁷ and can be obtained from multiple fetal and adult tissues [e.g. fetal lung and liver, amniotic fluid, umbilical cord (UC) and umbilical cord blood (UCB), placenta, dental pulp, adipose tissue, kidney, skeletal muscle, blood, menstrual blood, BM, skin and others^{2,13,15,18-21}]. MSC are capable of differentiating into cells of mesenchymal lineages including osteoblasts, adipocytes, chondrocytes and cells of the visceral mesoderm but also into non-mesodermal cell types such as neuronal cells, tenocytes, hepatocytes, skeletal myocytes *in vitro* and *in*

vivo^{2,13}. The multilineage and proliferative capacity of MSC changes depending on the source and donor^{19,22}.

Due to the fact that no antibody (Ab) is available allowing to uniformly characterize the heterogeneous population of MSC with regard to morphology and surface marker expression, the International Society for Cellular Therapy decided that human MSC under standard culture conditions must fulfil at least 3 criteria: (i) ability to evolve into adipocytes, osteoblasts and chondrocytes, (ii) adherence to plastic, (iii) presence of the markers CD73 (Src homology 3/4; SH3/4), CD90 (Thy-1) and CD105 (SH2), as well as absence of hematopoietic markers like CD34, CD45, CD11b or CD14, CD19 or CD79a and human leukocyte antigen (HLA) class II^{23,24}. Treatment with interferon- γ (IFN- γ) induces HLA class II expression on MSC and is associated with production of indolamine-2, 3-dioxygenase (IDO)^{13,19}. Other markers that are commonly admitted include stromal precursor antigen-1 (Stro-1), CD44, CD71, CD271 [low affinity nerve growth factor receptor (LNGFR)], stage-specific embryonic antigen-4 (SSEA-4), CD29, CD106 [vascular cell adhesion molecule (VCAM-1)], CD54 [intercellular adhesion molecule-1 (ICAM-1)] and CD166 [activated leukocyte cell adhesion molecule (ALCAM)]²⁴⁻²⁶. In the past, CD271 was used for the isolation of neural crest stem cells from peripheral nerves²⁷. Nowadays, CD271 is the most specific marker for BM-MSC and is suitable for the isolation of highly purified MSC subsets from the BM^{28,29}. CD271⁺ cells from the BM are also positive for the well-acknowledged MSC markers CD73 and CD105²⁸.

Furthermore, MSC express low levels of HLA class I but are negative for the costimulatory molecules CD80, CD86 or CD40, the endothelial cell marker CD31 [endothelial cell adhesion molecule-1 (PECAM-1)], CD56 [neural cell adhesion molecule-1 (NCAM-1)] and CD18 [leukocyte function-associated antigen-1 (LFA-1)]^{24,30}. Several chemokines, cytokines and growth factors are secreted by MSC enabling their expansion, migration, immunomodulatory capacity, coordination of cell death and angiogenesis and the assistance of engraftment and differentiation of HSC³¹. MSC are also positive for the expression of Toll-like receptors (TLR) 2, 3, 4, 7 and 9 and consequently the immunomodulatory activities of MSC can be influenced³². The activation of MSC through TLR ligands ensures alterations in differentiation, survival, proliferation, immunosuppressive potential and migration.

2.1.3 Immunological features of MSC

Even though numerous studies about the immunomodulatory characteristics of MSC have been reported, the exact mechanisms are not yet completely understood^{31,33,34}. A multitude of *in vitro* studies indicate that MSC possess immunosuppressive potential through interactions with cells of the adaptive and innate immune system³⁵. It has been widely demonstrated that MSC can inhibit T cell proliferation in response to allogeneic stimuli in a

mixed leukocyte reaction (MLR)³⁶ as well as by using a mitogenic system³⁷. Human MSC initiate an anti-inflammatory or tolerant milieu by changing at least in part the cytokine secretion pattern of natural killer (NK) cells, neutrophils, B cells, dendritic cells (DC) as well as naive and effector T cells^{17,38}.

2.1.3.1 Various cell types of the immune system can be affected by MSC

In humans, MSC are capable of inhibiting CD4⁺ and CD8⁺ T cell proliferation activated via mitogens, alloantigens, autologous antigens or anti-CD3 and anti-CD28 Ab *in vitro*, pointing out that there is no limitation by major histocompatibility complex (MHC)^{13,17}. The interaction between MSC and T cells is associated with a decrease of IFN- γ , interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α) and an increase of IL-4 and IL-10, resulting in an anti-inflammatory T helper 2 (T_h2) phenotype^{1,17}. The cytotoxicity of CD8⁺ T cells is also inhibited in the presence of MSC¹⁷. Additionally, MSC facilitate, both *in vitro* and *in vivo*, the accumulation of CD4⁺CD25⁺ or CD8⁺ regulatory T (T_{reg}) cells³⁹. Furthermore, NK cell proliferation stimulated by IL-2 or IL-15 is inhibited by MSC co-cultivation resulting in a decrease of IFN- γ secretion and cytolysis towards cells lacking HLA class I molecules¹⁷. This effect is passed on via both cell-to-cell interaction and production of soluble factors such as transforming growth factor-beta (TGF- β) and prostaglandin E₂ (PGE₂) by MSC¹⁹. IL-2-driven NK cells kill MSC induced by bonding of CD18 (NK cells) and CD54 (MSC)⁴⁰. The MSC/NK interplay is initiated by the natural cytotoxicity receptor NKp30. Killing of MSC is mediated upon binding of the receptor NKG2D and its ligands MHC class I-related stress-inducible molecule A and UL-16 binding protein 3 on MSC (**Fig. 1**).

Raffaghello and coworkers determined that *in vitro* the programmed cell death of IL-8-stimulated and resting neutrophils is prevented by MSC via an IL-6-mediated pathway combined with a decrease of reactive oxygen species. Production of adhesion molecules, phagocytosis and chemotaxis of neutrophils are not impaired by MSC⁴¹.

Another immune cell type affected by the presence of MSC is the DC^{13,17,19}. MSC block the generation and maturation of CD34⁺ progenitors and monocytes into DC through down-regulation of HLA class II, CD80 and CD86 *in vitro*^{13,17}. Additionally, both the accumulation of IL-12, IFN- γ , TNF- α and the ability to act as antigen-presenting cell (APC) is reduced, in turn, the secretion of IL-10 is stimulated^{17,19}. The production of IL-6 or PGE₂ is leading in part to the immunomodulatory effect¹⁷. MSC change DC into anti-inflammatory cells resulting in a diminished T cell response (**Fig. 1**)¹⁷.

B cells are also influenced by MSC¹⁶. MSC prevent B cell proliferation stimulated with cytokines such as IL-2 and IL-4, soluble CD40 Ligand and anti-immunoglobulin Ab. The generation of immunoglobulin (Ig) A, IgM and IgG can be considerably derogated due to suppression of B cell differentiation by MSC. Furthermore, the production of B cell

chemokine receptors CXCR4, CXCR5 and CCR7 are altered in the presence of MSC. Accordingly, the chemotactic features of B cells to their ligands CXCL12 and CXCL13 are diminished. Co-cultivation with MSC exhibit no influence on B cell accumulation of costimulatory molecules (CD86, CD40) and cytokine secretion (IFN- γ , IL-4, IL-10, TNF). A most recently published study provide evidence that MSC membrane vesicles are involved in the immunosuppressive actions on B cells (**Fig. 1**)⁴².

The MSC-mediated immunosuppression leads to a stop of cell division in the G₀/G₁ phase of the cell cycle, accompanied by an increase of p27 and a suppression of cyclin D2 output⁴³. No apoptosis is induced, hence, the viability is not affected¹⁶. This does not only apply to B cells, but is approved for many other immune cell types as well³⁷.

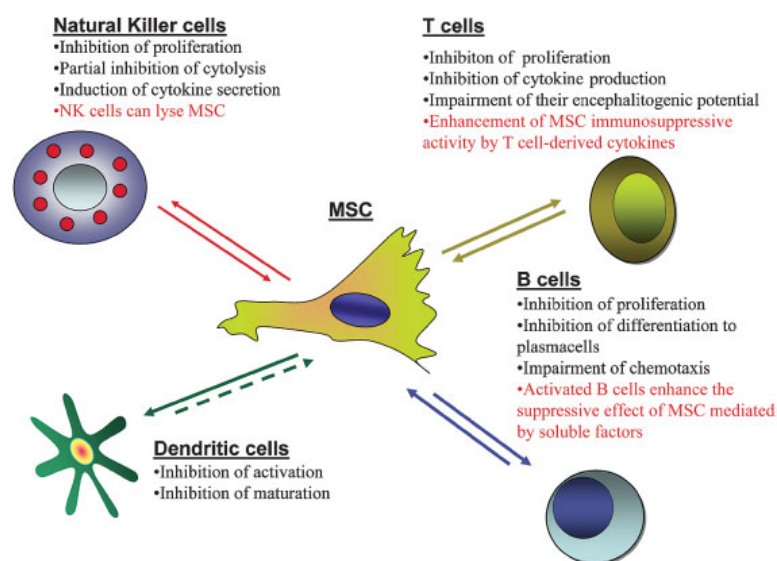


FIGURE 1. Immunoregulatory impact of MSC on multiple cell types of the immune system. Some of the consequences of MSC interactions with immune cells and vice versa are depicted⁴⁴.

2.1.3.2 MSC produce numerous soluble factors

Upon co-cultivation of immune cells and MSC, the latter become activated¹. Consequently, they produce various soluble factors that - apart from cell-cell contact – are responsible for the immunosuppressive activity of MSC. Soluble factors and cytokines which are implicated in the mechanism of immune modulation of MSC are heme oxygenase-1 (HO-1), nitric oxide (NO), TGF- β , IL-6, IL-10, HLA-G, hepatic growth factor (HGF), IDO and PGE₂^{45,46}. Several of these soluble molecules are located downstream of signalling pathways that are initiated by TLR, therefore the generation of anti-inflammatory factors in MSC which results in an immunosuppressive milieu may be provoked by TLR ligands³². Most recently, Sioud and coworkers determined that also galectins participate in the inhibitory effects of MSC^{47,48}. Investigating the secretion of immune modulators allowed a deeper insight into the pathogenesis of several diseases⁴⁹.

The enzyme HO causes the decomposition of heme to CO, biliverdin and free divalent iron⁵⁰. HO-1, the inducible isoform, has anti-apoptotic, cytoprotective, anti-oxidative, immunosuppressive and anti-inflammatory properties^{51,52}. The production of HO-1 is triggered by ultraviolet light, NO, stress factors, heat shock, heme, lipopolysaccharides or several inflammable cytokines⁴⁵. HO-1 and IL-10 are both involved in the induction of T_{reg} cells⁵³. This enzyme also prevents proliferation of NK cells and T cells as well as the generation of their cytokines⁴⁵. Furthermore, HO-1 is secreted by immature DC resulting in preventing DC differentiation via reduction of proinflammatory cytokine secretion and retaining IL-10 acquisition⁵¹.

NO synthases (NOS) generate NO, and can be subdivided into 3 groups - one of which is inducible NOS (iNOS)⁵⁴. NO play an important role in maintaining the balance of T_h1 and T_h2 cells which in turn control the production of NO⁵⁵. iNOS secretion is prevented via the T_h2 cytokines IL-10 and IL-4, whereas the T_h1 cytokine IFN- γ activates iNOS. The maturation of T_h1 cells and the secretion of IFN- γ and IL-2 is suppressed by NO, while IL-4 is stimulated probably ensuring a critical increase of T_h2 cells. The inhibition of T cell proliferation by MSC/macrophages is impaired due to suppression of STAT5 phosphorylation via NO⁵⁴. Additionally, NO suppresses TNF- α and the differentiation of DC in humans⁴⁵.

The pleiotropic growth factor TGF- β has 3 different isoforms and can control several cellular processes such as migration, cell proliferation, death, differentiation and organization^{56,57}. The IgM and IgG secretion by B cells and the differentiation of NK, B and T cells are negatively regulated by TGF- β ^{58,59}. It is crucial for the preservation of T cell homeostasis⁶⁰. The CD4⁺ T cell differentiation is prevented by TGF- β without having an effect on their cytokine pattern. TGF- β secreted by MSC³¹ is also implicated in the generation of T_{reg}¹³. The secretion of IL-1, TNF and IL-6 by monocytes is caused by TGF- β ⁶¹. TGF- β is also a key cytokine for Langerhans cell development and impairs keratinocyte growth⁶²⁻⁶⁴. Furthermore, this cytokine plays important roles for implantation, trophoblast invasion, modulation of the maternal immune system, wound healing and, in general, for development of the fetus^{65,66}.

IL-10 inhibits processes that amplify cellular immunity (IL-1 β , TNF- α) and stimulates procedures that result in the secretion of anti-inflammatory factors (IL-1RA)⁴⁹. Differentiation and growth of various cell types of the immune system like NK, B, T cells, granulocytes and APC as well as mast cells are controlled by IL-10⁶⁷. Additionally, IL-10 leads to a completion and limitation of inflammatory responses via IL-12 induction and subsequent IFN- γ secretion by T_h2 cells⁴⁹. Furthermore, DC and macrophages cannot act as APC because the expression of costimulatory and HLA class II molecules are inhibited. In contrast, upon increased expression of certain receptors, IL-10 promotes phagocytosis of macrophages and monocytes. IL-10 also facilitates the cytotoxic cell lysis of NK cells via augmented secretion

of granulocyte macrophage colony-stimulating factor, IFN- γ and TNF- α . In addition, CD4⁺ T cells can shift to T_{reg} cells in the presence of IL-10.

HLA-G belongs to HLA class Ib and shows low polymorphism⁴⁵. One of its receptors, the leukocyte Ig-like receptor (LILRB1/ILT-2) can be found on NK, T and B cells, monocytes and DC⁶⁸. Soluble HLA-G5 exerts tolerogenic properties via suppression of (i) NK cell cytotoxicity including IFN- γ production, (ii) lytic activity of CD8⁺ T cells, (iii) allogeneic T cell activation with a subsequent increased output of T_{reg} cells, and (iv) DC differentiation implicating an IL-6/STAT3 signaling pathway^{68, 69}. The HLA-G5 production in MSC is IL-10-dependent⁶⁸. Nasef and coworkers determined a positive correlation between HLA-G and leukemia inhibitory factor gene expression through MSC⁷⁰. The leukemia inhibitory factor is also involved in the induction of T_{reg} cells. Additionally, it is important in maternal tolerance of the fetus⁶⁸.

The multifunctional cytokine HGF is implicated to play a role in cell mobility and growth, angiogenesis and morphogenesis⁷¹⁻⁷³. Di Nicola and coworkers determined that the MSC-mediated inhibition of the T cell response could be fully re-established upon using neutralizing Ab against TGF- β and HGF⁷⁴. Hence, they supposed that the MSC released cytokines operate in a synergistic way. TNF- α amplifies the generation of HGF through TNF receptor II and certain signaling pathways⁷⁵.

Another promising candidate, that is supposed to be involved in the immunomodulatory mechanism of MSC, is IDO⁷⁶. This heme-containing enzyme is responsible for the reduction of L-tryptophan to kynurenine⁷⁷. IDO is permanently expressed and can be found in macrophages, DC, fibroblasts, eosinophils, endothelial/epithelial cells and the placenta. Its secretion is mainly mediated by IFN- γ during inflammatory responses but also lipopolysaccharides, TGF- β , IFN- α/β , cytotoxic T-lymphocyte antigen-4 and IL-10 are involved. Via suppression of alloreactive T cells, IDO ensures that no repulsion of the fetus occurs during gestation⁷⁸. IDO elicits an inhibition of T cell proliferation owing to depletion of tryptophan from the local surrounding area⁵³. Kynurenines cause T cell apoptosis through caspase-8 activation and secretion of cytochrome c from mitochondria. Further, the tryptophan metabolite may also lead to the induction of T_{reg} cells. T cell proliferation could be restored through the addition of 1-methyl tryptophan, a suppressor of IDO synthesis⁷⁹. Via manipulation of the IDO activity (stimulating or suppressing), IDO acts as a crucial intracellular immune modulatory enzyme for therapeutic treatments⁷⁷.

PGE₂ has been proposed to have a key role in the immunosuppressive properties of MSC⁸⁰. PGE₂ impairs apoptosis, proliferation and cytokine secretion of CD4⁺ T cells and exerts its effects through PGE₂ receptors and other intracellular targets⁸¹. Proliferation is partly prevented by inhibition of intracellular calcium output, polyamine synthesis and protein tyrosine kinase p59. Additionally, IL-2 production is diminished by PGE₂ receptors. Resting, mature and double positive T cells undergo apoptosis in the presence of PGE₂. In contrast,

PGE₂ prevents apoptosis upon reducing Fas ligand secretion on the surface of T cells. The cytokine profile of T cells is strongly influenced by PGE₂ as it augments the numbers of T_H2 and T_H17 cells due to suppression of IFN- γ , IL-12 and IL-12p70, while amplifying the secretion of IL-4, IL-5 and IL-23⁸². In addition, the activation of PGE₂ receptors 2 and 4 via induction of the signalling pathway NF- κ B causes the generation of T_{reg} cells. The proliferation of immature B cells is inhibited and cell death of these cells is promoted by PGE₂. In contrast, the proliferation of mature B cells is not influenced by PGE₂ and apoptosis is not favoured. Furthermore, PGE₂ induces Ig-class switching following enhanced generation of IgG1 and IgE. PGE₂ favours activation of DC but suppresses their maturation. In addition, PGE₂ prevents accumulation of TNF- α , IL-12R, IL-1 β and IL-12 but stimulates the generation of IL-10 (**Fig. 2**). Yañez and colleagues have demonstrated that the inhibition of PGE₂ with indomethacin (indo) ensures that the maturation of plasmacytoid DC but not myeloid DC is restored⁸³. Upon inhibition of PGE₂ by indo, proliferation of T cells could be retained however not the cytokine pattern.

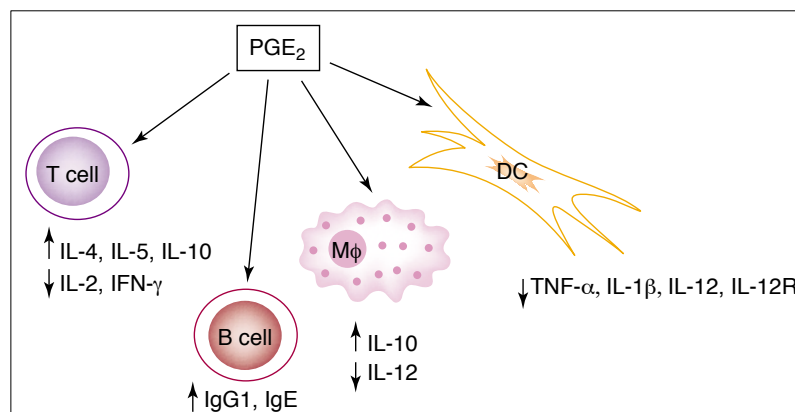


FIGURE 2. PGE₂ induces most of the immunomodulatory effects of MSC via proliferation, apoptosis and cytokine profile on immune cells⁸¹.

The exact molecular mechanism(s) of MSC-mediated immunomodulation still has to be further explored and evidence exists that also the ratio of immune cells and MSC can influence the results¹.

2.2 UC(B)-MSC AND UC-ENDOTHELIAL COLONY FORMING CELLS (ECFC)

BM is a common source of MSC and most of the information regarding MSC emerged from BM investigations⁸⁴. The clinical application of BM-MSC is restricted due to an invasive harvesting process, their low incidence in BM, their limited proliferative capability and the considerable decrease of their differentiation capacity with age^{85,86}. Thus, an increasing interest has arisen in defining alternate sources for MSC such as UC and UCB⁸⁷⁻⁸⁹. Most recently, a group of researchers described the subepithelial layer of UC tissue as a new

source for MSC, which can generate microvesicles (exosomes) and do not express STRO-1⁹⁰. Usually UC and UCB are considered as medical waste and are thrown away after childbirth^{89,91}. Therefore, the isolation procedure of these MSC is facile, non-invasive, ethically accepted, occurs without risk, is pain-free for the donor and can be found almost unlimited as compared to BM^{89,92}. Additionally, they have a faster growth rate and their expansion and differentiation capability is sustained longer *in vitro*, in contrast to adult SC^{87,89}. The immaturity of UC(B)-derived MSC is advantageous causing low rejection after transplantation, which in turn is the result of their lower immunogenicity^{89,91}. Furthermore, the immunophenotype of UC(B)-MSC resembles that of adult MSC^{87,89}. Also fetal-type MSC are capable of crossing lineage borders as they can differentiate into adipocyte, chondrocyte, osteoblast, neuronal, myocyte, hepatocyte and pancreatic lineages *in vitro*⁸⁷. Besides their immunomodulatory potential⁹³⁻⁹⁵ which is comparable to that of BM-MSC, they are also capable of providing trophic assistance to neighbouring cells⁹⁶. UC-MSC are currently applied for the treatment of immune-mediated disorders like graft versus host disease (GvHD), cancer, support of HSC expansion *ex vivo*⁸⁸ and tissue repair and reconstruction⁹⁷.

In 1997 Asahara and coworkers described for the first time the presence of circulating endothelial progenitor cells in adult human peripheral blood⁹⁸. Endothelial progenitor cells are descended from BM⁹⁹ and participate in postnatal neovascularization¹⁰⁰. Even though their incidence is rare they are normal elements of circulating blood¹⁰⁰. They migrate to injured sites (e.g. ischemia), acquire an endothelial phenotype and facilitate new blood vessel formation via paracrine excitation^{100,101}. Also a transplantation of endothelial progenitor cells into injured tissues may be helpful for the treatment of disorders combined with blood vessel diseases¹⁰¹. Several factors such as chemokines and growth factors are responsible for the recruitment of endothelial progenitor cells¹⁰¹. Additionally, they are used as biomarkers to evaluate cardiovascular disease risk¹⁰². Endothelial progenitor cells are still ill-defined, but known to be positive for CD34, CD133 and the endothelial marker protein vascular endothelial growth factor receptor 2 (VEGFR2)^{101,103}. UCB were recently discovered as a possible source to isolate endothelial progenitor cells because their frequency is higher compared with peripheral blood⁹⁹. Indeed, Ingram and colleagues described ECFC in human UCB¹⁰². ECFC are endothelial progenitor cells¹⁰⁴ with a strong clonal proliferative capacity *in vitro*, provoking functional and long-lasting blood vessels when implanted *in vivo* and displaying successive expansion in culture whereby the endothelial character and high levels of telomerase activity are preserved^{100,105}. ECFC express CD31, CD34, CD105, CD144, CD146, von Willebrand factor and VEGFR2^{105,106}. Hence, cell progeny lack expression of CD45 and the cell adhesion molecules CD62E and CD54¹⁰⁵. In culture ECFC show typical cobblestone morphology. ECFC may be significant in vascular renewal or reconstruction such as myocardial infarction, vascular damage or retinopathy¹⁰⁰.

2.3 CLINICAL APPLICATIONS OF MSC

The therapeutic capability of MSC have been investigated in many clinical trials¹⁰⁷. The promising therapeutic efficacy of MSC is based on various key characteristics: (i) multilineage potential, (ii) immunoregulatory capacity including the production of soluble factors, (iii) property to migrate to tumor and injured sites, (iv) potential for a great ex vivo expansion and (v) ability to self-renew^{107,108}. These unique biological features have allowed their use in many clinical settings (averting and care of repulsion after solid organ transplant and treatment-resistant GvHD, therapy of immune-mediated and inflammatory distresses, tissue regeneration, gene therapy, improvement of HSC engraftment and convalescence after allogeneic HSC transplantation)^{107,108}. A serious obstacle after BM and HSC transplantation is GvHD which is accompanied by high mortality and morbidity, especially in those who are refractory to corticosteroid treatment^{107,109-111}. Besides corticosteroids, intravenous infusion of BM-MSC is another possibility to medicate steroid-resistant GvHD^{84,110}. The elevated production of proinflammatory cytokines, tissue injury of the host and activation of a multitude of cells of the immune system are side effects of GvHD⁸⁴. In several studies in which MSC have been applied for the treatment of GvHD, the life expectancy of the patients has been improved, independent of the MSC origin, grade of GvHD intensity and HLA compatibility and family relations^{107,110,112}.

The immunomodulatory potential of MSC have been exploited concerning the therapy of autoimmune disorders¹¹³. Advantageous effects of MSC were reported for a few trials such as diabetes mellitus¹¹⁴, chronic pancreatitis, systemic lupus erythematosus¹¹⁵, asthma, amyotrophic lateral sclerosis¹¹⁶, multiple sclerosis¹¹⁷, rheumatoid arthritis¹¹⁸ and Crohn's disease^{107,119}. Furthermore, MSC are used for the treatment of cardiac, liver, kidney and bone disorders^{110,113}.

The skill of MSC to move to tumor sites has been successfully applied for various types of anti-cancer treatments like the transport of oncolytic viruses and anticancer therapeutics into tumors^{107,120,121}. Tumor cells attract MSC via secretion of specific molecules¹⁰⁷. Additionally, prodrug-converting enzymes such as herpes simplex virus-thymidine kinase were conveyed by MSC into tumors. The prodrug ganciclovir is then transformed into its toxic form by herpes simplex virus-thymidine kinase leading to an effective antitumor response.

Another characteristic of MSC is their capacity to migrate to injured sites and to support the reconstruction via the secretion of trophic factors leading to a decrease of inflammation and restoration of the affected area¹²². Paracrine factors are responsible for several tasks including anti-fibrosis, -apoptosis, angiogenesis, anti-bacterial effect, chemoattraction, immunomodulation, proliferation, neuroprotection and HSC support which can be fulfilled directly or indirectly^{109,123-125}. Recent trials exhibited outstanding results regarding the

treatment of wound healing by MSC, but further research will be necessary to fully understand the molecular action regarding migration and homing^{107,113,122}.

The use of MSC is ethically accepted and the risk concerning the formation of a teratoma is low in contrast to ESC and induced pluripotent SC¹²⁵. Nevertheless, they are not fully free from malignant capability¹²⁶.

Although immense progresses have been made regarding the clinical applications and the biological features including the underlying molecular mechanisms of MSC, several hurdles are still to be overcome: (i) difficulty of safety, (ii) allogeneic or autologous MSC, (iii) quality monitoring, and (iv) establishment of standardized methods¹¹⁰.

2.4 SKIN

2.4.1 Functions of the skin

The skin is the largest organ of the human body and very complex¹²⁷. It provides a first line of defence against external environmental offenses (biological, chemical, physical)¹²⁷, microbial pathogens and develops an inside out barrier in order to prevent fluid loss¹²⁸. Furthermore, the skin operates as a sense organ, thermo regulator¹²⁹ and plays an important role in the innate and adaptive immunity¹³⁰.

2.4.2 Structure of the skin

Human skin is divided into three layers: the epidermis, the dermis and the hypodermis (**Fig. 3**)¹²⁷. The epidermis emerges from the ectoderm while the dermis and the hypodermis evolve from the mesoderm. The dermal-epidermal junction combines the epidermis with the dermis. The skin is not uniformly constructed as there are several deviations like epidermal attachments, thickness (1-4 mm) and frequency of melanocytes.

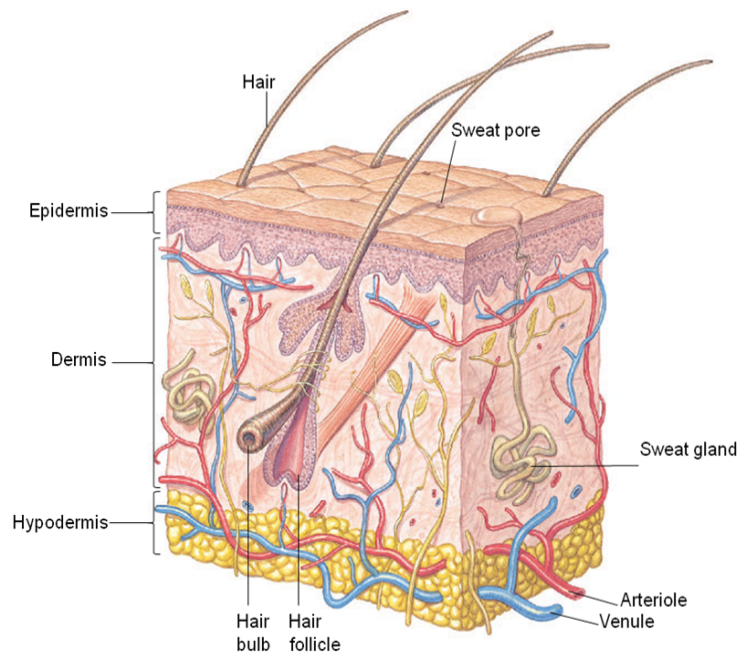


FIGURE 3. Assembly of human skin consisting of 3 compartments¹³¹.

2.4.3 Epidermis

The epidermis is an external, stratified, non-vascularized epithelium that regenerates itself constantly¹²⁷ by proliferation of resident stem cells^{132,133} that differentiate during their migration through the different epidermal layers¹³⁴. Its thickness ranges from 20-560 μm ¹³⁵ and comprises several cell types. Keratinocytes form the largest cell population (90-95%) and undergo a process called keratinization, resulting in the generation of corneocytes that shed from the skin surface in approximately 30 days¹²⁷. Other cell populations with specific functions are Langerhans cells (resident APC)¹³⁶, Merkel cells (responsible for the modulation of mechanotransduction and preservation of the 3-dimensional epidermal structure)¹³⁷, and melanocytes (involved in the production of the pigment melanin)^{127,138}. Furthermore, a small percentage (~1.3%) of lymphocytes mainly expressing a T memory/effector phenotype are present in human epidermis^{127,139}.

The epidermis comprises several cutaneous appendages including nails, hairs, sweat glands and pilosebaceous follicles^{127,140}. These epithelial structures are linked to the surface epidermis, but can be found inside the dermis and hypodermis.

Epidermal cells are organized in continuous layers (**Fig. 4**)¹²⁷ from the inside to the outside starting with the basal layer (*stratum basale* or *stratum germinativum*)¹³⁴. This single layer of cells is connected to a noncellular basement membrane, separating the epidermis from the dermis and mainly arranged by keratinocytes, some of which show stem cell-like characteristics by dividing continuously through mitosis and thereby migrating towards the skin surface^{131,134}. In addition, epidermal cell-derived Merkel cells, neural crest-derived melanocytes^{141,142} and BM-derived leukocytes are located in this layer¹²⁷. The *stratum*

spinosum (5-15 layers) comprises irregular polyhedral keratinocytes with a confined capability for cell division and Langerhans cells^{127,134}. Flattened, polyhedral, non-dividing keratinocytes with keratohyalin granules, are situated in the *stratum granulosum* (1-3 layers). The organelles and nuclei of these cells degrade and also become flattened due to the consecutive pressure towards the skin surface¹³⁵. The water content of the body is conserved by tight junctions in this layer (**Fig. 4**)¹⁴³. The *stratum corneum* (5-10 layers) is assembled of scale like, nonviable, flattened corneocytes that exert a protective function^{127,128}. In some body areas (palmoplantar region) an additional layer, the *stratum lucidum*, can be identified which is located between the stratum granulosum and the stratum corneum¹²⁷. Moreover, the superficial part of eccrine sweat glands and hair follicles are localized in the epidermis.

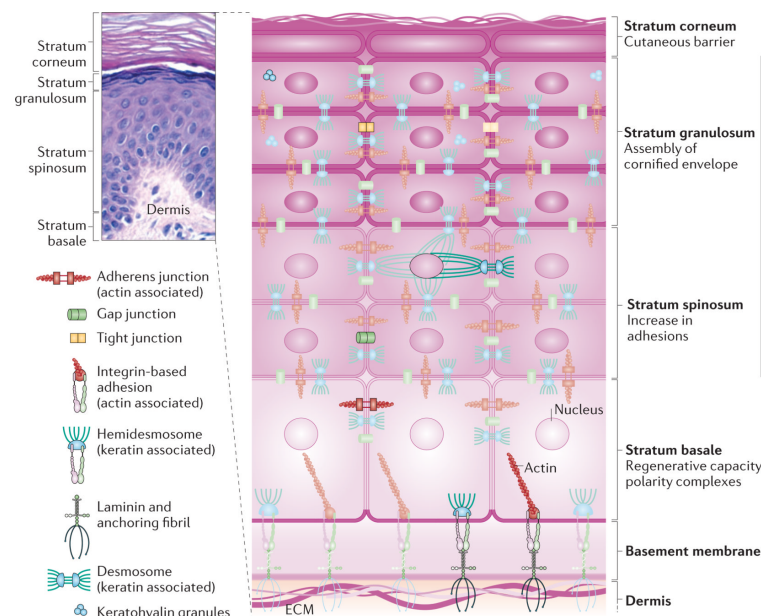


FIGURE 4. An illustration of epidermal strata¹⁴⁴.

2.4.4 Dermis

The dermis is much thicker (2-4 mm) than the epidermis and can be separated into an upper papillary (superficial) part and thereunder a reticular (deep) dermis^{127,134}. It is a supporting, compressible and flexible connective tissue that exerts protective functions with respect to the epidermis, its appendages and the vascular and nervous plexuses¹²⁷. The papillary dermis develops cone-shaped bulges (dermal papillae) that reach into the epidermis and foster further stability¹²⁷. The dermis is composed of fibres that consist of interstitial collagen to withstand mechanical stress, elastic fibres ensuring retractile features and the extrafibrillar matrix comprising glycoproteins (fibronectin, laminin, matrilin), proteoglycans (perlecan, versican), glycosaminoglycans (chondroitin sulphate, dermatan sulphate, keratin sulphate, heparin, heparin sulphate), hyaluronic acid and water^{127,134}. Furthermore, excretory and

secretory glands, lymphatic vessels, muscles, nails, hair follicles and nerves are located in the dermis^{127,134}. The main cell type of the dermis, the spindle-shaped fibroblasts, are responsible for the production of fibres and ground substance¹²⁷. Besides, it also hosts other cells of the immune system including mast cells, DC, T cells, NK cells and macrophages^{140,145}.

2.4.5 Hypodermis

The innermost layer of the skin, the hypodermis (subcutaneous fat) is a subcutaneous tissue that actually doesn't belong to the skin¹²⁷. It is responsible for connecting the dermis to the underlying bones or muscles, the control of body temperature, storage of lipids, isolation and body cushioning. Adipocytes are the major cell type of the hypodermis.

2.4.6 Progenitor cells and MSC in the skin

In 2001 skin derived-precursors¹⁴⁶ were described to be present in human skin as embryonic neural crest-related progenitor cells that develop during embryogenesis^{147,148}. They exhibit a self-renewing potential and are able to differentiate into cells of mesodermal (smooth muscle cells, adipocytes, osteocytes, chondrocytes), neural (glia, neurons) and hematopoietic origin (erythroid, myeloid lineage)¹⁴⁷⁻¹⁵⁰. Neural cell types express markers such as neurofilament-M, neuron-specific enolase, β -III-tubulin, CD271 and CD56^{148,151}. Additionally, they can differentiate into hepatocytes, insulin-producing pancreatic cells and keratinocytes¹⁵²⁻¹⁵⁴. The dermal papilla of hair follicles and whisker follicles represent a niche for skin derived-precursors^{148,155,156}. Unlike skin derived-precursors, dermal MSC are mainly isolated from hairless skin such as foreskin¹⁴⁷. Dermis-derived MSC might be located in the perivascular niche like BM-MSC or in the interfollicular dermis¹⁵⁷. Numerous studies demonstrated the presence of MSC in the dermis¹⁵⁸⁻¹⁶². The immunoregulatory capacity of human dermal MSC is functionally comparable with that of BM-MSC¹⁶³. They are capable of (i) initiating DC maturation¹⁶⁴, (ii) preventing activation of peripheral blood mononuclear cell (PBMC) and allogeneic T cells *in vitro*³⁷, (iii) affecting the cytokine profile of T cells¹⁶³, (iv) inducing cell cycle standstill in PBMC³⁷, and (v) impeding cell death. Dermal MSC express CD13, CD29, CD44, CD49, CD59, CD73, CD90, CD105, CD133, CD166, CD271, STRO-1, VCAM-1 and ICAM-1 and do not express hematopoietic and endothelial markers¹⁶⁵. Furthermore, they have the potential to differentiate into adipocytes, chondrocytes, osteoblasts, neurons, insulin-producing pancreatic cells and hepatocytes¹⁶⁶. Due to the fact that skin is a very easily accessible source, they might become a potentially effective tool in therapeutic options in particular recovery of damaged tissue¹⁶².

3 OBJECTIVE OF THE THESIS

MSC are located in various organs and tissues. Recently, we identified diverse cell populations in the human dermis, which expressed MSC markers and showed a differentiation potential comparable to that of BM-MSC. To better understand the role of MSC in the skin, the aims of this thesis were to:

- ✓ comparatively assess the immunoregulatory capacity of dermal MSC with BM-MSC, UC-MSC and UC-ECFC
- ✓ elucidate the mechanism(s) underlying the immunomodulatory activity of dermal MSC

4 MATERIALS & METHODS

4.1 ISOLATION OF HUMAN T CELLS

Buffy coats from peripheral blood of healthy volunteers were purchased from the local transfusion service (Rotes Kreuz Vienna, Austria). Cells were transferred in Vacutainer cell preparation tubes [CPT; 8 millilitre (ml)], and centrifuged [25 minutes (min); 1750 rzb]. Subsequently, the layer containing PBMC was collected with a pipette (5 ml), washed twice with phosphate-buffered saline (PBS) and counted using a hemocytometer. Trypan blue (0.4%) was used to visualize dead cells. Afterwards T cells were isolated using an appropriate isolation kit according to the instructions of the manufacturer. Furthermore, one part of the cells was stained with monoclonal (m)Ab directed against CD3, CD4, CD8 and CD45RA (**Table 1**) and analyzed for purity by fluorescence-activated cell sorting (FACS). In the common fraction around 66% of the cells expressed CD3 (**Fig. 5A**). Thereof, about 43% were positive for CD4, about 19% expressed CD8 and about 39% expressed CD45RA (**Fig. 5B**). Upon enrichment, the purity of CD3⁺ T cells was about 99% (**Fig. 5C**). Thereof, approximately 67% expressed CD4, 27% were positive for CD8, and about 65% expressed CD45RA (negative fraction; **Fig. 5D**).

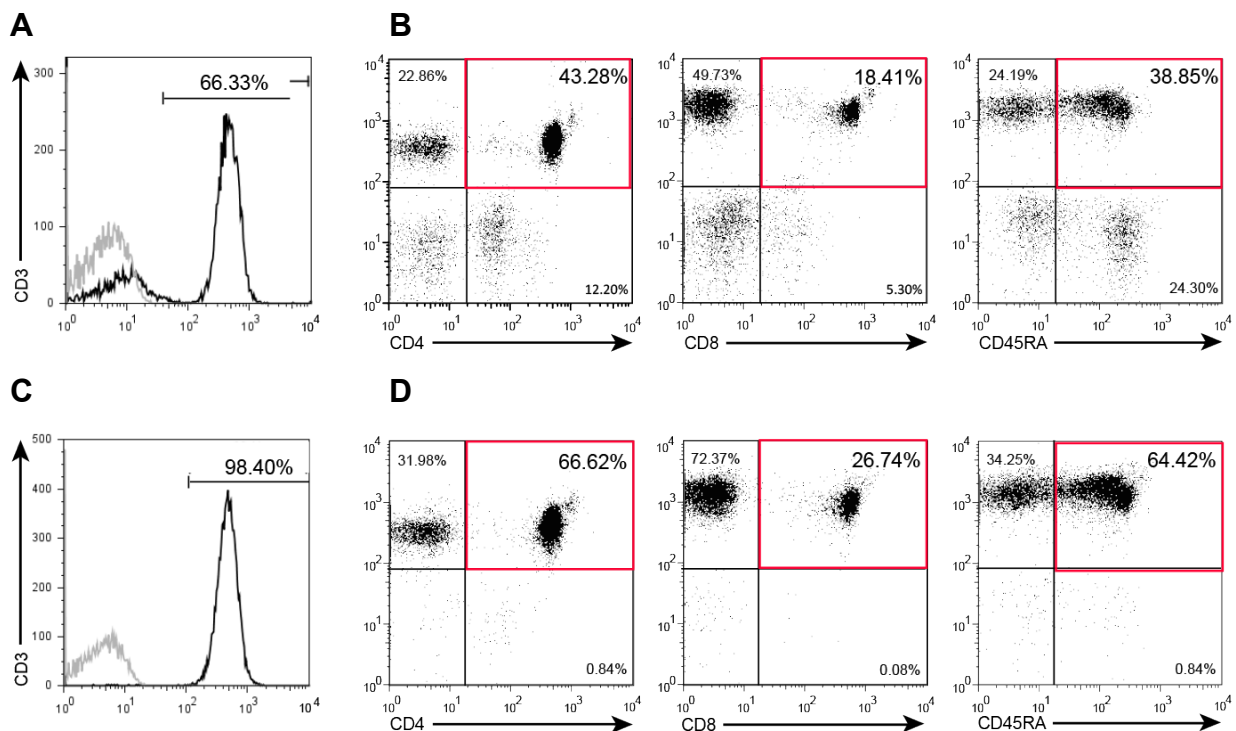


Figure 5. Purity of human T cells isolated from PBMC. Cells were stained with the indicated markers and analyzed by flow cytometry. Histogram (**A**) and dot plots (**B**) display the common PBMC fraction. Upon enrichment using an appropriate T cell isolation kit, the purity of T cells was evaluated and is demonstrated as histogram (**C**). The percentage of T cell populations within the purified negative fraction is illustrated in (**D**). Specific staining is displayed in histograms (black profile) in comparison to isotype-matched control (grey profile). Dot plots/histograms show 100,000 cells and are representative for 3 separate experiments.

4.2 HUMAN SKIN PREPARATION AND CELL ISOLATION

Non-inflamed human foreskins were obtained from routine circumcisions as discarded material after the ethical committee approval of the Medical University of Vienna. Skin samples were either immediately further processed or stored no longer than 6 hours at 4°C. Tissue samples obtained comprised whole skin including epidermis, dermis and hypodermis. For this study it was of great importance to free the dermis thoroughly from other tissue cell types, which may influence the outcome of the functional experiments. For this purpose, subcutaneous tissue was excised and remaining skin was disinfected (Betaisodona solution, 10 min, 70% ethanol, 1 min) and finally dried on sterile gauze. Subsequently, samples were cut into small stripes (2x4 mm) and incubated epidermal side up on 1.2 units/ml Dispase II in PBS overnight at 4°C. Epidermis was separated from dermis with forceps. Then the dermis was cut into small pieces and digested in a Liberase Blendzyme 3 solution (10.7 units in 15 ml PBS) in a shaking water bath (2 hours, 37°C). The dermal cell suspension was filtered through a 70 µm cell strainer and the enzymatic digestion was stopped by addition of normal culture medium (NCM; see Appendix). After several washings in NCM, cells were transferred to one culture flask [225 cm²; α -maintenance medium (α -MEM); see Appendix] and cultured until they were confluent.

4.2.1 Isolation of CD271⁺ dermal cells

Upon removal of medium, plastic-adherent dermal cells were washed with pre-warmed PBS. Afterwards, pre-warmed trypsin-ethylenediaminetetraacetic acid (EDTA; 0.05% trypsin) was applied to detach the cells from the bottom of the culture flask (5 min, 37°C). After washing with α -MEM, cells were resuspended in 1 ml α -MEM and counted using a hemocytometer. Magnetic-activating cell sorting (MACS) isolation of CD271⁺ dermal cells was performed according to manufacturer's instructions with minor adaptations of the procedure. Briefly, dermal cells were incubated with a biotinylated mAb directed against CD271 (1:60) in MACS buffer (30 min, 4°C; see Appendix). Subsequently, they were washed with MACS buffer, resuspended in 80 µl MACS buffer and incubated with 20 µl of anti-biotin microbeads (15 min, 4°C). After washing, cells were resuspended in 500 µl MACS buffer. A cell separation column was attached to a magnet, pre-washed with 500 µl MACS buffer, loaded with labelled cells and washed twice with 500 µl MACS buffer. For retrieval of bound cells, the column was removed from the magnet and flushed with 1 ml MACS buffer. Cell yield and viability were determined with trypan blue exclusion. Isolated cells were analyzed by flow cytometry as described below. To evaluate the percentage of enrichment, cells were incubated with a phycoerythrin (PE)-conjugated mAb directed against CD271 and subsequently analyzed by flow cytometry.

4.3 CULTIVATION OF MSC AND ECFC

BM-MSC, UC-MSC as well as corresponding UC-ECFC were prepared according to a published protocol¹⁶⁷ and stored in liquid nitrogen until use (kindly provided by Dr. Dirk Strunk, Department of Blood Group Serology and Transfusion Medicine, Division of Hematology and Stem Cell Transplantation, Medical University of Graz). Upon thawing, cells were seeded in culture flasks (225 cm²) and cultured in α -MEM (MSC) or endothelial cell growth medium (EGM; ECFC; see Appendix) at 37°C (5% CO₂). When 70-80% confluence was accomplished, adherent cells were detached from the bottom of the culture flask using pre-warmed PBS and trypsin-EDTA (0.05% trypsin for MSC; 0.1% trypsin for ECFC). After washing with the respective culture media, cells were resuspended in 1 ml α -MEM/EGM and counted using a hemocytometer to evaluate optimal cell numbers for further expansion. Cells were reseeded at selected cell densities in culture flasks (225 cm²) and cultivated in α -MEM/EGM at 37°C (5% CO₂).

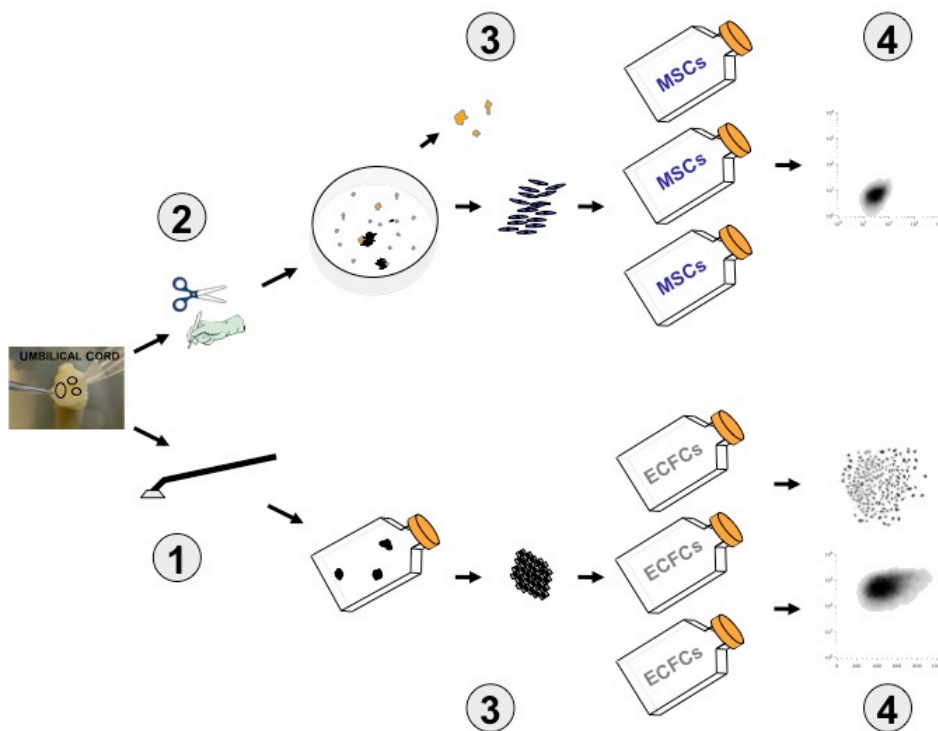


FIGURE 6. Isolation, expansion and phenotypic analysis of UC cells¹⁶⁷.

4.4 EVALUATION OF OPTIMAL MITOGEN CONCENTRATIONS FOR T CELL PROLIFERATION

To test the immunomodulatory capacity of plastic-adherent dermal cells, we have compared them with BM- and UC-MSC (positive controls). First, we had to set up a robust T cell proliferation assay. Since the mitogen phytohaemagglutinin (PHA) has already been successfully used in a similar study investigating the immunomodulatory function of lung-

resident MSC¹⁶⁸, we decided to use PHA also for our assays. To evaluate the optimal PHA concentration for T cell proliferation, human T cells (1.5×10^5 cells/well) were cocultured with selected PHA concentrations (15, 10, 7.5, 5 and 2.5 $\mu\text{g/ml}$) in 200 μl of NCM per well in a 96-well round-bottom microtiter plate for 3 and 5 days at 37°C (5% CO₂). Eighteen hours before harvest, ³H-thymidine [³H-TdR; 1 μCi (μCi)/37 kBecquerel (kBq)/well] was added and proliferation was determined with a 1450 Microbeta liquid scintillation counter. ³H-TdR incorporation is expressed as the mean of triplicates $\pm$ standard deviation (SD) in counts per minute (cpm). The purity of the isolated T cells was assessed as described. After 48 hours, T cell proliferation could be observed under the microscope in the presence but not absence of PHA (**Fig. 7A**). A PHA concentration-dependent T cell proliferation was observed on days 3 and 5 (**Fig. 7B, C**). T cell proliferation at all PHA concentrations tested was higher on day 3 as compared with day 5 (**Fig. 7B, C**). Marginal proliferation occurred in cultures without PHA (negative control) on both days (**Fig. 7B, C**).

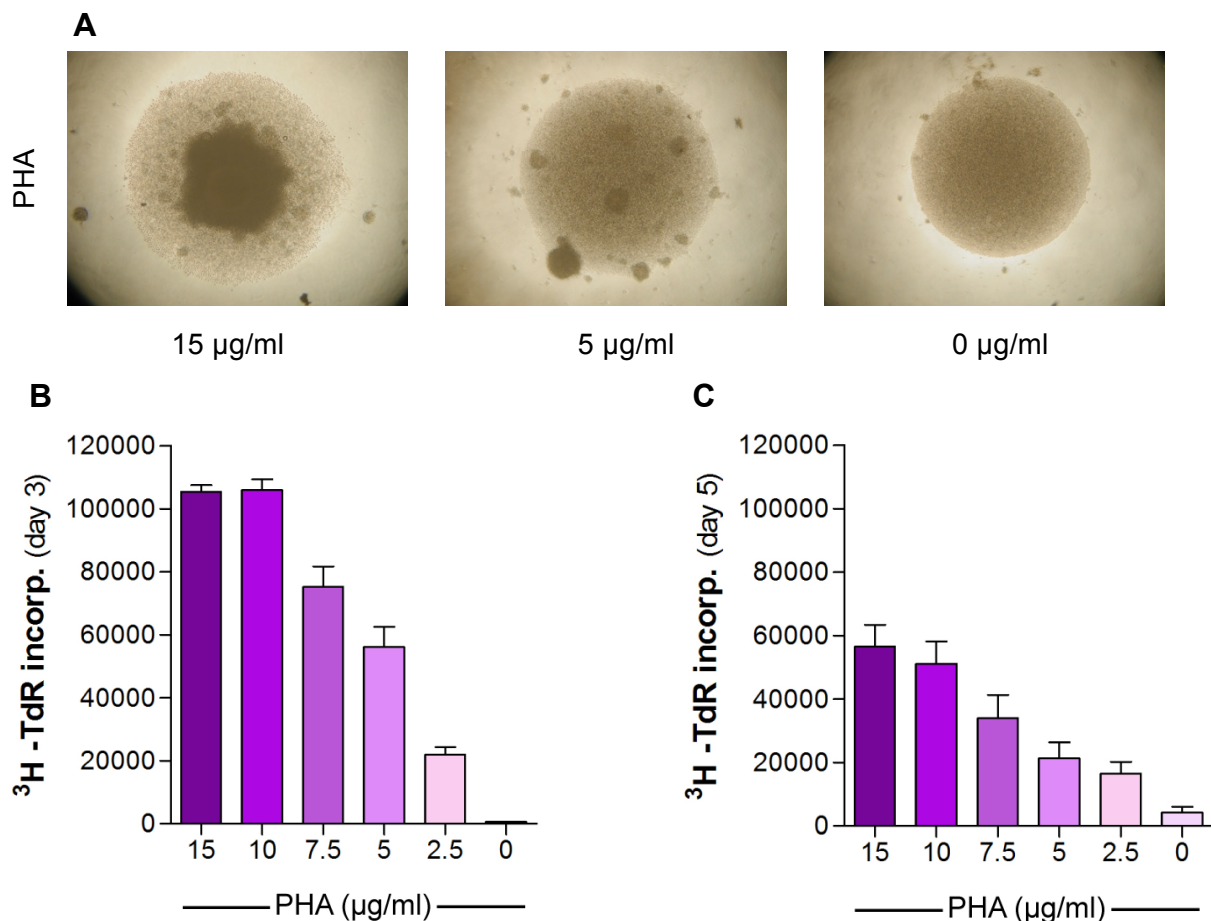


FIGURE 7. Mitogen concentration-dependent stimulation of T cells. T cells (1.5×10^5 cells/well) were incubated with the indicated concentrations of PHA and proliferation could be observed under the microscope after 48 hours (**A**) and was assessed on days 3 (**B**) and 5 (**C**) of culture by ³H-TdR incorporation. Data are expressed as mean $\pm$ SD of triplicate cultures (cpm). Results are representative for 3 independent experiments.

Based on these observations we decided to use 10 µg PHA/ml for future functional experiments.

4.5 PROLIFERATION ASSAYS

For allogeneic stimulation, human BM-MSC, UC-MSC as well as plastic-adherent dermal cells were irradiated [30 Gray (Gy)], seeded in triplicates in 96 well round-bottom microtiter plates (5×10^3 , 2.5×10^3 , 1×10^3 cells/well) and allowed to adhere to the plate for at least 4 hours before addition of irradiated (30 Gy) PBMC (1×10^5 /well) and allogeneic responder T cells (1×10^5 /well). Cells were cultured in a final volume of 200 µl NCM for 8 days at 37°C (5% CO₂). Eighteen hours before harvest, ³H-TdR (1 µCi/37 kBq/well) was added and proliferation was determined as described above.

For mitogenic stimulation, human BM-MSC, UC-MSC and corresponding UC-ECFC as well as plastic-adherent dermal cells were irradiated (30 Gy), seeded in triplicates in 96 well flat-bottom microtiter plates (5×10^3 , 2.5×10^3 , 1×10^3 cells/well) and allowed to adhere to the plate for at least 4 hours before the addition of human T cells (1.5×10^5 or 1×10^5 cells/well). PHA (10 µg/ml) was added in a final volume of 200 µl NCM and cultured for 5 days at 37°C (5% CO₂).

In certain experiments irradiated (30 Gy) human plastic-adherent dermal cells were plated in triplicates in 96 well flat-bottom microtiter plates (5×10^3 , 2.5×10^3 , 1×10^3 cells/well) and allowed to adhere to the plate for at least 4 hours before the addition of human T cells (1.5×10^5 cells/well) and 10 µg/ml PHA either in the presence or absence of the PGE₂ inhibitor indo (5 µM) or the solvent (5 µM methanol; negative control). Cells were cultured in a final volume of 200 µl NCM for 5 days at 37°C (5% CO₂) and proliferation was determined as described.

4.6 FLOW CYTOMETRY

For flow cytometric analyses, cells were resuspended in FACS buffer (see Appendix) and incubated with the respective allophycocyanin (APC)-, PE- and fluorescein isothiocyanate (FITC)-conjugated mAb. Appropriate isotype-matched control mAb were included. Dead cells were excluded by 7-aminoactinomycin D (7-AAD; 1 µg/ml) uptake. Cells were acquired using a LSRII flow cytometer and data were analyzed with FlowJo software. To evaluate cells for the expression of costimulatory/maturation markers, they were incubated with mAb directed against CD80, CD83 and CD86 (**Table 1**) as shown as an example in **Fig. 8**.

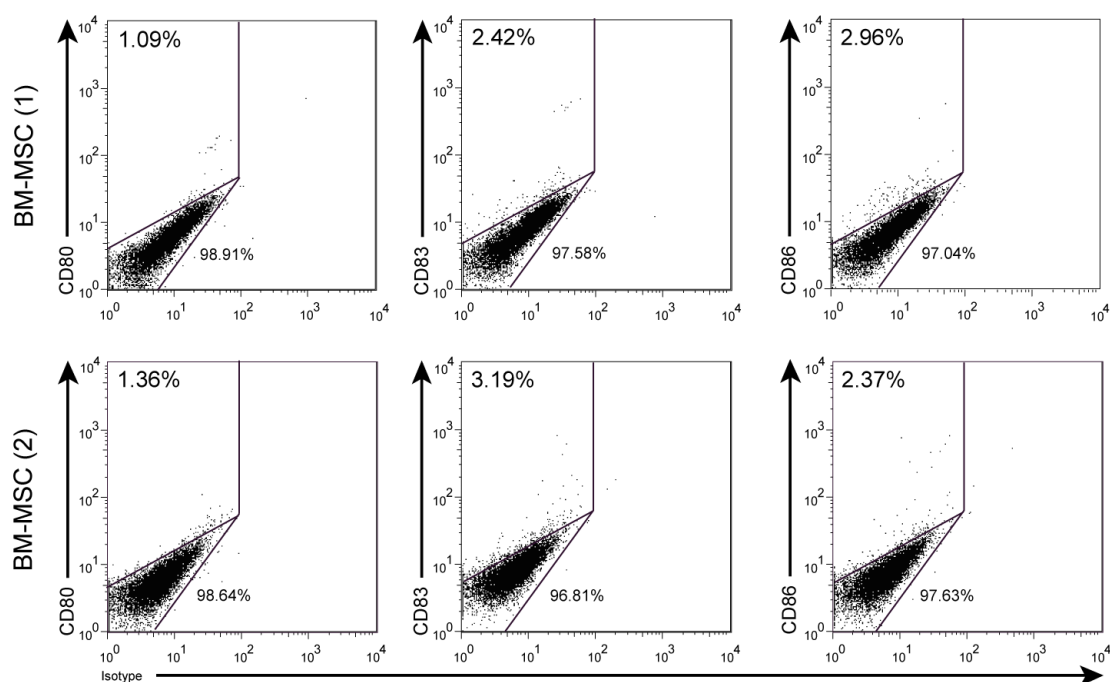


FIGURE 8. BM-MSC neither express costimulatory molecules nor maturation markers. Cultured BM-MSC (donors 1 and 2) were stained for the indicated markers. Dead cells were excluded by 7-AAD uptake. Dot plots show 100,000 cells.

Table 1. ANTIBODIES

AB SPECIFICITY	CLONE	Ig CLASS	WORKING DILUTION	SOURCE*	CONJUGATE
CD3	UCHT1	mouse IgG1	1:50	An der Grub	FITC
CD3	SK7	mouse IgG1	1:50	Becton Dickinson	APC
CD4	M-T466	mouse IgG1	1:50	Miltenyi	PE
CD8	BW 135/80	mouse IgG2a	1:50	Miltenyi	FITC
CD45RA	T6D11	mouse IgG2b	1:200	Miltenyi	FITC
CD80	L307.4	mouse IgG1, κ	1:20	BD Pharmingen	FITC
CD83	HB15e	mouse IgG1, κ	1:20	BD Pharmingen	FITC
CD86	2331 (FUN-1)	mouse IgG1, κ	1:20	BD Pharmingen	FITC
CD271 (=NGFR)	C40-1457	mouse IgG1, κ	1:50	BD Pharmingen	Biotin, PE

* An der Grub, Vienna, Austria; Becton Dickinson, San Jose, CA, USA; BD Pharmingen, San Diego, CA, US; Miltenyi, Bergisch Gladbach, Germany;

5

RESULTS AND DISCUSSION

5.1 PLASTIC-ADHERENT DERMAL CELLS SUPPRESS T CELL PROLIFERATION IN RESPONSE TO ALLOGENEIC STIMULI

To investigate whether plastic-adherent dermal cells inhibit T cell proliferation in response to allogeneic stimuli, we performed a MLR. For this purpose, irradiated PBMC were used to stimulate allogeneic T cells in the presence or absence of plastic-adherent dermal cells and the positive controls (UC-MSC, BM-MSC). Indeed, T cell proliferation was inhibited by plastic-adherent dermal cells as well as UC-MSC and BM-MSC in a cell density-dependent manner. When comparing dermal cells with UC-MSC we found that both cell types exerted their immunosuppressive effects in a cell density-dependent manner even though dermal cells had a higher immunosuppressive effect on T cell proliferation in response to allogeneic stimulation as UC-MSC (**Fig. 9A**). BM-MSC showed a similar immunosuppressive effect at the two cell densities tested (**Fig. 9B**).

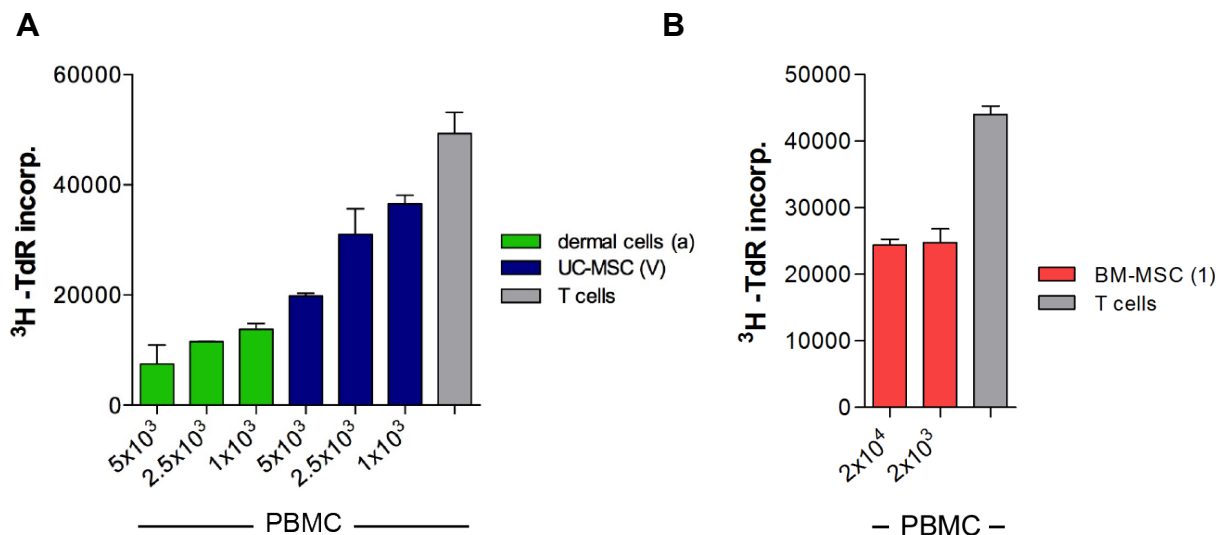


FIGURE 9. Plastic-adherent dermal cells inhibit the proliferative capacity of T cells in response to alloantigens. Irradiated (30 Gy) dermal cells, UC-MSC (**A**) and BM-MSC (**B**) were seeded at the indicated cell densities into a 96 well round-bottom microtiter plate and were allowed to adhere to the plate for at least 4 hours before addition of responder T cells (1×10^5 cells/well) in the presence or absence of irradiated (30 Gy) allogeneic PBMC (1×10^5 cells/well). Cells were cocultured in a final volume of 200 μ l for 8 days at 37°C. ³H-TdR (1 μ Ci/37 kBq/well) was added for the last 18 hours of culture. Data are displayed as mean $\pm$ SD of triplicate cultures and one representative of 3 independent experiments is shown. T cells alone, PBMC alone and wells containing T cells or PBMC and dermal cells, UC- or BM-MSC exhibited only a low proliferation (< 1000 cpm; data not shown).

5.2 BM-MSC SUPPRESS T CELL PROLIFERATION IN RESPONSE TO MITOGENIC STIMULI

To test the immunosuppressive capacity of plastic-adherent dermal cells, we had to set up a reproducible assay system. For this purpose, we decided to use a mitogenic system as described previously^{74,169}. BM-MSC (positive control) were cocultured at different cell

densities with T cells in the presence or absence of PHA. Upon examination of the cocultures under the microscope, we observed that wells containing T cells alone in the presence of PHA displayed typical T cell clusters after 24 hours. In contrast, wells containing T cells, PHA and BM-MSC lacked T cell clusters. After 36 hours, wells containing a monolayer with different cell numbers of BM-MSC in addition to T cells and PHA showed a minimal proliferative response. The size of T cell clusters decreased with increasing BM-MSC cell densities (data not shown). Upon quantitative analysis using ^3H -TdR incorporation, we confirmed that BM-MSC revealed an immunosuppressive effect in a cell density-dependent manner on the proliferation of T cells. Again, the highest immunosuppressive effect could be observed with the highest density of BM-MSC (**Fig. 10**).

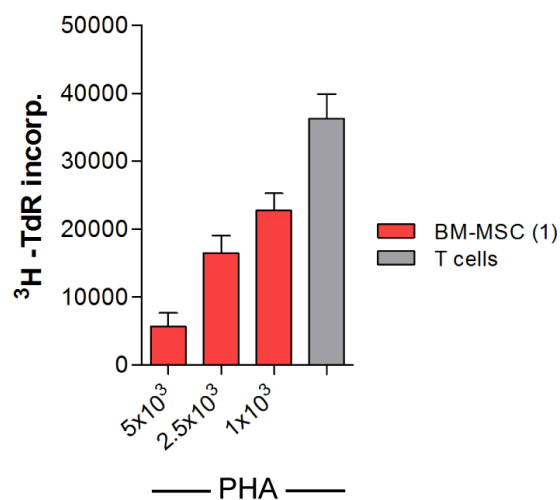


FIGURE 10. BM-MSC inhibit the mitogen-induced proliferative T cell response in a cell density-dependent manner. Irradiated (30 Gy) BM-MSC were seeded into 96 well flat-bottom microtiter plates at the indicated cell densities 4 hours before the addition of T cells (1×10^5 cells/well) in the presence or absence of PHA (10 $\mu\text{g}/\text{ml}$). Cells were cocultured in a final volume of 200 μl NCM for 5 days at 37°C . ^3H -TdR (1 $\mu\text{Ci}/37$ kBq/well) was added for the last 18 hours of culture. Data are displayed as mean $\pm$ SD of triplicate cultures and one representative of at least 6 independent experiments is shown. T cells alone, and wells containing T cells and BM-MSC without PHA exhibited only a low proliferation (< 1000 cpm; data not shown).

To investigate whether various T cell densities may alter the results, we have used two T cell densities from the same donor, and in addition, BM-MSC from two different donors which morphologically look quite similar (**Fig. 11A, B**). Comparable to data described in **Fig. 10**, we observed an immunosuppressive effect with both BM-MSC donors in a cell density-dependent manner. The T cell density did not influence their inhibitory effects, irrespective of the BM-MSC donor (**Fig. 11C, D**). On the basis of these data, we decided to use 1×10^5 T cells/well in further experiments except for experiments otherwise noted. In experiments with plastic-adherent dermal cells always 1.5×10^5 T cells/well were employed.

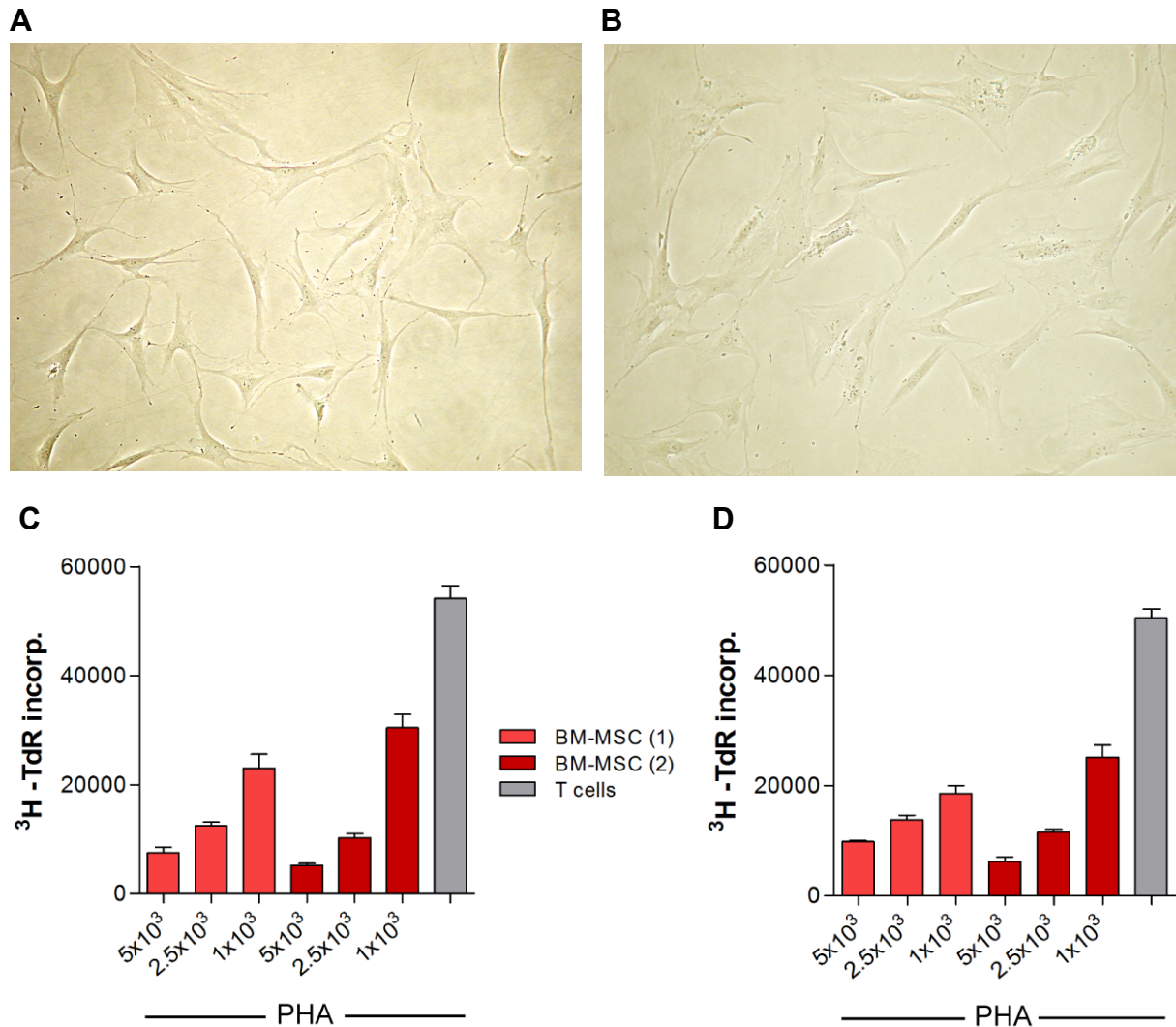


FIGURE 11. Comparable suppression profile of the mitogenic-induced T cell proliferation by BM-MSC using different T cell numbers. Upon thawing, BM-MSC from two different donors were seeded in culture flasks and cultured until they reached confluence. Photomicrographs of BM-MSC [(A), donor 1, passage 3, and (B), donor 2, passage 3] were taken on day 4 of culture (10x magnification). BM-MSC (donors 1 and 2) were irradiated (30 Gy) and seeded into 96 well flat-bottom microtiter plates at the indicated cell densities 4 hours before the addition of responder T cells [(C) 1×10^5 cells/well; (D) 1.5×10^5 cells/well] in the presence or absence of PHA (10 $\mu\text{g}/\text{ml}$). Cells were cocultured in a final volume of 200 μl NCM for 5 days at 37°C . ^3H -TdR (1 $\mu\text{Ci}/37 \text{ kBq}/\text{well}$) was added for the last 18 hours of culture. Data are displayed as mean $\pm$ SD of triplicate cultures. T cells alone, T cells plus BM-MSC exhibited only low proliferation ($< 1000 \text{ cpm}$; data not shown).

5.3 UC-MSC BUT NOT UC-ECFC SUPPRESS T CELL PROLIFERATION IN RESPONSE TO MITOGENIC STIMULI

The BM donation procedure is highly aggressive and additionally, the number and the differentiating capacity of BM-MSC is reduced with age¹⁷⁰. Therefore, alternative cell sources are needed. A good substitute is to use UC-MSC because they can also differentiate into cells of different connective tissue lineages like cartilage, bone and adipose tissues and represent a useful tool for tissue engineering. The immunophenotype of UC-MSC is similar to that described for BM-MSC¹⁷¹.

While the immunomodulatory potential of BM-MSC is well known¹⁷² and is confirmed in this study, little information is available in this respect regarding UC-MSC. Vellasamy et al. showed that UC-MSC inhibited T cell proliferation in response to mitogen treatment¹⁷³.

UC is also a resource for ECFC, which play a role in preserving the integrity of tissue and possibly also participate in regenerative processes and tumor formation¹⁶⁷. Due to the unique opportunity of receiving UC-MSC and UC-ECFC from the same donors, which look morphologically completely different (**Fig. 12A, B**), we aimed to test also the immunomodulatory potential of UC-ECFC in addition to UC-MSC and BM-MSC.

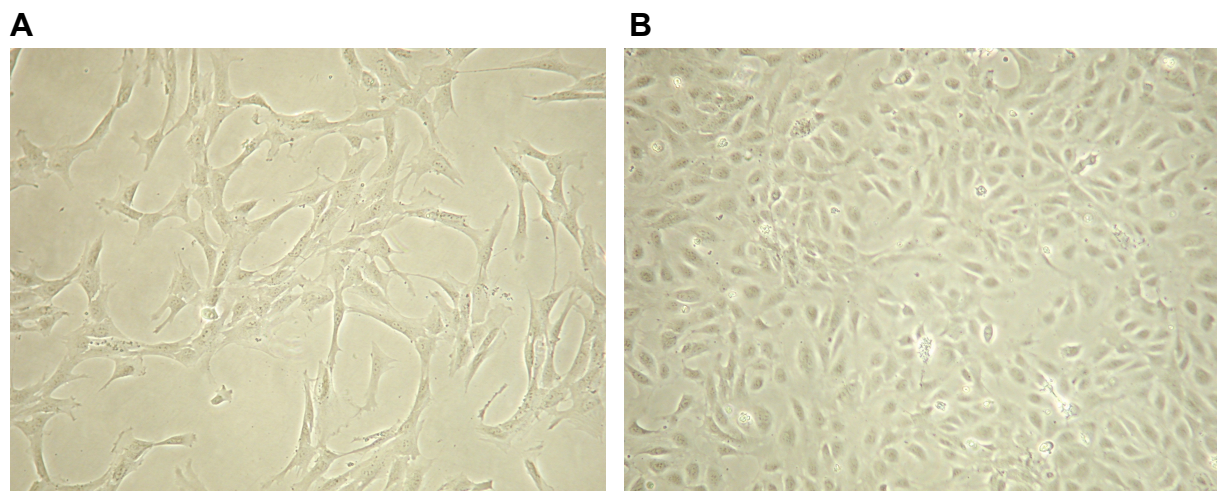


FIGURE 12. Different morphology of UC-MSC and UC-ECFC in culture. Upon thawing, cells were seeded in culture flasks and cultured in α -MEM (UC-MSC) or EGM (UC-ECFC) until 70-80% confluence was achieved. Photomicrographs of UC-MSC [(A), donor 1, passage 1] and of UC-ECFC [(B), donor 1; passage 1] were taken on day 4 of culture (10x magnification).

For this purpose, BM-MSC, UC-MSC and UC-ECFC were cocultured with T cells (1×10^5 , 1.5×10^5) in the presence and absence of PHA. Upon examination of the cocultures under the microscope we observed that the proliferation of T cells was inhibited in a similar way in the presence of BM-MSC as well as UC-MSC, but not by UC-ECFC after 36 hours (data not shown). Using ^3H -TdR incorporation, we confirmed that the inhibitory potential of UC-MSC was comparable to the immunosuppressive effect of BM-MSC, irrespective of T cell numbers employed in the assay (**Fig. 13A, B**). Similar to data described in **Fig. 11C** and **D**, BM-MSC from 2 different donors and in addition, UC-MSC inhibited the proliferative response of T cells in a cell density-dependent manner in a similar way. In contrast, UC-ECFC only marginally inhibited the proliferative response of T cells even at the highest cell densities, but instead rather induced T cell proliferation (**Fig. 13A, B**). From these data we conclude that UC-ECFC have no inhibitory potential.

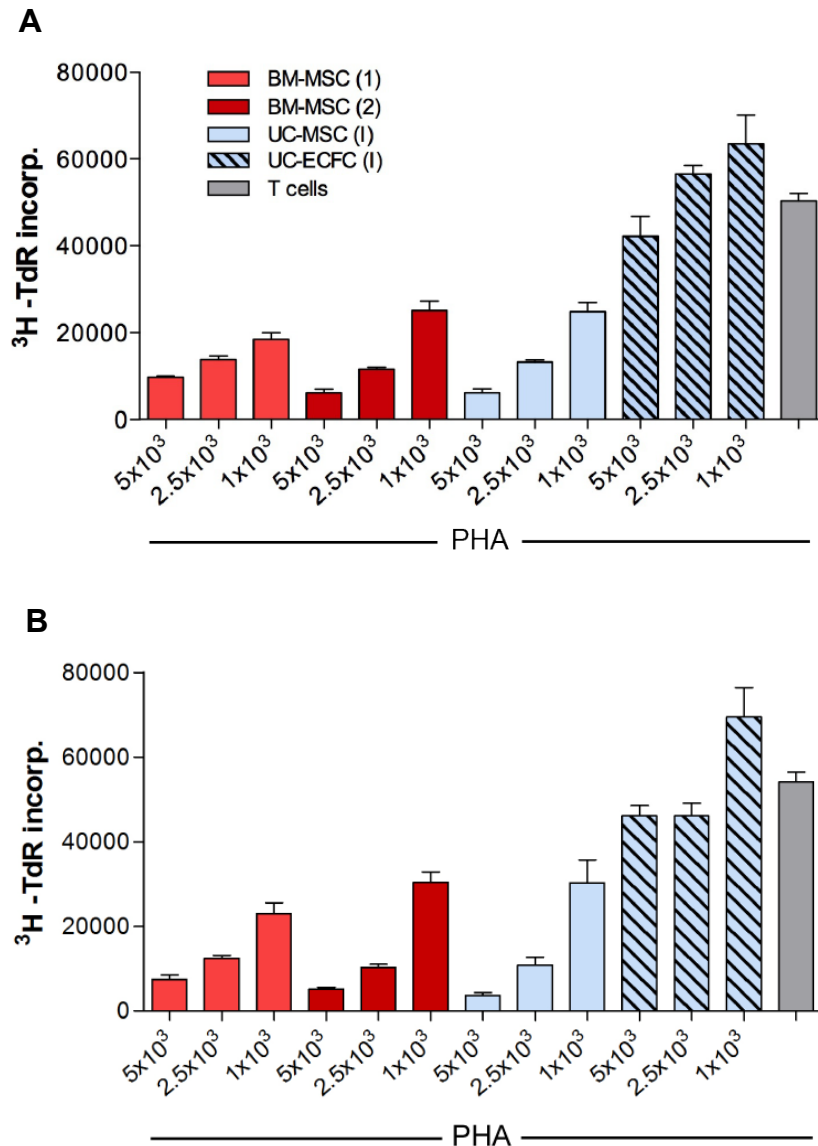


FIGURE 13. BM-MSC and UC-MSC but not UC-ECFC inhibit the proliferative capacity of T cells in response to mitogenic stimulation. Cells were irradiated (30 Gy) and seeded at the indicated cell densities/well 4 hours before the addition of responder T cells [(A) 1.5×10^5 cells/well; (B) 1×10^5 cells/well] in the presence or absence of PHA (10 $\mu\text{g/ml}$). Cells were cocultured in a final volume of 200 μl NCM for 5 days at 37°C. $^3\text{H-TdR}$ (1 $\mu\text{Ci}/37$ kBq/well) was added for the last 18 hours of culture. Data are displayed as mean $\pm$ SD of triplicate cultures. T cells alone, T cells plus BM-MSC, UC-MSC or UC-ECFC exhibited only low proliferation (< 1000 cpm; data not shown).

To further confirm the immunosuppressive effect of BM-MSC and UC-MSC we used additional donors. Similar to our results described in **Fig. 13**, the highest immunosuppressive effect could be identified with the highest cell density, which decreased with lower cell numbers. UC-MSC showed a similar tendency concerning the suppression of T cell proliferation and revealed at high cell densities an even higher suppressive effect compared to BM-MSC (**Fig. 14**).

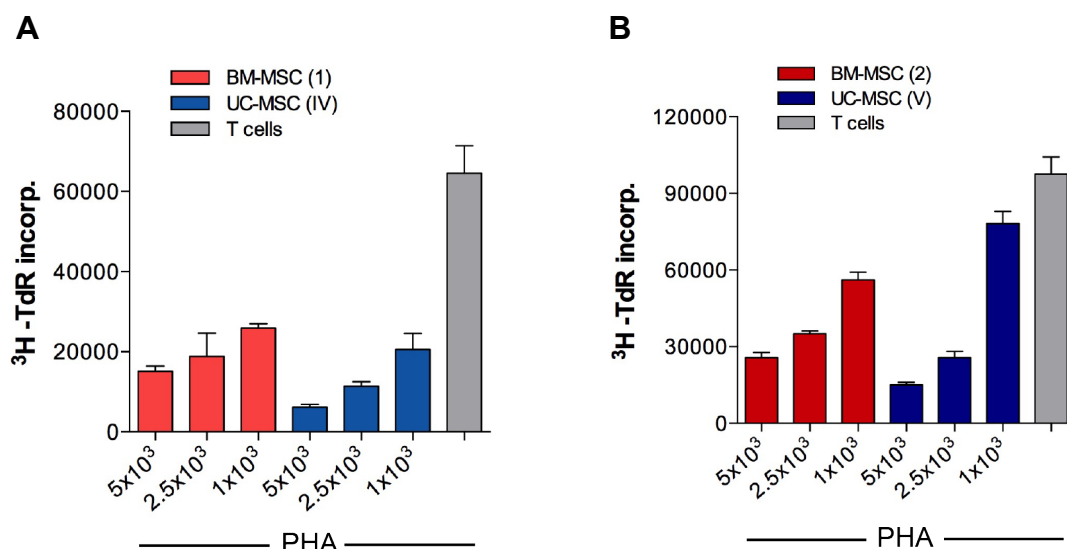


FIGURE 14. Comparable suppression profile of T cell proliferation by BM-MSC and UC-MSC. Results of the proliferation assay are displayed as mean $\pm$ SD of triplicate cultures with BM-MSC and UC-MSC and one representative of at least 5 independent experiments is shown. T cells alone and wells containing T cells and BM-MSC or UC-MSC exhibited only low proliferation (< 1000 cpm; data not shown).

To further strengthen our observation that ECFC do not have immunomodulatory capacities, we have included 5 different UC-ECFC donors in one experiment and have used UC-MSC from one donor as a positive control. Results obtained confirmed our previous observation showing that UC-ECFC exhibit no suppression but rather induce a proliferation of T cells in response to mitogenic stimulation compared to UC-MSC (**Fig. 15**).

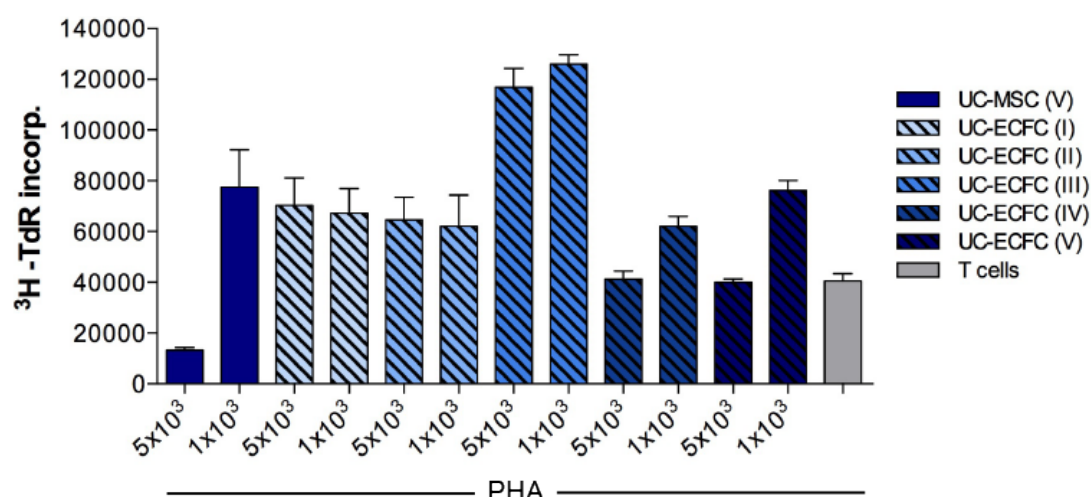


FIGURE 15. UC-ECFC do not inhibit T cell proliferation. Results of the proliferation assay are displayed as mean $\pm$ SD of triplicate cultures and one representative of at least 3 independent experiments is shown. T cells alone and wells containing T cells and UC-MSC or UC-ECFC exhibited only low proliferation (< 1000 cpm; data not shown).

As UC-ECFC induced T cell proliferation rather than inhibition, we determined whether they express costimulatory molecules and maturation markers such as CD80, CD86 and CD83. We found that neither UC-MSC (**Fig. 16**) nor corresponding UC-ECFC (**Fig. 17**) expressed significant levels of these markers, indicating that these cells do not represent classical APC.

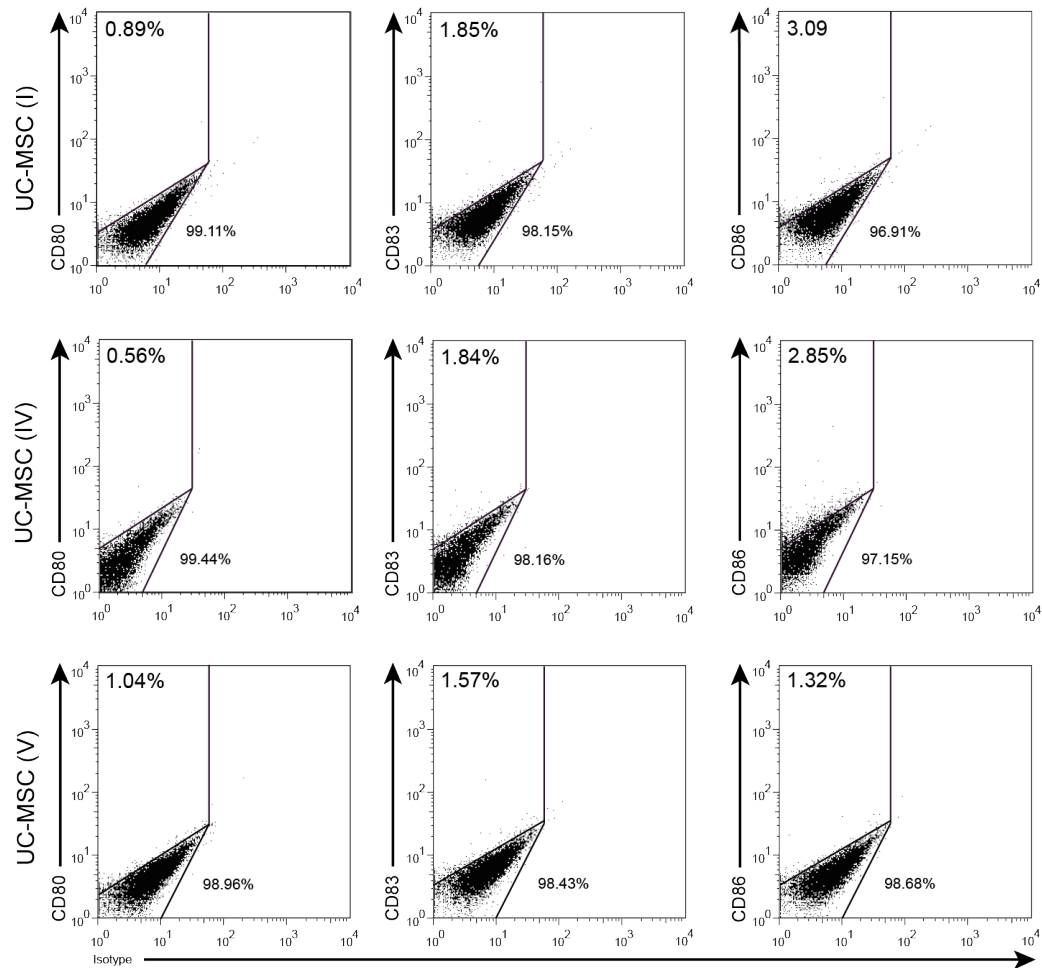


FIGURE 16. UC-MSC do not express costimulatory and maturation markers. UC-MSC from 3 different donors (I, IV, V) were analyzed by flow cytometry for the indicated markers. Dead cells were excluded by 7-AAD uptake. Dot plots show 100,000 cells.

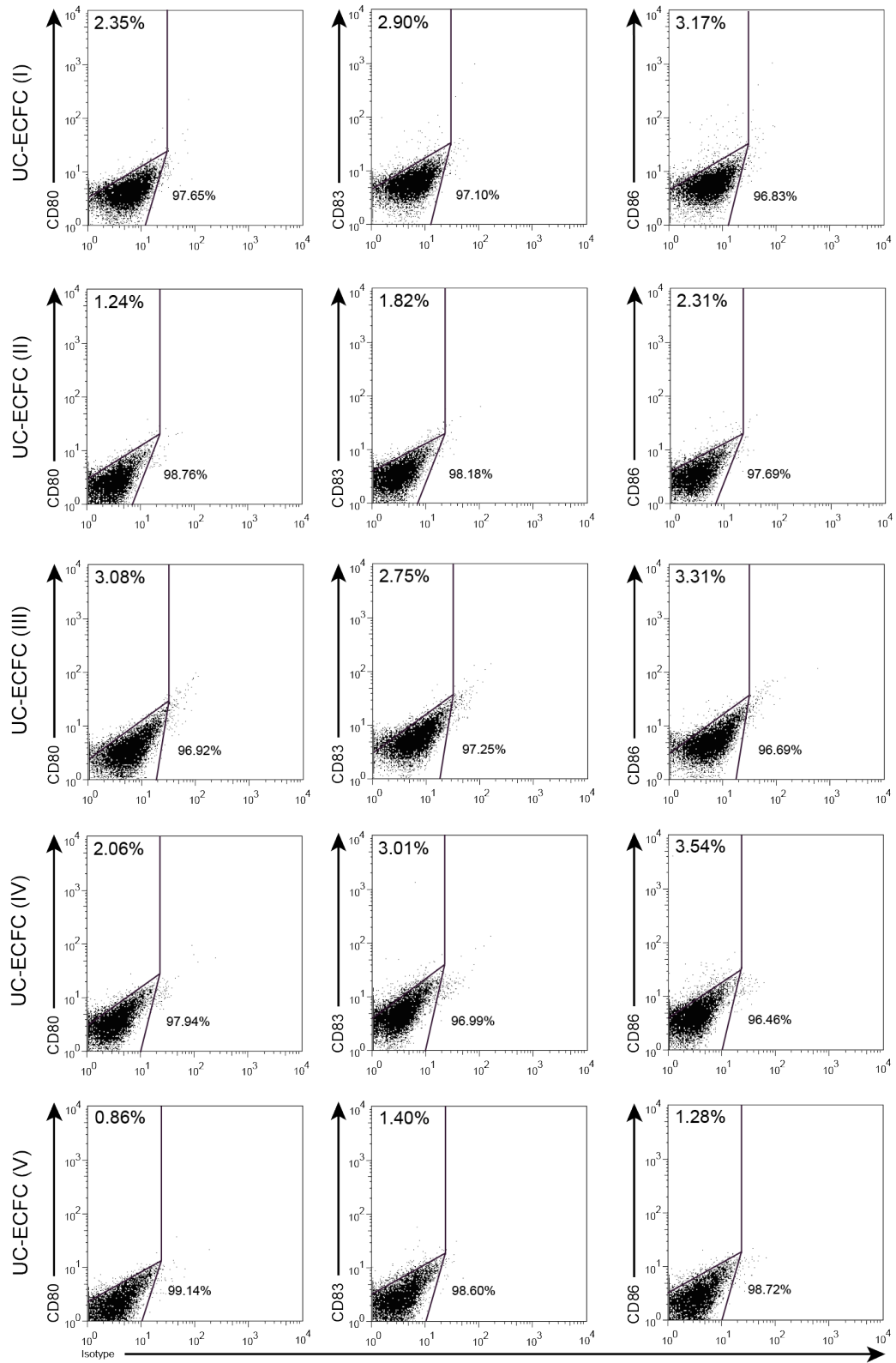


FIGURE 17. UC-ECFC do not express costimulatory molecules and maturation markers. UC-ECFC from 5 different donors (I-V) were analyzed by flow cytometry for the indicated markers. Dead cells were excluded by 7-AAD uptake. Dot plots show 100.000 cells.

5.4 PLASTIC-ADHERENT DERMAL CELLS SUPPRESS T CELL PROLIFERATION IN RESPONSE TO MITOGENIC STIMULI

In our laboratory techniques for the characterization and isolation of MSC and precursors from human dermis have been established. We reported recently that plastic-adherent dermal cells express the MSC markers CD73, CD90, CD105 and CD271¹⁷⁴ and, thus, express a profile which is typical for BM-MSC¹⁷⁵. To elucidate the immunomodulatory properties of plastic-adherent dermal cells, we employed the assays that were established with BM-MSC and UC-MSC. For this purpose, dermal cells were isolated from foreskins and cultured until they were confluent (**Fig. 18A, B**). With regard to morphology they were comparable with BM-MSC (**Fig. 18C**) and UC-MSC (**Fig. 18D**).

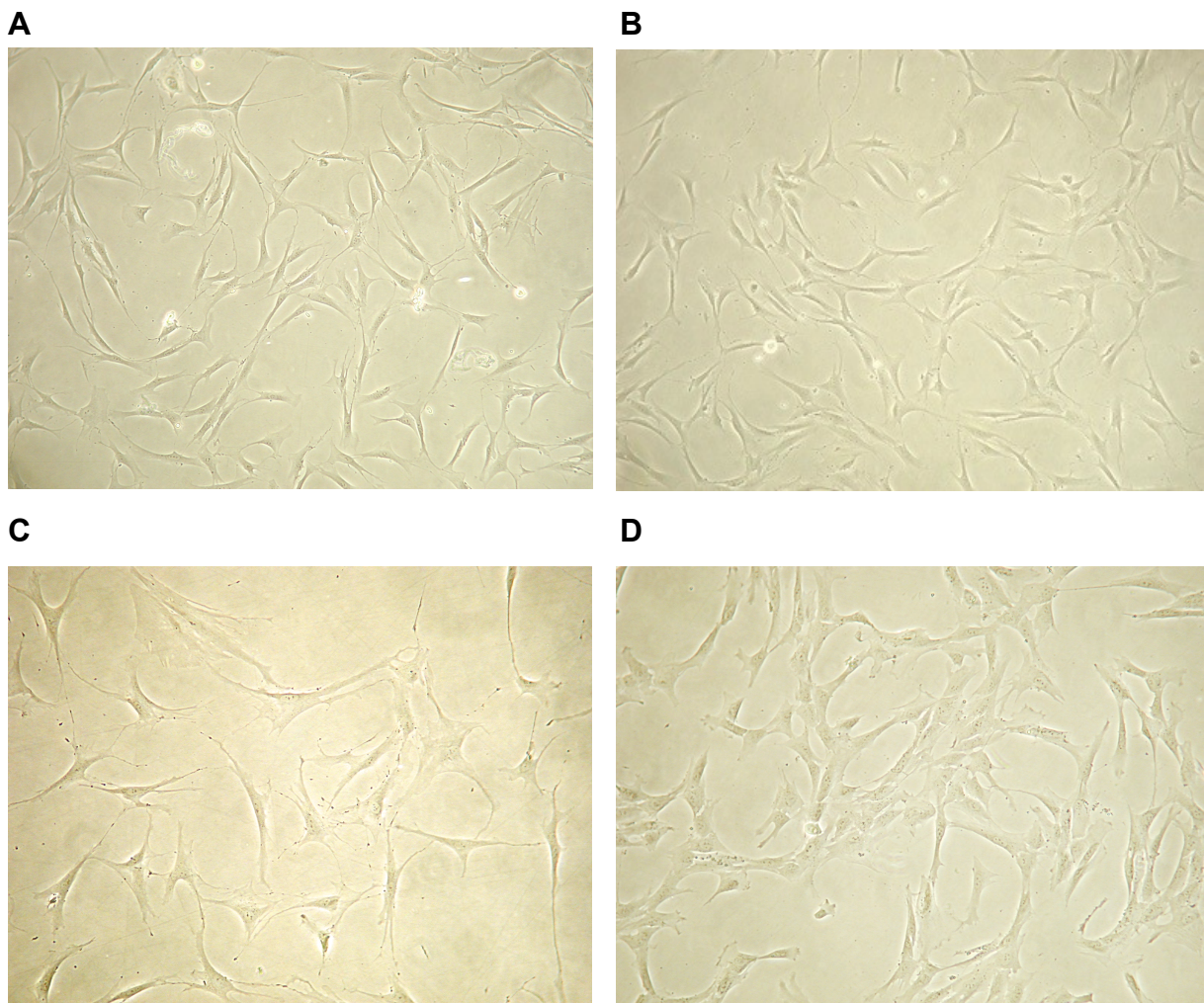


FIGURE 18. Plastic-adherent dermal cells have a comparable morphology to BM-MSC and UC-MSC in culture. Dermal cells (**A, B**) were prepared from human foreskins of 2 different donors and cultured until they reached confluence. Upon thawing, BM-MSC (**C**) and UC-MSC (**D**) were seeded in culture flasks and cultured until 70-80% confluence was accomplished. Photomicrographs of adherent dermal cells [(**A**) passage 0, 2 years old and (**B**) passage 0, 2 years old], BM-MSC [(**C**), donor 1, passage 3], and UC-MSC [(**D**), donor 1; passage 1] were taken on day 4 of culture (10x magnification).

Upon coculture of plastic-adherent dermal cells with T cells and PHA, we found a similar behaviour of dermal cells as previously shown for BM-MSC and UC-MSC. Also dermal cells revealed an immunosuppressive effect in a cell density-dependent manner on the proliferation of T cells. The highest immunosuppressive effect could be determined with the highest density of dermal cells. The tendency of the immunomodulatory profile for 2 different donors was quite similar even though the PHA-induced T cell proliferation rate was different for each dermal cell donor (**Fig. 19A, B**).

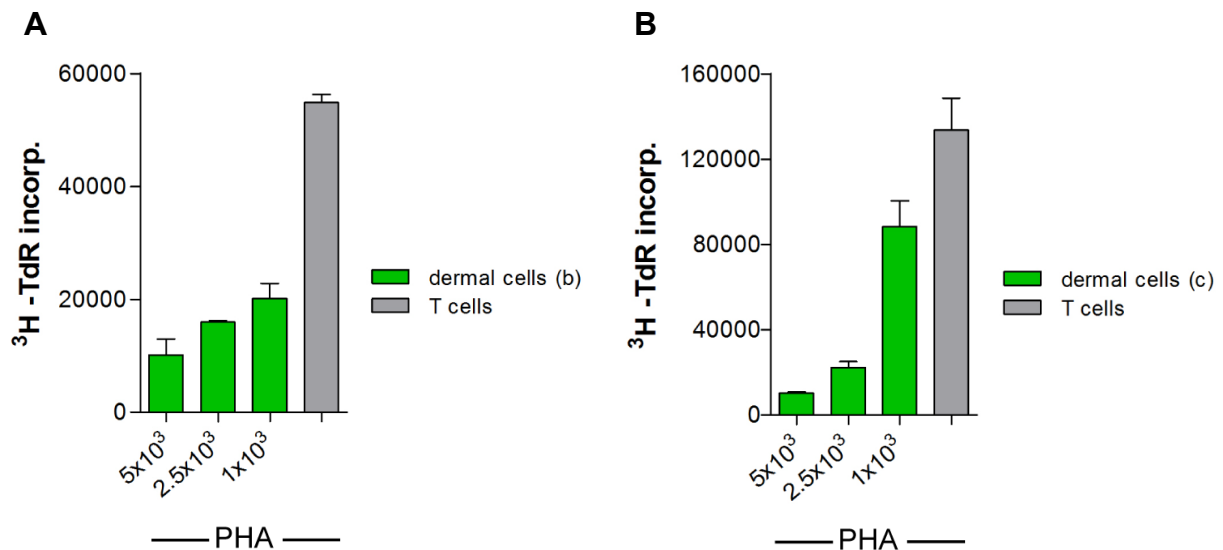


FIGURE 19. Inhibition of T cell proliferation by plastic-adherent dermal cells in response to mitogenic stimulation. Dermal cells from two different donors (**A**, donor b; **B**, donor c) were irradiated (30 Gy) and seeded into 96-well flat-bottom microtiter plates at the indicated cell densities 4 hours before the addition of T cells (1.5×10^5 cells/well; different T cell donors) in the presence or absence of PHA (10 μ g/ml). Cells were cocultured in a final volume of 200 μ l NCM for 5 days at 37°C. 3 H-TdR (1 μ Ci/37 kBq/well) was added for the last 18 hours of culture. Data are displayed as mean $\pm$ SD of triplicate cultures and one representative of at least five independent experiments is shown. T cells alone and wells containing T cells and dermal cells exhibited only low proliferation (< 1000 cpm; data not shown).

Next we comparatively assessed the immunomodulatory capacity of plastic-adherent dermal cells with BM-MSC and UC-MSC. The immunosuppressive profile of dermal cells was similar to that of BM-MSC (**Fig. 20A**) and UC-MSC (**Fig. 20B**). Again, T cell proliferation decreased with increasing cell densities of dermal cells, BM-MSC and UC-MSC.

Experiments are planned to explore whether dermal cells modify the cytokine profile of T cells in the same way as BM-MSC.

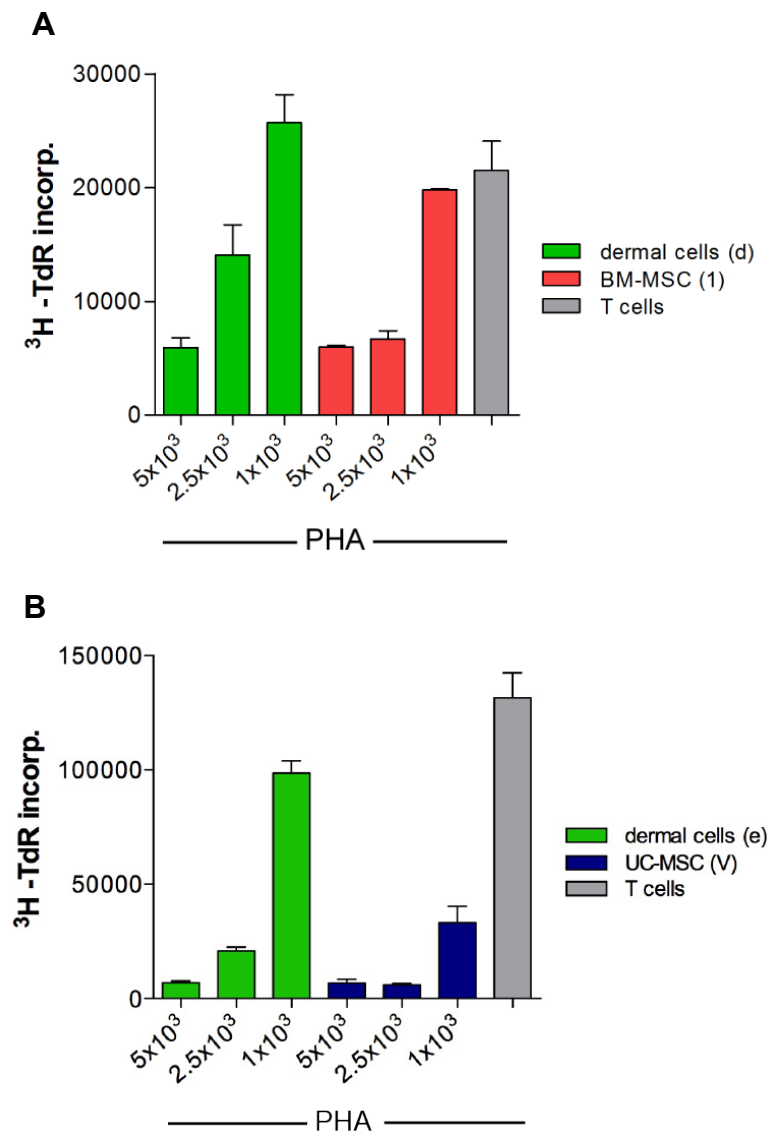


FIGURE 20. Comparable immunomodulatory capacity of plastic-adherent dermal cells, BM-MSC and UC-MSC for T cell proliferation in response to mitogenic stimulation. The assay was performed as described in Fig. 19. Results of the proliferation assay are displayed as mean $\pm$ SD of triplicate cultures and one representative of at least 5 independent experiments is shown. T cells alone and wells containing T cells and dermal cells exhibited only low proliferation (< 1000 cpm; data not shown).

As we observed that plastic-adherent dermal cells at lower cell densities occasionally induced a proliferation of T cells, we tested whether costimulatory molecules and maturation markers are upregulated. Staining with mAb directed against CD80, CD83 and CD86 and analysis by flow cytometry revealed that plastic-adherent dermal cells did not express significant levels of these markers, indicating that they cannot act as classical APC (**Fig. 21**), thus, confirming our previous reported results¹⁷⁴.

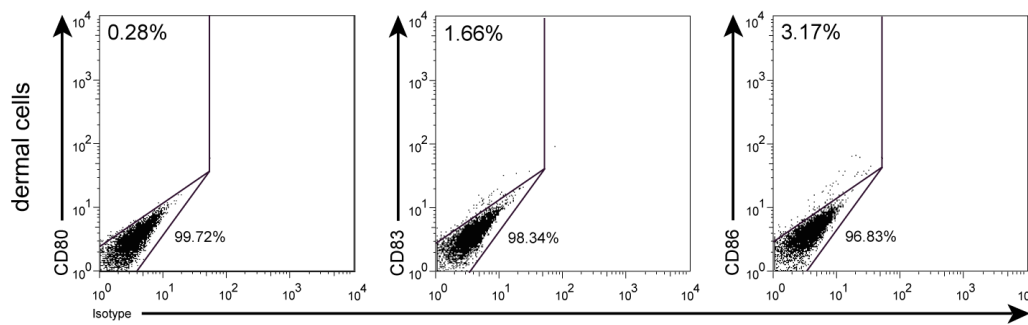


FIGURE 21. Plastic-adherent dermal cells do not express costimulatory molecules and maturation markers. Dermal cells were cultured under similar conditions as for the functional assays. Dead cells were excluded by 7-AAD uptake. Dot plots show 100,000 cells and are representative for 3 separate experiments.

Vaculik et al. reported that on a small subpopulation of plastic-adherent dermal cells the marker CD271 is expressed¹⁷⁴. Subsequent differentiation assays revealed that purified CD271⁺ dermal cells exhibited a high adipogenic, osteogenic and chondrogenic differentiation capacity in contrast to CD271⁻ or total plastic-adherent dermal cells. Based on this observation, we investigated the immunomodulatory potential of all three populations. For this purpose, total plastic-adherent dermal cells were purified for CD271⁺ cells using MACS sorting. The positive and negative cell fractions were tested for purity and compared to total plastic-adherent dermal cells (**Fig. 22**).

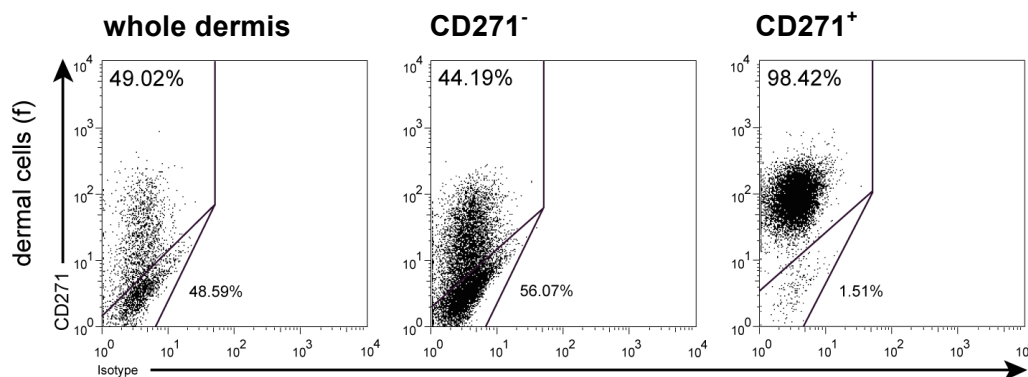


FIGURE 22. CD271⁺ dermal cells can be isolated with high purity. Cells were purified as described in M&M, stained with an anti-CD271 mAb and analyzed by flow cytometry. The left panel represents the whole fraction, the middle panel the negative fraction and the right panel the positive fraction. The purity of the CD271⁺ dermal cells was repeatedly 99%. Dot plots show 100,000 cells and are representative for 3 separate experiments.

When applying our established mitogen-induced proliferation assay, we found that all three dermal cell fractions as well as BM-MSC (positive control) revealed a cell density-dependent immunosuppressive effect on the proliferation of T cells. The highest inhibition of T cell proliferation in response to mitogenic stimuli could be observed in the CD271⁺ fraction at high cell densities. Again, the superior immunosuppressive effect could be observed with the highest dermal cell and BM-MSC density. The general trend of inhibition was the same even

though the PHA-induced T cell proliferation rate was different for each dermal cell population indicated (**Fig. 23**). In further experiments we plan to elucidate which factors are responsible for the superior immunosuppressive effect of CD271⁺ cells. Furthermore, we plan to assess other dermal MSC subpopulations (e.g. SSEA-4⁺ dermal cells) for their immunosuppressive activity.

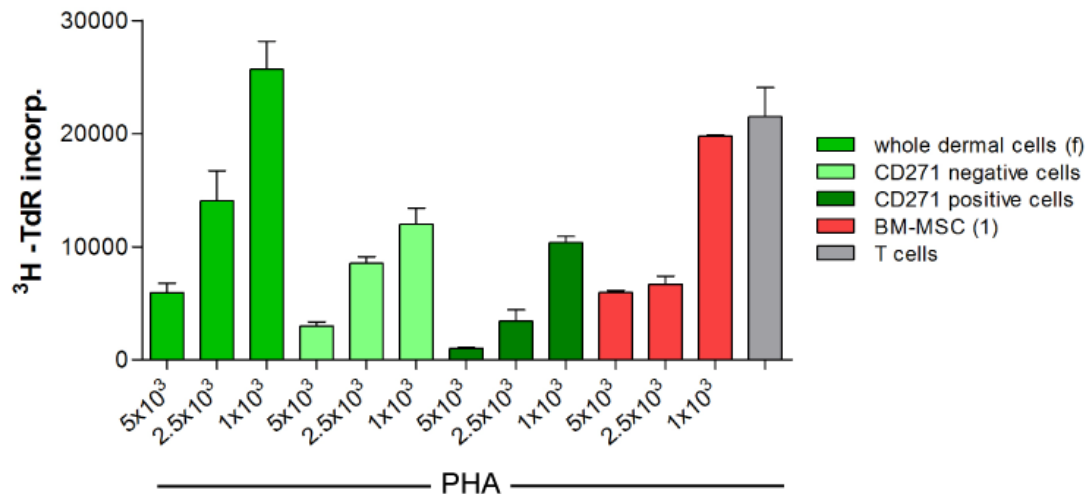


FIGURE 23. CD271⁺ dermal cells showed a higher immunosuppressive effect in comparison with CD271⁻ and total plastic-adherent dermal cells. The assay was performed as described in Fig. 19. Results of the proliferation assay are displayed as mean $\pm$ SD of triplicate cultures and one representative of at least 3 independent experiments is shown. T cells alone and wells containing T cells and dermal cells exhibited only low proliferation (< 1000 cpm; data not shown).

Besides other factors, PGE₂ is a well-established suppressor of T cell proliferation¹⁶³. To investigate whether the dermal cell-mediated T cell suppression can be reversed by inhibitors of PGE₂ synthesis, irradiated plastic-adherent dermal cells were cocultured with PHA-stimulated T cells in the presence of Indo or the solvent. In the presence of the solvent we observed a cell density-dependent inhibition of T cell proliferation that was comparable to that described without solvent (**Fig. 24**). In contrast, Indo partially reversed the inhibitory effect of dermal cells, suggesting that the dermal cell-mediated immunosuppression operates through secreted soluble factors and that PGE₂ is an important component of the suppressive effect. It would be interesting to investigate whether PGE₂ act in a synergistic way with other soluble molecules to alter T cell proliferation as already shown by Di Nicola and coworkers⁷⁴ with regard to TGF- β and HGF in BM-MSC. Previous studies support a role of PGE₂ in the inhibition of T cell proliferation *in vitro* and *in vivo*^{38,176-178}, but the signalling pathways need further investigation to improve the use of dermal MSC in clinical therapies¹⁷⁹.

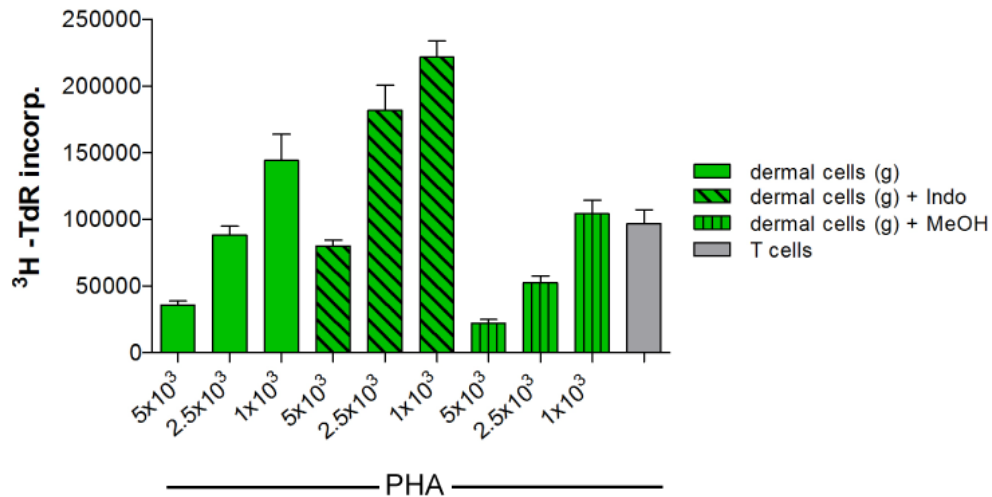


FIGURE 24. The dermal cell-mediated immunosuppression partially operates via PGE₂. Dermal cells were irradiated (30 Gy) and seeded into 96 well flat-bottom microtiter plates at the indicated cell densities 4 hours before the addition of T cells (1.5×10^5 cells/well) in the presence or absence of PHA (10 μ g/ml) and/or indo (5 μ M) and/or methanol (MeOH; 5 μ M). Cells were cocultured in a final volume of 200 μ l NCM for 5 days at 37°C. ³H-TdR (1 μ Ci/37 kBq/well) was added for the last 18 hours of culture. Results of the proliferation assay are displayed as mean $\pm$ SD of triplicate cultures and one representative of at least 4 independent experiments is shown. T cells alone and wells containing T cells and dermal cells exhibited only low proliferation (< 1000 cpm; data not shown).

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7 APPENDIX

7.1 APPARATUSES AND INSTRUMENTS

- Centrifuge 4K15C (Sigma Laboratory Centrifuges, Munich, Germany)
- CO₂ incubator (Heraeus, Vienna, Austria)
- FloJo software (Tree Star Inc., Ashland, OR, USA)
- Fluorescence-activated cell sorter (FACS LSRII, Becton Dickinson, San Jose, CA, USA)
- Forceps
- Freezers (-20°C, -80°C)
- Fridges
- Cell Harvester (TomTec, Hamden, CT, USA)
- Hemocytometer
- Laminar flow (Holten, Allerød, Denmark)
- Magnetic Separator (MACS Multi Stand + Octo MACS Separator, Miltenyi Biotech, Bergisch Gladbach, Germany)
- MicroBeta 1450 Liquid scintillation counter (Wallac/PerkinElmer, Waltham, MA, USA)
- Microcentrifuges (Eppendorf, Hamburg, Germany)
- Microsealer 1495-021 (Wallac)
- Pipetaid (Costar, Cambridge, MA, USA)
- Pipetman (2, 20, 200 µl, 1 ml; Gilson, Middleton, WI, USA)
- Racks
- Scale (Sartorius, Vienna, Austria)
- Scalpels
- Scissors
- Sterile gauze
- Vacutainer CPT (Becton Dickinson)
- Vortex Genie 2 (Lactan, Graz, Austria)
- Water bath

7.2 PLASTIC MATERIAL

- Bottle-top filters (0.2, 0.4 µm; Costar)
- Cell separation columns MS (MACS, Miltenyi Biotech)
- Cell strainer (70 µm; Falcon, Lincoln Park, NJ, USA)
- Cryotubes (1.8 ml; Nunc, Langenselbold, Germany)
- Culture dishes (35 mm; Stem Cell Technologies, St. Katharinen, Germany)
- Culture flasks (225 cm²; Nunc)

- Culture plates (96 well round- and flat-bottom; Costar)
- Eppendorf tubes (1.5 ml; Eppendorf)
- Gloves
- Microtubes for flow cytometry (Micronic; Lelystad, The Netherlands)
- Petri dishes for tissue culture (100 x 20 mm; Corning, NY, USA)
- Pipettes (5, 10, 25, 50 ml; Corning)
- Polypropylene tubes (15, 50 ml; Falcon)
- Sterile tips (0.5-10, 1-100, 200-1000 µl; Corning)

7.3 REAGENTS

- 7-AAD (Sigma, St. Louis, MO, USA)
- Ab (**Table 1**)
- Betaisodona (Mundipharma, Vienna, Austria)
- Bovine serum albumin (Sigma)
- Buffy coat (Rotes Kreuz, Vienna, Austria)
- Dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany)
- Dispase II (neutral protease, grade II; Roche Applied Science, Basel, Switzerland)
- Ethanol (Merck)
- EDTA (Merck)
- Fetal calf serum (FCS; PromoCell, Heidelberg, Germany)
- Indo (Sigma)
- Liberase Blendzyme 3 (Roche Applied Science)
- Methanol (Merck)
- Microbeads (anti-biotin; Miltenyi Biotech)
- Pan T cell isolation kit (Miltenyi Biotech)
- PBS (Invitrogen, Lofer, Austria)
- PHA (Sigma)
- ³H-TdR (1 µCi/37 kBq/well; Hartmann Analytic, Braunschweig, Germany)
- Trypan blue (0.4%; Sigma)
- Trypsin-EDTA (0.05%; Invitrogen)
- Trypsin-EDTA (0.1%; Sigma)

7.4 MEDIA, BUFFERS, CELL ISOLATION KIT

MEM (α -MEM; Invitrogen) supplemented with:

10% FCS (PromoCell)

1X penicillin/streptomycin (Invitrogen)

100 µg/ml gentamycin (Invitrogen)

EGM (Lonza, Cologne, Germany) supplemented with:

- 2% fetal bovine serum
- recombinant human epidermal growth factor
- vascular endothelial growth factor
- GA-1000
- recombinant human fibroblast growth factor
- hydrocortisone
- insulin-like-growth factor-1
- heparin
- ascorbic acid

NCM RPMI 1640 (Invitrogen) supplemented with:

- 10% FCS
- 1X penicillin/streptomycin
- 100 µg/ml gentamycin
- 1 mM sodium pyruvate (Invitrogen)
- 0.1 mM non-essential amino acids (Invitrogen)
- 2 mM L-glutamine (Invitrogen)
- 100 µM 2-mercaptoethanol (Invitrogen)
- 25 mM N-2-hydroxyethylpiperazine-N`-2-ethanesulfonic acid (HEPES; Invitrogen)

FACS BUFFER 1X PBS supplemented with:

- 1% FCS
- 0.5 mM EDTA

MACS BUFFER 1X PBS supplemented with:

- 0.5% bovine serum albumin
- 2 mM EDTA

PAN T CELL ISOLATION KIT

Pan T cell biotin-Ab cocktail containing biotin-conjugated mAb against CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin A. Anti-biotin MicroBead: conjugated to an anti-biotin mAb (isotype: mouse IgG1). Both are supplied in a solution containing a stabilizer and 0.05% sodium azide.

8 CURRICULUM VITAE

Personal information

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Education

02/2009 – 02/2010	Diploma Thesis at the Department of Dermatology, Medical University of Vienna; Thesis Title: Immunomodulatory potential of human skin stem cells Supervisor: Ao. Univ.-Prof. Dr. Adelheid Elbe-Bürger
10/2003 – present	Studies of genetics and microbiology with specialization in immunology at the University of Vienna
1995 – 2003	Grammar School Bundesgymnasium, 4780 Schärding, Austria
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