DIPLOMA THESIS

Characterisation of the expression profile of pattern recognition receptors in TGF-β1- vs. BMP7-driven Langerhans cells

Submitted by

Melanie Spies B.Sc.

For the academic degree of

Doktorin der gesamten Heilkunde / Doctor of medicine

(Dr. med. univ.)

At the

Medizinische Universität Graz

Conducted at the

Otto Loewi Research Center for Vascular Biology, Immunology and Inflammation

Under supervision of Univ.-Prof. Dr.med. univ. Herbert Strobl and Izabela Borek, MSc

Graz, 01.August 2019

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 01. August 2019 *Melanie Spies eh*

Acknowledgements

Zunächst möchte ich mich bei all denjenigen bedanken, die mich bei der Anfertigung dieser Arbeit unterstützt haben. Des Weiteren bin ich dankbar für die freundliche Aufnahme in die Arbeitsgruppe.

An erster Stelle möchte ich Herrn Univ.-Prof. Dr.med. univ. Herbert Strobl für die Vergabe dieses interessanten Themas und die engagierte Betreuung danken.
Auch bei Izabela Borek, MSc möchte ich mich für die Betreuung im Labor sowie die persönliche Unterstützung bedanken. Außerdem danke ich ihr dafür, dass sie mir immer mit Rat und Tat zur Seite stand und die Umsetzung dieses Themas überhaupt erst möglich gemacht hat.

Frau Christina Passegger, MSc, Frau Elke Schwarzenberger und Frau Anika Stracke danke ich für die fachliche Hilfsbereitschaft und Unterstützung im Labor.

Zusammenfassung

Langerhans Zellen sind Dendritische Zellen, welche in der Epidermis liegen und eine wichtige Rolle in dem erworbenen Immunsystem spielen. Sie exprimieren ein spezifisches Oberflächenprofil, welches eine eindeutige Abgrenzung von anderen dendritischen Zellen ermöglicht (z.B. $CD1a^{+}CD207^{+}CD324^{+}$). Langerhans Zellen exprimieren ebenfalls Toll-like-Rezeptoren (TLR), welche zur Gruppe der Pattern Recognition Rezeptoren gehören und wichtig für die Erkennung von Pathogenen sind. Die regulative Rolle von Langerhans Zellen auf das Immunsystems ist noch umstritten, da ihr Verhalten stark situationsabhängig ist, denn Langerhans Zellen können sich sowohl Immunsuppressiv verhalten als auch eine Entzündungsreaktion bzw. zytotoxische T-Zell Antwort auslösen.

Der Transforming Growth Factor Beta 1 (TGF-β1) und das Bone Morphogenetic Protein 7 (BMP7) sind Zytokine aus der TGF-Familie, welche aus sich strukturell ähnelnden Zell-regulatorischen Proteinen bestehen. Kürzlich wurde herausgefunden, dass BMP7 den TGF-ß1 bei der Bildung von Langerhans Zellen vollständig ersetzten kann. Ziel dieser Studie war die Charakterisierung von Langerhans Zellen und ein Vergleich der Expression von Toll-Like-Rezeptoren zwischen BMP7 und TGFß1 Langerhans Zellen.

Die qPCR Analyse zeigte, dass durch BMP-7 gebildete Langerhans Zellen signifikant höhere Level von TLR2 im Vergleich zu TGF-ß1 Langerhans Zellen exprimieren. Im Vergleich dazu exprimierten TGF-ß1-generierte Langerhans Zellen höhere Level von TLR1, TLR6 und TLR10. Beide Zelltypen zeigten keine messbare Expression der Rezeptoren 4, 5 und 9.

Um Langerhans Zellen mit anderen Dendritischen Zellen vergleichen zu können wurde eine zusätzliche Analyse der TLR Expression von CD14 positiven, aus Monozyten generierten Dendritischen Zellen (MoDCs) durchgeführt. Es zeigte sich, dass moDCs die TLR1, TLR2, TLR3, TLR4, TLR6 und TLR8 exprimierten, TLR5, TLR7, TLR9 und TLR10 jedoch nicht exprimiert wurden. Eine Stimulation mit Lipopolysachariden führte zu einer verminderten Expression aller untersuchten Rezeptoren.

Abstract

Langerhans cells (LCs) are dendritic cells (DCs), which reside in the epidermis and play an important role in the adaptive immunity. LCs exhibit a unique marker profile, which enables their precise distinction from other DCs (e.g. $CD1a^{+}CD207^{+}CD324^{+}$). They also express Toll like receptors (TLRs), which are pattern recognition receptors important for the detection of pathogens. The role of LCs in the regulation of the immune system responses is still debated as their behavior is heavily context dependent: LCs can be immunosuppressive as well as they can induce an inflammatory and cytotoxic T-cell response.

Transforming growth factor beta 1 (TGF-β1) and bone morphogenetic protein 7 (BMP7) are cytokines that belong to the TGF-beta family composed out of structurally related, cell regulatory proteins. We recently found that BMP7 fully replaced TGF-β1 in LC generation cultures. The aim of this study was to characterize and compare the TLRs expression pattern of BMP7-dependent LCs vs. TGF-dependent LCs.

qPCR analysis reviled that BMP7-generated LCs expressed significantly higher levels of TLR2 compared to TGF-β1-generated LCs. In contrast TGF-β1-generated LCs expressed higher levels of TLR1, TLR6 and TLR10 than BMP7-generated LCs. Both cell types had no detectable expression of TLR4, TLR5 and TLR9.

To compare LCs to other DCs, we additionally analyzed the TLR expression profile of $CD14^{+}$ monocyte derived dendritic cells (MoDCs). We showed that MoDCs express TLR1, TLR2, TLR3, TLR4, TLR6 and TLR8 and they had no detectable expression of TLR5, TLR7, TLR9 and TLR10. Stimulation with lipopolysaccharide (LPS) resulted in downregulation of all analyzed receptors.

Table of Contents

Acknowledgements....ii
Zusammenfassung....iii
Abstract....iv
Table of Contents....v
List of abbreviations....vii
List of figures....ix
List of tables....x
1 Introduction....11
1.1 Immune system – innate and adaptive immunity....11
1.2 Antigen-presenting-cells (APCs) – gate keepers between innate and adaptive immunity....13
1.3 Skin is a first line defense against the outer environment....15
1.4 LCs are a subpopulation of DCs....15
1.5 Development of LCs in humans....17
1.6 TGF-β family signaling: Role of TGF-β1 and BMP7 in LCs biology....18
1.7 In vitro generation of LCs from CD34⁺ hematopoietic stem and progenitor cells with TGF-β1 or BMP7....19
1.8 In vitro generation of MoDCs from Peripheral blood CD14⁺ monocytes....19
1.9 Toll-like-Receptors (TLRs) as a subclass of Pattern-Recognition Receptors (PPRs)....19
2 Material and Methods....22
2.1 Objective of the work....22
2.2 Materials....23
2.2.1 Chemicals and Reagens....23
2.2.2 Cell Culture....23
2.2.3 Primers for Real-time PCR....24
2.2.4 Consumable Materials....24
2.2.5 Laborartory Equipement....25
2.2.6 Programms....26
2.3 Methods....27
2.3.1 Isolation of CD34⁺ hematopoietic stem and progenitor cells from cord blood....27
2.3.2 Isolation of CD14⁺ monocytes from buffy coats....28

2.3.3 *In vitro* differentiation of LCs ... 28
2.3.4 *In vitro* differentiation of MoDCs and stimulation with LPS ... 29
2.3.5 Magnetic cell sorting (MACS) ... 29
2.3.6 Flow-cytometry ... 30
2.3.7 RNA-Isolation ... 31
2.3.8 Quantification and quality control of RNA ... 31
2.3.9 RT-PCR ... 32
2.3.10 qPCR ... 33
2.3.11 Statistical Analysis ... 34
3 Results ... 35
3.1 Experimental workflow ... 35
3.2 Isolation of $CD34^+$ hematopoietic stem and progenitor cells from human cord blood and in vitro generation of Langerhans cells (LCs) ... 36
3.3 MACS sorting of LCs ... 37
3.4 Analysis of RNA integrity on agarose gel and spectrophotometer ... 38
3.5 BMP7-generated LCs express higher level of TLR2 relative to TGF-β1-generated LCs ... 39
3.6 MoDCs express a greater variety of TLRs than LCs ... 40
4 Discussion ... 42
5 Literature ... 47

List of abbreviations

°C	Degree Celsius
Aq. dest.	*Aqua destillata*
APC	Antigen-presenting-cell
BG	Birbeck granules
BMP7	Bone morphogenetic protein 7
cDNA	Complementary DNA
CLRs	C-type lectin receptors
Ct-value	*Cycle threshold*
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
dNTP	Deoxyribonucleic triphosphates
et al.	And others
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSPC	Human hematopoietic stem and progenitor cell
l	Liter
LC	Langerhans cell
LNs	Lymph nodes
LPS	Lipopolysaccharide
µg	Microgram
µl	Microliter
M	Molar
M-CSF	Macrophage colony stimulation factor CSF-1
mg	Milligramm
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
mM	Millimolar
MPs	Mononuclear phagocytes
nm	Nanometer
nM	Nanomolar
NLRs	NOD-like receptors
OD	Optical density
PAMPs	Pathogen-associated molecular patterns

PBS	Phosphate buffered saline
PCR	Polymerase-Chain-reaction
PGN	Peptidoglycan
pH	Potentia hydrogenii
PRR	Pathogen Recognition Receptor
qPCR	Quantitative PCR
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase-chain-reaction
sec	Seconds
Tab.	Table
TCR	T cell receptor
TGF-β1	Transforming growth factor beta 1
TLR	Toll-like-receptor
UV	Ultraviolet light

List of figures

Figure 1. Innate and adaptive immune system.. 12
Figure 2. Antigen presenting cells (APCs). 14
Figure 3. LCs are characterized by the expression of specific surface markers... 16
Figure 4. Signaling of the TGF beta superfamily. 18
Figure 5. Pattern recognition receptors (PRRs)... 20
Figure 6. Pattern Recognition Receptors (PRRs): Toll-like receptors.................. 21
Figure 7. Workflow.. 35
Figure 8. Purity of MACS sorted CD34$^+$ hematopoietic stem and progenitor cells isolated from umbilical cord blood. 36
Figure 9. During the process of differentiation LCs form typical E-cadherin mediated clusters. 37
Figure 10. MACS-sorting allows significant enrichment of CD207$^+$ LCs from heterogeneous cultures. 38
Figure 11. RNA integrity of TGF-ß1/BMP7 generated LCs visualized on agarose gel... 39
Figure 12. BMP7-dependent LCs differ in TLR expression profile from TGF-β1-dependent LCs.. 40
Figure 13. MoDCs express TLR1, TLR2, TLR3, TLR4, TLR6 and TLR8. After the stimulation with LPS moDCs downregulate the expression of TLRs. 41
Figure 14. Micro array analysis of TGF-β1 (TGF) and BMP7 (BMP) LCs. 42

List of tables

Table 1. Primers for Real-time PCR 24
Table 2: Flow cytometry antibodies 30
Table 3. Preparation of RT-PCR tubes. 32
Table 4. RT-PCR incubation program... 32
Table 5. Preparation of qPCR plates. 33
Table 6. qPCR incubation program.. 33

1 Introduction

1.1 Immune system – innate and adaptive immunity

The immune system is the body's defense system involved in the protection against pathogens and the removal of dying cells. Furthermore, the immune system plays an important role in the development of cancer where it can have a protective as well as a supportive role [1]. The immune system can be divided into innate and adaptive branches. It can detect a broad variety of microorganisms like bacteria, parasites, fungi and viruses. Inflammation is one of the first reactions the immune system is carrying out upon stimulation e.g. by pathogens [2].

The first defense line of the body is its surface (the skin and the mucosa) comparable to a physical barrier which prevents the entrance of pathogens. The epithelial barrier is part of the innate immune system and whenever pathogens cross the barrier and enter the organism the innate immune system is activated [3]. The immune system offers a non-specific but immediate reaction to different pathogens. The innate immunity makes use of different mechanisms of protection like the complement system, antimicrobial peptides and a broad variety of cells carrying out phagocytosis and antigen presentation. These cells, belonging to the innate immune system, are phagocytic cells (such as macrophages, neutrophils and dendritic cells (DCs)), mast cells, eosinophils, basophils and natural killer cells. [4].

In cases where the pathogen cannot be eliminated by the innate immune system the adaptive immune system gets activated. The adaptive immune system is a more specialized system consisting of different cell types like T- and B-lymphocytes. Certain cells of the adaptive immune system are able to memorize antigens after their detection. This so-called immunological memory enables faster and stronger reaction in case of reoccurring infections. This effect is also responsible for the effectiveness of vaccinations [4]. Finally, the innate and adaptive immune system are tightly connected and regulated on multiple levels.

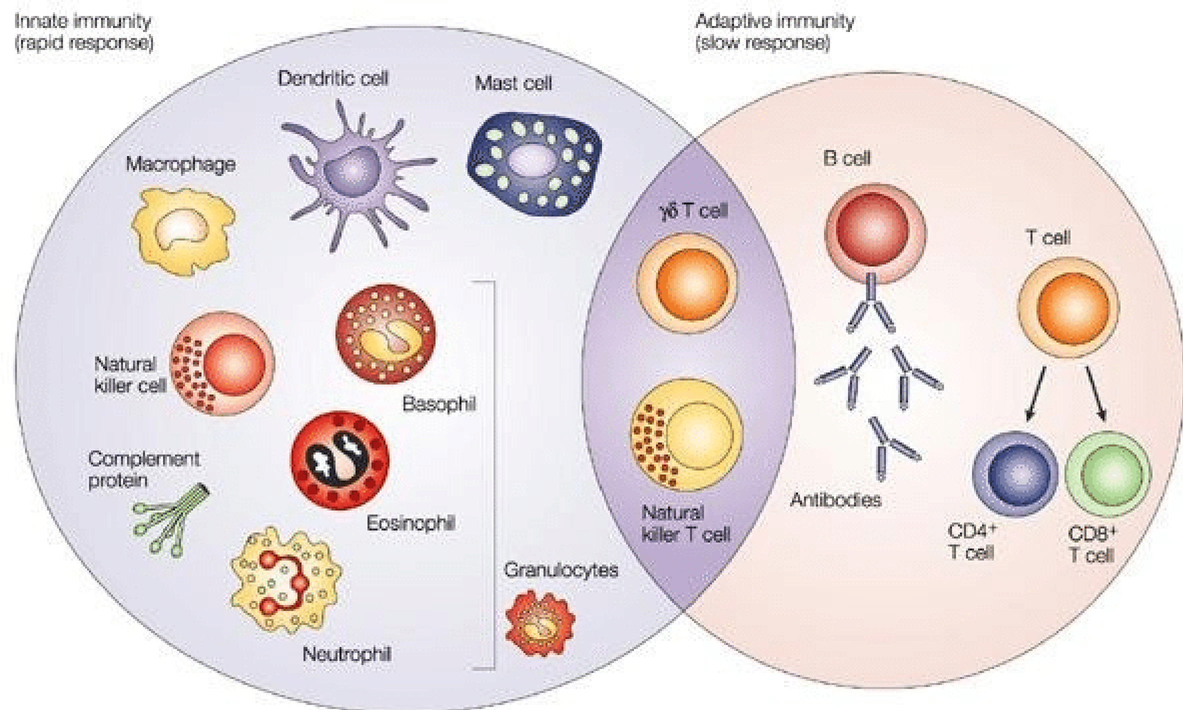


Figure 1. Innate and adaptive immune system. Granulocytes, macrophages, DCs, natural killer cells and mast cells belong to the innate immunity. B and T cells are part of the adaptive immunity. Some specialized T cells and Natural killer T cells belong to both systems (Dranoff et al.) [5].

To work properly it is important that the immune system can distinguish between foreign structures, like pathogens and the body's own structures, like cells. Beside the detection of pathogens, that may cause infections, the immune system can also detect body's own danger signals. Those signals occur, when cells get stressed or damaged, and are significant for a well-regulated apoptosis [6]. In case the immune system is not working properly and is not able to distinguish between its own and foreign structures, autoimmune diseases can occur. Autoimmunity is caused by a hyperreactive immune system, not able to properly differentiate between external and the body's own structures and therefore attacks its own healthy tissues [4]. Other common diseases, like different types of cancer, are also partly caused by a dysfunction of the immune system. In a well working body, cells which exhibit hallmarks of cancer transformation either undergo the process of apoptosis or they are recognized and destroyed by the immune system. Mechanisms of cancer protection by the immune system are based on the detection of antigens or the specific targeting of those cancer cells. Beside those

mechanisms, the immunological memory by the adaptive immune system is also part of cancer protection [2, 7].

1.2 Antigen-presenting-cells (APCs) – gate keepers between innate and adaptive immunity

To carry out its protective task the immune system is utilizing different mechanisms like phagocytosis. On the one hand, phagocytosis fulfills the task of body defense from pathogens. On the other hand, phagocytosis is one of the most important processes in removal of cell debris generated during apoptosis [2].
Antigen presenting cells (APCs) use phagocytosis for antigen uptake and presentation [8]. Therefore, APCs keep the task of immune surveillance on body surfaces by the detection of pathogens.
After processing of the phagocytized antigens, APCs present the microbial fragments on their surface via major histocompatibility complexes (MHCs). Those presented antigens can be detected by other immune cells, like T-cells using their T cell receptors (TCRs) [2]. In the end this cascade leads to the development of the adaptive immune response.
Dendritic cells, as well as macrophages and B-cells, are called professional APCs due to their superior antigen presentation efficiency. Professional APCs present extracellular antigens to helper/regulatory T-cells ($CD4^+$ T-cells) [2] in the context of MHC class II [9]. MHC class I is expressed on all nucleated cells and on platelets. Antigen presentation in context of MHC class I, so-called cross presentation, is used for the presentation of intracellular antigens directly to cytotoxic T-cells ($CD8^+$ T-cells) [2, 9]. The cross presentation is very well performed by LCs and also plays an important role in the body's own tumor defense [9].

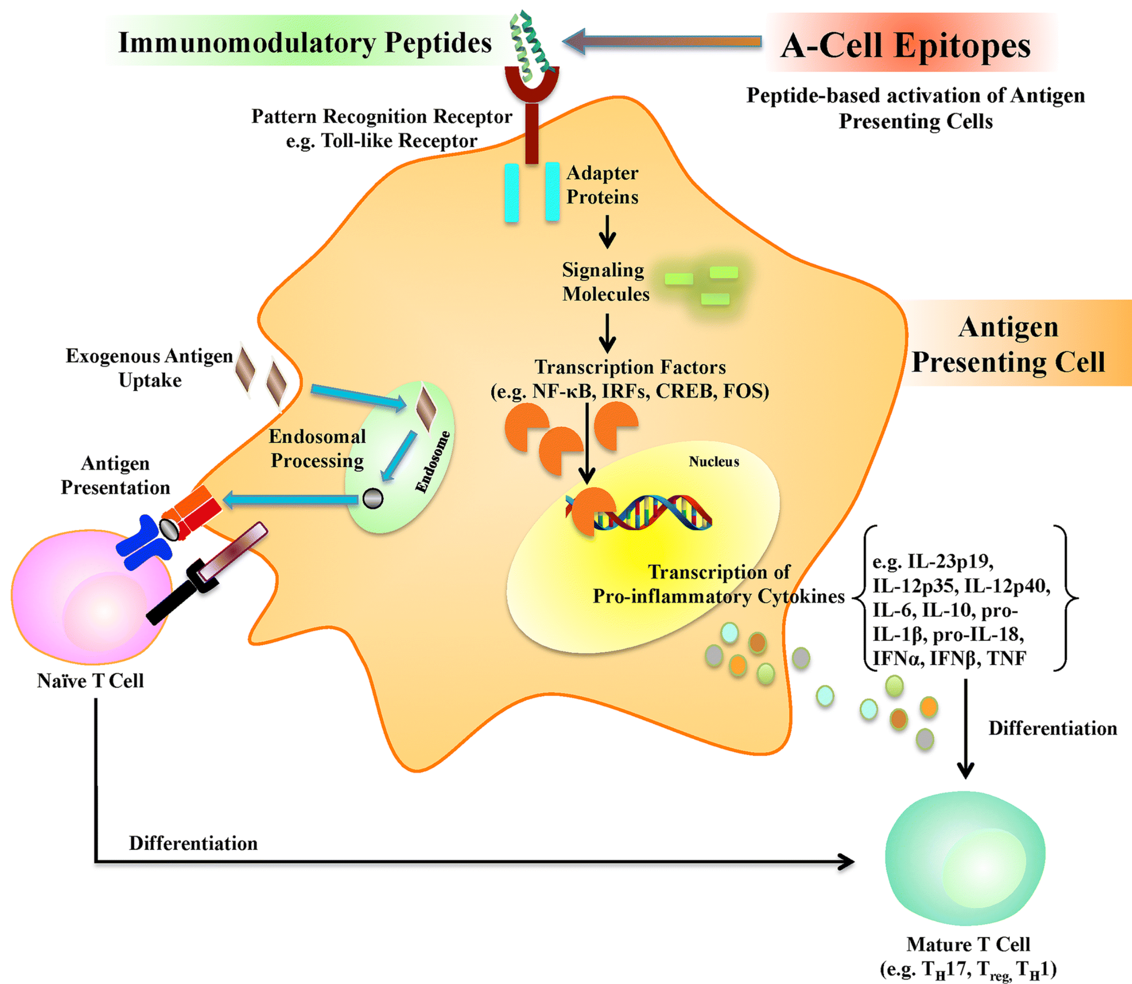


Figure 2. Antigen presenting cells (APCs). APCs have specialized receptors on their surface for the detection of pathogens. After the processing of the ingested antigens they present them via MHCs on their surface to induce further cellular mechanisms. Those mechanisms are performed by different immune cells, especially T-cells. The detection of immunomodulatory peptides also leads to cytokine production by the modulation of transcription factors (Nagpal et al.) [10].

After the detection of pathogens APCs get activated and migrate to the draining lymph nodes (LNs). Activated APCs secrete cytokines, essential for an appropriate immune reaction.

During the migration APCs undergo a process of maturation. Arrived in the LNs APCs interact with T- and B-cells and induce adaptive immunity.

1.3 Skin is a first line defense against the outer environment

Skin creates an effective physical barrier against the outer environment and hosts an important part of the immune system. The two main layers of skin are the epidermis and the dermis, divided by the basement membrane. The skin immune system consists of different DCs populating both in the epidermis and dermis [11]. The epidermal DCs are called Langerhans cells (LCs), compared to the dermal DCs that belong to the major group of interstitial DCs [11].

The anatomical barrier of the epidermis is maintained by the keratinocytes (KCs) which are the most abundant cells in the epidermis. They form cornified layers and are essential for the buildup of a tight epidermal barrier against the dehydration and the invasion of pathogens [12]. Beside the KCs, around 3-5% of all nucleated cells in the epidermis are LCs. Those LCs can be found throughout the epidermis, but they are most abundant in the stratum spinosum [12]. LCs protrude their dendrites via tight junctions through the stratum spinosum up to the stratum corneum where they can detect antigens without destroying the epidermal cell arrangement [12]. One can say, LCs form a great cellular network in the epidermis fitting tightly and interlinking with KCs [11-13].

Concerning the mononuclear phagocytes (MPs), the two cell types populating the skin are DCs and macrophages. The DCs in the skin have the task of transporting antigens to the skin draining lymph nodes where they induce T cell responses [9].

In general MPs lack the expression of surface markers which are found on other cells like neutrophils, T and B cells (CD66b, CD3, CD19 and CD20) but they express MHC class II [9, 14]. As previously mentioned, in human’s MHC class II so far was only found to be expressed by APCs.

1.4 LCs are a subpopulation of DCs

DCs and LCs as Antigen-presenting cells connect the adaptive and innate immune system by their special task of antigen-presentation. DCs reside all over the body in the multiple tissues like the skin and mucosa of respiratory, gastrointestinal and urogenital tissues and they can also be found in the blood and the cornea [15]. The specialized DCs in the skin are named LCs. LCs are environmentally exposed sentinel cells which conduct effective immune surveillance on the body surfaces.

Beside the immunological function, LCs also play an important role in homeostasis [12]. They are involved in the development of pathologies, either by the mediation of inflammation or induction of tolerance [9, 12]. LCs are specialized to perform effective antigen presentation and under normal conditions LCs are the only APCs that maintain the immunosurveillance of the epidermis [9, 16]. In the epidermis they form a cellular network and within this network LCs are the first immunological barrier to the external environment. Human LCs are a subset of DCs defined by the expression of specific surface markers: MHC II^{+} / CD1a^{+}/ CD207^{+}(langerin) / E-cadherin^{+} / CD1c^{+} / CD 11c^{+} and TROP2^{+} [12, 15].

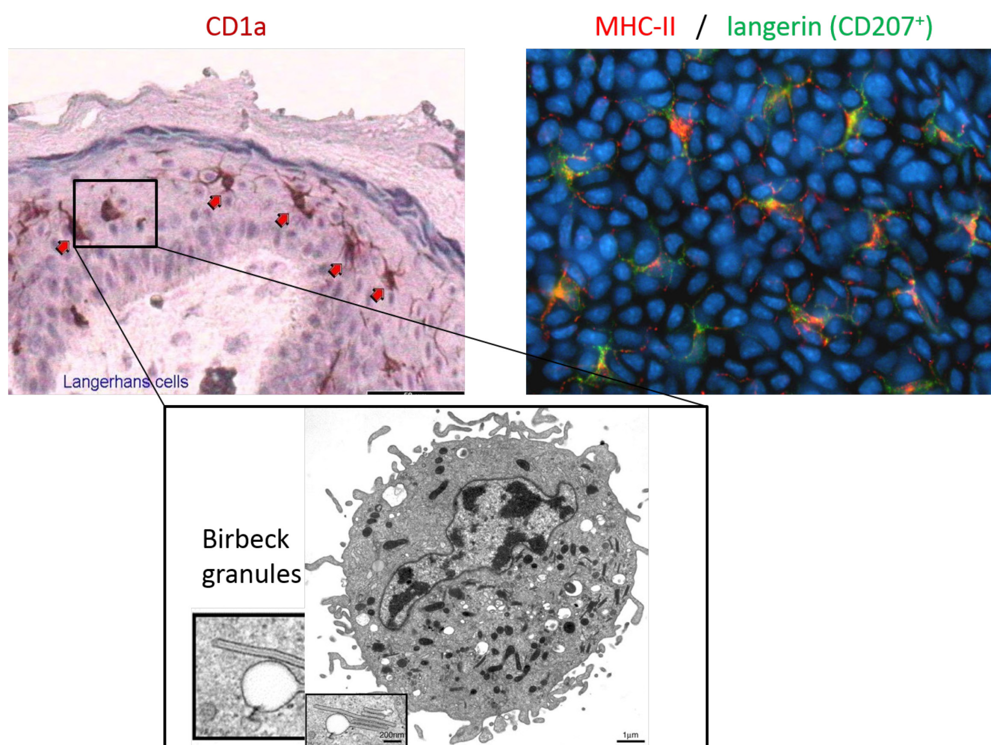


Figure 3. LCs are characterized by the expression of specific surface markers. LCs express CD1a, MHC class II and CD207/langerin responsible for the formation of cytoplasmic ultrastructures called Birbeck granules (images Borek I.).

CD1a and CD1c, both expressed by LCs, are MHC class I related surface molecules associated with the presentation of lipids [12]. Langerin (CD207) is a C-type lectin receptor highly expressed by human and mouse LCs. Sometimes CD207 is also expressed by other DCs connected to the skin immune response and by the immune cells found in the lungs [11]. The CD207 receptor binds

mannose and other related sugars [17]. Additionally LCs contain specialized organelles called Birbeck granules (BG), which aren't identified in any other cell types so far [17]. There is evidence that langerin is stored inside of the BG and it is responsible for their formation [17]. In general epithelial adhesion molecules are essential for the adhesion of LCs to KCs [18]. Therefore, E-Cadherin, Trop2 and epithelial cell adhesion molecule (EpCAM) are important for the regulation of epidermal residence and migration of LCs to the draining LNs [12].

1.5 Development of LCs in humans

Dynamic of the LCs network during steady state and inflammation differ. LC precursors seed the epidermis early in embryonic development [9] and create a network which in the steady state is maintained independently from the bone marrow, by the proliferation of local precursors [19, 20]. Fetal LC precursor cells come from the yolk sac or the fetal liver [9, 21]. Inflammation leads to the reduction in LC number due to their egress from the skin. LCs that have left the epidermis accumulate in the T-cell area of the LNs when they arrive, strikingly this accumulation also occurs during steady state [11, 22]. Still the number of LCs migrating to the LNs increases during inflammation [11]. LC numbers are restored by circulating progenitor cells which are recruited to the inflammatory site [23].
The replenishing progenitor cells are part of the MPs cell system of the bone marrow origin [16, 24]. These cells have the typical LC phenotype, but they still maintain some monocyte characteristics [24]. After the resolution of the inflammation activated LCs disappear, and the steady state network is re-established by the epidermal precursors [20]. In contrast to the self-renewing of macrophages and epidermal LCs in the steady state, there is evidence that maintenance of cDCs and dDCs depends on bone marrow precursors [25].

1.6 *TGF-β family signaling: Role of TGF-β1 and BMP7 in LCs biology*

Transforming growth factor beta 1 (TGF-β1) and bone morphogenetic protein 7 (BMP7) are cytokines that belong to TGF-β family composed out of structurally related cell regulatory proteins. Those cytokines signal via transmembrane type 1 receptors that associate with type 2 receptors [26]. TGF-β1 signals via ALK 5 receptor (TGFβR1) by the activation of SMAD 2/3 (so called canonical signaling) [15]. BMP7 on the other hand signals via ALK3 (BMPR1a) to activate SMAD 1/5/8 [15]. TGF-β1 is also able to coactive the BMP receptor ALK3 [27]. The signaling of both mentioned cytokines via ALK3 leads to *in vitro* human LC differentiation [28].

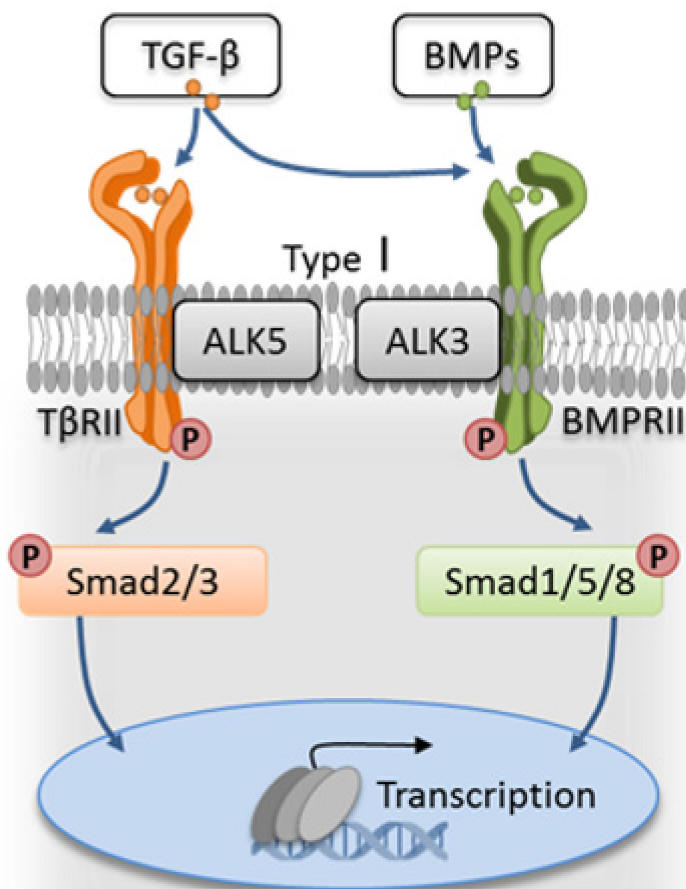


Figure 4. Signaling of the TGF beta superfamily. BMP7 signals via ALK 3 and activates SMAD 1/5/8 compared to TGF-β1 which can signal via ALK 3 and additionally via ALK 5 receptor by the activation of SMAD 2/3 (Strobl et al.) [15].

1.7 *In vitro generation of LCs from CD34⁺ hematopoietic stem and progenitor cells with TGF-β1 or BMP7*

Human hematopoietic stem and progenitor cells (HSPCs) can be used as progenitor cells for the generation of different leukocytes [29]. Isolated and purified HSPCs can be cultured in medium supplemented with cytokines that favor the LC differentiation. The common way of generating LCs *in vitro* is via TGF-β1 [30]. The underlying biology of TGF-β1 for the transformation of LCs is well known and understood. In comparison, our group recently identified BMP7, another factor from the same cytokine family, to be sufficient to fully replace TGF-β1 in the induction of LC differentiation [28].

1.8 *In vitro generation of MoDCs from Peripheral blood CD14⁺ monocytes*

Most of the DCs stem from precursor cells of the bone marrow origin and the two major subgroups of DCs are conventional DCs (cDCs) and plasmacytoid DCs (pDCs) [31]. Each subgroup is characterized by specific surface marker profile. Monocyte derived dendritic cells (MoDCs) are DCs that are generated from monocytes. So far, the moDCs were not associated with one specific subgroup but they gain interest because of their potential function in adaptive immunity [32].
As moDCs are quite similar to cDCs when it comes to morphology and physiology, they are often used for research on dermal DCs [33]. For the generation of MoDCs from peripheral $CD14^+$, monocytes cultures need to be supplemented with interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [34].

1.9 *Toll-like-Receptors (TLRs) as a subclass of Pattern-Recognition Receptors (PPRs)*

APCs are characterized by the expression of specific marker molecules including Pattern-Recognition-Receptors (PRRs) [35]. Membrane bound PRRs are Toll-like-receptors (TLRs) and C-type lectin receptors (CLRs). Besides that, cytoplasmic

PRRs exist, such as NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs) [36, 37].

TLRs are important for the recognition of microbial patterns. They play an important role in the innate immunity. Activation of TLRs by microbial stimuli leads to the development of a broad range of immune responses [38].

PPRs are expressed by large number of different immune cells like DCs, macrophages, monocytes, neutrophils and epithelial cells. They can detect microbial pathogens, so called pathogen-associated molecular patterns (PAMPs) and Damage-associated molecular patterns (DAMPs) [39]. DAMPs are molecules produced by damaged host cells. During apoptosis/necrosis cells can release cellular fragments which can induce a non-infectious, inflammatory response [40].

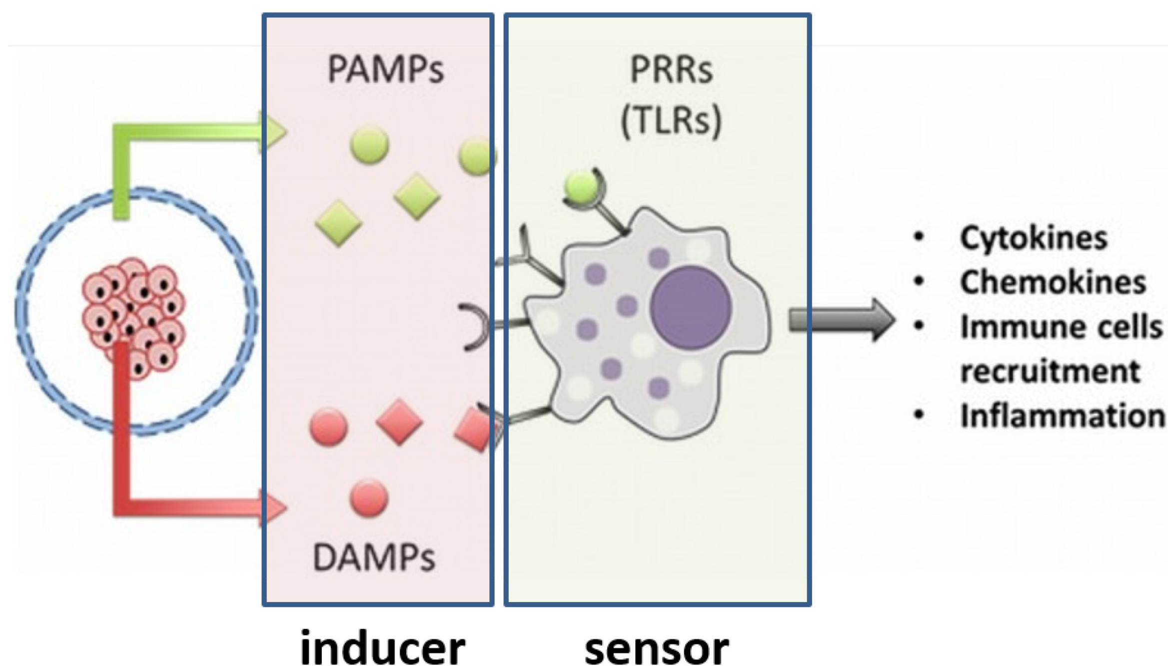


Figure 5. Pattern recognition receptors (PRRs). PPRs expressed by the variety of immune cells can detect PAMPs and DAMPs. Activation of PPRs leads to the release of different cytokines and chemokines which induce the recruitment of immune cells and mediate inflammatory reaction (Paredes Juarez et al.) [39].

So far 10 types of TLRs are known in humans. TLRs can either be extracellular or intracellular: TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 belong to the group of

extracellular and TLR3, TLR7, TLR8, TLR9 to the intracellular receptors [38]. TLR1 is a receptor for peptidoglycan (PGN) and lipoproteins in collaboration with TRL2. For the detection of viruses TLR3 as an intracellular receptor plays an important role. TLR4 is specialized for the recognition of the Lipopolysaccharide (LPS) from bacteria and TLR5 for the identification of flagellated bacteria. TLR6 detects bacterial lipoproteins together with TRL2. TLR7 and TLR8 are also intracellular receptors specialized for the detection of viruses, in comparison TLR9 which is also an intracellular receptor detects bacteria [38, 41-44]. TLRs are considered to be immunostimulatory. Exception from this rule is TLR10 which is believed to have an anti-inflammatory and therefore suppressive function [45, 46]. Some of the TLRs tend to dimerize. As already said before, TLR2 can dimerize with TLR1 and TLR6 for the detection of bacteria and parasites [42].

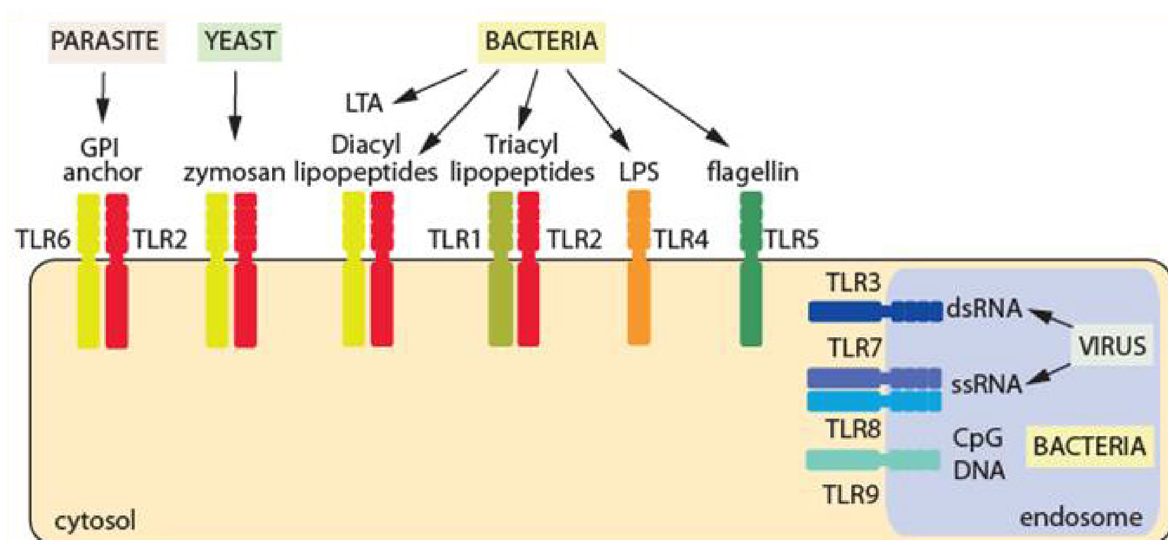


Figure 6. Pattern Recognition Receptors (PRRs): Toll-like receptors (Zaru et al.). [47]

2 Material and Methods

2.1 Objective of the work

There is still a lot of controversy around the role of LCs in immune regulation as their behavior is heavily context dependent. LCs can play an immunosuppressive function as well as induce a potent inflammatory and cytotoxic T-cell response. Hence, there is a need for new models in studies of LC biology. We recently established a novel *in vitro* system where for LC generation we replaced transforming growth factor beta 1 (TGF-β1) with bone morphogenetic protein 7 (BMP7). BMP7 is sufficient to fully replace TGF-β1 in the generation of LCs *in vitro*. This culture conditions give rise to fully differentiated $CD1a^{+}CD207^{+}CD324^{+}$ LCs. The detailed phenotype of these BMP7-dependent LCs is not well characterized, and their biological relevance is not understood.

Hence, we focused on characterization of BMP7-driven LCs and compared them to well-described TGF-β1-dependent LCs. TLRs are an important receptor family that determines responsiveness of LCs and dictates the nature of an adaptive immune response they induce.

To better understand their functional properties, we therefore performed a comparative analysis of the TLR expression profile between TGF-β1 and BMP7 instructed LCs. For the analysis of the expression of TLRs, RNA was isolated from both types of LCs (TGF-β1 and BMP7 generated). After reverse transcription, cDNA was used for quantitative PCR (qPCR).

2.2 Materials

2.2.1 Chemicals and Reagens

2-Mercaptoethanol	Sigma-Aldrich®
Agarose LE	Biozym
Ethanol, abs. 100%, HPLC grade	Chem-Lab nv
Fc Block	BD Biosciences
BD™FACSRinse	BD Biosciences
BD™FACSClean	BD Biosciences
GelRed	FisherBiotec
Nuclease free water	Quiagen
Phosphate buffered Saline (PBS Buffer)	ThermoFisherScientific, USA
RNase Zap®	Sigma-Aldrich®
RNase-free DNase Set	Quiagen
NH_4Cl	Sigma-Aldrich®
KHCO3	Sigma-Aldrich®
Na2EDTA	Sigma-Aldrich®
HCL	Sigma-Aldrich®
BSA 2%	ThermoFisherScientific, USA

2.2.2 Cell Culture

BMP7	ImmunoTools, Germany
FLT3L	PeproTech, UK
GM-CSF	PeproTech, UK
SCF	PeproTech, UK
TGF-β1	R&D Systems, Germany
TNF α	PeproTech, UK
TPO	PeproTech, UK

2.2.3 Primers for Real-time PCR

Primer	Orientation	Sequence 5' -> 3'
HPRT	Fw	GACCAGTCAACAGGGGACAT
	Rev	AACACTTCGTGGGGTCCTTTTC
TLR1	Fw	GGCACCCCTACAAAAGGAATC
	Rev	TGAAGATAATGGCAAAATGGAAG
TLR2	Fw	GCTGCCATTCTCATTCTTCTG
	Rev	GCCACTCCAGGTAGGTCTTG
TLR3	Fw	TCCACCACCAGCAATACAAC
	Rev	AAGCCAAGCAAAGGAATCG
TLR4	Fw	TCATTGTCCTGCAGAAGGTG
	Rev	AGATGTTGCTTCCTGCCAAT
TLR5	Fw	TTGCTCAAACACCTGGACAC
	Rev	CACCACCATGATGAGAGCAC
TLR6	Fw	GACCTACCGCTGAAAACCAA
	Rev	CTCACAATAGGATGGCAGGA
TLR7	Fw	TCCTAAAACTCTGCCCTGTGA
	Rev	GGGAGATGTCTGGTATGTGG
TLR8	Fw	GGGGATCAAAGAGGGAAGAG
	Rev	TTGGGATGTGGAAAGAGACC
TLR9	Fw	CTGCCTTCCTACCCTGTGAG
	Rev	AGAATCATGGAGGTGGTGGA
TLR10	Fw	TGGTTGGATGGTCAGATTCA
	Rev	AGGGCAGATCAAAGTGGAGA

Table 1. Primers for Real-time PCR

2.2.4 Consumable Materials

6 well tissue culture plate	ThermoFisherScientific, USA
24 well tissue culture plate	ThermoFisherScientific, USA
Hard-Shell® PCR Plates 96 wells	Applied Biosystems, USA
Micro Amp™ Optical Adhesive Film	Applied Biosystems, USA
Nitrilhandschuhe	Semper-care

PCR Tube strips 0.2 ml	Eppendorf
PCR tubes 0.2 ml	Eppendorf
Pipette tips 10 µl	Biozym
Pipette tips 200, 1000 µl	Sarstedt
Falcon™ 5ml Polysterene Round-Bottom Tube	ThermoFisherScientific, USA
Reaktionsgefäß 1,5 ml	Eppendorf
Safe Seal-Tips ® 10, 20, 200, 1000µl	Biozym
Serologische Pipette 5, 10, 25ml	Sarstedt
Tube 15, 50 ml	Sarstedt

2.2.5 Laborartory Equipement

Fume hood, ice machine, scales, fridge, freezer, microwave, beaker, flask and cylinder

Autoklave- Thermo Scientific Varioclav	ThermoFisherScientific, USA
Centrifuge 5424R	Eppendorf
Beckmann GS-6 Centrifuge (cell culture)	ThermoFisherScientific, USA
CASY®1 Schärfe System	OLS-Bio
CASYton	OLS-Bio
Biozym sprout Mini Centrifuge	Biozym
CFX96™ Real-Time System	Bio-Rad
Electrophoresis power supply EPS300	Pharma Biotech
Hoefer Scientific Instruments HE99X	Hoeferinc
BioRad ChemiDoc XRS System, PC version	BioRad
BD™CSRFortessa™	BD Bioscience, USA
Grobwaage Kern 572	Kern-Sohn
Magnetrührer Heidolph MR3001	Heidolph-Instruments
Nikon TMS Inverted Microscope	Nikon ®
Ph-electrode SenTix ®	WTW
Pipetierhilfe Pipetus ®	Hirschmann Laborgeräte
Pipetten 10, 20, 100, 200, 1000 µl	Eppendorf
Spectrometer ND-100C	Nano Drop®
HeraSafe KS 18 Mikrobiology Safety Cabinet	ThermoFisherScientific, USA
Inkubationsschrank Binder 37°C 5%CO2 95% rH	Binder

Vortex Schüttler VF2	IKA
Waterbath Haake DC5	

2.2.6 Programms

Bio-Rad CFX Manager 2.0	Bio-Rad
MATLAB	Mathworks
Microsoft Excel	Microsoft
Microsoft Power Point	Microsoft
Microsoft Word	Microsoft
FlowJo software	Tree Star, Inc. USA

2.3 Methods

2.3.1 Isolation of $CD34^+$ hematopoietic stem and progenitor cells from cord blood

Cord blood samples were collected during scheduled caesarian sections at the LKH Graz. First samples were diluted 1:2 with PBS (PBS-solution: 9,55 g PBS in 1 l aq. dest., autoclave, pH 7,2-7,4; filtered sterile). For density gradient centrifugation 20ml of Lymphoprep™ (Axis Shield, Norway) was overlaid with a maximum of 25ml PBS-diluted cord blood, followed by centrifugation for 30min at 400g without break. Buffy coat containing mononuclear cells (MNCs) was collected with a pasteur pipette. For platelet removal MNCs were washed with PBS and centrifuged for 8min at 500g with break.

Next, supernatant was removed, and pellet was resuspended in 5ml ACK lysis buffer (8.29g of 0.15M NH_4Cl, 1g 0.01M KHCO3, 37.2mg 0.1MNa2EDTA, 800ml aq. dest., adjusted pH 7.2-7.4 with HCL, filtered sterile). Samples were incubated for 10 min on ice than washed with PBS and centrifuged for 10min at 100g with low brake. The supernatant was removed, and the pellet was resuspended in 1ml PBS. In case of incomplete erythrocyte lysis (red cell pellet) incubation with AKC lysis buffer was repeated.

$CD34^+$ cells were isolated using EasySep™ Human CD34 Positive Selection Kit (StemCell Technologies™): Isolated cells were counted with CASY®1 (OLS-Bio) and for a maximum of $5x10^8$ cells per preparation, the mononuclear cell suspension was resuspended in 1ml of MACS buffer (250ml of PBS, 0.930g of 1mM EDTA and 50g of 2% BSA, filtered sterile) and transferred to a 12x75mm polystyrene tube. 100µl/ml of EasySep® Positive Selection Cocktail (StemCell Technologies™) was added, properly mixed and incubated at room temperature (RT) for 15min. Next 50µl of EasySep® Magnetic Nanoparticles (StemCell Technologies™) were added, mixed well and incubated for another 10min at RT.

Recommended medium was added to the cell suspension for a total volume of 2.5ml and after mixing, the tube was placed into the EasySep® Magnet (StemCell Technologies™). The following washing step was performed for a total of five times: 5min incubation at RT and removing of the supernatant by one continuous invert motion and keeping the tube upside down for 3 s, while the tube remains in the magnet to ensure that labeled cells stay inside the tube. After removing the

tube from the magnet, 2.5ml of medium were added, the suspension was mixed properly, and the tube was placed back in the magnet and again incubated at RT for 5min. After the 5th washing, cells were resuspended in an appropriate amount of MACS buffer, counted again with CASY and washed one time with PBS (400g, 5min). After sorting the phenotype of the cells was assessed with flow cytometry. For the following experiments only cells with purity ≥90% were used.

2.3.2 Isolation of $CD14^+$ monocytes from buffy coats

For the isolation of $CD14^+$ monocytes, buffy coats from the Transfusion Medicine Department of the Medical University of Graz, Austria were used. For density gradient centrifugation 20ml of Lymphoprep™ was overlaid with a maximum of 25ml heparinized blood, followed by centrifugation for 30min at 400g without break on RT. Monocytes were isolated using the magnetic sorting technique using human anti-CD14 MicroBeads (Miltenyi Biotec, Germany) according to the manufacturer's instructions. After sorting, the phenotype of the cells was assessed with Flow cytometry. For the following experiments only, cells with purity ≥90% were used.

2.3.3 *In vitro* differentiation of LCs

To recover from the isolation $CD34^+$ hematopoietic stem and progenitor cells were cultivated for 3 days in serum free medium (X-vivo™15 medium, Lonza, Switzerland) supplemented with recombinant human cytokines: 50ng/ml stem cell factor (SCF), 50ng/ml human trombopoietin (TPO) and 50ng/ml Fms-related tyrosine kinase 3 ligand (FLT-3L).

After the recovery cells were counted with CASY counter and placed in LC differentiation conditions. Therefore, 40 000 - 50 000 cells were plated in 24 well tissue culture plates in serum-free medium (CellGenix® GMP DC Medium, CellGenix, Germany) supplemented with 2.5 mM GlutaMax (Gibco) and the following human recombinant cytokines: 50ng/ml FLT-3L, 100ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), 2.5ng/ml tumor necrosis factor alpha (TNFα), 25ng/ml SCF, and either 1ng/ml TGF-β1 or 200ng/ml BMP7. Cells in BMP7 supplemented cultures were split during the course of differentiation on

day 3 and 6 due to significant proliferation in contrast to TGF-β1 generated LCs. For the splitting of cells 1ml of fresh BMP7-containing differentiation medium was added and then after mixing, cells were split into two separate wells.

2.3.4 *In vitro* differentiation of MoDCs and stimulation with LPS

For the generation of monocyte derived dendritic cells (MoDCs) 60 000 of peripheral blood $CD14^+$ monocytes were plated in 6-well tissue culture plates in RPMI-1640 (Sigma-Aldrich, USA) supplemented with 10% FBS, 35ng/ml interleukin 4 (IL-4) and 100ng/ml GM-CSF. Cells were cultivated for 6 days. The culture conditions provide a selective pressure where on the last day of culture ≥80% of the cells have a moDC phenotype ($CD1a^+CD11b^+$).
For the stimulation of MoDCs, cells were washed with 1ml PBS and afterwards stimulated with 100ng/ml LPS (LPS-EK Ultrapure, InvivoGen, tlrl-peklps) for 12 hours (overnight). Cells were harvested from culture plates after their incubation on ice for 30min to disturb the clusters. Cells were washed two times with PBS and the supernatant was removed after centrifugation on 300g for 5min.

2.3.5 Magnetic cell sorting (MACS)

Magnetic cell sorting was used to positively select different cell populations according to their surface markers. First, cells were harvested from culture plates after 7 days of differentiation and rinsed two times with PBS. To disrupt the LC clusters, cells suspended in PBS were incubated on ice for 15min and mixed by pipetting. Disruption step was repeated two times followed by centrifugation at 300g for 5min.
Up to $1x10^7$ cells, were resuspended in 1ml MACS buffer (Miltenyi Biotec, Germany) and washed one more time. Samples were resuspended in 80µl MACS buffer and 20µl of $CD207^+$ (Langerin) MicroBeads human (Miltenyi Biotec, Germany) were added, followed by incubation of 15min in 4°C. After another washing with 2ml of MACS buffer (500g, 10min) cells were resuspended in 500µl MACS buffer. Cells were directly put on the activated column and washed three times with 3ml of MACS buffer. Finally, the column was removed from the magnet and MACS labeled LCs were flushed out with 5ml of MACS buffer. Collected LCs

were washed one more time (500g, 5min) and resuspended in calcium and magnesium-free PBS (CMF-PBS, ThermoFisher Scientific) before the further processing.

2.3.6 Flow-cytometry

$CD34^+$ cord blood stem cells, peripheral blood $CD14^+$ monocytes, MACS-sorted LCs and monocyte derived dendritic cells (MoDCs) were stained for surface markers to determine the success of the enrichment steps. Cells were harvested and washed one time with PBS (300g, 5min). Pellet was resuspended in 100µl PBS and transferred to polystyrene tubes. At least 100 000 cells were used for each staining. 10µl of F_c-block (50% human serum) was added to each tube to block unspecific binding and tubes were incubated on ice for 10min. For the staining a master mix of antibodies was prepared, and all antibodies were pre-diluted with PBS:

$CD34^+$ cord blood stem cells:	
CD34-PE Antibody (BD Biosciencies)	1µl
peripheral blood $CD14^+$ monocytes:	
CD14 PE (Miltenyi)	1µl
MACS-LCs:	
CD1a-Pacific Blue/Brilliant Violet 421 421 = CD1a HI149 (BD Biosiences)	1µl
CD207(langerin)-PE = CD207 DCGM4 (Beckman Coulter)	1µl
CD11b-PE-Cy7 = ICRF44 (BioLegend)	1µl
CadE-APC = CD324/E-cadherin 67A4 (BD Biosiences)	1µl
Monocyte derived dendritic cells (MoDCs):	
CD1a BV421 (Biolegend)	1µl
CD11b PE (Biolegend)	1µl

Table 2: Flow cytometry antibodies

Antibodies were added to the samples, mixed and incubated for 25min in 4°C. All tubes were washed with 2ml of FACS wash buffer (0.1% BSA + azide in PBS) and centrifuged at 500g for 5min. Supernatant was discarded, cells were resuspended in 300µl of PBS and analyzed on BD LRS Fortessa (BD Biosciences) flow cytometer.

2.3.7 RNA-Isolation

At first, the workplace and the instruments were cleaned with RNase Zap (Sigma-Aldrich®). For isolation of total RNA from MACS-purified LCs, RNeasy® Micro Kit (50) (Quiagen) was used. For each isolation $1x10^5$ cells were pelleted and resuspended in 350 µl RLT Buffer with 3.5 µl β-Mercaptoethanol and homogenized by pipetting up and down 8-10 times. After addition of 350 µl of 70% EtOH and another mixing, the sample was transferred to a RNeasy column and centrifuged at 8000g for 15 s. After washing the column with RW1 Buffer (8000g, 15 s), 80 µl DNase (10 µl DNase stock solution + 70 µl RDD buffer) was added directly on the column and incubated for 15min at RT. Samples were washed with 350 µl RW1 buffer one more time (8000g, 15 s) and 500 µl buffer RPE was added on the column and centrifugated 15 s at 8000g. After addition of 500 µl of 80% EtOH, samples were spun down 2 min at 8000g. To remove any residual liquid, columns were centrifugated with open lid 5 min at full speed. To eluate RNA, 14 µl RNase free water was added directly on the column and spun down for 1 min at full speed. Eluted RNA was directly subjected to the reverse transcription.

2.3.8 Quantification and quality control of RNA

To determine the concentration of isolated RNA, 1.5 µl of each sample was analyzed with Spectrometer ND-100C (Nano Drop®). Only RNA with 260/280 ratio 1.8 - 2.1 was used for further steps.
Gel electrophoresis was used to determine integrity of isolated RNA. Therefore, 1% agarose gel was made, using TAE buffer (4.844 g 40mM Tris base, 1.21 ml 20mM Acetic Acid, 0.372g 1mM EDTA). Agarose in TAE buffer was heated up until completely dissolved. For the visualization of RNA GelRed (FisherBiotec) was

added to the agarose mix. Electrophoresis was run at 70 V for 40 min and gel was pictured with Bio-Rad ChemiDoc XRS System.

2.3.9 RT-PCR

After isolation and quality check of RNA, cDNA was generated. For the reverse transcription High-Capacity cDNA Reverse Transcriptase Kit was used (Thermo Fisher). Reagents and samples were always stored on ice. Samples were prepared in 0.2ml PCR tubes according to table 3.

RNA template	2000ng
10x RT Random Primers	2 µl
10x RT Buffer	2 µl
25x dNTP Mix (100mM)	0.8 µl
Reverse Transcriptase	1 µl
Aq. dest.	Ad 10 µl
Final volume	20 µl

Table 3. Preparation of RT-PCR tubes.

The transcription was performed without the use of RNAse inhibitor. Samples were incubated in the C1000™ Thermal Cycler (Bio-Rad) according to the following program (table 4).

Incubation program	
Temperature of lid	105.3°
Total volume of reaction	20µl
Denaturation	25° for 10min
Primer extension	37° for 120min
cDNA synthesis	85° for 5min
Reaction termination	4°

Table 4. RT-PCR incubation program.

For negative control samples without RNA and without enzyme were used. All samples were run with template concentration of 2000 ng. Depending on the

efficiency of reverse transcription all samples were diluted 1:5 before processing with qPCR.

2.3.10 qPCR

For qPCR Applied Biosystems Fast SYBR Green Kit (Applied Biosystems, USA) was used. Reactions were carried out in CFX96™ Real-Time System (BioRad). All steps were performed on ice and samples were prepared in a 96 well plate. Each reaction contained ingredients listed in table 5.

Template-cDNA	1 µl
Primer (f*orward* and *reverse*)	2 µl
aq. dest.	2 µl
iQ SYBR-Green Supermix	5 µl
Final volume	10 µl

Table 5. Preparation of qPCR plates.

Samples were incubated in the CFX96™ Real-Time System Bio-Rad according to the following program (table 6).

Incubation program	
Denaturation	95°C 5min
40 cycles of:	
Hybridization	95°C 15sec
Elongation	60°C 60sec

Table 6. qPCR incubation program.

For the relative quantification Bio-Rad CFX Manager 2.0 was used and genes were normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Therefore, difference of Ct-values of TLR genes and HPRT were calculated. The ∆Ct-values show how strong genes are expressed in relation to the house keeping gene. The lower the value is, the higher the gene is expressed.

2.3.11 Statistical Analysis

Statistical analysis was performed using the paired, 2-tailed Student *t* test; *P* values less than <0.05 were considered significant.

3 Results

3.1 Experimental workflow

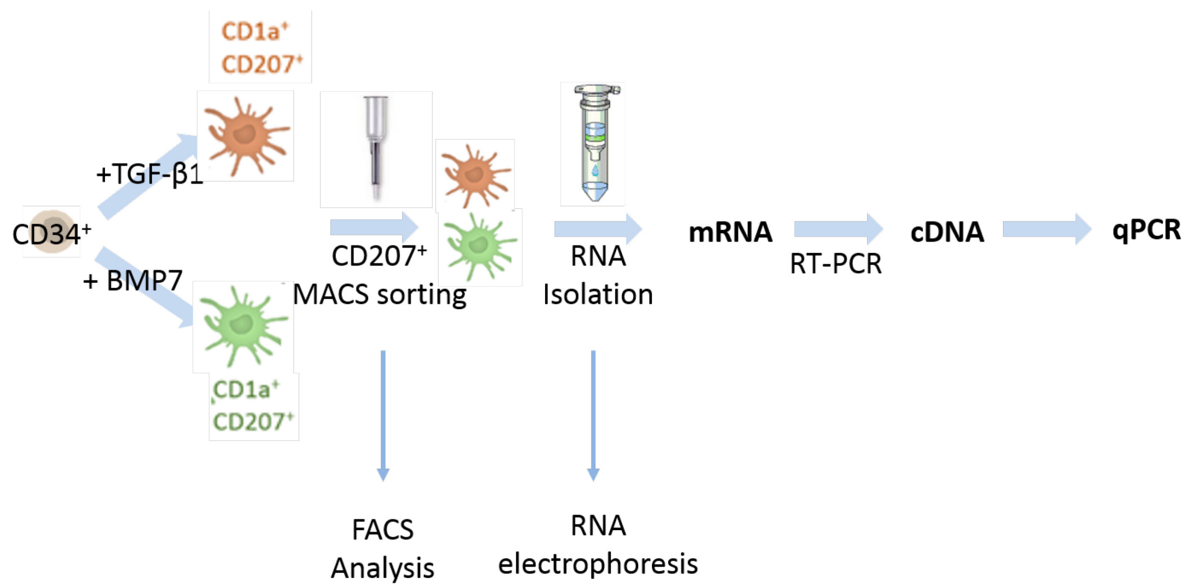


Figure 7. Workflow. CD34+ hematopoietic stem and progenitor cells were cultivated either with TGF-β1 or BMP7 to generate LCs. For the analysis of the expression of TLRs, RNA was isolated from both types of MACS sorted LCs. Reverse transcription was performed, and the cDNA was used for quantitative PCR (qPCR).

3.2 Isolation of CD34⁺ hematopoietic stem and progenitor cells from human cord blood and in vitro generation of Langerhans cells (LCs)

In this work, the expression profile of the Toll like receptors (TLRs) between TGF-β1 and BMP7-driven LCs were compared. For the *in vitro* generation of LCs $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) isolated from cord blood were used. HSPCs were isolated by magnetic-activated cell sorting (MACS) and their phenotype was confirmed by using flow cytometry. Only cells with purity 90% were used for the LC generation cultures (Fig.8).

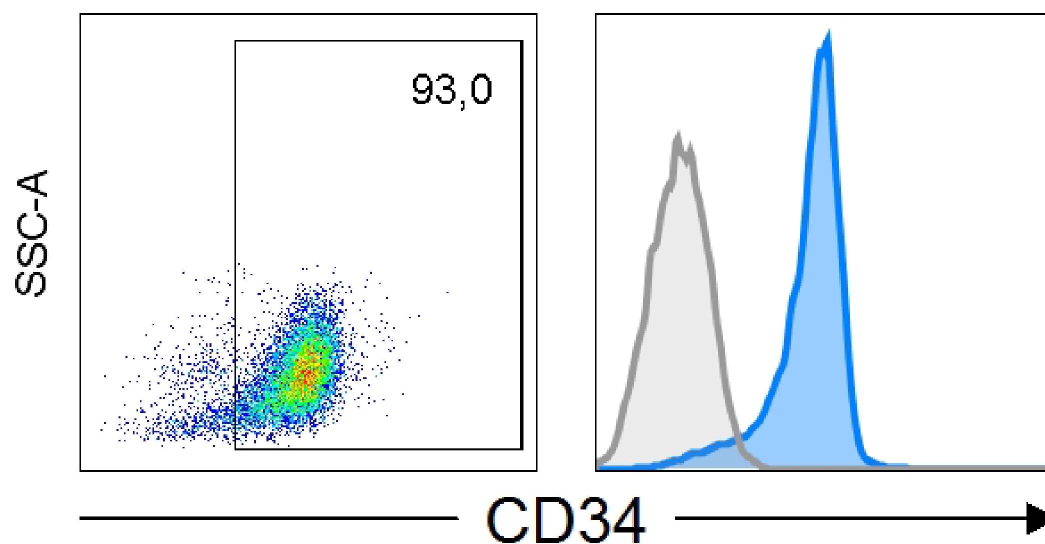
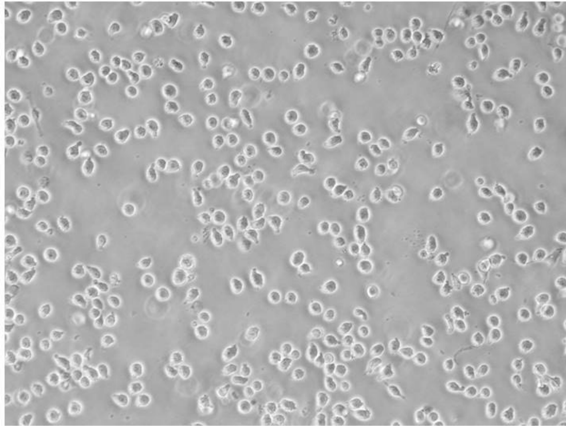


Figure 8. Purity of MACS sorted $CD34^+$ hematopoietic stem and progenitor cells isolated from umbilical cord blood. Stem cells were isolated from human cord blood with MACS sorting. Purity was measured by flow cytometry. Dot plot (left) and histogram (right) show average purity assed based on CD34 expression of isolated cells.

In the next step LCs were generated from purified $CD34^+$ cells. The cells were cultured for 3 days in expansion mix (serum free medium supplemented with SCF, FLT3-L and TPO). For LC differentiation cells were re-plated and cultured for additional 7 days in LC-generating mix with either TGF-β1 or BMP7. During differentiation both types of LCs formed E-cadherin mediated clusters which contain $CD1a^+CD207^+$cells (Fig. 9).



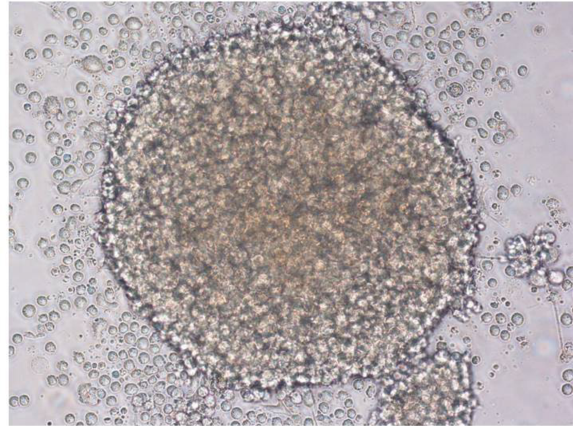


Figure 9. During the process of differentiation LCs form typical E-cadherin mediated clusters. Bright field microscopy of $CD34^+$ hematopoietic stem and progenitor cells on day 0 (left side) and differentiated LCs on day 7 (right side). On day 0 $CD34^+$ stem cells appear in single cell suspension compared to day 7 were differentiated LCs form clusters.

3.3 MACS sorting of LCs

Since not all $CD34^+$ cells differentiate into LCs, cells need to be sorted after differentiation. We used magnetic beads which target $CD207^+$ surface epitopes on LCs.

TGF-β1 and BMP7 generated LCs were MACS sorted according to their CD207 expression. The purity of the sorted cells was analyzed by flow cytometry. The purity of the cells before and after the sorting was compared: Prior to the sorting TGF-β1 supplemented cultures contained ± 50% of $CD207^+$ cells compared to BMP7 supplemented cultures which on average yielded much lower percentages of LCs (around 15%). After the sorting there was an enrichment of up to 90% for both conditions (Fig. 10).

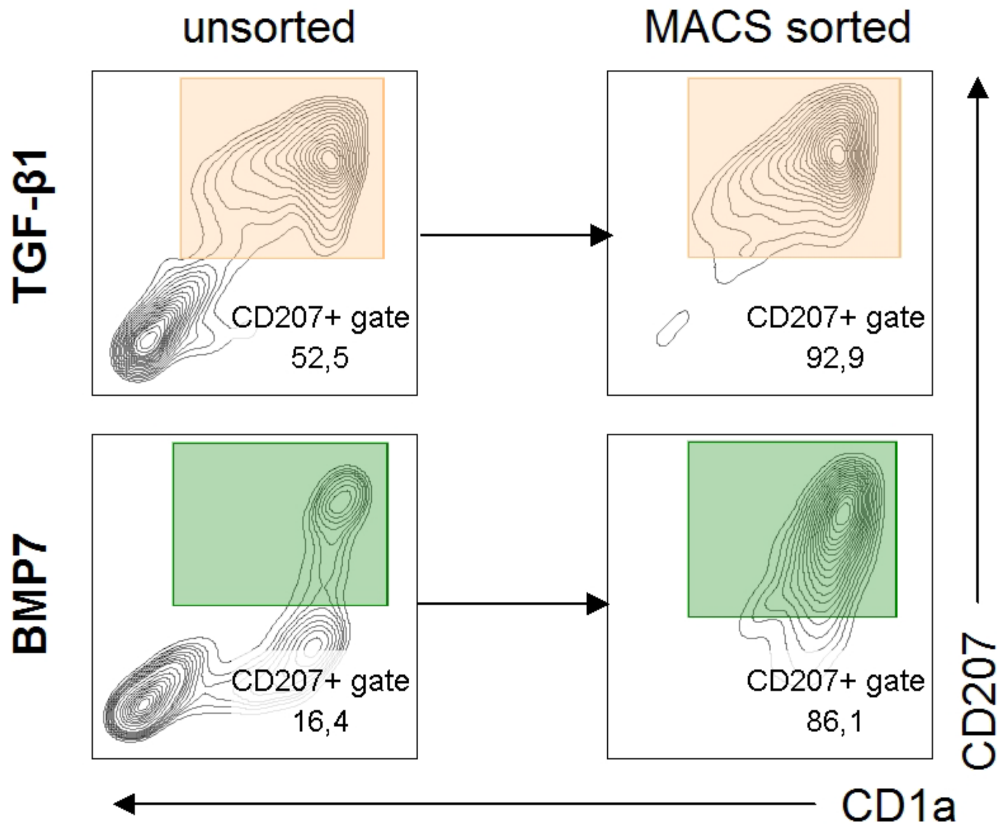


Figure 10. MACS-sorting allows significant enrichment of CD207⁺ LCs from heterogeneous cultures. Cells harvested on day 7 after cultivation in LC-generation mix either with TGF-β1 or BMP7 were sorted with CD207⁺ MicroBeads. Purity was determined by flow cytometry. Cells positive for both CD207 and CD1a were considered as LCs.

3.4 Analysis of RNA integrity on agarose gel and spectrophotometer

The spectrophotometer measures RNA concentration via UV-absorption. RNA has a maximum absorption at 260 nm (an A260 of 1.0 is equivalent to 40 µg/mL of RNA) and A280 measures the contamination with aromatic proteins. The purity of the sample is stated as a 260/280 ratio, which must be between 1.8 - 2.1 to ensure high quality of RNA.

Total RNA was isolated from purified LCs. Afterwards, the concentration and purity were measured by spectrophotometer to ensure only good quality RNA template was introduced to the downstream analysis. We analyzed RNA integrity on agarose gel. Non-degraded RNA presents on the agarose gel as two clear bands,

the upper band reflecting the 28S large rRNA subunit and the lower band reflecting the 18S small rRNA subunit. In analyzed samples, two bands were clearly visible (Fig. 11). Only samples yielding to clear bands were processed for further use.

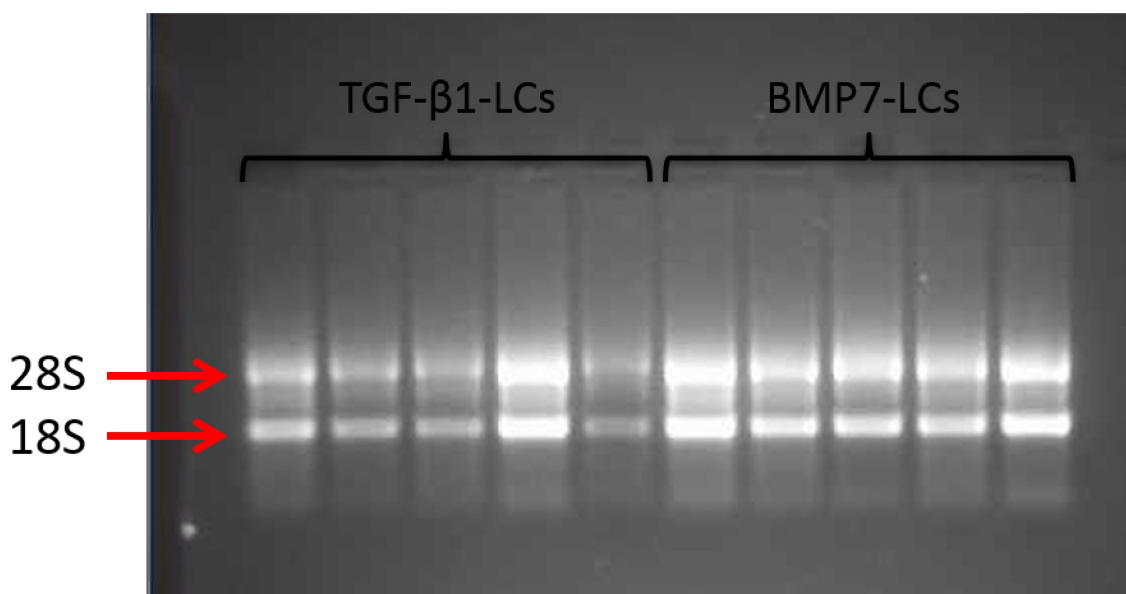


Figure 11. RNA integrity of TGF-ß1/BMP7 generated LCs visualized on agarose gel. RNA was isolated from MACS sorted LCs with RNase Minelute Kit. For each sample 2µl of isolated mRNA was run on GelRed Agarose Gel (70V, 40min). Upper band reflects 28S and lower band 18S ribosomal RNA (rRNA). Gel was visualized with Bio-Rad ChemiDoc XRS System.

3.5 BMP7-generated LCs express higher level of TLR2 relative to TGF-β1-generated LCs

RNA from TGF-β1/BMP7 generated LCs was reverse transcribed to obtain cDNA template for quantitative PCR (qPCR). In qPCR during the process of amplification, the amount of PCR product is quantified by the increase in florescence. SYBR green is a fluorescent dye that intercalates into double stranded DNA what enables detection of newly synthesized double-stranded DNA. When the signal passes a threshold a certain Ct-value is given. As an endogenous control housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used. qPCR was performed to assess expression of TLR1–10 in both cell types. In BMP7 generated LCs RNA for TLR2 was significantly more

expressed compared to TGF-β1 LCs. On the other hand, TLR1, TLR6 and TLR10 had a higher expression in TGF-β1 LCs relative to BMP7 LCs. For the other Toll like receptors (i.e. TLR3, TLR4, TLR5, TLR7, TLR8 and TLR9) there was no detectable expression neither in TGF-β1 nor in BMP7 dependent LCs (Fig. 12). In summary, BMP7-generated LCs on the one hand express significantly higher levels of TLR2 compared to TGF-ß1-generated LCs. On the other hand, TGF-β1-generated LCs express higher levels of TLR1, TLR6 and TLR10, in comparison to BMP7-LCs.

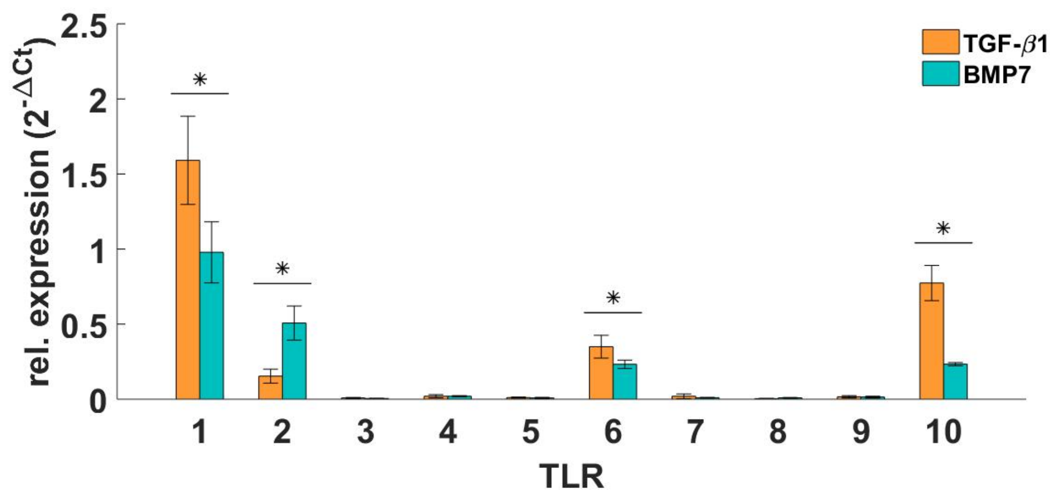


Figure 12. BMP7-dependent LCs differ in TLR expression profile from TGF-β1-dependent LCs. CD34+ cells were cultivated in LC generation mix either with TGF-β1 or BMP7. After 7 days RNA was isolated, and reverse transcribed into cDNA. qPCR was performed for TLR1 - 10. Values were analyzed with ΔCt method and normalized to HPRT. qPCR was performed in triplicates (n=4, ±SD, 2-tailed students t-test, p≤0.05*).

3.6 MoDCs express a greater variety of TLRs than LCs

RNA from monocyte derived dendritic cells (MoDCs) stimulated/or not with LPS was reverse transcribed to obtain cDNA template. Quantitative real-time PCR (qRT-PCR) was performed to assess relative expression of TLR1–10 in both conditions. In general, MoDCs express TLR1, TLR2, TLR3, TLR4, TLR6 and TLR8 and they lack the expression of TLR5, TLR7, TLR9 and TLR10. Stimulation with LPS leads to downregulation of all analyzed TLRs. (Fig. 13).

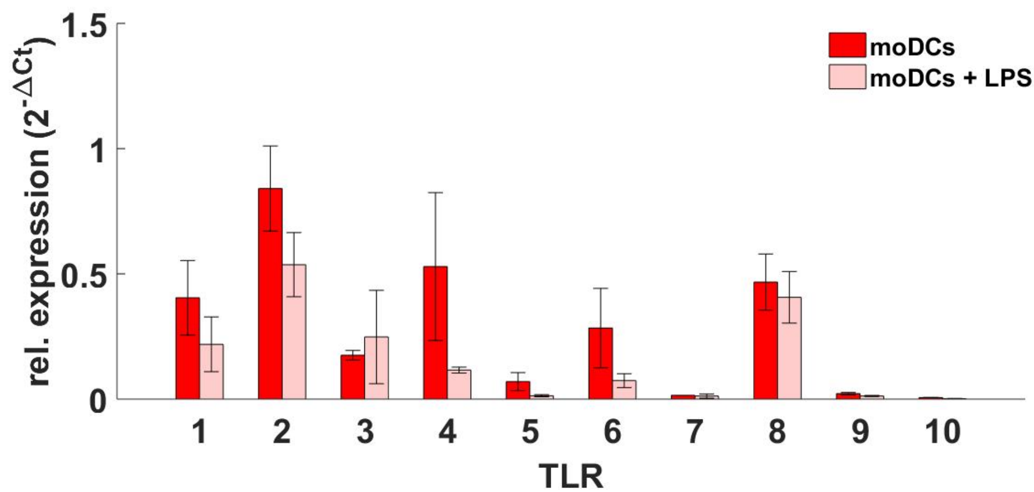


Figure 13. MoDCs express TLR1, TLR2, TLR3, TLR4, TLR6 and TLR8. After the stimulation with LPS moDCs downregulate the expression of TLRs. MoDCs were generated from $CD14^+$ peripheral blood monocytes by IL-4 and GM-CSF cultures. After 6 days either RNA was isolated directly or moDCs were stimulated with LPS and RNA was isolated afterwards. RNA was reverse transcribed into cDNA and qPCR was performed for TLR1-10. Values were analyzed with ΔCt method and normalized to HPRT. qPCR was performed in triplicates (n=3, ±SD, 2-tailed students t-test, no significance).

4 Discussion

To verify the collected data concerning the expression of TLRs, the results of the qPCR were compared to previously published micro array profiling of TGF-β1- vs. BMP7-generated LCs.

The micro array can be found in Yasmin N. at el, data set GSE49085 and was provided and analyzed by Borek I.(http://jem.rupress.org/content/210/12/2597)

	TGF			BMP		
	d1	**d2**	**d3**	**d1**	**d2**	**d3**
TLR1	3650	3401	4907	3522	4329	4334
TLR2	1215	276	229	1341	758	1598
TLR3	2	6	37	27	6	30
TLR4	103	76	42	42	40	66
TLR5	6	57	9	59	9	115
TLR6	426	322	394	455	479	458
TLR7	13	146	283	76	547	1009
TLR8	126	77	8	102	112	137
TLR9	62	74	12	18	65	78
TLR10	388	395	354	409	783	585

Figure 14. Micro array analysis of TGF-β1 (TGF) and BMP7 (BMP) LCs. Unpublished data from ,,Identification of bone morphogenetic protein 7 (BMP7) as an instructive factor for human epidermal Langerhans cell differentiation'' [28] by Yasmin N. at el data set GSE49085.

The micro array analysis showed that both TGF-β1 and BMP7 generated LCs express TRL1, 2, 6 and 10. TLR2 levels were significantly higher in BMP7-LCs compared to TGF-β1-LCs. Other TLRs are not expressed by either of analyzed LCs. Those microarray results were confirmed by the subsequent qPCR analysis performed in this work (Fig. 12).

TLRs are not only expressed by LCs but also by other DCs in the skin. TLRs are Pattern recognition receptors and for the detection of microorganisms invading the body [35]. Within the skin the LCs of the epidermis constantly check the outer environment for microorganisms, like bacteria and participate in the epidermal barrier homeostatic maintenance [15].

As stated before TLR1, TLR2, TLR4 and TLR5 are important for the extracellular and TLR9 for the intracellular detection of microorganisms. For the detection of viruses TLR3, TLR7 and TLR8 are of great importance.

Dermal DCs express a different TLR profile than epidermal LCs. The different TLR profiles results in variable responsiveness to microbial ligands. In this work the TLR expression profile of both LCs and moDCs was analyzed. As already said before, moDCs are often used as a model of dermal DCs as they have similar phenotype and function.

On one hand, to promote tolerance towards commensal bacteria and prevent improper immune activation, LCs lack TLR4, TLR5 and TLR9 which are important for the recognition of bacterial ligands [15, 48]. Conversely, they express high levels of other TLRs involved in detection of viruses.

On the other hand, DCs in the dermis express all TLRs except TLR9 and TLR10. Therefore, they can detect both bacteria and viruses. Especially TLR4 which is critical for the detection of bacterial LPS is highly expressed by dermal DCs.

Different responsiveness to TLR stimulation between LCs and DCs is of particular importance for the skin homeostasis. LCs seem to have a tolerance to wide diversity of bacteria which colonize the skin surface. In case bacteria breach the skin barrier and reach a deeper layer of the skin, they are immediately detected by dermal DCs.

One of the major bacteria colonizing the skin is *Staphylococcus epidermidis which* fulfills a protective function [49]. Iwase et al. showed, that *Staphylococcus epidermidis* secretes a serine protease called Esp. This serine protease inhibits biofilm formation and nasal colonization by *Staphylococcus aureus* [50]. Therefore, it is of great importance that LCs in the epidermis have a certain tolerance to commensal bacteria like *Staphylococcus epidermidis* colonizing the skin.

Peptidoglycan (PGN) is a polymer forming the cell wall of most bacteria. In gram-positive bacteria this PGN layer is much thicker than in gram-negative bacteria but it is present in both. Detection of PGN by immune cells leads to their stimulation and subsequent anti-microbial immune response. TLR2 dimerizes with TLR1 and detects PGN and lipoproteins. Yasmin et al. showed that PGN-stimulated BMP7-LCs produce much higher amounts of cytokines (e.g. TNF, IL-1ß, IL-6, IL-10, IL12p40 and IL12p70) than TGF-β1-LCs [28]. The higher expression of TLR2 by BMP7-LCs explains the more effective stimulation with PGN compared to TGF-β1-LCs shown by Yasmin et al. As BMP7-LCs have a higher expression of TLR2 (Fig. 12) and increased cytokine production compared to TGF-β1-LCs they might be more sensitive in the detection of bacteria.

Autoimmune diseases are systemic diseases characterized by over-reactiveness of the immune system. Psoriasis is a systemic autoimmune disease with inflammatory skin lesions. Psoriasis is driven by inflammatory cytokines involved in T-cell responses: TH17 cells are specialized T helper cells which produce IL-17 after stimulation [51] and seem to be involved in many inflammatory and autoimmune diseases [52]. Above all Th1 and Th17 cells seem to participate in the pathology of psoriasis [53]. Th17 cells are also important in the defense against extracellular microbiomes and participate in the pathogenesis of cancer [54, 55].

Aliahmadi et al. showed that LCs activated via TLR2 are potent inducers of Th17 T-cell response [56]. Stimulation of LCs with TLR2 agonists like PGN leads to the production of IL-6, and the stimulation of DCs with PRR agonists like LPS leads to the production of IL-6 and IL-23 by DCs [57, 58]. Both cytokines, IL-6 and IL-23 are important for the regulation of Th17 cells. IL-6 affects polarization of naïve Th cells into Th17 cells and IL-23 might be important for the outgrowth of differentiated Th17 cells [54-56, 59]. The higher expression of TLR2 by BMP7-LCs might make them more potent inducers of Th17 T-cell responses compared to TGF-β1-LCs.
Another approach for the explanation of the more sensitive reaction of BMP7-LCs to bacteria was made by Chandran et al [60]. They compared TLR2 and TLR4 signaling on APCs in primary and memory CD4 T cell responses. They showed that in vivo engagement of TLR2 by the TLR2 agonist Pam3CysK resulted in the

activation of influenza hemagglutinin (HA)-specific CD4 T cells that produced IL-2 and IL-17 as a sign of stimulation. In comparison the stimulation with HA peptide and the TLR4 agonist LPS leads to a lower frequency of HA-specific naive CD4 T cells and less production of IFN-gamma [60]. As BMP7-LCs have a higher expression of TLR2 they might be more sensitive to bacterial stimulation and upon stimulation react with a higher cytokine secretion.

Typically, ligation of TLR receptors mediates proinflammatory responses. TLR10 is an orphan receptor with a unique anti-inflammatory function [46]. As demonstrated in this work, TGF-β1-LCs have a high expression of TLR10 compared to BMP7-LCs (Fig. 12). Therefore, TGF-β1-driven LCs might exhibit more immunomodulatory properties

Oosting et al. showed that TLR10 with its mainly suppressive function is the only known anti-inflammatory PRR so far, and the only one able to reduce TLR2 responsiveness [46]. Oosting et al. showed that blocking of TLR10 by antagonistic antibodies resulted in a higher production of inflammatory cytokines especially after TLR2-mediated stimulation. They also showed that human TLR10 transgenic mice have less sings of inflammation after stimulation with the TLR2 ligand pam3CSK4 [46]. As TGF-β1-LCs have significant higher levels of TLR10 one could assume that among the LCs especially the TGF-β1-LCs have an anti-inflammatory position.

After microbial stimulation of TLRs few downstream signaling cascades can be activated. TLR signaling can be divided into MyD88-dependent and MyD88-independent signaling, for example by TIRAP/Mal signaling [61]. The MyD88-dependent signaling is common in almost all TLR pathways and there is evidence that it might contribute to the induction of cell death [62].

In mice MyD88 is an adapter protein which plays an crucial role in the activation of the transcription factor NF-κB which is essential in the signaling of TLRs [61]. TIRAP/mal is another factor in the TLR signaling pathway and Arancibia, et al. showed that TIRAP-deficient mice had less production of cytokines and activation of NF-κB when TLR2 and TLR4 were activated [61, 63, 64].

Jiang et al. showed in stably transfected human myelomonocytic U937 cells that TRL10 suppressed the production of many cytokines and that this suppression

affects both MyD88-dependent and -independent pathways [45]. They also showed that human mononuclear cells treated with a monoclonal anti-TLR10 antibody had impaired production of proinflammatory cytokines after stimulation with LPS [45]. Therefore, Jiang et al. showed that TRL10 is a negative regulator of the MyD88-dependent but also the MyD88-independent signaling of TLRs [45]. The fact that TLR10 Is a negative regulator of both signaling ways supports the idea that TGF-β1-LCs with a higher TLR10 expression are anti-inflammatory.

In summary BMP7- and TGF-β1-generated LCs behave unequal in their response to microbial simulation. This difference, at least in some part, is due to their different TLR-receptor profile. BMP7-LCs show a high expression of TLR2 what makes them very PGN-responsive. This could result in a higher cytokine production and therefore more extensive inflammation. In comparison TGF-β1-LCs with a higher TLR10 expression might play an anti-inflammatory role in the epidermis.

5 Literature

1. Vesely, M.D., et al., *Natural innate and adaptive immunity to cancer.* Annu Rev Immunol, 2011. **29**: p. 235-71.
2. Charles A Janeway, J., Paul Travers, Mark Walport, and Mark J Shlomchik., *Immunobiology, 5th edition The Immune System in Health and Disease*. 2001, New York: Garland Science.
3. Abdallah, F., L. Mijouin, and C. Pichon, *Skin Immune Landscape: Inside and Outside the Organism.* Mediators Inflamm, 2017. **2017**: p. 5095293.
4. Nicholson, L.B., *The immune system.* Essays Biochem, 2016. **60**(3): p. 275-301.
5. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy.* Nat Rev Cancer, 2004. **4**(1): p. 11-22.
6. Rock, K.L., J.J. Lai, and H. Kono, *Innate and adaptive immune responses to cell death.* Immunol Rev, 2011. **243**(1): p. 191-205.
7. Pandya, P.H., et al., *The Immune System in Cancer Pathogenesis: Potential Therapeutic Approaches.* J Immunol Res, 2016. **2016**: p. 4273943.
8. Rosales, C. and E. Uribe-Querol, *Phagocytosis: A Fundamental Process in Immunity.* Biomed Res Int, 2017. **2017**: p. 9042851.
9. Kashem, S.W., M. Haniffa, and D.H. Kaplan, *Antigen-Presenting Cells in the Skin.* Annu Rev Immunol, 2017. **35**: p. 469-499.
10. Nagpal, G., et al., *Computer-aided prediction of antigen presenting cell modulators for designing peptide-based vaccine adjuvants.* J Transl Med, 2018. **16**(1): p. 181.
11. Merad, M., F. Ginhoux, and M. Collin, *Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells.* Nat Rev Immunol, 2008. **8**(12): p. 935-47.

12. Deckers, J., H. Hammad, and E. Hoste, *Langerhans Cells: Sensing the Environment in Health and Disease.* Front Immunol, 2018. **9**: p. 93.
13. Valladeau, J. and S. Saeland, *Cutaneous dendritic cells.* Semin Immunol, 2005. **17**(4): p. 273-83.
14. Tamoutounour, S., et al., *Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin.* Immunity, 2013. **39**(5): p. 925-38.
15. Strobl, H., C. Krump, and I. Borek, *Micro-environmental signals directing human epidermal Langerhans cell differentiation.* Semin Cell Dev Biol, 2018.
16. Ginhoux, F., et al., *Langerhans cells arise from monocytes in vivo.* Nat Immunol, 2006. **7**(3): p. 265-73.
17. Valladeau, J., et al., *Langerin, a novel C-type lectin specific to Langerhans cells, is an endocytic receptor that induces the formation of Birbeck granules.* Immunity, 2000. **12**(1): p. 71-81.
18. Tang, A., et al., *Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin.* Nature, 1993. **361**(6407): p. 82-5.
19. Kanitakis, J., et al., *Self-renewal capacity of human epidermal Langerhans cells: observations made on a composite tissue allograft.* Exp Dermatol, 2011. **20**(2): p. 145-6.
20. Chorro, L., et al., *Langerhans cell (LC) proliferation mediates neonatal development, homeostasis, and inflammation-associated expansion of the epidermal LC network.* J Exp Med, 2009. **206**(13): p. 3089-100.
21. Ginhoux, F. and S. Jung, *Monocytes and macrophages: developmental pathways and tissue homeostasis.* Nat Rev Immunol, 2014. **14**(6): p. 392-404.
22. Randolph, G.J., J. Ochando, and S. Partida-Sanchez, *Migration of dendritic cell subsets and their precursors.* Annu Rev Immunol, 2008. **26**: p. 293-316.
23. Merad, M., et al., *Langerhans cells renew in the skin throughout life under steady-state conditions.* Nat Immunol, 2002. **3**(12): p. 1135-41.

24. Sere, K., et al., *Two distinct types of Langerhans cells populate the skin during steady state and inflammation.* Immunity, 2012. **37**(5): p. 905-16.
25. Merad, M., et al., *The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting.* Annu Rev Immunol, 2013. **31**: p. 563-604.
26. Mueller, T.D. and J. Nickel, *Promiscuity and specificity in BMP receptor activation.* FEBS Lett, 2012. **586**(14): p. 1846-59.
27. Daly, A.C., R.A. Randall, and C.S. Hill, *Transforming growth factor beta-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth.* Mol Cell Biol, 2008. **28**(22): p. 6889-902.
28. Yasmin, N., et al., *Identification of bone morphogenetic protein 7 (BMP7) as an instructive factor for human epidermal Langerhans cell differentiation.* J Exp Med, 2013. **210**(12): p. 2597-610.
29. Caux, C., et al., *CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to granulocyte-macrophage colony-stimulating factor plus tumor necrosis factor alpha: II. Functional analysis.* Blood, 1997. **90**(4): p. 1458-70.
30. Riedl, E., et al., *TGF-beta 1 promotes in vitro generation of dendritic cells by protecting progenitor cells from apoptosis.* J Immunol, 1997. **158**(4): p. 1591-7.
31. Solano-Galvez, S.G., et al., *Human Dendritic Cells: Ontogeny and Their Subsets in Health and Disease.* Med Sci (Basel), 2018. **6**(4).
32. Chow, K.V., et al., *Heterogeneity, functional specialization and differentiation of monocyte-derived dendritic cells.* Immunol Cell Biol, 2017. **95**(3): p. 244-251.
33. Posch, W., C. Lass-Florl, and D. Wilflingseder, *Generation of Human Monocyte-derived Dendritic Cells from Whole Blood.* J Vis Exp, 2016(118).

34. Grassi, F., et al., *Monocyte-derived dendritic cells have a phenotype comparable to that of dermal dendritic cells and display ultrastructural granules distinct from Birbeck granules.* J Leukoc Biol, 1998. **64**(4): p. 484-93.
35. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* Int Rev Immunol, 2011. **30**(1): p. 16-34.
36. Hedayat, M., M.G. Netea, and N. Rezaei, *Targeting of Toll-like receptors: a decade of progress in combating infectious diseases.* Lancet Infect Dis, 2011. **11**(9): p. 702-12.
37. Kumar, S., et al., *Recognition of bacterial infection by innate immune sensors.* Crit Rev Microbiol, 2013. **39**(3): p. 229-46.
38. Miller, L.S., *Toll-like receptors in skin.* Adv Dermatol, 2008. **24**: p. 71-87.
39. Paredes Juarez, G.A., et al., *Immunological and technical considerations in application of alginate-based microencapsulation systems.* Front Bioeng Biotechnol, 2014. **2**: p. 26.
40. Amarante-Mendes, G.P., et al., *Pattern Recognition Receptors and the Host Cell Death Molecular Machinery.* Front Immunol, 2018. **9**: p. 2379.
41. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annu Rev Immunol, 2003. **21**: p. 335-76.
42. Jimenez-Dalmaroni, M.J., M.E. Gerswhin, and I.E. Adamopoulos, *The critical role of toll-like receptors--From microbial recognition to autoimmunity: A comprehensive review.* Autoimmun Rev, 2016. **15**(1): p. 1-8.
43. Xagorari, A. and K. Chlichlia, *Toll-like receptors and viruses: induction of innate antiviral immune responses.* Open Microbiol J, 2008. **2**: p. 49-59.
44. McGuire, V.A. and J.S. Arthur, *Subverting Toll-Like Receptor Signaling by Bacterial Pathogens.* Front Immunol, 2015. **6**: p. 607.

45. Jiang, S., et al., *TLR10 Is a Negative Regulator of Both MyD88-Dependent and -Independent TLR Signaling.* J Immunol, 2016. **196**(9): p. 3834-41.
46. Oosting, M., et al., *Human TLR10 is an anti-inflammatory pattern-recognition receptor.* Proc Natl Acad Sci U S A, 2014. **111**(42): p. E4478-84.
47. Rossana Zaru, E.B.I.E.-E., UK, https://www.immunology.org/public-information/bitesized-immunology/receptors-and-molecules/pattern-recognition-receptors-prrs.
48. van der Aar, A.M., et al., *Loss of TLR2, TLR4, and TLR5 on Langerhans cells abolishes bacterial recognition.* J Immunol, 2007. **178**(4): p. 1986-90.
49. Kong, H.H., *Skin microbiome: genomics-based insights into the diversity and role of skin microbes.* Trends Mol Med, 2011. **17**(6): p. 320-8.
50. Iwase, T., et al., *Staphylococcus epidermidis Esp inhibits Staphylococcus aureus biofilm formation and nasal colonization.* Nature, 2010. **465**(7296): p. 346-9.
51. Yao, Z., et al., *Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor.* Immunity, 1995. **3**(6): p. 811-21.
52. Yasuda, K., Y. Takeuchi, and K. Hirota, *The pathogenicity of Th17 cells in autoimmune diseases.* Semin Immunopathol, 2019.
53. Chularojanamontri, L., et al., *Proteomics in Psoriasis.* Int J Mol Sci, 2019. **20**(5).
54. Bettelli, E., M. Oukka, and V.K. Kuchroo, *T(H)-17 cells in the circle of immunity and autoimmunity.* Nat Immunol, 2007. **8**(4): p. 345-50.
55. Stockinger, B. and M. Veldhoen, *Differentiation and function of Th17 T cells.* Curr Opin Immunol, 2007. **19**(3): p. 281-6.
56. Aliahmadi, E., et al., *TLR2-activated human langerhans cells promote Th17 polarization via IL-1beta, TGF-beta and IL-23.* Eur J Immunol, 2009. **39**(5): p. 1221-30.
57. Peiser, M., et al., *Human Langerhans cells selectively activated via Toll-like receptor 2 agonists acquire*

migratory and CD4+T cell stimulatory capacity. J Leukoc Biol, 2008. **83**(5): p. 1118-27.
58. Acosta-Rodriguez, E.V., et al., *Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells.* Nat Immunol, 2007. **8**(9): p. 942-9.
59. Aggarwal, S., et al., *Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17.* J Biol Chem, 2003. **278**(3): p. 1910-4.
60. Chandran, S.S., et al., *TLR2 engagement on dendritic cells promotes high frequency effector and memory CD4 T cell responses.* J Immunol, 2009. **183**(12): p. 7832-41.
61. Arancibia, S.A., et al., *Toll-like receptors are key participants in innate immune responses.* Biol Res, 2007. **40**(2): p. 97-112.
62. Aliprantis, A.O., et al., *Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2.* Science, 1999. **285**(5428): p. 736-9.
63. Yamamoto, M., et al., *Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4.* Nature, 2002. **420**(6913): p. 324-9.
64. Horng, T., et al., *The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors.* Nature, 2002. **420**(6913): p. 329-33.