

EAACI Long-term Fellowship - Final Report – Short description

1. Project title: Priming of keratinocytes under Th2 conditions

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4. Background: Epithelial cells can undergo type-2 differentiation processes. While this has been shown for airway epithelial cells, it is still unclear whether is also true for skin epithelial cells. Even less is known about the precursors of these cells, the skin stem cells. Regulating immunological mechanisms, hypothetical resilience to pro-inflammatory (including type-2) stimuli and the significance of the expression of some type-2 traditionally associated molecules in these cells are currently considered a hot topic and in the same time a matter of debate in the fascinating scientific field of stem cells. Observations deriving from the simulation of environmental conditions on keratinocyte and the skin stem cell (SSC) 2D and 3D cultures have a considerable potential to impact on our current understanding of the pathophysiology and impliedly on the management of disorders such as atopic skin diseases and skin oncological diseases in which the SSCs seem to play a key role. Differential, tailored treatment of already committed skin may become a very useful tool in an era dominated by personalized medicine. Note: Originally proposed fellowship topic was changed.

5. Introduction: Epithelial cells, covering the body surface and internal cavities, represent our interface with the environment, conferring mechanical protection and bearing immunological functions. It is known today that keratinocytes are not bystander cells waiting for their differentiation, but active cells. Constantly exposed to environmental factors, they possess the ability to produce effector molecules in response to stimuli. Keratinocytes originate in the SSCs. Different types of SSCs can be found within distinct niches which are notorious for their potential to give rise to progeny, the transit amplifying cells (TA). TA have limited

proliferative capacity and are able to terminally differentiate.

Recent publications have uncovered a role for IL-4 and interferon-gamma (IFN- γ) in the airway epithelial cell's commitment towards a type-2 (E2) or type-1 (E1) phenotype. To the moment it remains uncertain, whether keratinocytes and the SSCs can also undergo type-2 or type-1 differentiation processes although for some diseases such as atopic dermatitis and psoriasis, an intrinsically abnormal and different chemokine production profile has been described.

The aim of the project was to identify, whether type-2 regulation differentiation mechanisms occur in keratinocytes or the SSCs.

For this purpose SSCs cultures from skin excisions (ethical approval obtained during the fellowship) and primary keratinocytes (obtained from suction blisters and available at ZAUM Institute) have been established and later stimulated with IL4 and IFN γ and IL-22 in order to analyse gene expression profile changes and differentiation processes.

Hypothesis: The study addresses the question whether keratinocytes differentiate into type-2 imprinted epithelial cells.

6. Study design

1. Cell cultures:

- a. **2D (6-well plates)** - From **skin excisions**; ethical approval; three protocols for isolation of **SSCs** from the hair shaft, epidermis and dermis have been developed.

- b. **2D (6-well-plates)** - From primary keratinocytes (**PK**) isolated from suction blisters.
- c. **3D cultures** of **PK** and **SSCs** –
 - a and b - **Achieved**
 - c - **Partially Achieved**

2. Histological analysis of the 3D PK and SSCs model with regards to proving the potential of these cells to differentiate and form multilayered tissue-like structures (functional aspect). **Partially Achieved.**

3. Stimulation of the keratinocytes in order to mimic a complex immunological micromilieu with IL-4, IL-22, IFN γ and a combination of these – **Achieved.** Later, after evaluating the first results, stimulation of PK, SSCs 2D and 3D cultures with FICZ (6-Formylindolo [3, 2-b] - carbazole), an AhR ligand has been added to the protocol – **Achieved** (for 2D cultures and PK 3D cultures).

4. Transcription factor and surface marker profile of human skin stem cells and primary

keratinocytes before and after stimulation – **Partially Achieved.**

5. Microarray for gene expression profiling – **Not achieved.**

6. Evaluation of the cytokine profile in keratinocytes after stimulation (MesoScaleDiscovery's Multiplex-Assay) - **Partially Achieved.**

7. Analyses of epigenetic changes in keratinocytes after stimulation – **Not achieved.**

Biostatistics: For the data analysis, we have used GraphPad Prism, FlowJo, and Diva for the FACS results.

Data management: Data have been stored on the internal computer and on printouts which will be kept for further verification for at least 10 years after closure of patient files

7. Brief Summary of Results

Cell cultures:

2D (6 well plates):

- **PK obtained from suction blisters** (n=5, 08.2017; n=3, 02.2018). Mean number of days until >80 % confluence, when cells are ready for stimulation, was achieved in 3.2 days.
- **SSCs cultures from skin excisions** (n=21, 09.2017-03.2018). Cultures obtained from the hair shaft cells grew faster (80% confluence in 10 - 14 days for the first passage), were more stable, organized and generated more cells (2 - 5 million cells - passage 3-5) compared with the ones obtained from isolated epidermis or dermis.

3D cultures, on 2 types of inserts placed in 6 well plates:

- **PK obtained from suction blisters** (n=3, 02-03.2018) on two types of inserts (different surface) cultured on an air-liquid interface, for 28 days, to allow complete differentiation. The cells arranged initially in a relatively uniform layer covering the whole surface of the insert membrane which, but over a 28 days interval became web-like, with connecting areas in which cells grew in a stratified manner forming a thick surface and areas free of cells (resembling holes in a web).

Histology

- **PK** (n=3, 02-03.2018), cultured for 28 days, on 2 types of **3D inserts**, in differentiation stimulating media developed a multi-layered architecture which, during Microtomy and Paraffin Section Preparation, detached from the insert membrane on which the cells were grown, potentially rendering this result less useful.

Transcription factor and surface marker profile of human skin stem cells and primary keratinocyte

PCR results

- In **2D cultures from PK** (n=5, 08.2017), **stimulated with IL4, IFN γ and IL-22 and a combination of these, IL-4 strongly induced type 2 associated** genes such as **CCL26** (4.89-fold increase, p=0.0556), **GATA-3** (2-fold increase, p=0.0556) and **AhR** (2 fold increase, p<=0.05). **IL-22 had synergistic effects with IL-4** on the expression of two traditionally associated type-2 genes: **CCL26** (5.44-fold increase, p=0.0556) and **GATA-3** (3-fold increase, p<0.05).

Flow cytometry results

- Unstimulated 2D SSCs (n=3, 02.2018-03.2018) and 2D PK cultures (n=3, 02.2018) were analyzed by flow cytometry and the results were compared with FICZ stimulated cultures (SSCs and PK) (n=1, 02-03.2018).
- The initial FACS Panel was modified in order to include more surface and internal markers for stem cells but also the transcription factors GATA-3, p63 and AhR, regulators of interconnected pathways, controlling the fate of epithelial cells, thus the “**stem**” cell quality was defined by the presence of CD34, CD200, ITGB1, ITGA6, KRT15, LGR5 and p63 as well as the absence of involucrin.
- Based on the distribution of CD34 at least two populations of stem cells were observed: CD34 high and CD34 low SSCs.
- Interestingly the presence of GATA-3 has been identified in SSCs, both CD34 high and CD34 low, while IL-4RA (CD124) seemed to be present only in a minority of the CD34 high SSCs.
- P63 was present in SSCs.
- AhR was not constantly present in SSCs. The AhR ligand FICZ exerted a negative effect (feedback loop) on its receptor but also influenced other surface and intracellular SSC markers.

- The PK obtained from suction blisters showed a **transit amplifying cell profile**, keeping some of the SSCs markers such as **ITGA6+**, **ITGB1+**, **KRT15+**, and **p63+**. **Two populations** have been identified based on the distribution of **ITGB1**. AhR was present, although not constantly, in only one of the PK populations. The same negative effect (feedback-loop) of FICZ on AhR could be observed in this population.
- GATA-3 was present inconstantly only in one of the PK populations.

Evaluation of the cytokine profile in keratinocytes after stimulation (MesoScaleDiscovery's Multiplex-Assay)

- In **PK 2D cultures** (n=5, 08.2017), a strong IL-4-related induction has been observed on protein level for CCL-26 (8-fold increase, p<0.05).

8. Conclusions

- **PK:** In primary keratinocytes obtained from suction blisters, we have been able to show induction of type-2 genes such as GATA-3 and CCL-26 by IL-4, which was also confirmed on a protein level for CCL-26. Less clear is the impact of IFN- γ on keratinocytes a task to be demonstrated by future experiments.
- AhR is IL-4 inducible in keratinocytes. To our knowledge, this is the first time when a group has been able to demonstrate the IL4-AhR dependence in keratinocytes. Interestingly, IL-22 appeared to have synergistic effects with IL-4 on the expression of two traditionally associated type-2 genes (GATA-3 and CCL26).
- Overall the project revealed a relatively comparable expression pattern for type-2 regulated genes in PK and airway epithelial cells.
- The multicolor flow cytometry analysis of PK showed a TA phenotype for these cells which were positive for some of the SSCs markers: ITGA6+, ITGB1+, KRT15+, and p63+.
- GATA-3 was absent in one of the PK population and discretely present in the other one.
- **SSCs:** Stem cell quality has been established on a complex combination of markers. Many of these markers were co-expressed dropping hints to interconnected pathways which regulate the fate and behavior of epithelial cells.
- Through flow cytometry, at least two populations of SSCs have been identified based on the CD34 distribution (CD34 low and high).
- **GATA-3 was present in the SSCs, where we postulate that it probably auto-activated its expression.**
- **In the SSCs, the presence of GATA-3 does not seem to be a mark/fingerprint of a type 2 inflammation.** This remark is supported by our FACS finding that IL4Ra was discretely present (difficult to exclude an artefact in CD34 high stem cells) or even absent (CD34 low stem cells) on the skin stem cells in which GATA-3 might actually be unresponsive to IL4 external influences.
- **P63** a transcription factor essential for the commitment of simple ectoderm to epidermal lineages, was also present in the SSCs, illustrating the potential of these cells to differentiate into keratinocytes.
- **AhR** could be identified in SSCs. Stimulation with the AhR endogenous agonist FICZ controlled the AhR expression through a negative feedback loop, an effect which is frequently seen in ligands and their receptors. Also, FICZ exerted effects on other surface and internal SSC markers.
- Skin stem cells appear to act by different rules, compared to the differentiated keratinocytes, with regard to their immunological response (e.g the presence of GATA-3 in these cells).

The project started in April 2017 with the Fellowship and it is ongoing. Thus during the Fellowship, the bases for later experiments involving PK and SSCs have been laid.

9. Achievements

April – end of August 2017 (before the Ethical Committee approval of the Project),

- **Type of cells - PK** obtained from suction blisters: cell cultures on coated plates and stimulation of the cells with cytokines (IL4, IL22, IFN- γ), RNA purification and control of RNA quality, high-Capacity cDNA Reverse Transcription Kit, qPCR, MesoScaleDiscovery's Multiplex-Assay technology for 10 biomarkers

Ethical approval Form for the use of human skin excision material has been **written** and submitted to the Ethical Committee of the Klinikum Rechts der Isar together with the Dermatology Clinic (Dr. Thomas Volz)

In parallel with the main project the student actively contributed to the analysis of clinical samples of sputum cells (sputum processing) as part of an **ongoing project of the Airway Immunology Group**. Beside data assessment, the student learned the use of multicolor flow cytometry (staining, readings) for more than 80 sputum samples.

September 2017- December 2017 (After the Ethical Committee approval)

- **Skin excisions - stem cell isolation and analysis:** development of three protocols for the isolation of skin stem cells (from the hair shaft, epidermis, and dermis), FACS Panel design for the evaluation of surface and intracellular markers of skin stem cells.

January 2018-March 2018:

- Initial FACS Panel for SSCs and PK was modified, FACS compensation, readings and analysis, Stimulation of PK and SSCs 2D cultures (FICZ) and FACS evaluation, 3D model design for PK, PK cultures 2D stimulation (IL4, IL22, IFN γ and FICZ), RNA isolation and purification, histological analysis of the PK 3D model.

Working program from 8.30-17.30 (with adjustment according to the needs of the Project – including weekend experiments or late evening/night experiments)

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