From the Department of Women's and Children's Health,
Karolinska Institutet, Stockholm, Sweden

HUMAN MYELOID CELLS IN CANCER, INFLAMMATION, AND INFECTION

Eglė Kvedaraitė

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By

Eglė Kvedaraitė

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Principal Supervisor:
Professor Jan-Inge Henter
Karolinska Institutet
Department of Women's and Children's Health
Childhood Cancer Research Unit

Co-supervisors:
Associate Professor Mattias Svensson
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Dr. Désirée Gavhed
Karolinska Institutet
Department of Women's and Children's Health
Childhood Cancer Research Unit

Dr. Magda Lourda
Karolinska Institutet
Department of Medicine, Huddinge
Center for Infectious Medicine

Dr. Maja Ideström
Karolinska Institutet
Department of Women's and Children's Health
Childhood Cancer Research Unit

Opponent:
Professor Marco Prinz
University of Freiburg
Faculty of Medicine
Institute of Neuropathology

Examination Board:
Professor Liv Eidsmo
University of Copenhagen
Department of Immunology and Microbiology
Skin Immunology Research Center

Professor Karin Loré
Karolinska Institutet
Department of Medicine, Solna
Division of Immunology and allergy

Professor Eva Sverremark-Ekström
Stockholm University
Department of Molecular Biosciences
The Wenner-Gren Institute

POPULAR SCIENCE SUMMARY OF THE THESIS

The human body is protected against attacks from pathogens, such as viruses and bacteria, by coordinated responses of various types of immune cells. A certain type of immune cells, so called myeloid cells, are our frontline soldiers fighting pathogens during infection. In addition, those cells are major players during development of other diseases, such as cancer or chronic inflammatory conditions. So, what happens when myeloid cells do not perform their duty as they should? How do they change when they travel into the tissues of the body and what is their origin? How exactly are they regulating immune responses? How do they communicate with other cells in our body? How do they switch off the inflammatory response so that it does not go on forever? Detailed answers to this kind of questions are necessary in order to significantly improve diagnostics and treatment options in infections, inflammatory diseases, and cancer.

In this thesis, we studied myeloid cells in three different clinical conditions: Langerhans cell histiocytosis that is a disease occurring in the boundary between cancer and inflammation; inflammatory bowel disease (IBD) that is an incurable disease of the intestine; and COVID-19 that every reader is most probably aware of due to the ongoing pandemic. Knowledge gained here on distinct types of myeloid cells improves our understanding on their features during health and disease. In addition, we gained general and specific insights in their communication with other cells that circulate in the blood, but also the ones that build up the tissues of different organs, such as the intestine. For example, by investigating those cells in a blood sample taken from the hospitalized COVID-19 patients, we could predict a group of patients with severe disease that also included all non-survivors. Notably, none of those markers are used in the clinic today, illustrating the potential that the detailed knowledge on myeloid cells have to offer to clinical medicine.

While follow-up studies are necessary to confirm our results, the data presented here provides important advances in our understanding of the role of human myeloid cells in cancer, inflammation, and infection.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Människokroppen skyddas mot attacker från smittämnen, såsom virus och bakterier, genom samordnade svar från olika typer av immunceller. En viss typ av immunceller, så kallade myeloida celler, är våra främsta soldater för att bekämpa bakterier och virus i samband med infektioner. Dessutom är dessa celler viktiga aktörer under utvecklingen av andra sjukdomar, såsom cancer och kroniska inflammatoriska tillstånd. Men vad är det som händer när myeloida celler inte utför sin funktion som de ska? Hur förändras de när de reser i kroppens vävnader och vilket är deras ursprung? Hur exakt reglerar de immunsvaret? Hur kommunicerar de med andra celler i vår kropp? Hur stänger de av det inflammatoriska svaret så att det inte fortsätter för alltid? Detaljerade svar på dessa frågor är nödvändiga för att avsevärt förbättra diagnostik och behandling vid infektioner, inflammatoriska sjukdomar och cancer.

I denna avhandling studerade vi myeloida celler i tre olika kliniska tillstånd: Langerhans cellhistiocytos som är en sjukdom som uppstår i gränsen mellan cancer och inflammation; inflammatorisk tarmsjukdom som är en obotlig sjukdom i tarmen; och COVID-19 som alla läsare troligen är medvetna om på grund av den pågående pandemin. Kunskap som erhållits i avhandlingen om olika typer av myeloida celler kan förbättra vår förståelse för deras egenskaper i hälsa och sjukdom. Dessutom har vi fått insikter om deras kommunikation med andra celler som cirkulerar i blodet, men också med de celler som bygger upp vävnaderna i olika organ, till exempel tarmen. Till exempel, genom att undersöka dessa celler i blodprov som tagits från sjukhusvårdade COVID-19-patienter kunde vi påvisa ett sätt att i förväg identifiera en grupp av svårt sjuka COVID-19 patienter som senare gick bort i sjukdomen. Ingen av dessa markörer som vi studerade används idag i ordinarie klinisk vård, vilket illustrerar den potential som detaljerad kunskap om myeloida celler har att erbjuda till klinisk medicin.

Även om uppföljningsstudier är nödvändiga för att bekräfta våra resultat, så innebär data som presenteras i avhandlingen viktiga framsteg i vår förståelse av myeloida cellers roll i cancer, inflammation och infektion.

DISERTACIJOS APŽVALGA POPULIARIAJAM MOKSLUI

Žmogaus kūną nuo ligų sukėlėjų, tokių kaip virusai ir bakterijos, saugo koordinuotas įvairių tipų imuninių ląstelių atsakas. Vienos jų – mieloidinės ląstelės, yra mūsų priešakinės linijos kariai, tiesiogiai kovojantys su virusais ir bakterijomis infekcijos metu. Šios ląstelės dalyvauja ir kovoje su kitomis ligomis, tokiomis kaip vėžys ar lėtinės uždegiminės ligos. Kas atsitinka, kai mieloidinės ląstelės deramai nevykdo savo funkcijų? Kaip jos pakinta keliaudamos į kūno audinius? Kokia jų kilmė? Kaip tiksliai jos reguliuoja imuninį atsaką? Kaip jos bendrauja su kitomis mūsų kūno ląstelėmis? Kaip jos išjungia uždegiminį atsaką, kad jis nesitęstų amžinai? Norint pagerinti infekcijų, uždegiminių ligų ir vėžio diagnostikos bei gydymo galimybes, būtini išsamūs atsakymai į šiuos klausimus.

Disertacijoje mieloidinės ląstelės buvo tiriamos trijuose skirtingose klinikiniuose kontekstuose: (1) Langerhanso ląstelių histiocitozės – ligos, turinčios tiek vėžio, tiek uždegimo požymių, (2) uždegiminių žarnyno ligų, pasireiškiančių ilgalaikiu žarnyno uždegimu, ir (3) COVID-19, apie kurią greičiausiai žino kiekvienas skaitytojas dėl šiuo metu vykstančios pandemijos, metu. Čia įgytos žinios pagerina mūsų supratimą apie mieloidinių ląstelių savybes tiek mums esant sveikiems, tiek sergant. Be to, svarbių įžvalgų įgijome tirdami mieloidinių ląstelių bendravimą su kitomis kraujyje cirkuliuojančiomis ir skirtinguose organų audiniuose, pavyzdžiui, žarnyne, randamomis ląstelėmis. Šias ląsteles ištyrę hospitalizuotų COVID-19 pacientų kraujo mėginiuose, galėjome patikimai nuspėti, kuriems pacientams gresia sunki ligos eiga ar mirtis. Nei vienas iš šių žymenų klinikinėje praktikoje dar nėra naudojamas, tad disertacijoje įgautų žinių apie mieloidines ląsteles potencialas medicinoje yra akivaizdus.

Nors tolimesni moksliniai tyrimai yra būtini rezultatams patvirtinti, šioje disertacijoje pateikti duomenys pagerina mūsų supratimą apie žmogaus mieloidinių ląstelių vaidmenį sergant vėžiu, infekcinėmis ligomis bei esant uždegiminiam procesui.

ABSTRACT

Myeloid cells are a part of innate immunity, playing a major role in orchestrating innate and adaptive immune responses. While work performed in experiment model systems has significantly increased our knowledge on fundamental myeloid cell functions, studies in well-designed clinical cohorts are important for understanding their functions in human health and disease, such as in cancer, infection and chronic inflammation. In this thesis, distinct populations of human myeloid cells were investigated in three different clinical contexts: Langerhans cell histiocytosis (LCH), an inflammatory myeloid neoplasia; inflammatory bowel disease (IBD), a chronic disorder of the gastrointestinal tract; and COVID-19, an acute viral infection.

We found that neutrophils, rather than antigen presenting mononuclear phagocytes (MNPs) such as monocytes and dendritic cells (DC), are the main cellular source of the regulatory cytokine IL-23 in colon tissue of newly diagnosed and treatment-naïve children with IBD (**paper I**). Moreover, we demonstrated that inflammation-responsive intestinal stroma has a capacity to shape the monocyte-derived macrophage pool, and that phenotypes modelled in fibroblast-macrophage co-culture systems were reflected in the IBD tissue (**paper II**). In addition, while the role of IL-23 is well-established in IBD, we also proposed its potential involvement in the immunopathogenesis of LCH (**paper III**). Furthermore, proficient delineation of LCH cells, that are neoplastic MNP found in LCH lesions, from the normal MNPs was performed at single-cell resolution, allowing identification of two major LCH cell populations, corresponding to DC type 2 and monocytes/DC type 3 lineages (**paper IV**). Lastly, a comprehensive map over major alterations in MNP responses in COVID-19 was depicted, where MNPs profile, alone, could predict a cluster of non-survivors (**paper V**).

Taken together, this data provides important input in our understanding of the role of MNPs in human disease, also showing that we only scratch the surface of myeloid cell functions in cancer, inflammation, and infection, as many outstanding questions remain.

LIST OF SCIENTIFIC PAPERS

This thesis is based on five publications, and the individual papers are referred to by Roman numerals.

I. **Egle Kvedaraite**, Magda Lourda, Maja Ideström, Puran Chen, Selma Olsson-Åkefeldt, Marianne Forkel, Désirée Gavhed, Ulrik Lindforss, Jenny Mjösberg, Jan-Inge Henter, Mattias Svensson. Tissue-infiltrating neutrophils represent the main source of IL-23 in the colon of patients with IBD.
Gut. 2016 vol 65 (10), 1632–1641.

II. **Egle Kvedaraite**, Magda Lourda, Natalia Mouratidou, Indranil Sinha, Efthymia Kokkinou, Tea Soini, Aline Van Acker, Nelly Rahkonen, Kirsten Moll, David Unnersjö-Jess, Mira Akber, Ruta Nadisauskaite, Jessica Jansson, Anastasios Damdimopoulos, Niels Vandamme, Chiara Sorini, Eduardo J Villablanca, Helena Jonsson Rolandsdotter, Maja Ideström, Jenny Mjösberg, Henrik Arnell, Jan-Inge Henter, Mattias Svensson. Inflammation responsive intestinal stroma shapes the macrophage pool.
Manuscript submitted.

III. **Egle Kvedaraite**, Magda Lourda, HongYa Han, Bianca Tesi, Jenée Mitchell, Maja Ideström, Natalia Mouratidou, George Rassidakis, Tatiana von Bahr Greenwood, Fleur Cohen-Aubart, Martin Jädersten, Selma Olsson Åkefeldt, Mattias Svensson, George Kannourakis, Yenan T. Bryceson, Julien Haroche, Jan-Inge Henter. Patients with both Langerhans cell histiocytosis and Crohn's disease highlight a common role of interleukin-23.
Acta Paediatrica. 2021 vol 110 (4), 1315–1321.

IV. **Egle Kvedaraite**, Ahad Khalilnezhad, Marion Chevrier, Paul Milne, Hong Kai Lee, Daniel W. Hagey, Tatiana von Bahr Greenwood, Nicole Yee Shin Lee, Lara Minnerup, Tan Yingrou, Charles-Antoine Dutertre, Nathan Benac, You Yi Hwang, Josephine Lum, Amos Hong Pheng Loh, Karen Wei Weng Teng, Shabnam Khalilnezhad, Xu Weili, Anastasia Resteu, Tey Hong Liang, Ng Lai Guan, Anis Larbi, Shanshan Wu Howland, Samir EL Andaloussi, Jorge Braier, Georgios Rassidakis, Laura Galluzzo, Andrzej Dzionek, Matthew Collin, Jan-Inge Henter, Jinmiao Chen, Florent Ginhoux. Senescent Langerhans cell histiocytosis cells arise from both dendritic cell (DC) and monocyte/DC3 lineages.
Manuscript submitted.

V. **Egle Kvedaraite**, Laura Hertwig, Indranil Sinha, Andrea Ponzetta, Ida Hed Myrberg, Magda Lourda, Majda Dzidic, Mira Akber, Jonas Klingström, Elin Folkesson, Jagadeeswara Rao Muvva, Puran Chen, Sara Gredmark-Russ, Susanna Brighenti, Anna Norrby-Teglund, Lars I. Eriksson, Olav Rooyackers, Soo Aleman, Kristoffer Strålin, Hans-Gustaf Ljunggren, Florent Ginhoux, Niklas K. Björkström, Jan-Inge Henter, Mattias Svensson, and Karolinska KI/K COVID-19 Study Group. Major alterations in the mononuclear phagocyte landscape associated with COVID-19 severity.
Proceedings of the National Academy of Sciences. 2021 vol 118 (6), e2018587118.

CONTENTS

LIST OF ABBREVIATIONS

AP-1	Activator protein 1
CD	Crohn’s disease
COVID-19	Corona virus disease 2019
COX	Cyclooxygenase
DC	Dendritic cell
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
cDC	classical DC
cDC1	classical DC type 1
cDC2	classical DC type 2
CLEC9A	C-type lectin domain containing 9A
CITE-seq	Cellular indexing of transcriptomes and epitopes by sequencing
DC1	DC type 1
DC2	DC type 2
DC3	DC type 3
CCL	C-C motif chemokine ligand
CCR	C-C motif chemokine receptor
CNS	Central nervous system
CGD	Chronic granulomatous disease
C/EBP	CCAAT-enhancer-binding proteins
CXCR	C-X-C motif receptor
DSS	Dextran sodium sulfate
EBV	Epstein-Barr virus
EEN	Exclusive enteral nutrition
ERBB3	Erb-B2 receptor tyrosine kinase 3
ERK	Extracellular signal-regulated kinase
FLT3L	FMS-like tyrosine kinase 3 ligand
GDPR	General Data Protection Regulation
G-CSF	Granulocyte colony-stimulating factor
GI	Gastrointestinal
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HLH	Hemophagocytic lymphohistiocytosis

IBD	Inflammatory bowel disease
IFN-α/β	Type I Interferons
IFN-γ	Interferon γ
IL	Interleukin
ILC	Innate lymphoid cell
infDC	Inflammatory DC
LCH	Langerhans cell histiocytosis
MAPK	Mitogen-activated protein kinase
MHC-II	Major histocompatibility complex class II
MNP	Mononuclear phagocyte
MMP	Metalloprotease
mregDC	Mature dendritic cells enriched in immunoregulatory molecules
MPO	Myeloperoxidase
mo-DC	Monocyte-derived DC
Mo-MDSC	Monocytic myeloid-derived suppressor cell
6-MP	Mercaptopurine
NET	Neutrophil extracellular trap
NF-kB	Nuclear factor-kB
NKT cells	Natural killer T cells
NLRP	Nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing
NOD2	Nucleotide-binding oligomerization domain 2
pDC	Plasmacytoid DC
pERK	Phosphorylated ERK
PBMC	Peripheral blood mononuclear cell
PDPN	Podoplanin
PD-L1	Programmed death-ligand 1
qPCR	Quantitative polymerase chain reaction
R	Receptor
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa-B ligand
ROS	Reactive oxygen species
RORγt	Retinoic acid receptor-related orphan receptor gamma-t
SCF	Stem cell factor
scRNA-seq	Single-cell RNA sequencing
STAT3	Signal transducer and activator of transcription 3

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptor
TNBS	Trinitro-benzene sulphonic acid
TNF	Tumor necrosis factor
Th	T helper
UC	Ulcerative colitis
XCR1	X-C motif chemokine receptor 1

1 INTRODUCTION

The immune system rests on two major cornerstones: innate and adaptive immunity. While adaptive immunity provides a highly specialized line of acquired protection against microbe invasion, the innate immunity is designed to respond broadly and rapidly, and thus represents the first line of defense against pathogens in tissues. The functions of innate immunity, such as recognition of microbial components, phagocytosis and production of cytokines, are performed primarily by specialized myeloid cells, namely phagocytes. Furthermore, the interplay between these immune cells and tissue specific cells, such as stromal cells, is crucial in maintaining tissue homeostasis, and therefore central in understanding the immunopathogenesis of infections, cancers and immune mediated chronic inflammatory diseases. Based on the appearance of their nuclei and, more importantly, their ontogeny and functions, phagocytes are subdivided in polymorphonuclear phagocytes, also called granulocytes, and mononuclear phagocytes (MNPs). The focus of this thesis is on mononuclear phagocytes (**papers II-V**), further subdivided into dendritic cells (DCs), monocytes, and macrophages; and on neutrophils (**paper I**), polymorphonuclear cells that are the most common granulocytes and the most common leukocytes in human blood (Figure 1). The immunopathological aspects of those cells will be addressed in human diseases, such as Langerhans cell histiocytosis (LCH), inflammatory bowel disease (IBD), and Corona virus disease 2019 (COVID-19).

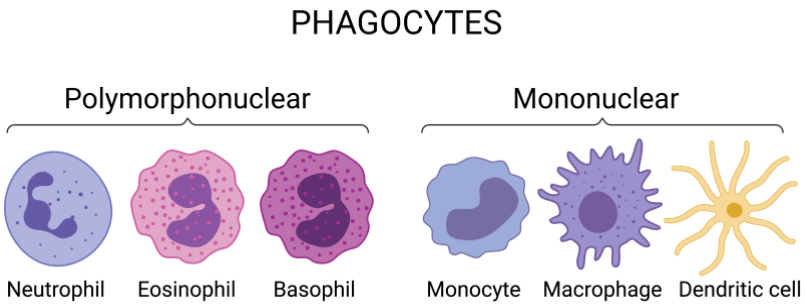

Figure 1: Phagocyte classification
Phagocytes are myeloid cells of the innate immune system, based on ontogeny and functions subdivided into polymorphonuclear phagocytes, that is neutrophils, eosinophils and basophils, and mononuclear phagocytes (MNPs), that is monocytes, macrophages, and dendritic cells. *Illustration was created with BioRender.com.*

1.1 MONONUCLEAR PHAGOCYTE SYSTEM

Based on ontogeny, but also location, phenotype and function, mononuclear phagocytes are subdivided into DCs, monocytes and macrophages (Guilliams et al., 2014). DCs were discovered more than 50 years ago, when a Canadian scientist Ralph M. Steinman and his mentor Zanvil A. Cohn first described DCs (Steinman and Cohn, 1973). By using basic cinematography, which allowed live recording of cells at that time was considered state-of-art and, the scientists distinguished DCs from the macrophages in the culture, and the first keystone for the research field of DCs was laid. Years of meticulous follow-up work by

Ralph M. Steinman and other scientists built a fundament to the immunology of DCs as we see them today – the major antigen-presenting cells organizing adaptive and innate immunity in cancer, chronic inflammation, and infection. Knowledge on anti-viral, anti-tumor, but also tolerogenic DC functions has grown thanks to studies performed in animal models, but recent advances in human DC biology open up new avenues for questions addressing their heterogeneity, ontogeny, and function in both homeostasis and during its disruption, such as infection, chronic inflammation, and cancer.

Following Steinman's discovery, DCs were included in the system of MNPs and are today classified as MNPs in the modern nomenclature. Classical DCs (cDCs), also known as conventional DCs, are subdivided into type 1 (cDC1) and type 2 (cDC2), and in humans develop from the circulating myeloid progenitor pre-DC population pre-cDC1 and pre-cDC2, respectively (See et al., 2017; Villani et al., 2017). In contrast to the previous view where a common DC precursor was thought to give rise both to cDCs and also to plasmacytoid DCs (pDCs), the majority of pDCs were recently suggested to derive from the lymphoid sources (Dress et al., 2019; Rodrigues et al., 2018), and the pDC human precursor (pre-pDC) remains to be described (Rodrigues and Tussiwand, 2020). Regarding the functional subset specific qualities, DC subsets specialize in responses against different pathogens, produce distinct cytokines, and foster certain types of T-cell mediated immunity (Collin and Bigley, 2018). With regard to pDCs, they specialize in the production of type I Interferons (IFN-α/β), that are important in anti-tumor and anti-viral immune responses, but their production of IFN-α/β in cancer seems to be impaired (Koucký et al., 2019; Mitchell et al., 2018). cDC1 are master regulators in immune responses against intracellular pathogens and anti-tumor immunity through cross-presentation to $CD8^+$ T cells, and cDC2 orchestrate responses to extracellular pathogens through antigen presentation to specialized subsets of helper $CD4^+$ T cells (Anderson et al., 2020). While distinct surface markers (e.g. C-type lectin domain containing 9A (CLEC9A) and X-C motif chemokine receptor 1 (XCR1)) identify the cDC1s, cDC2s are more heterogenous and divided into two populations, named DC2 and DC3 (Villani et al., 2017). Follow-up work strengthened the distinction between $CD5^-$ DC3 and $CD5^+$ DC2, on the phenotypic and functional levels (Bourdely et al., 2020; Cytlak et al., 2020; Dutertre et al., 2019) (Figure 2).
Monocytes are MNPs also found in circulation, approximately 10 times as abundant as DCs, and they are rapidly recruited to the site of inflammation or infection, through for example the C-C motif chemokine L (CCL)-2 and C-C motif chemokine receptor (CCR)-2. They are subdivided into three major subsets, that is classical $CD14^+CD16^-$ monocytes, $CD14^+CD16^+$ intermediate, and $CD14^{low}CD16^+$ non-classical monocytes (Geissmann et al., 2003; Ingersoll et al., 2010; Wong et al., 2011). Monocytes play essential roles in innate proinflammatory responses and are able to produce large amounts of proinflammatory cytokines, as well as contribute to immunosuppressive responses, crucial in the healing processes when the inflammatory reaction needs to be switched off (Chu et al., 2020; Mildner et al., 2013). Monocytes are able to become macrophages in the tissues, contributing to the local macrophage pool (Mildner et al., 2013). Regarding macrophage

origins, their embryonic sources are today well-described and it is established that yolk sac macrophages give rise to microglia, while fetal liver monocytes seed other organs, such as skin, liver, lung, and gut (Hoeffel et al., 2015). After birth, however, tissues are seeded with monocyte-derived macrophages at different rates, and for example in the gut the macrophage pool is constantly replenished and maintained by circulating monocytes (Bain et al., 2014). Macrophage functions in maintaining tissue homeostasis are well-recognized, and they are regarding as niche cells, defined by a specific tissue-related identity (Guilliams et al., 2020). In addition, macrophages and tissue resident cells fibroblasts form a stable cell circuit system, that is resilient to perturbations and ensures their population stability (Zhou et al., 2018b). The phenotypic and functional consequences of this cell-cell system remain to be determined in health and pathologic tissue conditions, such as in chronic tissue inflammation. We have addressed this in a co-culture system derived from healthy pediatric colon tissue in a setup that allowed us to interrogate how stromal cells affect macrophage development during health and inflammation (**paper II**).

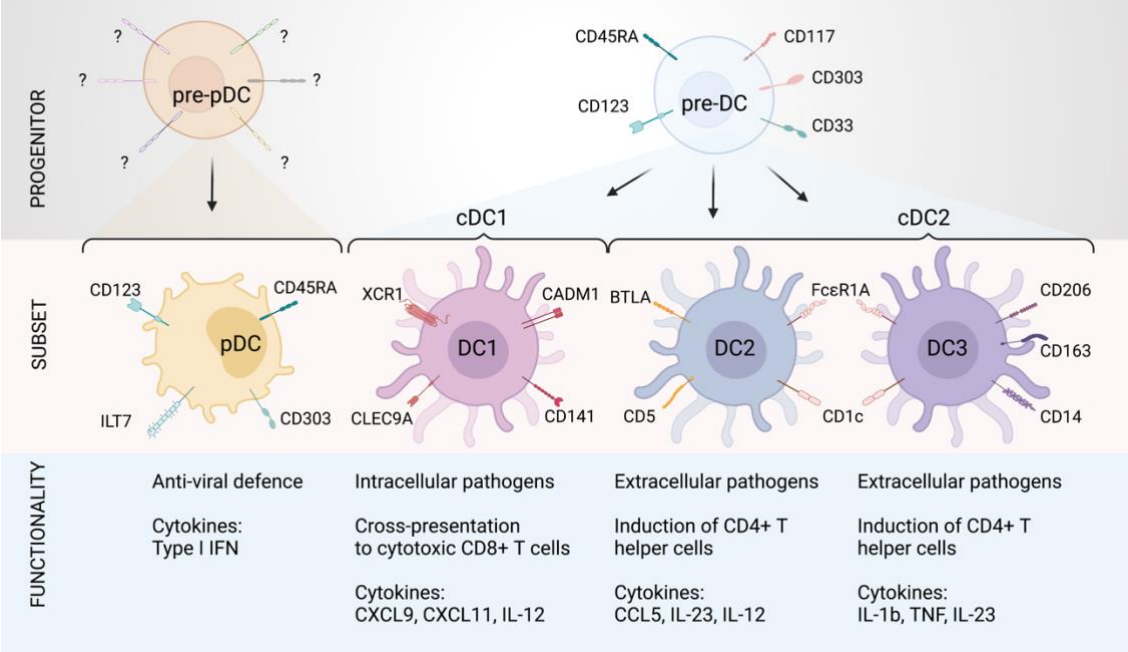

Figure 2: Human DC nomenclature: basic functions and ontogeny relationship
DCs in humans are divided into conventional DCs (cDC) and plasmacytoid DCs (pDCs) in a nomenclature, based on location, function, and ontogeny. While pDCs are suggested to develop from lymphoid progenitors, the pDC progenitor (pre-pDC) in humans remains to be identified. Circulating cDCs progenitors, named as pre-DC, have recently been identified; they share many features and phenotypical surface markers with pDCs. cDCs are sdivided into type 1 DC (cDC1) and type DC (cDC2), that comprise two major distinct entities, namely $CD5^+$ DC2 and $CD5^-$ DC3, on functional, phenotypic, and developmental level. In general, DC1 present antigens to cytotoxic $CD8^+$ T cells; DC2 and DC3 present antigens and $CD4^+$ T helper cells, all produce high levels of IL-12, and regulate immune responses to intracellular (DC1) and extracellular (cDC2) pathogens. Moreover, DC3 are able to produce IL-23 and may be important in chronic inflammatory conditions. pDCs produce type 1 interferons (IFN), crucial for anti-viral immunity. *Illustration was created with BioRender.com.*

1.2 IL-23 DRIVEN INFLAMMATION

Progress in the field of genetics has led to increased knowledge of the pathogenesis of immune mediated disorders by identifying alterations in susceptibility genes involved in the inflammatory response. Genome-wide association studies identified IL-23 receptor polymorphisms, associated with IBD, multiple sclerosis, psoriasis, ankylosing spondylitis, and psoriatic arthritis (Cho and Feldman, 2015). IL-23 is a proinflammatory cytokine consisting of two subunits, p40 and p19, and it belongs to the IL-6/IL-12 family of heterodimeric cytokines. This cytokine family is involved in a wide range of immune reactions and has a regulatory role in shaping the immune response, providing a link between innate and adaptive immunity (Hasegawa et al., 2016). While p40 is shared with another proinflammatory cytokine, IL-12, p19 is considered to be specific for IL-23. IL-23 binds to the transmembrane receptor, consisting of IL-12Rβ1 and IL-23R subunits, and through the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) the signal is transduced. IL-23 signaling, in combination with tumor necrosis factor (TNF), IL-6, and IL-1b, is essential for the expansion and stabilization of IL-17A-producing T helper cells (Th17) that belong to adaptive immunity and are potent inducers of tissue inflammation. Also, populations of innate cells of the lymphoid lineage, such as the gamma-delta (γδ) T cells and the retinoic-acid receptor related orphan receptor (ROR) γt innate lymphoid cells (ILCs), respond to IL-23 and mediate host immune defense and establishment of local inflammatory responses in the tissue by producing the downstream cytokines, such as IL-17A and IL-22 (Langrish et al., 2005). The main cellular source of IL-23 in tissue is considered to be antigen presenting mononuclear myeloid cells, such as macrophages and DCs (Teng et al., 2015). However, the data supporting this in humans remains to be confirmed and further explored. To address this in a human setting and while minimizing risks for confounding factors that are difficult to avoid in studies conducted in adult populations, we performed our analyzes on cellular source of IL-23 in fresh-frozen biopsies from newly diagnosed and treatment naïve pediatric patients with IBD, and found that neutrophils represent the main source of IL-23 (**paper I**).

Corroborating on cytokines downstream of IL-23, in addition to IL-17A, five other IL-17 family cytokines have been identified, namely IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. The IL-17 receptor family consists of five members and is expressed on non-hematopoietic tissue cells such as epithelial cells and fibroblasts (Patel and Kuchroo, 2015). IL-17 receptor signaling leads to activation of Nuclear factor-kB (NF-kB), CCAAT-enhancer-binding proteins (C/EBP), and Activator protein 1 (AP-1) signaling pathways (Fragoulis et al., 2016) and production of proinflammatory cytokines, such as IL-6, IL-1β, TNF, IL-8 as well as metalloproteases (MMPs), nitric oxide, Cyclooxygenase (COX) and upregulation of receptor activator of nuclear factor kappa-B ligand (RANKL), contributing to the establishment of the inflammatory environment in the tissue (Patel and Kuchroo, 2015). In addition to fibroblasts and epithelial cells, IL-17 targets T cells themselves, creating a positive feedback loop (Yosef et al., 2013). Another cytokine downstream of IL-23, namely IL-22, is a member of the IL-10 family cytokines, and mainly targets tissue

specific cells, such as fibroblasts and epithelial cells, and signals through STAT3 phosphorylation. As opposed to IL-17, IL-22 promotes tissue repair and enhances tissue regeneration and wound healing in response to injury. In addition to the protective role of IL-22 in ensuring tissue homeostasis, the cytokine has been associated with malignancies and inflammatory diseases through its capacity to induce cell proliferation and inhibit apoptosis (Dudakov et al., 2015). The precise role of the IL-23 driven inflammation through the production of downstream cytokines, such as IL-17A and IL-22, in the context of different immune mediated diseases remains to be further defined. Several clinical trials in which members of the IL-23 signaling cascade are targeted have been completed or are ongoing. Indeed, drugs targeting IL-23 and its related pathways, namely IL-17A (Secukinamab, Ixekizumab), IL-17 receptor (Brodalumab), IL-12/-23 (Ustekinumab, Briakinumab), and specifically IL-23 (Tildrakizumab, Guselkumab, BI-655066, AMG 139; MEDI-2070, LY3074828, LY2525623) are in different phases of clinical trials, and are also being gradually introduced in the clinics. In psoriasis, which is an inflammatory skin condition where these drugs have been studied extensively, the blockage of IL-17A and its receptor as well as IL-12/-23 and IL-23 led to amelioration of the disease (Patel and Kuchroo, 2015). Similar effects were seen in ankylosing spondylitis, an inflammatory disease that mainly affect the spine, where targeting of IL-17A and IL-12/-23 showed clinical efficacy (Fragoulis et al., 2016). Intriguingly, while trials targeting IL-17A and IL-17R in patients with IBD, an inflammatory condition in the gastrointestinal tract, were terminated due to exacerbation of the disease, the IL-12/-23 antagonist (Ustekinumab) showed clinical efficacy compared to the placebo group and is now a part of clinical routine (Teng et al., 2015). On the other hand, the benefit observed using IL-12/-23 antagonists in multiple sclerosis, an inflammatory condition engaging the central nervous system, was not considered to be large enough to motivate further development, while IL-17A blockage demonstrated promising effects (Teng et al., 2015). Based on the data collected from targeting the IL-23/IL-17A signaling pathway, it is likely that pathogenic versus protective effects of each cytokine is specific to the human disease setting and the site of inflammation. Further studies are warranted to address the contributions of individual members of the IL-23 pathway in immune-mediated inflammatory disorders. In a case series report that focused on clinical characteristics of patients affected by two granulomatous conditions, namely Langerhans cell histiocytosis and Crohn's disease, we have also addressed the role of IL-23 in unrelated LCH patients (**paper III**).

1.3 INFLAMMATORY BOWEL DISEASE: CHRONIC INFLAMMATION

IBD is a chronic incurable inflammatory disease mainly involving the gastrointestinal tract, and is divided into ulcerative colitis (UC), Crohn's disease (CD), and also IBD unclassified (IBD-u), the latter more common in children than in adults (Thurgate et al., 2019). This classification is based on clinical examination, laboratory tests, radiological and endoscopic findings as well as histological criteria. Up to 25% of all IBD patients are diagnosed during childhood, and in general IBD onset usually occurs in young adults (Roberts et al., 2020;

Sýkora et al., 2018). Our understanding of IBD immunopathogenesis stems from studies on microbiome research, genetics, and immunology. The disease mechanisms are multifactorial and involve environmental factors and genetic susceptibility, that lead to dysregulation of the commensal ecosystem and misdirected immune responses in the intestine. In more detail, the environmental triggers, such as dietary, infectious and microbial, in combination with dysfunction of epithelial and mucosal immunity in genetically susceptible individuals influence the development of IBD (Peloquin et al., 2016). Historically, CD was believed to be driven by interferon γ-producing T helper cells (Th1), and UC was associated with IL-4-producing T helper cells (Th2). A modern view of IBD immunopathogenesis stresses the importance of innate immunity and IL-23 driven inflammation, both in CD and UC, through maintenance of IL-17A, IL-17F, and IL-22-producing innate and adaptive cell populations, present in the mucosal compartment of gut. Mononuclear myeloid cells, such as DCs and macrophages ($CD14^{+}CD163^{low}$), are believed to be the main producers of IL-23 in human IBD gut mucosa (Kamada et al., 2008), maintaining the pathological Th17 responses.

A major role for the immune system in driving IBD is evident from the treatment options used in the clinic today, aiming to limit inflammation. These treatments include steroids, immunomodulatory drugs (methotrexate, 6-mercaptopurine (6-MP)), exclusive enteral nutrition (EEN), and biological therapies. Indeed, the biological drugs targeting TNF (infliximab, etanercept, adalimumab, golimumab, certolizumabpegol), T cells (α4β7 inhibitor vedolizumab), as well as IL-23 (ustekinumab) have revolutionized the treatment approaches and outcomes of IBD (Moschen et al., 2019). However, up to 40% of patients do not respond to anti-TNF therapies, or may lose the efficacy during the treatment (Kennedy et al., 2019). Interestingly, the non-responders could be predicted based on parameters related to intestinal stromal cells or fibroblasts (West et al., 2017). The IBD subtype, but also clinical responses and severity, are determined using a global strategy including clinical examination, endoscopy and radiology, histopathologic review, and laboratory tests. One of the most useful and non-invasive laboratory tests indicating intestinal inflammation is fecal calprotectin (Konikoff and Denson, 2006). Calprotectin is a protein found abundantly in neutrophils, and its levels are related to clinical IBD characteristics (Costa et al., 2005; Daniluk et al., 2019; Degraeuwe et al., 2015; Foster et al., 2019; Hanai et al., 2004; Konikoff and Denson, 2006; Lee et al., 2017; van Rheenen et al., 2010). In addition, neutrophil presence and localization in the gut are important for evaluation of histological IBD severity through multiple scoring systems used in the clinic (Neri et al., 2021). New insights into neutrophil biology, plasticity and heterogeneity depict them as sophisticated mediators of cellular immuniny (Ballesteros et al., 2020; Ponzetta et al., 2019). Knowledge of relationships between neutrophils and other immune and non-immune cells in the intestine, utilizing unbiased approaches, aiming to depict specific treatment targets, has potential to contribute to new generation IBD treatments aiming to cure.

1.4 LANGERHANS CELL HISTIOCYTOSIS: CANCER?

"Histiocytosis X", a designation previously used to describe Langerhans cell histiocytosis (LCH), unified three syndromes: single or multiple lytic granulomatous bone lesions called eosinophilic granulomas; Hand-Schüller-Christian disease, typically characterized by lytic bone and mucosal lesions, diabetes insipidus caused by granulomatous lesions in the pituitary gland, and exophthalmos caused by retroorbital granulomas; and Letterer-Siwe disease, with serious hepatosplenomegaly and hematopoietic involvement. Dr. Christian Nezelof, founding member and the first president of Histiocyte Society, suggested in 1973 that proliferating Langerhans cells, which currently are considered as epidermal resident macrophages (Ginhoux and Guilliams, 2016), but at that time, were thought to be cells continuously replenished by bone marrow derived monocytes, caused the disease (Nezelof et al., 1973). This led to the clinical praxis being used today: in LCH, granulomas, predominantly composed of eosinophils, macrophages, lymphocytes and multinuclear giant cells, and the positivity of the markers associated with Langerhans cells, namely CD1a and Langerin, is mandatory for the establishment of the diagnosis. LCH has prominent inflammatory features and a range of clinical presentations, varying from spontaneously resolving single lytic bone lesion or skin rash to life-threatening disease, organ failure, and death. LCH is the most common histiocytic disorder, with the annual incidence of 8.9 per million children per year (Stålemark et al., 2008), further subdivided into single system disease or multisystem disease, affecting several organ systems. Single system LCH often involves bone or skin, and organs affected by multisystem disease also include liver, spleen, lungs, or central nervous system (CNS), that latter remaining the cause of one of the most severe complications of LCH. Treatment strategies have been designed and conducted by the Histiocyte Society since 1991, in three large, prospective international studies (LCH-I, LCH-II and LCH-III), with the chemotherapeutic drug vinblastine in combination with prednisolone used as the basis for the therapy. Treating CNS-LCH remains one of the most difficult tasks, and intravenous immunoglobulin and cytosine arabinoside/cytarabine, drugs that have demonstrated some promising effects, will be evaluated in the ongoing LCH study, LCH-IV.

Since Dr. Barrett Rollins and his colleagues in 2010 found the BRAF V600E point mutation in approximately half of the studied LCH lesions (Badalian-Very et al., 2010), new treatment strategies targeting the mutant are being used in LCH (Donadieu et al., 2019), and recently have been proposed to represent a successful treatment strategy even for CNS-LCH (Henter et al., 2021). LCH is today classified as inflammatory myeloid neoplasia and the identification of BRAF V600E as well as LCH cell clonality (Halbritter et al., 2019; Willman et al., 1994; Yu et al., 1994), were arguments used to emphasize the cancerous or neoplastic nature of LCH cells, although the previous debate regarding the nature of LCH as an inflammatory condition (Fadeel and Henter, 2003; Laman et al., 2003) is still ongoing (Mitchell and Kannourakis, 2021). With respect to mutational characteristics, BRAF V600E is a well-known mutation found in various cancers, most frequently in melanoma, as well as in benign neoplastic conditions, such as colonic polyps and skin nevi. This mutation leads to a downstream signaling cascade through

phosphorylation and activation of MEK and ERK that belong to the MAPK signaling pathways, central for proliferation, differentiation, and survival of a cell. While 50-65 % of LCH lesions are positive for BRAF V600E, mutations in other upstream members of the ERK signaling pathway have been described: *MAP2K1* (encoding MEK1), as well as *ARAF* and *ERBB3* (Chakraborty et al., 2014). Interestingly, regardless of mutation status, all LCH lesions studied have been found positive for phosphorylated ERK (pERK) (Badalian-Very et al., 2010). According to a model of LCH pathogenesis developed by Dr. Carl Allen and his colleagues, ERK activation at specific stages of the myeloid mononuclear cell development determines clinical disease phenotype (Collin et al., 2015). This model is based on an observation that in high-risk patients with multisystem LCH the mutation is found in circulating mononuclear myeloid cells in blood (including $CD14^+$ monocytes, $CD16^+$ nonclassical monocytes, $CD1c^+$ DCs and $CD141^+$ DCs and, in some cases, hematopoietic stem and progenitor cells in bone marrow, whereas patients with single system LCH were positive for the mutation only in the lesion (Collin et al., 2015). The concept has been confirmed using animal models (Berres et al., 2014), but recently has been challenged as precursors from patients with single system LCH were demonstrated to carry mutations, and were able to develop to LCH cell-like cells (Xiao et al., 2020). With respect to origins, although an alternative hypothesis exists for LCH especially with CNS involvement (Mass et al., 2017), LCH is today believed to stem from the hematopoietic system rather than from Langerhans cells that are tissue resident macrophages, which may bring the "X" back to Histiocytosis "X". Indeed, the ontogeny, heterogeneity, and functional properties of the mutated LCH cells in relation to normal MNPs in tissue environment remain to be elucidated. To address this, we have performed profiling of LCH cells and other MNPs found in LCH lesions using single-cell RNA sequencing (scRNA-seq) combined with protein analyses and high-dimension microscopy, taking microenvironmental perspective into account (**paper IV**).

1.5 COVID-19: ACUTE INFECTION

At the time of the writing this thesis, all the world is aware of COVID-19, and the still ongoing pandemic caused by this disease will likely leave its wounding mark in our lives and health for the years to come. COVID-19 stands for "corona virus disease 2019", that first originated in the end of 2019 and started to spread around the globe, developing into a pandemic. It is caused by a new highly contagious zoonotic viral pathogen, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), that caused more than 1.5 million deaths during the first year of the pandemic (Guan et al., 2020). The symptomatology varies: the infection can be asymptomatic or cause mild cough, but may also develop into a life-threatening acute respiratory distress syndrome, coagulopathies, systemic thrombogenicity, a hyperinflammatory state, multiple organ failure, and death (Huang et al., 2020a; Mehta et al., 2020). Patients with diabetes, obesity, older age, or underlying immunosuppressive disease are at higher risk to develop severe COVID-19. While severe COVID-19 is rare in the pediatric population, and most COVID-19-related hospitalizations

in children occur in patients with a predisposing condition, such as immunosuppression or cancer, approximately half of the hospitalized children have no identified risk factor (Bogunovic and Merad, 2021). While the complete picture of COVID-19 and the associated hyperinflammation mechanisms remain to be elucidated, myeloid cells, that are able to produce large amounts of pro-inflammatory cytokines such as TNF and IL-6, appear to play a major role in disease pathogenesis (Merad et al., 2021). With regard to efficient treatment options in COVID-19, much have been learnt from earlier studies on hemophagocytic lymphohistiocytosis (HLH), a syndrome which embraces several virus-triggered life-threatening hyperinflammatory conditions, the most common being Epstein-Barr virus (EBV)-HLH (La Rosée et al., 2019). Although the use of include immunosuppressive drugs, such as corticosteroids (including dexamethasone), IL-1 inhibitors, and IL-6 inhibitors, may appear counter-intuitive in viral infections, they have shown remarkable clinical value in first HLH (Bergsten et al., 2017; Imashuku et al., 1999; 2021; La Rosée et al., 2019; Trottestam et al., 2011) and later also in COVID-19 (RECOVERY Collaborative Group, 2021; Somagutta et al., 2021; The RECOVERY Collaborative Group, 2020).

Many cellular players have been implicated in COVID-19 immunopathogenesis, ranging from epithelial, stromal, and immune cells. In general, MNPs play a crucial role in anti-viral defence, ranging from orchestration of adaptive and innate immunity, as well as restoration of tissue homeostasis and repair mechanisms. However, misguided or dysregulated immune responses by these cells may instead have detrimental consequences causing immunopathology and tissue damage, as it is also evident in COVID-19 (Merad et al., 2021). Monocyte, monocyte-derived cells, and DCs have been implicated in COVID-19 immunopathogenic mechanisms in early reports (Giamarellos-Bourboulis et al., 2020; Liao et al., 2020; Merad and Martin, 2020; Sánchez-Cerrillo et al., 2020; Schulte-Schrepping et al., 2020; Silvin et al., 2020; Wen et al., 2020; Wilk et al., 2020; Zhou et al., 2020), and it is now clear that aberrant MNP responses, such as expansion of immature subsets, highly contribute to the pathogenic inflammation in COVID-19. In addition, depletion of lung alveolar macrophages, that are key regulators of tissue repair mechanisms and maintenance of tissue homeostasis, has been reported (Merad et al., 2021). The engagement and contributions from the global research community are continuously increasing the large body of knowledge of disease mechanisms in COVID-19, and taking historical perspective into account, this process has been fuelled at a record speed. Broad single-cell sequencing efforts had taken our understanding of cellular players in immunopathogenesis to new levels, but a detailed and comprehensive understanding of different MNP subsets, including DCs, and their circulating progenitors in COVID-19 was still largely missing. To address this, we performed a deep profiling of the circulating MNPs, analyzing up to 10 million cells per patient, using 25-color flow cytometry, followed by integration of our data with soluble factor data and publicly available single-cell data from tissue (**paper V**). This was done as part of The Karolinska KI/K COVID-19 Immune Atlas project, aiming to provide a comprehensive overview of how immune system respond to COVID-19 (https://covid19cellatlas.com/).

2 RESEARCH AIMS

The general aim of this thesis was to improve the understanding of the role of myeloid cells in human health and disease. More specifically, the role of neutrophils and MNPs were addressed in inflammation, cancer and infection, focusing on patients with IBD, LCH, and COVID-19.

3 RESULTS AND DISCUSSION

The content of the scientific papers included in this thesis will be discussed in the following sections. The first section will cover the methods used here and future perspective for studying myeloid cells in human health and disease, followed by a second section focusing on ethical considerations. The next two sections will focus on myeloid cells in IBD, more specifically: IL-23 producing neutrophils (**paper I**) and their role in IBD immunopathogenesis; and the role of inflammation-responsive stroma in shaping intestinal macrophage pool (**paper II**). The following section will cover IL-23 in LCH, including a brief discussion of case series of patients affected both by CD and LCH (**paper III**). Next, the focus will lay on the MNPs, and in particular DCs, in relation to the composition of the LCH lesions and the origin of neoplastic LCH cells (**paper IV**), including a perspective of general DC functions in cancer. Finally, the last section includes a discussion on circulating MNP landscape in COVID-19 (**paper V**). An extensive description of materials, methods, patient characteristics, results, and discussion, are provided in the thesis articles.

3.1 METHODOLOGICAL DISCUSSION

Questions asked and answers delivered depend on the methods and clinical research platforms available at that time. In this section the methodological context of the thesis will be discussed, also presenting an outlook for future research.

In **paper I** and **paper III**, proteins of interest were detected using immunohistochemistry, immunofluorescence and flow cytometry, and qPCR was used to detect mRNA. The strengths of the methodological pipeline applied include quantification, such as the one of neutrophils and IL-23^+ neutrophils, using confocal microscopy, that was performed on the whole tissue sections. Also, the analyses were performed *ex vivo* in colon tissue biopsies of newly diagnosed and treatment pediatric patients with suspected IBD, included prior to their first diagnostic colonoscopy. This allowed analyses relatively early in the development of IBD, and minimizing the risk for biases related to medication and comorbidities that are otherwise difficult to avoid in adults, especially with many years of IBD. After the diagnostic work-up, patients who were not diagnosed with IBD, were considered as controls, providing an important reference. The limitations of the study include a relatively low number of patients, meaning that stratification with respect to subtypes of IBD, other clinical characteristics, and disease severity within those subgroups was not assessed. Overall, hypothesis-based methodological approaches were taken in **paper I**, which have both strengths and limitations. Future work addressing neutrophil frequencies and functions in IBD tissue would benefit from multi-omics approaches, both on single cell protein and transcriptional levels, taking spatial perspective into account.

In **paper II**, in addition to microscopy, the flow cytometry analyses were developed further in this study, including the 25-color flow cytometry, allowing simultaneous analyses of MNPs and stromal cells in the colon tissue. In addition, samples from inflamed and non-

inflamed colonic areas were compared also utilizing single cell sequencing, teasing out specific inflammation-related effects without variance introduced by interindividual comparisons. Moreover, co-culture systems allowed functional analyses of primary stromal cell subsets as well as monocytes. These are the strengths of the study, with limitations including challenges related to expansion of all the individual subsets of stromal cells given relatively small amounts of starting material. Moreover, studies on effects of specific intestinal stromal populations on macrophage development should be addressed in different intestinal layers, as well as with respect to proximal to distal direction in the gut.
In **paper III**, a similar laboratory setup was used as in **paper I**, and main limitations of the applied setup were related to the rareness of the samples and affected patients. To start with, deeper characterization of molecular status of the initial LCH lesion of the patient, whose family was also included in the study, was necessary in order to have a chance to track eventual clones during the intestinal manifestation. Second, IL-23 levels and its functional consequences in other case series patients were not addressed since samples were not available for this type of analyses. Nevertheless, even if increasing patient numbers would remain challenging in future studies of similar design, this type of case report series plays an important role in reminding us of differential diagnoses, such as CD in **paper III**, and diagnostic biases that may hamper both diagnostic and therapeutic strategies.

The main strengths of techniques utilized in **paper IV** include index-sorting combined smart-seq2 protocol, that is deeper than the one used in **paper III**, namely 10x. On the other hand, higher number of cells can be interrogated using 10x, which presented a limitation in **paper IV**. This was overcome by integrating our smart-seq2 data set with the already published 10x, with the benefit of protein-based annotation available due to index-sorting. Moreover, high-dimensional microscopy, employing 30 different antibodies was utilized in this study, taking the spatial perspective into account. Future work would benefit from spatial analyses utilizing even broader approaches on different levels ranging from RNA to protein, and comparing different disease stages, treatments, and outcomes. In addition, a robust methodological pipeline allowing unbiased detection of DNA, RNA, and protein in the same cell, would take our understanding of LCH pathogenesis and heterogeneity to new levels, and may be important also in other neoplastic and inflammatory conditions.

In the last study of the thesis, **paper V**, 25-color flow cytometry was performed on freshly isolated mononuclear cells from the blood. In the context of DC biology, and especially in COVID-19, where they disappear from the circulation, it was crucial to analyze relatively high number of cells, which was further facilitated by the fact that the analyses were performed on the fresh blood. This allowed phenotypic analyses of different DC subsets, and also their circulating progenitors. The work was designed and performed in an atlas-like fashion, meaning that a variety of parameters were measured and compared between the cohorts, but no functional tests on isolated cells were performed, which is an important

limitation. However, analyzing the frequencies and phenotype in relation to soluble factors, already published tissue data sets, and detailed clinical data, allowed us to provide an important reference for MNP responses in moderate and severe COVID-19. Other strengths of the study include a prospective study design, and defined inclusion and exclusion criteria. This may also represent major drawbacks, as the conclusions made cannot be directly translated to the general population, and should be regarded in the light of the inclusion and exclusion criteria applied.
Overall, all five studies, **papers I-V**, were performed in well-defined patient populations, which has a great potential to contribute to translational platforms aiming to improve diagnostic and therapeutic approaches in relevant clinical contexts. *In vivo* data and hypothesis testing utilizing experimental models allowing for further mechanistic insights will be important for future work exploiting concepts studied in this thesis.

3.2 ETHICAL CONSIDERATIONS

The research of this thesis focuses on myeloid cells in children with IBD and LCH as well as adults with COVID-19, and is covered by six ethical applications that have been approved by the Ethical Review Board in Stockholm (2010/32-31/4, 2018/323-31/1 for IBD; 2009/1937-31/1, 2012/530-31/2, 2019-03956 for LCH; 2020-01558 for COVID-19). Investigations involve collection of sensitive personal information, such as information regarding the patient's health status, and in addition to general information such as age and gender, also includes information on different treatments, clinical examinations, and outcomes. This data is combined with immunological parameters obtained from the analyzed biological patient samples, such as blood and tissue samples. All research samples are taken during examinations that are a part of clinical care, which also means no additional needle sticks for children. Clinical samples are coded prior to reaching the laboratory, and patient data is only accessible to authorized personnel directly involved in the studies.

In IBD, a central part of our studies is based on colon tissue biopsy investigations. The collection of the biopsies is performed during routine colonoscopy, and the risks associated with the collection of additional biopsies (always associated with a routine biopsy) are considered to be minimal for the patients participating in the study. The incidence and prevalence of pediatric IBD are increasing globally and there is still no cure, thus better understanding of the mechanisms underlying pediatric IBD are warranted.
With regard to LCH, our knowledge of the mechanisms leading to disease development is limited. The immunobiology studies of LCH are hampered by the rareness of the tissue samples, and LCH even today may have fatal consequences for the affected children.

With regard to COVID-19, the knowledge on disease pathogenesis is even more limited and although the literature related to COVID-19 is growing, new diagnostic and therapeutic tools are urgently needed. As our studies potentially can provide novel knowledge for the

development of treatment strategies and diagnostic tools for LCH, IBD and COVID-19 patients, potential benefits of the studies are considered to be greater than risks related to the studies.

Patients are included in studies after informed consents are provided. It is assured that patients, and their parents if the participant is younger than 18 years of age, receive the information regarding participation in the study so that they have enough time for reflection and discussion of their decision. It is crucial to confirm that the information is well-comprehended, which would allow patients and parents to make an informed decision regarding the participation. Importantly, even the written part of the study information is adjusted for different ages of the pediatric participants, in order to assure their consent is always given on a well-informed basis. Of note, in the middle of the research work performed in this thesis, a new European data protection law, namely the General Data Protection Regulation (GDPR) has been implemented. In line with this, all new ethical applications composed after 2018 were in accordance with the GDPR, and it could be noted that this did not incorporate drastic deviations in the way the patient data is being handled, since the highest standard for assuring patient safety and privacy was always a priority in our research.

3.3 IL-23 PRODUCING NEUTROPHILS AND THEIR ROLE IN IBD

Neutrophils are polymorphonuclear myeloid cells, granulocytes, that belong to the innate immune system, and they are the most frequent immune cells in blood. Neutrophils are absent in non-inflamed tissue, and are rapidly recruited to sites of inflammation to clear microbial pathogens. Thus, the recruitment and presence of neutrophils in tissue is an early sign of inflammation. Although recruited neutrophils contribute to the host defense by translocating across the epithelial barrier and killing luminal microbes, it is also evident that neutrophils exacerbate the inflammation by creating microscopic gaps between epithelial cells and release toxic granule contents while transmigrating (Fournier and Parkos, 2012). The classical view of neutrophils as terminally differentiated effector cells, exclusively involved in acute cellular inflammatory response, has been complemented by other features during recent decades. For example, it is now apparent that neutrophils interact with innate and adaptive immune cells, contribute to cytokine production and thereby play an important role in cancer, chronic inflammation, and autoimmunity (Mayadas et al., 2014). In the current thesis, IL-23-producing neutrophils were described in the colon tissue of newly diagnosed and treatment naïve pediatric IBD patients (**paper I**). This provides an additional perspective for neutrophil ability to orchestrate adaptive immune cells, such as T cells, in IBD. In fact, the neutrophil influence on adaptive immunity through interactions with T cells, and in particular Th17 cells, and neutrophil antigen presenting capacities and their role in IL-23-driven inflammation may be of key importance for IBD development in humans. In addition, studies performed in a non-IBD context suggest that neutrophils and T cells indeed interact (Silvestre-Roig et al., 2019), and focusing on their interaction in

tissues, the communication between neutrophils and T cells also involve other cell types. For example, stromal cells found in tissues respond to T cell derived IL-17 by producing granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-8, crucial for neutrophil recruitment and survival (Laan et al., 1999; Park et al., 2005; Witowski et al., 2000; Ye et al., 2001). In addition to Th17 cells, that are named after their IL-17 producing capacities, also other T cell types, such as subsets of $CD8^+$ T cells, γδT cells, CD1d-restricted natural killer T cells (NKT) cells, and mucosal associated invariant T cells, are able to produce IL-17 (Zimmer et al., 2021). Although neutrophils lack IL-17 receptor and thus can cannot directly respond to IL-17, Th17 cells can interact with human neutrophils through production of other cytokines, such as IFN-γ, GM-CSF, and TNF (Pelletier et al., 2010). In addition, it has been shown that human neutrophils cultured with lipopolysaccharide and IFN-γ induce migration of Th17 cells, in a CCL20- and CCL2-dependent fashion (Pelletier et al., 2010). Notably, a reciprocal chemotactic relationship where IL-8-producing Th17 cells induced migration of neutrophils has been described (Pelletier et al., 2010). In line with these observations, the highest expression of both IL-8 receptors, namely C-X-C motif receptor (CXCR)1 and CXCR2 (found on neutrophils), and IL-8 were detected in patients with severe disease in colonic tissue from children with newly diagnosed treatment naïve IBD (**paper I**). In addition, a strong correlation between expression levels of the ligand IL-8 and its receptors was detected (**paper I**). This suggests that in IBD, an IL-8/CXCR1/CXCR2-dependent migration of neutrophils into the intestine occurs. Moreover, compared to controls, a higher frequency of $IL\text{-}8^+$ cells was detected in mucosa of IBD patients, while epithelial IL-8 expression remained unchanged (**paper I**). So, what are those cells in IBD mucosa that via IL-8 production contribute to neutrophil influx? Broader studies employing single cell sequencing depicted myeloid and stromal sources of IL-8 in IBD, including both CD and UC (Friedrich et al., 2021; Martin et al., 2019; Smillie et al., 2019), while there is little evidence to suggest that T cells themselves are major IL-8 producers in IBD (Brandt et al., 2000). Indeed, isolated human fibroblasts from colon tissue respond to IL-17 and IL-22 with increased expression of neutrophil chemo-attractants (Andoh et al., 2005; Hata et al., 2002; Kerami et al., 2014).

Corroborating of the role of neutrophils in IBD, and especially taking into consideration the chronicity aspect of this disease, discussion regarding neutrophil capacity to activate and maintain the adaptive immunity is relevant. Notably, the neutrophils are capable to participate in this process by presenting antigens to T cells (Beauvillain et al., 2007; Vono et al., 2017). Using a mouse model, it was demonstrated that colonic neutrophils induced proliferation of antigen specific $CD4^+$ T helper cells, in a major histocompatibility complex class II (MHC-II) and antigen-dependent fashion (Ostanin et al., 2012). Moreover, they expressed MHC-II and the co-stimulatory CD86 in inflamed mucosa and synergistically increased cytokine production in co-cultures with T cells (Ostanin et al., 2012). However, while this suggest that neutrophil antigen presentation capacity to T cells may be important in driving pathological colonic inflammation in mice, the insights on neutrophil antigen

presenting abilities at the mucosa as well as in the secondary lymphoid organs of IBD patients is lacking. Nevertheless, human neutrophils are able to present antigens and induce proliferation of antigen-specific memory $CD4^+$ T cells, as this has been demonstrated in non-IBD contexts (Vono et al., 2017), (Beauvillain et al., 2011). More specifically, and perhaps not surprisingly, antigen presentation by human neutrophils was MHC-II dependent (Vono et al., 2017) and migration to lymph nodes was CCR7 dependent (Beauvillain et al., 2011). Other clinical contexts where neutrophil antigen presenting phenotypes have been adressed include cancer (Saha and Biswas, 2016), parasitic skin infection (Davis et al., 2017), allergy (MSc et al., 2019), and rheumatoid arthritis (Sandilands et al., 2006). To conclude, there is reason to suspect that neutrophils are able to maintain and activate adaptive immunity in human IBD also by directly presenting antigens, but future studies addressing this in clinical IBD contexts are warranted.

Apart from IL-23 production by neutrophils, reported by us in **paper I**, IL-17A-and/or IL-22-expressing neutrophils have been described in tissue inflammatory contexts both in mice and humans (Campillo-Gimenez et al., 2014; Chen et al., 2016; Ferretti et al., 2003; Hoshino et al., 2008; Li et al., 2010; Lin et al., 2011; Taylor et al., 2014; Werner et al., 2011; Zindl et al., 2013). Regarding the intestinal inflammation, both IL-22 and neutrophils had a protective role in pathogenic inflammation in a model of microbiota antigen-specific T cell–mediated colitis, although protection mediated specifically by IL-22-producing neutrophils was not established (Chen et al., 2016). Instead, in a mouse model of dextran sodium sulfate (DSS)-induced acute colitis, a protective role of IL-22-producing neutrophils has been demonstrated (Zindl et al., 2013). In more detail, the TNF potentiated IL-22 production by neutrophils, that lead to higher levels of protective antimicrobial peptides, produced by colonic epithelium (Zindl et al., 2013). In a human IBD context, IL-22 mRNA expression was higher in a subset of $CD177^+$ neutrophils, which, compared to controls, were more abundant in colonic lamina propria and peripheral blood of CD and UC patients (Zhou et al., 2018a). Moreover, the $CD177^+$ neutrophils had increased bactericidal activity and produced higher levels of myeloperoxidase (MPO), antimicrobial peptides, reactive oxygen species (ROS), and neutrophil extracellular traps (NETs). In addition, this subset of neutrophils also exhibited lower levels of proinflammatory cytokines, such as IL-6, IL-17A, and IFN-γ. A protective role of this neutrophil subset was also addressed in a DSS colitis model, where intestinal disease was worsened by depletion of CD177-expressing cells (Zhou et al., 2018a). It should, however, be noted that IL-17 producing capacities by human neutrophils have been debated and technical issues regarding the specificity of antibodies recognizing IL-17 and IL-17 family member cytokines, and that are commercially available, have been raised (Tamassia et al., 2018). This emphasizes the importance of reproduction of findings in different studies, also by including multiple detection techniques on mRNA and protein level. Likewise, comparisons of results from different contexts with respect to tissues, species, experimental setups, and diseases, needs to take those different the contextual platforms into account. An exciting question regarding the maintenance of IL-17A and IL-22 producing capacities by neutrophils and of course T cells, is the cellular IL-23 source at the site of inflammation (**paper I**). The general and

established answer to this question is that antigen presenting cells, such as macrophages or DC (Kamada et al., 2008; Ogino et al., 2013; Schmitt et al., 2019), is the dominant source. It was thus intriguing to identify neutrophils as the main producers of IL-23 in newly diagnosed and treatment naïve pediatric IBD patients (**paper I**). Actually, other studies in humans have also demonstrated that bacteria derived neutrophil-activating proteins are able to induce the IL-23/IL-12 production/secretion by neutrophils (Amedei et al., 2006; Codolo et al., 2008). Furthermore, TNF potentiates toll-like receptor (TLR) 8-dependent production of IL-23 by human neutrophils, and supernatants from TLR8-primed neutrophils drove Th17 phenotype in naïve T cell (Tamassia et al., 2019). Together, this may propose that neutrophils contribute to production of IL-17A and IL-22, and has a regulatory, IL-23-dependent capacity, not only with respect to T cells, but also in an autocrine fashion. Future studies will be needed to further dissect the neutrophil contributions to the IL-22, IL-23, and IL-17 family cytokines, as well as their functional consequences in IBD, considering different clinical IBD contexts, ranging from early disease stages to its advanced manifestations. Indeed, the role of IL-23 and its efficient targeting may depend on the cellular context/status/stage of IBD.

3.4 MACROPHAGES ARE SHAPED BY STROMA

Stromal cells, such as fibroblasts, myofibroblasts, glia, pericytes and endothelium, are tissue resident cells, widely distributed in human tissues and important for tissue development and homeostasis. In addition to classical fibroblast functions, such as mechanical tissue support and production of extracellular matrix proteins, a role of fibroblasts in mediating immune response through production of cytokines and chemokines, phagocytosis as well as tissue remodeling, has emerged in multiple inflammatory disorders. Fibroblast-leukocyte interactions are best studied in the immunopathology of rheumatoid arthritis (RA), an inflammatory joint condition, where inflammatory changes in synovial RA fibroblasts appeared to be persistent (Korb-Pap et al., 2016). Mechanistically, with respect to colonic stroma, it is known that colonic fibroblasts express pattern recognition receptors, such as TLRs, nucleotide-binding oligomerization domain 2 (NOD2), nucleotide-binding oligomerization domains, leucine rich repeat and pyrin domain containing (NLRPs) (Owens et al., 2013) and respond to pro-inflammatory cytokines, such as TNF, IL-1b, IL-17A and IL-22 (Andoh et al., 2005; Owens and Simmons, 2013). It has been suggested that a subset of fibroblasts, situated in subepithelial regions in the gut, support growth of epithelial stem cells, contributing to the maintenance of epithelial integrity and providing a link between mucosal and epithelial immunity (Lei et al., 2014). Recent broad sequencing efforts has brought up the role of colonic stromal cells in IBD pathogenesis (Huang et al., 2019; Martin et al., 2019; Smillie et al., 2019). A small subset of inflammatory fibroblasts, expressing proinflammatory cytokines and higher levels of a glycoprotein podoplanin (PDPN), were identified in IBD patients in those studies, while our data point to general remodeling of all fibroblast clusters investigated, and increase of PDPN on all major fibroblast subtypes, including myofibroblasts (**paper II**). There are a couple of differences

in the experimental design and questions asked that may explain these differences. For example, in contrast to studies performed in adult population with years of IBD prior to biopsy collection/surgical resection (Martin et al., 2019; Smillie et al., 2019), or comparisons made between IBD patients and healthy controls (Elmentaite et al., 2020; Huang et al., 2020b; Kinchen et al., 2018), our scRNA-seq experimental pipeline was designed to take interindividual variation into account and instead compare mesenchyme in inflamed and non-inflamed intestinal biopsies from newly diagnosed and treatment naïve pediatric patients with IBD (**paper II**). In addition, we focused on stromal cells in particular by enriching for stromal compartment using flow cytometry-based sorting, that allowed analyses of higher number of cells. Future studies will be needed to address functional consequences of stroma remodeling in IBD, also with respect to stroma potential to shape all the different immune cell populations.

Interestingly, it has been demonstrated *in vitro* that cytokines implicated in IBD pathogenesis, e.g. TNF and IL-17A, enhance colonic myofibroblast production of GM-CSF, a cytokine involved in myeloid cell differentiation (Andoh et al., 2005). In line, a concept of fibroblast-macrophage cell circuit has recently been presented, where an interdependency relationship was described between these two cell types (Zhou et al., 2018b). While one may suspect that such circuit systems in a complex tissue environment involve multiple cell types, our understanding of how stromal cells, such as fibroblasts, shape immune cells, such as macrophages, in health as well as in IBD, is limited. To gain insights in this process, we investigated stromal cells and monocytes/macrophages in **paper II**. Interestingly, colonic stroma, more specifically fibroblasts, had a potential to induce a macrophage phenotype on monocytes in co-culture systems, as evident by loss of the chemokine receptor CCR2 and increased expression of CD206. Moreover, when monocytes were cultured with $PDPN^+$ fibroblasts, the derived macrophages from these co-cultures expressed lower levels of the immunoregulatory molecule PD-L1. This observation, that was both inflammation and contact dependent, was reflected in the *ex vivo* monocytes from newly diagnosed and treatment naïve patients with IBD. This data points to stroma-mediated impairment of monocyte capacities to regulate inflammatory response in IBD mucosa. To illustrate the functional consequences of insufficient regulatory capacity mediated through PD-L1, the development of enterocolitis in cancer patients treated with PD-L1 blockade could be brought up (Ibraheim et al., 2020). Indeed, complications related to this type of enterocolitis, that often requires hospitalization and might be life-threatening (Ibraheim et al., 2020), illustrates the degree to which PD-L1 participates in the homeostatic balance of mucosal gut immunity. Moreover, stromal capacity to shape tumor-associated macrophages may be of interest to explore in a cancer setting. For example, high levels of PD-L1 is a biomarker for a poor prognosis in colorectal cancer (Li et al., 2019), and an important source of PD-L1 in cancer involving large intestine is tumor-associated $CD14^+$ monocytes/macrophages (Cantero-Cid et al., 2018). Nevertheless, it remains to be understood how this effect of $PDPN^+$ fibroblasts is mediated, and what (if any) function PDPN has in shaping the macrophage pool. In addition, little is known on how these

processes are governed with respect to different layers of the intestine, and also with respect to the proximal to distal direction. To sum up, it appears to be so that we are only scratching the surface of understanding the stromal cells capacity to regulate intestinal immunity. Studies combining multi-omics techniques and functional testing in well-characterized clinical cohorts of IBD, and other disorders, ranging from cancer to infection, has potential to open up new therapeutic avenues.

3.5 THE ROLE OF IL-23 IN LCH

The chronic inflammatory context addressed in this thesis focus on two major pediatric patient groups: patients with LCH, that is an inflammatory myeloid neoplasia, and IBD, a chronic inflammatory condition mainly affecting the gastrointestinal tract. Both in LCH and in a subtype of IBD, namely in CD, organized inflammatory nodules, so called granulomas, can be found. In general, granulomas are characterized by accumulation of immune cells in affected tissues and they are a hallmark of granulomatous diseases. They can be infectious by nature, such as in tuberculosis, or apparently non-infectious, such as in sarcoidosis, LCH or CD, where one particular pathogen triggering the formation of granuloma and leading to tissue destruction has not been identified. The study of **paper III** in this thesis focused on a family affected by both LCH and CD, which leads to the question: how common are these two conditions? LCH is less common than CD, with a minimal incidence in children of 4.6-8.9 per million with the highest incidence so far reported in a population-based study from Sweden (Allen et al., 2018; Stålemark et al., 2008). The incidences of both pediatric CD and UC have increased over the last 50 years, and for pediatric CD is instead 9-10 per 100,000 person-years in parts of Europe, including Scandinavia (Roberts et al., 2020). Although there are reasons to believe that the incidence of LCH often may be underestimated and that pediatric CD is not as rare as it was 50 years ago, it is nevertheless very unusual to see patients affected by both LCH and CD. This may explain why the diagnosis of CD in LCH patients in the case series presented in **paper III** has been delayed up to three years. Also, due to this rarity, our experimental design started by whole genome sequencing of the family in order to investigate and exclude the possible monogenetic causes. On the other hand, the patient, affected by LCH as a toddler followed by the CD diagnosis 12 years later, and his brother, both had their CD onset in the age range that would fit more with the polygenetic disease phenotype, that is the most common in all IBD cases. Corroborating on the challenges related to identifying a CD diagnosis in patients with LCH, the gastrointestinal involvement in LCH should be discussed. Indeed, LCH has a broad range of clinical manifestations, ranging from single system self-resolving disease in bone and skin, to multisystem risk organ disease that may be life-threatening (Allen et al., 2018). Keeling and Harries first reported a case of gastrointestinal (GI)-LCH in an autopsy study (Keeling and Harries, 1973), and clinical symptoms of GI-LCH include abdominal pain, failure to thrive, anemia, hypoalbuminemia, bloody stool, and diarrhea, and is typically associated with systemic disease and poor prognosis (Minkov et al., 2021; Singhi

and Montgomery, 2011). Thus, the overlap of clinical symptoms between GI-LCH and CD represents another diagnostic and therapeutic challenge.

When LCH is suspected in patients with inflammatory gastrointestinal involvement, CD1a and CD207 immunostainings as well as BRAF V600E status may facilitate the diagnostics significantly. That being said, cases with CD1a-negative LCH in children have been described (Powell et al., 2017), which further exemplifies the diagnostic challenges. Even if the etiology of CD and LCH may be completely different, it is interesting to investigate the possibly common downstream immunological traits between these two diseases, as this may have potential therapeutic implications. IL-23-driven disease caught our attention in the patient presented in **paper III**. In this case, rather not surprisingly, it was detected during the CD manifestations. But the question regarding the role of IL-23, for which an efficient therapeutic inhibition with limited side effects exists, in the general LCH population was raised. Interestingly, elevated IL-23 plasma levels were detected in patients with LCH, and IL-23 levels in the circulation correlated with the percentage of neoplastic cells, namely LCH cells, in the lesions (**paper III**). These observations suggest that IL-23 has a role in LCH pathogenesis, and in line with our findings, higher levels of cytokines downstream of IL-23, as well as the subunit p40, which is shared by IL-12 and IL-23, have been reported in LCH (Coury et al., 2008; Morimoto et al., 2017). It remains to be understood how IL-23 could be used for diagnostic and/or therapeutic purposes, and questions of IL-23 biology and signaling events in relation to disease severity in LCH remain to be addressed. From the immunobiology perspective, it would be important to understand which the cellular source of IL-23 in LCH is, and how IL-23 producing cells are related to LCH cells in the lesion microenvironment, taking the spatial perspective into consideration. This might be interesting from the point of view related to tissue destruction, that presents a clinical problem in LCH, as a role for IL-23 in an IL-17-independent bone resorption through osteoclastogenesis has been described (Moschen et al., 2019). In addition, questions on regulatory IL-23 functions and maintenance of adaptive responses in LCH lesions remain to be addressed. Last but not least, LCH cells themselves carry transcripts for the receptor of an IL-23 downstream cytokine, namely IL-22 (Halbritter et al., 2019) (and also **paper IV**), which allows a speculation that signaling through the IL-23/IL-22/IL-22R axis may present an additional survival signal for LCH cells, contributing to their persistence in the lesions.

3.6 DENDRITIC CELLS IN LCH AND IN CANCER

LCH is today recognized as an inflammatory myeloid neoplasia characterized by $CD1a^{+}/CD107^{+}$ histiocytes that often carry BRAF V600E, but its origin as well as the precise MNP composition in LCH lesions remains to be elucidated. In **paper IV** we addressed the composition of lesion MNPs at single-cell level to define the LCH specific core signature, that pointed to senescence, that has recently been demonstrated in LCH using animal models (Bigenwald et al., 2021), and several tumor escape mechanisms. It has

been suggested that LCH originate from the DC lineage (Lim et al., 2020), but normal DCs may also be identified in the lesions, given their key roles in cancer surveillance. Indeed, in addition to LCH cells, monocytes/macrophages and DC1, DC2, DC3, pre-DC, and newly described mregDCs (Maier et al., 2020) were identified in LCH lesions using index-sort combined with single-cell sequencing (**paper IV**). Moreover, proficient separation of LCH cells from the rest of the MNPs allowed us to address LCH heterogeneity, which revealed two major clusters, phenotypically related DC2 and monocyte/DC3 lineages (**paper IV**). While lineage specific as well as general programs in antitumor as well as tolerogenic DC responses in LCH lesion tissue microenvironment remain to be elucidated, it is evident that high-dimensional single-cell techniques opened up new horizons for the DC heterogeneity and functions at the tumor site.

Deeper characterization of tumor-infiltrating myeloid cells has recently identified a common program in myeloid DCs detected in lung cancer (Lavin et al., 2017; Maier et al., 2020; Zilionis et al., 2019), hepatocellular carcinoma (Zhang et al., 2019), as well as in LCH (**paper IV**). Those LAMP3^{+} DCs found in tumors displayed maturation and an immunoregulation profile, evident by expression of CD200, PD-L1, PD-L2, and IL-12, CCR7, CD40, respectively, and were named ‘mature DCs enriched in immunoregulatory molecules’ (mregDC) (Maier et al., 2020). Notably, upon tumor encounter, both cDC2 and cDC1 upregulated mregDC markers (PD-L1, CD40, IL-12) in an animal model (Maier et al., 2020). Furthermore, by using cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) the scientists confirmed that the mregDC pool in mice included cells from both cDC2 (XCR1^{-}CD103^{-}CD11b^{+}) and cDC1 (XCR1^{+}CD103^{+}) lineages (Maier et al., 2020). In human tumors, the mregDC pool consists of cells that are transcriptionally similar to cDC2 or cDC1 (Maier et al., 2020; Zilionis et al., 2019), and in LCH samples it included cells, identified by index sorting (18-color flow cytometry), as cDC1, cDC2 and pre-DCs (**paper IV**). Recent atlas-like integration efforts confirmed the existence of mregDCs in various human tumors (Cheng et al., 2021; Gerhard et al., 2021), and, using computational approach, suggested that mregDC originate from both cDC1 and cDC2 (Cheng et al., 2021). Functionally, the mregDC program is suggested to be driven by IL-4, and resulting into reduced potential of anti-tumor responses through lower levels of IL-12 (Gerhard et al., 2021; Maier et al., 2020; Zhang et al., 2020). In fact, higher levels of LAMP3 (marker for mregDCs) in human lung cancer tissue (Dieu-Nosjean et al., 2008; Germain et al., 2014) and in the sentinel nodes of metastatic melanoma patients (Elliott et al., 2007; Movassagh et al., 2004) were associated with better survival. From the spatial perspective, human LAMP3^{+} DCs in tumors were associated with tertiary lymphoid structures (Dieu-Nosjean et al., 2008; Germain et al., 2014), or accumulating together with T cells at the tumor margins, suggesting a strategic localization with respect to their regulation of adaptive immunity at the site of the tumor (Liu et al., 2010). In line with these observations, formation of tertiary lymphoid structures also in LCH lesions pointed to better outcome (Quispel et al., 2016), but mregDC positioning in relation to these structures in LCH, as well as other neoplastic lesions, remains to be further defined. Moreover, the

mechanistic insights that may be important for modulation of this program from the therapeutic point of view are also warranted. A recent study using animal models revealed that loss of TIM-3, that is an immune checkpoint that recently gained more attention, in DCs resulted into a strong anti-tumor immunity (Dixon et al., 2021). In more detail, TIM-3 deficient DCs exhibited a weaker IL-4 driven mregDC program (Maier et al., 2020), and lower levels of receptor for IL-4 were detected on TIM-3 deficient DCs (Dixon et al., 2021). Future work will be required to prove and consolidate if this mechanism could represent a relevant target in human cancer, and in LCH. Conceptually, it is intriguing to map contributions of DC lineage/ontogeny and tumor microenvironment effects on DC mediated anti-tumor immunity.

With respect to functional specifications of different DC subsets during anti-tumor and tolerogenic responses in the contexts of neoplastic transformation, higher levels of cDC1 signature and frequencies predict better survival and responsiveness to treatments in human cancer (Barry et al., 2018; Böttcher et al., 2018; Michea et al., 2018; Spranger et al., 2017). It is evident that cDC1 is a the principal regulator of anti-tumor immunity cross-presenting tumor antigen to cytotoxic $CD8^+$ T cells, though cDC1 capability to skillfully present tumor antigens to $CD4^+$ T cells has also been demonstrated *in vivo* using animal models (Ferris et al., 2020). With respect to tumor-induced immune escape, modulation of cDC1 cross-presentation has recently been described through diminished CLEC9A binding to dead cell fragments, resulting in impairment of cross-presentation of dead cell-associated tumor antigens (Giampazolias et al., 2021). In addition, it was demonstrated that higher levels of CLEC9A predicted better survival in patients with head and neck squamous cell carcinoma, liver hepatocellular carcinoma, and stomach adenocarcinoma (Giampazolias et al., 2021). It remains to be understood whether a similar mode of action could be relevant for LCH lesions, as a subset of LCH cells was previously suggested to express CLEC9A (Halbritter et al., 2019). However, we could not confirm this observation, as the only cluster expressing CLEC9A in LCH lesions were DC1s (**paper IV**). There might be a couple of reasons explaining this discrepancy: while patient heterogeneity may be one, although less likely explanation, the use of different technical pipelines, i.e. a deeper smartseq2 protocol in our study allowing more accurate cell annotation, and a less deep 10x protocol instead allowing for analysis of higher number of cells in the previous study, present more likely explanations (Halbritter et al., 2019).

With respect to human cDC2, that share many phenotypic features with the neoplastic histiocytes in LCH, a DC2/DC3 cluster distinct from the LCH cells were identified among the lesion MNPs (**paper IV**). There is convincing evidence that certain aspects of normal cDC2 functional features in cancer are species-specific, since in contrast to the mice equivalent, human cDC2 secrete high levels of IL-12, that has a potential to prime cytotoxic T cell anti-tumor responses (Mittag et al., 2011; Nizzoli et al., 2013). In humans, cDC2 represents a heterogenous population, comprised of DC2 and DC3, and DC3 often corresponds to so called inflammatory DC (infDC). InfDCs were found to accumulate in

ascites from patients with ovarian cancer and in patients with breast cancer, prior to initiation of treatment (Segura et al., 2013). In line with DC3 characteristics, infDC share molecular signature both with monocytes and DCs, and are positive for DC3 markers, such as CD206, FcεRI, CD1c, and CD14 (although the CD14 expression on DC3 population is approximately one log lower compared to monocytes) (Segura et al., 2013). In line with myeloid DC functionality, DC3/infDC have capacity to efficiently induce naïve $CD4^+$ helper T cells responses and their production of IL-17, but could not be identified in tonsils of healthy controls or cancer-free lymph nodes from breast cancer patients (Segura et al., 2013). Hence, it is less probable that the major task of DC3/infDC is induction of immune responses in secondary lymphoid organs, where their existence seems to be related to context/cancer/tissue type (Abolhalaj et al., 2018; Segura et al., 2013). With respect to the prognostic role of cDC2, while higher levels of DC1 signature was associated with better survival in both triple negative breast cancer (that is considered to be more aggressive) and luminal breast cancer, a DC2 signature was instead only associated with better prognosis in the luminal type (Michea et al., 2018). In contrast, higher macrophage/monocyte signature was associated with worse outcome in both cancer types (Michea et al., 2018). It will therefore be exciting to explore the role of DC3, that share qualities of DC2s and monocytes, and understand the functional properties of different LCH subsets, related to either DC2 or DC3/monocytes, identified in **paper IV**. At this point, it was not possible to tell whether one of the LCH clusters had more in common with DC3 or monocytes, due to their transcriptional similarity. However, when exposed to LCH-phenotype-inducing culture conditions dependent on Notch ligation, DC3 showed more similarity with this particular *ex vivo* LCH cluster. In addition, receptor-ligand interactions between the different subsets revealed Notch related communication between the LCH subsets pointing to the relevance of the *in vitro* culture system used (**paper IV**). Future research will be needed to further define developmental trajectory of LCH subsets, and their interdependency relationship with each other and with other MNPs at the lesions. For example, there is a large body of literature to suggest that cancer cells alter monocyte phenotype and functions, and in a study where primary non-small cell lung cancer cells, derived from patients, were cultured with monocytes, higher levels of anti-inflammatory cytokines and lower levels of co-stimulatory molecules were detected on monocytes (Lu et al., 2019). This report, in line with a substantial amount of supporting data, depicts the immunosuppressive profile of *in vitro* monocyte-derived cells often referred to as monocyte-derived DCs (mo-DCs) (Brown et al., 2003; Laoui et al., 2016). Due to seemingly immunosuppressive nature, and also with respect to the ontogeny, these cells in tumors may rather correspond to tumor-associated macrophages. Yet, it becomes clear that DC3, in contrast to monocyte-derived cells *in vitro*, denotes a separate lineage on functional, phenotypical, and probably also on developmental level (Bourdely et al., 2020; Cytlak et al., 2020; Dutertre et al., 2019; Villani et al., 2017). Moving forward, it will be important to further dissect the heterogenous cDC2 compartment and understand its functional contributions during the neoplastic transformation such as the one in LCH.

Regarding pDCs, their higher frequencies at the site of the tumor associate with poor prognosis in human cancer (Aspord et al., 2013; Han et al., 2017; Jensen et al., 2012; Labidi-Galy et al., 2011; 2012; Treilleux et al., 2004). With respect to function, pDCs are the key producers of IFN-α/β, that is crucial in anti-tumor immunity. However, in cancer, pDC IFN-α/β-producing capacity is impaired, leading to perpetuation of the immunosuppressive tumor microenvironment. This has been demonstrated in multiple human tumors, ranging from cervical (Demoulin et al., 2015), ovarian (Labidi-Galy et al., 2011), head and neck (Bruchhage et al., 2018; Hartmann et al., 2003), melanoma (Aspord et al., 2013), to breast cancer (Sisirak et al., 2012; 2013). Mechanistically, inhibition of IFN-α/β production by pDCs appears to be mediated by TNF and immunosuppressive cytokines, such as IL-10 and TGF-β, at the tumor site (Sisirak et al., 2012), (Sisirak et al., 2013), (Bruchhage et al., 2018). It is expected that tumor environment contains immunosuppressive cytokines such as TGF-β, and many sources contributing to their production are well-described also in LCH by us and others (Allen et al., 2010; Mitchell et al., 2020; Quispel et al., 2015; Senechal et al., 2007; Tong et al., 2014). With respect to cellular mediators, it has been suggested that pDCs are able to stimulate IL-10 producing cellular sources, such as $Foxp3^+$ T regulatory cells (Conrad et al., 2012; Faget et al., 2012; Pedroza-Gonzalez et al., 2015; Sisirak et al., 2012), that are present at LCH lesions as well (Mitchell et al., 2020). On the contrary, better survival in patients with ductal pancreatic adenocarcinoma is associated with higher circulating pDC frequencies (Tjomsland et al., 2010). Essentially, the better outcome was in this study linked with higher pDC frequencies among the circulating peripheral blood mononuclear cells (PBMCs), and one may suspect that these patients as a result had lower levels at the tumor site, possibly clarifying the better outcome. Interestingly, we have recently shown that lower pDC frequencies in the circulation predict a more severe disease phenotype in LCH (Shi et al., 2021). This type of comparison was not performed in **paper IV** due to specific enrichment of MNPs, as well as the fact that PBMCs were not included in the study, but it remains an important question to be further explored in future studies.

3.7 CIRCULATING MONONUCLEAR PHAGOCYTES IN COVID-19

Circulating MNPs, both DCs and monocytes, are at the frontline during infections, and excel in pathogen antigen recognition, processing, presentation to adaptive immune cells, and production of cytokines, shaping the inflammatory response. Both pro-inflammatory and immunosuppressive functions are necessary during a course of a balanced immune response to a pathogen. In other words, the aim to efficiently clear the pathogen needs to be harmonized and achieved without causing extensive damage to the host and the role of immune cells during the later phases of resolution of the inflammatory reaction are important keystones in the healing process. So what does go right and wrong with respect to viral clearance and immunosuppression in COVID-19? To gain insight in this question, we studied COVID-19 patients that were prospectively recruited to groups with moderate and severe disease activity, respectively (**paper V**). In addition, a group of healthy donors, that were

matched for age and sex and were negative for SARS-CoV-2 IgG, provided the healthy reference in the analytic pipeline. Compared to healthy controls, an expansion of intermediate $CD14^{+}CD16^{+}$ monocytes was observed in both moderately and severely affected patients. This observation went in hand with previous reports in COVID-19 patients (Gatti et al., 2020; Merad and Martin, 2020), and appeared to be a general feature of clinical SARS-CoV-2 infection, given the more significant differences in moderately sick patients. Indeed, intermediate monocytes contain a pronounced IFN-signature in COVID-19 (Schulte-Schrepping et al., 2020), and one may hypothesize that a sufficient response from the intermediate monocytes helps to clear the SARS-CoV-2 infection in an efficient way, but *in vivo* evidence would be required to strengthen this hypothesis. While intermediate monocytes expand, most significantly in the moderately affected patients, human leukocyte antigen (HLA)-DR^{low} monocytes emerge in the severely affected COVID-19 patients (**paper V**). This observation in is line with previous reports (Agrati et al., 2020; Giamarellos-Bourboulis et al., 2020; Schulte-Schrepping et al., 2020), that in severely COVID-19 patients detected these immunosuppressive subsets, corresponding to monocytic myeloid derived suppressor cells. While expansions of the immature monocytes and intermediate monocytes are evident in COVID-19, with most pronounced changes in severely and moderately affected patients, respectively, a loss of non-classical $CD14^{low}CD16^{high}$ is also well-documented (Gatti et al., 2020; Hadjadj et al., 2020; Schulte-Schrepping et al., 2020; Wilk et al., 2020). Lower frequencies of non-classical monocytes is not a COVID-19-specific feature, but rather well-described in other inflammatory and infectious settings (Burbano et al., 2014; Naranjo-Gómez et al., 2018; Poehlmann et al., 2009; Scholz et al., 2017). Myeloid differentiation bias, suggested by the presence of high numbers of immature monocytes and instead inadequate expansion of intermediate monocytes, that may be able to clear the virus more efficiently due to a strong IFN-response, may underlie manifestations of severe COVID-19. To support this, a positive correlation between frequencies of monocytic myeloid-derived suppressor cell (mo-MDSC)-like cells and hepatocyte growth factor (HGF), a factor known to support mo-MDSCs (Yen et al., 2013), and a negative correlation between these cells stem cell factor (SCF) and FMS-like tyrosine kinase 3 ligand (FLT3L), required for myeloid cell development, were detected (**paper V**). Also, this phenomenon would fit with a model of sequential monocyte development, proposed by the lab of Dr. Simon Yona, where by using human *in vivo* deuterium labelling it was suggested that a certain proportion of classical monocytes become intermediate monocytes, and finally develop into non-classical monocytes (Patel et al., 2017). Notably, the patient cluster that was strongly linked to the HLA-$DR^{low}CD163^{high}$ monocytic MDSC-like clusters, included all COVID-19 non-survivors. It will be important to tease out the beneficial predictive value of the HLA-DR^{low} monocyte quantification in larger cohorts of patients, stratifying them according to, for example, incidence of secondary infections and other clinical parameters.

Regarding DCs, our understanding of the alterations in frequencies and functions of the different DC subsets, and especially of their circulating precursors, during acute viral human infection is limited. We detected lower frequencies of DCs and their progenitors in the

circulation of patients with COVID-19, and this observation is in line with previous reports focusing on acute viral infections (Hadjadj et al., 2020; Scholz et al., 2017; Wilk et al., 2020). Similar to observations made in monocytes, DCs in severely affected COVID-19 patients were less mature, as evident by lower levels of HLA-DR and CD86. While this was true for all DC subsets studied, no such pattern could be detected in the cross-presenting DC1, that play a key role in anti-viral defense (**paper V**). In support of an altered developmental phenotype, an expansion of the DC2 progenitor pre-DC2 and higher levels of c-KIT, a stem cell marker, was detected in pre-DCs. With respect to lineage specific alterations, it is interesting to note that lower levels of IL-6R were detected on DC1 patients with severe COVID-19. Given the importance of IL-6 in COVID-16 pathogenesis and beneficial effects of anti-IL-6 treatment (RECOVERY Collaborative Group, 2021), a recent study dissecting the DC1-mediated IL-6 buffer system (Yousif et al., 2020) may explain the lower IL-6R levels of DC1 in COVID-19. In brief, Yousif et al demonstrated that DC-derived soluble IL-6R captured IL-6 secreted after viral or bacterial challenge, functioning as a neutralizing buffer system, possibly relevant even in COVID-19. Regarding the cDC2 lineage, one of the largest effects with respect to disease severity, detected on all cDC2 subsets including the progenitors, was lower CD200R expression in severely affected COVID-19 patients. Decreased levels of CD200R may mirror an immature state, and may also be of particular importance given its immunoregulatory functions controlling tissue destruction mediated by myeloid cells (Hoek et al., 2000; Vaine and Soberman, 2014). While functional consequences of the changes described here remain to be validated on the functional level, it can be noted that, in general, the immunological parameters related to MNP frequencies and functions that in **paper V** predicted a patient cluster with all non-survivors, are underutilized in the clinical contexts. These contexts range from COVID-19 to also other infectious and inflammatory conditions, where more knowledge on MNP profiles may be important in clinical decision making with respect to prognosis and therapeutic approaches.

4 CONCLUDING REMARKS

This thesis provides new insights in the immunobiology of myeloid cells, with focus on their phenotype and functions in human tissue. The pathological contexts studied in this thesis include IBD, LCH, and COVID-19, but knowledge gained has potential to contribute to translational platforms aiming to improve diagnosis and therapy also in other infectious, chronic inflammatory, or neoplastic conditions. More specifically, the following key points may be concluded:

- The major source of the regulatory cytokine IL-23 in colon of newly diagnosed and treatment naïve children with IBD are neutrophils (**paper I**)
- Neutrophils infiltrate colon tissue through the IL-8/CXCR1/CXCR2 axis, and IL-8^{+} extra-epithelial cellular sources are augmented in IBD tissue (**paper I**)
- Global alterations are detected in intestinal stroma of newly diagnosed and treatment naïve children with IBD (**paper II**)
- Intestinal fibroblasts shape the monocyte-derived macrophage landscape (**paper II**)
- In patients with inflammatory gastrointestinal involvement, both CD and LCH should be considered, and IL-23 signaling pathway represents a common immunological trait between CD and LCH (**paper III**)
- Deep single-cell sequencing combined with index-sorting allows a proficient LCH cell separation from the lesion MNPs, enabling identification of LCH core signature pointing to senescence and tumor immune surveillance escape (**paper IV**)
- Two major subsets of neoplastic LCH cells are identified, corresponding to the DC2 and DC3/monocyte lineages (**paper IV**)
- The MNP landscape in COVID-19 is altered with respect to absolute numbers, frequencies, and phenotype, and association with soluble factors (**paper V**)
- Cells from monocyte and DC lineages, include circulating DC progenitors, in COVID-19 exhibit an impaired developmental phenotype in severely affacted patients, and changes detected in the circulation are mirrored in the lung (**paper V**)
- Immunological MNP parameters, alone, may predict a cluster with COVID-19 non-survivors (**paper V**)

Many outstanding questions related to myeloid cell functions in tissue pathology remain to be addressed. Tackling those questions in well-designed patient cohorts and using unique material, while applying state-or-art laboratory techniques, that are continuously improving, may help us target myeloid cells, aiming to cure diseases that today are considered incurable.

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