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SKIN INFECTIONS ARE ELIMINATED BY COOPERATION OF THE FIBRINOLYTIC AND INNATE IMMUNE SYSTEMS

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Abbreviation List

APC: antigen presenting cell

cDC: conventional dendritic cell

CLP: common lymphoid progenitor

CLR: C-type lectin receptor

CMP: common myeloid progenitor

DC: dendritic cell

ECM: extracellular matrix

IFN: interferon

IL: interleukin

ILC: innate lymphoid cell

LC: Langerhans cell

LPS: lipopolysaccharide

LTA: lipoteichoic acid

MHC: major histocompatibility complex

MMP: matrix metalloproteinase

moDC: monocyte-derived dendritic cell

MRSA: Methicillin-Resistant *Staphylococcus aureus*

NET: neutrophil extracellular trap

NFAT: nuclear factor of activated T cell

NK: natural killer

NLR: NOD-like receptor

PAI-1: plasminogen activator inhibitor type-1

PAMP: pathogen associated molecular pattern

pDC: plasmacytoid dendritic cell

PGE_2: prostaglantin E 2

PRR: pattern recognition receptor

RLR: RIG-I like receptor

ROS: Reactive oxygen species

TCR: T cell receptor

TGF-β: transforming growth factor-β

Th1: T helper 1

Th2: T helper 2

TLR: Toll like receptor

tPA: tissue-type plasminogen activator

uPA: urokinase-type plasminogen activator

α-SMA: α smooth muscle actin

Chapter 1: Introduction

1.1. Physical barriers

Human beings are at constant risk of infection by a variety of microorganisms. The human body can fight these microorganisms with two main lines of defense: the first, the skin and the mucosae, are physical barriers that mechanically prevent the microorganisms from entering the body. The second line of defense is composed of immune cells that directly fight microorganisms that have been able to penetrate the skin or the mucosae.

In a healthy individual, the mechanical barriers are intact and the organism becomes infected. The skin and the mucosae are covered with billions of microorganisms, some of which can be beneficial for the host. These microorganisms are part of the so-called microbiota, the composition of which varies among individuals and on the localization of the body's surface. If there is an alteration on the surface of the skin or the mucosae, then all these microorganisms can have easy access to the host and cause several pathological conditions such as bacteremia and sepsis. Once they have passed the first physical barrier, these microorganisms, even those that are usually beneficial, can become detrimental, inducing an inflammatory response in the host through the activation and the involvement of the immune system.

1.1.1. The skin

The skin is the widest and the most extended organ of the body with a surface that covers an average dimension of 1,8 m^2 (*1*). The structure of this organ is clearly thought to form an almost impenetrable barrier. The skin is divided into three main layers which are the epidermis, the dermis and the hypodermis.

The epidermis is further composed of different layers (Fig. 1), each of which is composed of a specific cellular type, called keratinocyte, at a different stage of maturation. The epidermis starts from the bottom with the *stratum basale* that is composed of stem cells responsible to give birth to keratinocytes. The stem cells present in the *stratum basale* are necessary for the continuous renewal of the skin by constantly originating keratinocytes. The keratinocytes then start migrating toward the surface undergoing a process of maturation at every layer. From the *stratum basale* they form a second layer called *stratum spinosus* where keratinocytes start creating inter-cellular connections. The third layer is the *stratum granulosum* where keratinocytes display a granular phenotype due to an elevated production of keratin. Finally, they end up in the *stratum corneum*, the last layer, that is composed of dead keratinocytes, flat and nonnucleated, extremely close one to each other (Figure 1)(*2*).

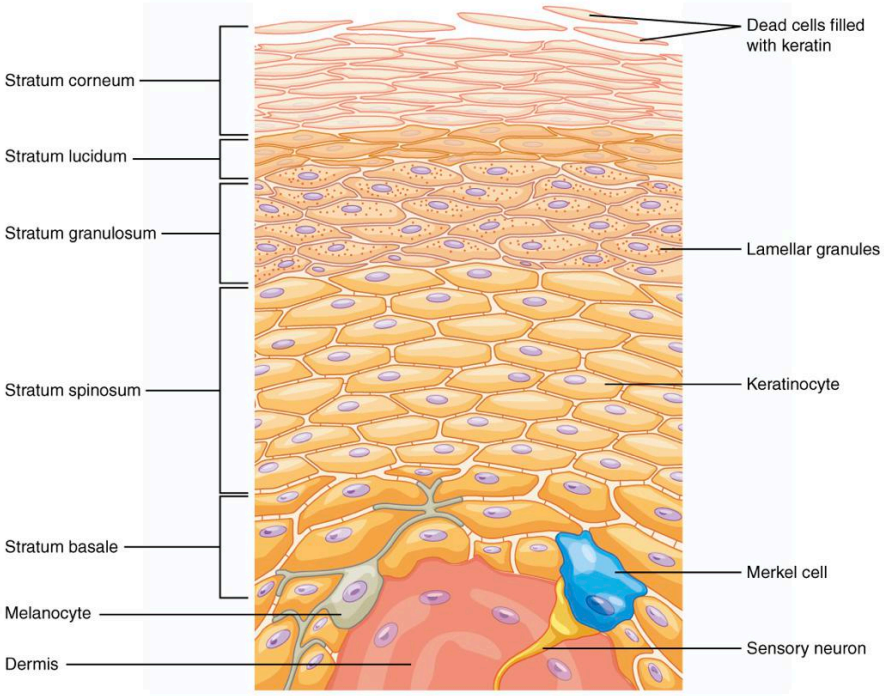

Figure 1. Structure of the epithelium. Each layer is composed of keratinocytes at different stages of maturation (*3*).

Below the epidermis there is the dermis, made of a fibrous tissue that confers elasticity to the entire organ. Inside the dermis there is a high number of glands, such as the sebaceous and the sweat glands, the hair follicles and, most importantly, the first innervations and both blood and lymphatic vessels. These vessels are necessary for both bringing nutrients to the skin, and draining the organ in order to facilitate the identification of those microorganisms that have penetrated the mechanical barrier.

Finally, the last layer that comprises the skin is the hypodermis. Inside the hypodermis there is a high component of fat tissues, muscles and

both blood and lymphatic vessels. This layer is the one that is directly connected with the rest of the body.

An elevated number of cells of the immune system resides within these layers, acting as sentinels of the organism, with the ability to detect the presence of foreign organisms and therefore starting an immune response to confine and eliminate the infectious agents. These cells are necessary to respond to the infection of the microorganisms that penetrate the upper layers.

1.1.2. The mucosa

Another physical barrier that stands between the external environment and the inside of the organism is the mucosa. The two main mucosal sites of the human body are the airway mucosa and the gastrointestinal mucosa.

The gastrointestinal tract faces, as the skin, the presence of millions of different microorganisms that live permanently on it. In the gut it has been estimated the presence of 10^{14} microorganisms (*4*). Sender *et al.* recently reported that the ratio between microorganisms and host cells is about 1,3:1 (*5*). Since most of these microorganisms resides in the gut, this means that the gastrointestinal mucosa has to deal with an extraordinarily elevated number of foreign organisms and therefore must be extremely efficient in maintaining them outside of the body.

The main role of the intestine is to absorb nutrients and, to do so, the surface of the intestine is not regular but is made of different

extensions, named villi, to amplify the surface exposed to the outer environment. Every villus is composed of enterocytes that project smaller structures, called microvilli, in order to enhance nutrient absorption (Figure 2). These structures, however, cause an increment in the area exposed to microorganisms. Most of the microorganisms that resides in the gut is composed of commensal microorganisms and their presence is not just non-detrimental for the host but, on the opposite side, it is required for a healthy individual for several reasons. These commensal bacteria are necessary for the host not only to uptake nutrients that, otherwise, would be inaccessible, but also to create the so-called microflora which is important for the maintenance of a healthy environment. Inside the epithelium there is a specific cellular type involved in the maintenance of a healthy environment. These cells, called Globlet cells, are responsible for the production of mucin, a protein that is released in the intestinal lumen and gives raise to the formation of a mucus layer. This layer comprises an external and ulterior defense against pathogens and can be divided into an inner mucus layer and an outer mucus layer. The microbiota takes advantage of this outer layer by using it as a medium inside of which grow and proliferate.

Paneth cell is another cellular type, present within the epithelium, involved in the maintenance of homeostasis through the production of different antimicrobial peptides that are released in the intestinal lumen thus keeping under control a possible overgrowth of the microorganisms.

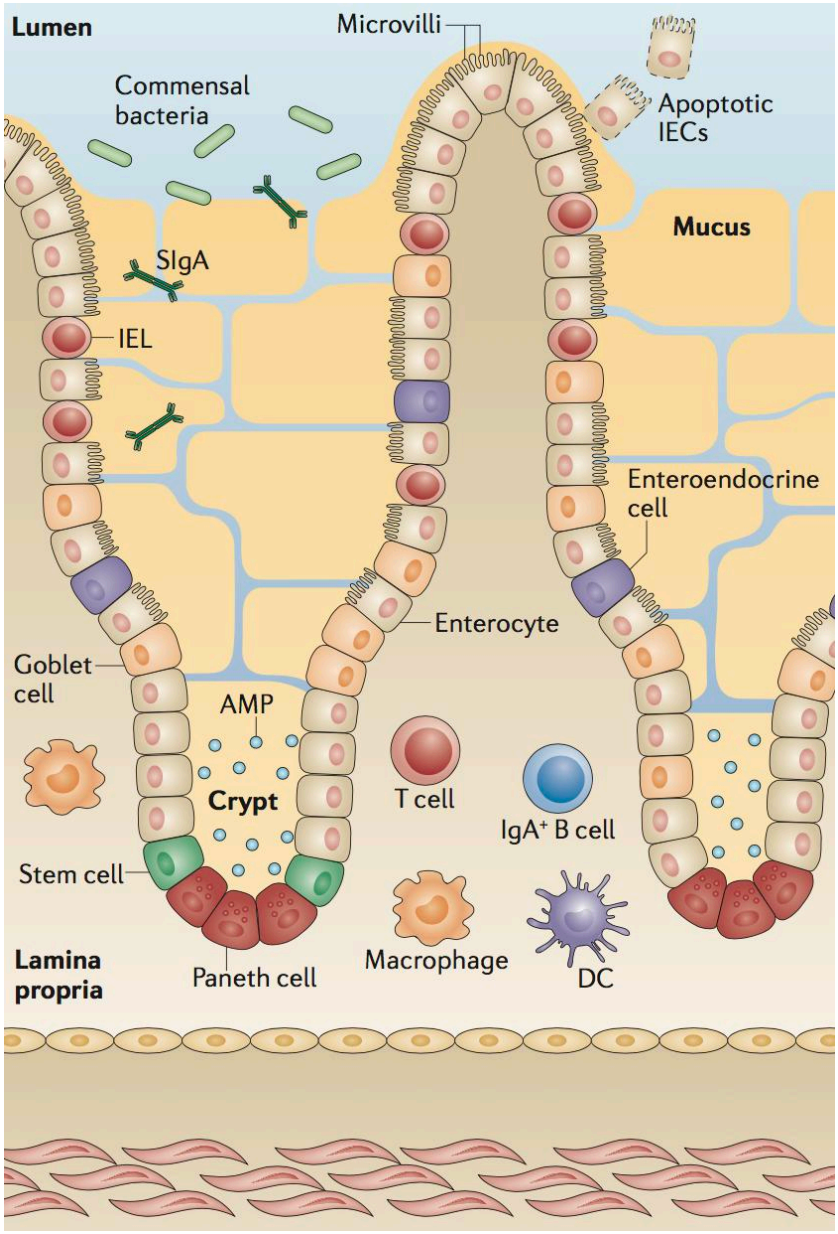

Figure 2. Structure of the intestinal mucosa, with the identification of villi and microvilli. The mucosal layer of the intestine is composed of different cell type, each of which with a specific role in maintaining a homeostatic condition (*6*).

In order to maintain a homeostatic condition, cells of the immune system are present along the epithelium and have been described to be able to sense the extracellular environment through projections. Among these cells we can find dendritic cells that can extrude protrusions of their bodies into the lumen in order to uptake bacteria and bacterial products therefore activating an immune response (*7*).

1.2. Innate immunity

When a microorganism reaches in crossing the physical barriers of the body, then a second line of defense takes place. This second line is made of the immune system, which is divided into two branches: the innate immune system and the adaptive immune system.

The adaptive immunity is the most recent form of immunity an it has evolved in vertebrates about 500 million years ago (*8*), while the innate immune branch is more primitive and it is important for the early encounter with the pathogens. The innate immune system detects pathogens, and in general foreign organisms, through the recognition of common structures shared by different classes of microorganisms, that have been called pathogen associated molecular patterns (PAMPs), thanks to the expression of specific receptors both on their membranes and inside their cytoplasms. These receptors, called pattern recognition receptors (PRRs) can be divided into different families based on their molecular structure. PRRs will be investigated later since they deserve a specific chapter.

The role of innate immune system's activation is to impede the development of an infection and its reaction is non-pathogen-specific. The innate immune system, in fact, directly fights pathogens through the production of pro-inflammatory cytokines as well as through a process, called phagocytosis, where innate immune cells engulf the pathogens in order to physically eliminate them. The innate immune system is also necessary for a proper activation of an adaptive immune response, strictly pathogen-specific, thus leading to an immune response mostly restricted to the infection in action. Diversely from the innate immune system, the adaptive immune system doesn't recognize common microbial structures but pathogen-specific antigens. These cells express clonally distributed receptors that are generated following genetic rearrangement, making them different one another with an uncountable number of possible combinations (*9*).

An innate immune response is represented by the presence of both soluble mediators, such as the complement system, and immune cells. Among the innate immune system, we can find a very diversified number of cellular types.

These cells can act by directly killing microbes through the release of granules containing proteases, enzymes, antimicrobial peptides and cytokines like the so-called granulocytes. Among granulocytes we can find neutrophils, the most abundant cellular type, basophils and eosinophils. These cells are the very first line of defense and react to the presence of the pathogen by either phagocytic activity or release of extracellular mediators.

Other cells with phagocytic activity are macrophages and dendritic cells. These cells are those necessary for the connection between the innate and the adaptive immune systems. They are present almost in every district of the body and serve as sentinels of the host. Upon the recognition of a pathogen, they phagocytose it and migrate through the lymphatic vessels into the lymph nodes where they can induce an adaptive immune response specific for the pathogen of interest. In addition to the activation of the adaptive immunity, macrophages and dendritic cells can start fighting the infection through the production of pro-inflammatory cytokines and extracellular mediators able to recruit other cells of the immune system (*10*).

There is, at the end, another category that comprises different, newly discovered, cells: innate lymphoid cells (ILCs).

Among these cells, the first to be described are Natural Killer (NK) cells (*11*) which have been studied for the last decades because of their role not just during microbial infections but for their ability in recognizing cancer cells.

In the last years, this category became bigger and bigger due to the identification of other cellular types identified with the general name of ILCs. These cells, discovered for the first time in 2008 (*12*), are now classified depending on their cytokines expression in ILCs 1, ILCs 2 and ILCs 3.

1.2.1. Dendritic cells

In 1973 Ralph Steinman, together with Zanvil Cohn, described a large stellate cell type from mouse spleen able to adhere to glass and plastic surfaces (*13*). These cells were named dendritic cells (DCs). For his discovery, Ralph Stainman was awarded with the Nobel Prize in 2011. During the last decades DCs gained more and more importance in the immunological research. In homeostatic conditions, DCs patrol the organism in order to sense the presence of microorganisms through the expression of an extremely diversified repertoire of PRRs. Upon recognition of a foreign organism, DCs undergo transcriptional and morphological changes that enable them to phagocytose the microorganisms, release pro-inflammatory mediators and, most importantly, confer them the ability to migrate to the draining lymph nodes (*14*). The microorganisms that are internalized by DCs are processed and peptides derived from them are charged on the major histocompatibility complex (MHC). This machinery is required for the presentation of the antigens to T cells. T cells recognize the complex MHC-peptide through their T cell receptor (TCR) and this recognition, together with the binding of co-receptors, on T cell surface, with costimulatory molecules, on DCs' membrane, leads to the activation and proliferation of T cells. DCs express two different classes of MHC: MHC class I and class II. The MHC class I usually present endogenous antigens, antigens derived from the cytoplasm of DCs, while MHC class II present antigens that derive from the endosomal compartments, *i.e.* products of phagocytosis (Figure 3) (*15*).

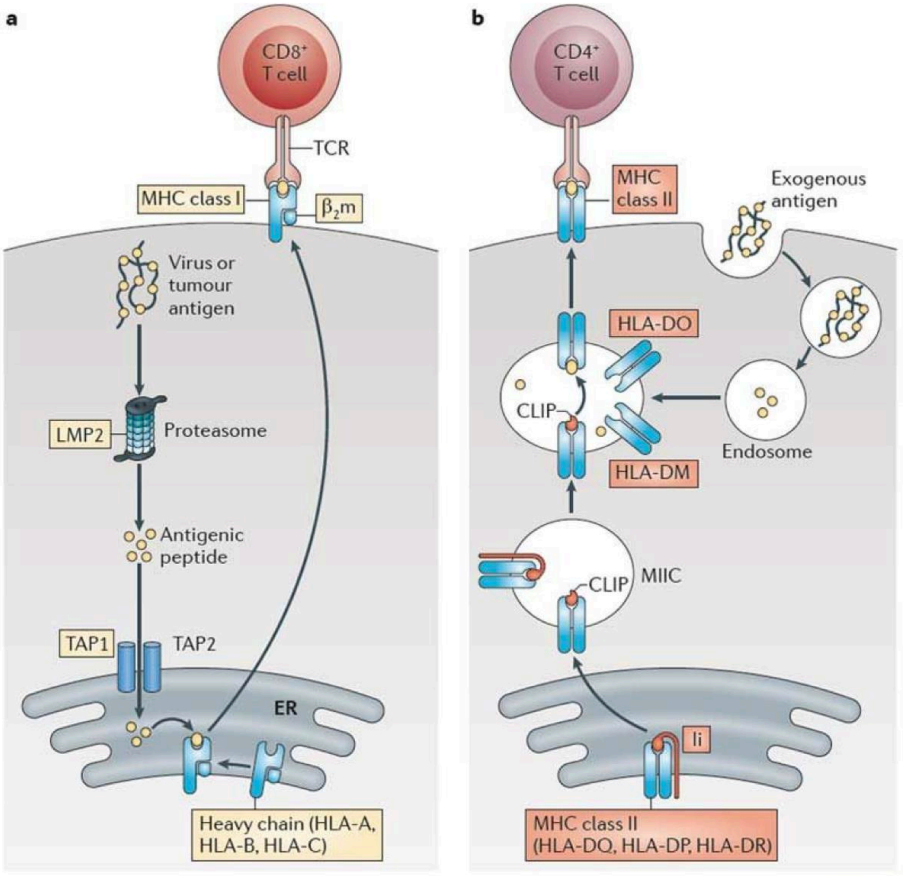

Figure 3. MHC processing. (A) MHC class I molecules present endogenous antigens on the surface of DCs. (B) Antigens derived from the extracellular environment are charged on MHC class II molecules (*16*).

Since their first discovery, 4 different DCs subsets have been identified: conventional Dendritic Cells (cDCs), plasmacytoid Dendritic Cells (pDCs), Langerhans cells and monocyte-derived Dendritic Cells (mo-DCs).

DCs have usually a short lifespan in non-lymphoid tissues so they require a continue substitution with newly originated cells. They

originate from bone marrow starting from the haematopoietic stem cell compartment. Among this compartment we can find the common dendritic progenitor (CDP) that is able to give raise to almost all the DCs subsets (*17*). These precursors can start their late differentiation toward a cDCs fate, that is a terminal commitment. Once the differentiation process has begun, cells are named pre-cDCs and can only differentiate into cDCs. These precursors migrate through the blood stream toward lymphoid and non-lymphoid tissues where they persist and terminally differentiate into cDCs.

1.2.1.1. Conventional Dendritic Cells

cDCs are able to phagocytose pathogens and present antigens on their surface to activate T cells. cDCs include different subpopulations that are identified on the basis of a differential expression of surface markers.

cDCs comprise different subsets that are differentially distributed among lymphoid and non-lymphoid organs. In lymphoid organs 5 DCs subtypes have been identified, characterized by different surface markers. Three of these subtypes can be found in the spleen and are divided, based on the diverse expression of 4 surface proteins, in: $CD4^-CD8^{hi}CD205^{hi}CD11b^-$, $CD4^+CD8^-CD205^-CD11b^+$ and $CD4^-CD8^-CD205^-CD11b^+$. Lymph nodes, instead, comprise two more subtypes $CD4^-CD8^-CD205^+$ $CD11b^+$ and $CD4^-CD8^{low}CD205^{hi}CD11b^+$. This last subtype is believed to be the mature form of Langerhans cells since it expresses also the CLR Langerin (*18*). $CD8^+$ cDCs have been described to be able

to cross-present exogenous antigens on MHC class I therefore activating a cytotoxic $CD8^+$ T cells activation (*19*) while $CD4^+$ cDCs are mainly involved in antigen presentation on MHC class II molecules, thus involved in a $CD4^+$ T cell response (*20*).

Other than lymphoid organs, DCs are present in almost all non-lymphoid tissues. Even in non-lymphoid organs DCs express on their surfaces distinct combination of markers based on their localization. At the skin level, DCs can be divided in two subtypes of dermal DCs: $langerin^+CD103^+$ DCs and $langerin^-CD103^-$ DCs. The differential expression of CD103 as a marker of DCs subset is found also in the intestinal lamina propria where DCs are defined by its positivity together with another marker, CX3CR1. Here cDCs are therefore divided into $CD103^+CX3CR1^-CD11b^+$ and $CD103^-CX3CR1^+CD11b^+$ subsets. Another organ that is in continuous contact with foreign antigens, thus requiring the presence of DCs to sense the presence of microorganisms, is the lung. Here cDCs are divided in two classes: $CD103^+CD11c^{high}CD11b^-$ DCs, present in the intraepithelial network, and $CD103^-CD11c^{high}CD11b^+$ DCs that reside in the lamina propria of conducting airways (*21*).

When DCs uptake a pathogen, they undergo a process of maturation. During their maturation, DCs upregulate the expression of MHC I and II molecules but also the expression of costimulatory molecules that will be necessary for the activation of T cells after antigen recognition. In addition to the upregulation of activation markers, DCs undergoing maturation start releasing pro-inflammatory cytokines in the

extracellular environment, a key step in order to boost an inflammatory process.

cDCs are then able to activate a T cells response thank to 3 signals: 1) recognition between MHC-peptide complex and TCR; 2) engagement between co-receptors on T cells with costimulatory molecules expressed by DCs; 3) production of soluble mediators like cytokines by DCs (Figure 4) (*22*).

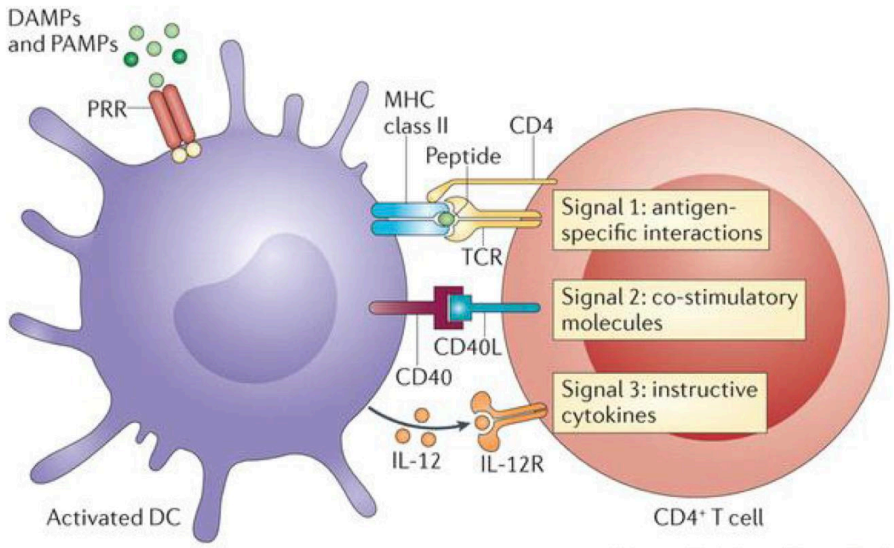

Fig. 4. Activation of T cells require three signals from DCs. Signal 1 is made of the formation of the complex MHC-TCR, signal 2 by the binding of co-receptors with co-stimulatory molecules present on the DC's surface. Finally, signal 3 comes from the release of cytokines by DCs (*23*).

One of the most important characteristics of cDCs is the ability to migrate to the draining lymph nodes charged with self and non-self antigens. cDCs present in non-lymphoid tissues uptake antigens from

the surrounding environment and migrate to the lymph nodes in order to present those antigens to T cells. This process occurs at a very low rate in non-inflammatory conditions, and it is exacerbated in an inflammatory environment. In inflammatory conditions, the PRR-mediated activation of DCs induces the upregulation of CCR7 and DC trafficking to lymph nodes (*24*).

1.2.1.2. Plasmacytoid dendritic cells

Plasmacytoid DCs (pDCs) have been identified in 1999 by Siegal *et al*. analyzing the already known "Natural IFN-producing cells (IPC)" isolated from human blood. They described these cells with a plasmacytoid morphology as the main source of type I interferons (IFNs) among peripheral blood mononuclear cells (Figure 5) (*25*).

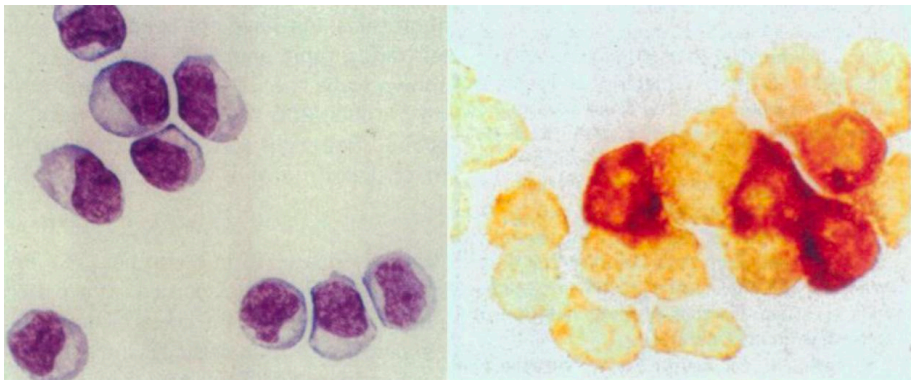

Fig. 5. Identification of "Natural IFN-producing cells" (IPCs) with a plasmacytoid morphology (on the left) that stain positive for IFN-α after stimulation with herpes simplex virus (on the right) (*25*).

Two years later similar cells were also identified in mice (*26*).

Diversely from cDCs, pDCs are poor antigen presenting cells (APCs) and inefficient activators of $CD4^+$ T cells. This is not due to a lack of MHC expression but rather to a much lower stability of MHC-peptide complexes at the cell surface compare to cDCs (*27*). The continuous MHC molecule turnover makes difficult the interaction with antigen specific T cells (*28*).

pDCs are constantly released from the bone marrow from where they migrate to the lymph nodes through the blood stream.

Even though pDCs' origin has been difficult to be determined at the very beginning, now pDCs are considered to derive from the same common progenitor of cDCs in the bone marrow (*17*). Their development requires, like cDCs, the presence of Flt3L without which the number of pDCs decreases dramatically. Another cytokine mandatory for the complete commitment of pDCs is GM-CSF which is necessary for their mobilization from the bone marrow.

The most important and specific transcription factor for pDCs development in the bone marrow is E2-2. It has been demonstrated that E2-2 knock down in the human haematopoietic progenitors causes the lack of pDCs differentiation while its overexpression induces an increase in pDCs development (*29*).

pDCs are an extremely important tool of the innate immune system particularly in fighting viral and bacterial infections. pDCs express a specific repertoire of PRRs, like TLR7 and TLR9, required for the recognition of nucleic acids. TLR7 is able to recognize single strand RNA, therefore involved in the detection of viral RNA, while TLR9 binds to double strands DNA recognizing non-methylated bacterial and viral

CpG-DNA. Unlike cDCs, pDCs do not express other receptors of the TLR family, therefore are unable to respond to bacterial products, such as LPS and peptidoglycan. The characteristic for which pDCs have been identified, the production of type I IFNs, is a direct consequence of the engagement of these two PRRs (*30*).

1.2.1.3. Langerhans cells

Langerhans cells have been known for almost a century before the identification of DCs by Steinman. Langerhans cells (LCs) were identified by Langerhans in 1868. After the identification of DCs, Steinman's group realized that the two cell types shared some properties (*31*).

LCs reside in the skin and, diversely from cDCs and pDCs, are long lived and their number in the skin is maintained by a low rate proliferation of these cells in situ, with no further recruitment of precursors from the bone marrow (*32*, *33*).

LCs have a unique ontogeny since they derive mainly from embryonic fetal liver monocytes, and the cytokine Flt3L, essential for cDCs and pDCs development, is unnecessary for LCs formation (*34*). Several studies have demonstrated the persistence of LCs in the skin of irradiated mice, as well as the persistence of the host LCs in mice that had undergone bone marrow transplantation (*35*).

LCs can act as APCs. They have phagocytic activity, express MHC class II and, under inflammatory conditions, they acquire the ability to

migrate to the draining lymph nodes (Figure 6) where they can activate an adaptive immune responses (*36*).

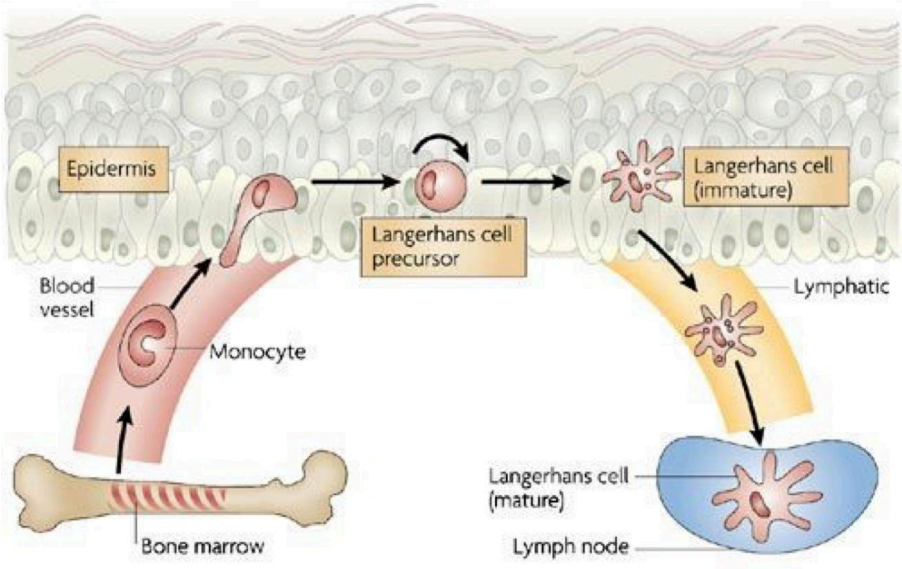

Figure 6. Langerhans cells are present in the epidermis and, upon pathogen recognition, migrate to the lymph nodes to activate an adaptive immune response (*37*).

After the recognition of a microorganism, LCs gain the ability to migrate to the lymph nodes thanks to the upregulation of CCR7 (*38*). This migration process is also assisted by the reduced expression of E-cadherin which is necessary for the attachment of LCs to keratinocytes (*39*). Once reached the draining lymph nodes, LCs upregulate the expression of the MHC class II and costimulatory molecules on their surface. This maturation process is mandatory for T cells activation.

Phenotypically, LCs can be identified for the co-expression of different markers such as CD11c, MHC II, Langerin, CD11b and for the absence of CD103, which is, instead, a marker for dermal DCs.

LCs express on their surface several PRRs making them able to play an important role in innate immunity against pathogens. Among TLRs, LCs express TLR1, 2, 3, 6 and 7 while they lack TLR4, 5 and 9. This expression profile confers LCs a specific role in viral (TLR 3, 6 and 7) and Gram-positive bacterial recognition (TLR 1 and 2) while they seem to be unable to detect Gram-negative bacteria. TLRs expression profile was analyzed only in the skin compartment (*40*, *41*), but their expression could be different in the gut and other mucosae where LCs are also found. Among the CLRs, LCs express low levels of Dectin-1 and high levels of Langerin, the principal marker of LCs able to detect several monosaccharides such as mannose, fucose and N-acetyl-glucosamine (*42*, *43*).

1.2.1.4. Monocyte-derived dendritic cells

The last subset of DCs comprises the monocyte-derived Dendritic Cells (moDCs). This subset arises from monocytes that are present in the blood stream and that are recruited during inflammation at the site of interest. In 2003 Serbina *et al*. found in the spleen a monocyte subset able to differentiate, during bacterial infections, in a particular DC subset they called Tip-DCs (TNFα/iNOS producing DCs) during bacterial infections (*44*). In 2006, Naik *et al*. demonstrated how, in the presence of an inflammatory condition and with the requirement of

GM-CSF, Ly6C^{hi} monocytes, isolated from both murine spleen and bone marrow, were able to differentiate into DCs with the uniquely signature CD11c^{int}CD11b^{hi}Mac-3^{+}, upregulated MHC II and were as good as steady state-DCs in stimulating T cells proliferation (*45*).

From 2006 on, these cells have been characterized by different laboratories and it is now clear that moDCs are able to induce a CD4^{+} T cell response and can also cross-present antigens to CD8^{+} T cells (*46*, *47*).

Literature about moDCs is still controversial. They are closely related to the CD11b^{+} DCs subsets but they express CD64, Mac-3 and FcγRI which are a reminiscence of their monocyte origin (*48*, *49*); however, their actual role during an inflammatory process is still under evaluation. Depending on the inflammatory environment, they can induce T cells differentiation into Th1 (*50*) or Th2 (*48*).

Copin *et al*. demonstrated how, during infections with the Gram-negative bacterium *Brucella*, moDCs are able to control the infection through the activation of TLR4 and TLR9 in a MyD88 dependent manner (*51*). Studies about moDCs are at the very early stages so more information will be available in the next years.

1.2.2. Granulocytes

More than 100 years ago, in the late 19th century, Paul Ehrlich identified three different population of white blood cells based on their differential staining and he named these cells on the basis of the dyes used: basophils, that stained with basic dyes, eosinophils, with

acidophilic dyes, and neutrophils, with neutral dyes. Still in the same years he, together with Metchnikoff, realized that these cells, particularly neutrophils, were able to engulf bacteria (*52*). This discovery led to an increased interest for these cells in the immunological field.

Phenotypically all these three cellular types possess an elevated number of preformed granules that occupy most of the cytoplasm. Due to this characteristic, these cells have been named granulocytes.

Granulocytes originate from the bone marrow. Here the haematopoietic stem cells can differentiate into two types of cells: the common lymphoid progenitors (CLPs) and the common myeloid progenitors (CMPs). The CLP gives rise to all the lymphoid lineages such as T cells and B cells. On the other side, the CMPs can generate almost all the myeloid lineages like red blood cells, platelets as well as granulocytes and macrophages (Figure 7). Granulocytes are then released into the blood stream already in a fully active form and they start patrolling the body (*53*).

Granulocytes are the very first cells involved in an immune response. Metchnikoff himself realized, soon after the discovery, that these cells were recruited almost immediately at the site of an injury. Their predominant role is to fight the infection, by phagocytosing microorganisms and producing anti-microbial effector molecules.

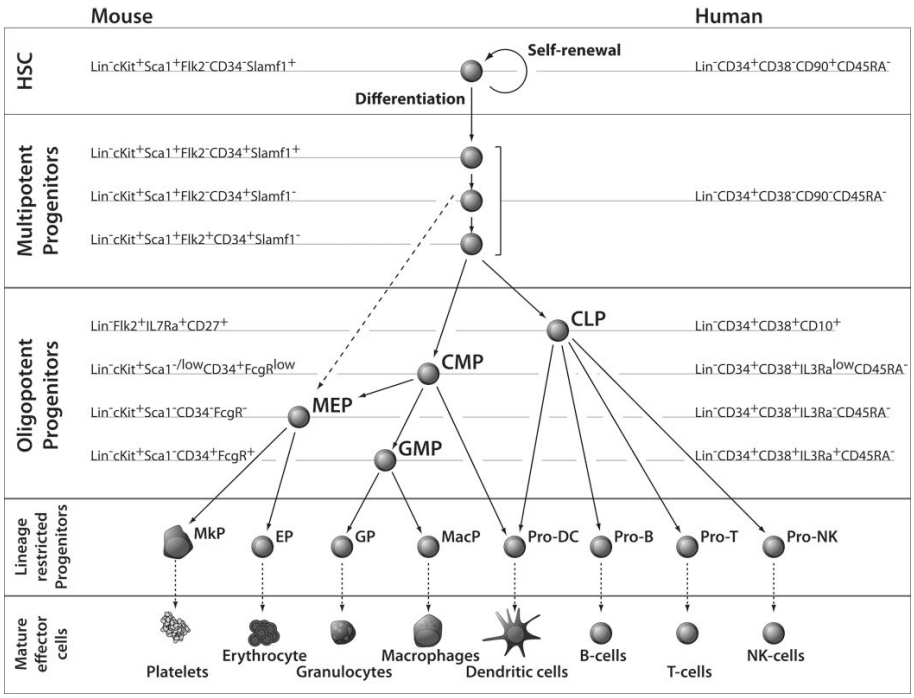

Figure 7. Schematic view of the origin of the myeloid and lymphoid branches of the immune system starting from the haematopoietic compartment (*53*).

In addition to the high number of granules present inside the cytoplasm, another aspect that connect all the three subsets of granulocytes is the short life span. After they leave the bone marrow, they persist in the blood stream, or in the tissues, for less than one week. The short life span has been a difficult task to overcome for the in vitro studies of these cells (*54*).

1.2.2.1. Neutrophils

Neutrophils are the first cells to be recruited at the site of an infection. After leaving the bone marrow, they patrol the body through the blood vessels in search of alterations in the homeostasis.

When an inflammatory process occurs, it has consequences on endothelial cells. When endothelial cells detect either inflammatory mediators or pathogens, they increase the expression on their surfaces of protein of the selectin family, *i.e.* P-selectin and E-selectin (*55*). These proteins are then recognized by receptors on neutrophils' surfaces. This binding thus leads to the extravasation of neutrophils which is divided into 4 phases (Figure 8):

_ rolling: neutrophils bind to E- and P-selectin on endothelial cells, they start "rolling" on the endothelial surface until a stronger binding is formed.

_ firm adhesion: during the rolling phase, neutrophils are activated by either the detection of pro-inflammatory cytokines or the presence of chemoattractants but also by the binding of activated endothelial cells. This activation leads to the upregulation of specific integrins, such as LFA1, which is able to bind molecules on the endothelial surface such as ICAM1 in order to form a stronger binding and therefore firm adhesion.

_ crawling: after firm adhesion, some neutrophils continue to form pseudopods in order to find a way to exit the bloodstream.

_ extravasation: neutrophils are able to migrate through the endothelial barrier in two ways. The most common and fast one is

through the endothelial junctions but it has been described that neutrophils can also pass the endothelial barrier in a transcellular way. This last process is less common and it can take up to 30 minutes, while the first one requires only few minutes to be completed (*56*)

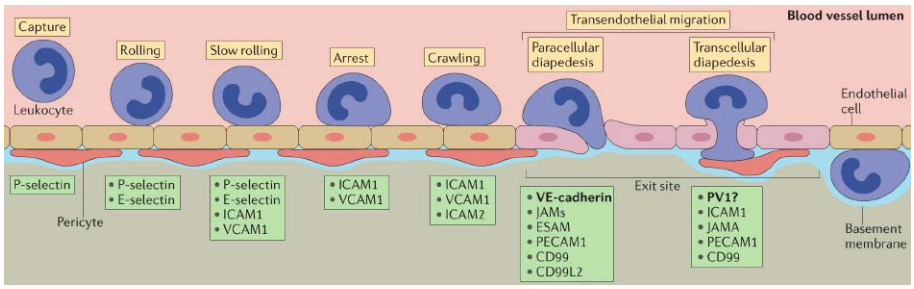

Figure 8. Neutrophil's extravasation requires several phases. Upon binding with the endothelial cells, neutrophils start rolling and then stop during the firm adhesion. Once firmly arrested, neutrophils can start extravasate in a process that can happen both paracellularly and transcellularly (*56*).

Once out of the bloodstream, neutrophils follow the chemotactic gradient in order to reach the inflammatory site. Neutrophils express several PRRs of different families. They express almost the entire repertoire of TLRs, except for TLR3, as well as a huge variety of other PRRs such as CLRs and NLRs (*57*). This elevated number of different receptors expressed by neutrophils give them the ability to respond to several infections. They can, actually, react to bacterial infections but also to fungal infections and their responses are tightly regulated and depend also on the stimuli they receive.

After the detection of a microorganisms, neutrophils can activate several pathways in order to eliminate the infection. On one hand,

they can directly phagocytose the microorganisms, on the other hand they are able to release a repertoire of cytokines and antimicrobial peptides in order to kill the pathogen. Furthermore, through the activation of the NADPH oxidase machinery, neutrophils produce of reactive oxygen species (ROS). These ROS can be released both in the phagolysosome as well as outside of the cell where they react with other molecules thus damaging cells and microbes. For the activation of the respiratory burst, the fusion of specific granules with the cellular membrane is mandatory. Granules' fusion is important in order to release their contents within the tissue. Neutrophils, in particular, possess four types of granules: primary granules, secondary granules, tertiary granules and secretory vesicles (*58*). These granules have specific contents and are release in a different manner. The azurophilic granules are poorly exocytosed and their role is thought to be mainly the activation of the phagolysosome. Their content is principally composed of myeloperoxidase and α-defensins; the former is important for its reaction with ROS in order to increase their toxicity, the latter have antimicrobial activity since they can create holes in microorganisms' membranes. Secondary and tertiary granules contain a very diversified set of proteins, enzymes and antimicrobial peptides. Secondary granules' probably most important and known content is Lactoferrin, a glycoprotein whose principal role is to sequester iron thus depriving bacteria of a vital resource. The principal component of tertiary granules is gelatinase, also known as metalloproteinase 9 (MMP9). Its role in degrading the extracellular matrix is important for neutrophils migration within the tissues as well as for the activation of

several other inflammatory mediators in the extracellular environment such as IL-8 and IL-1β (*59*). Finally, secretory vesicles contain different receptors important in the early phases of an inflammatory response driven by neutrophils. Exocytosis of these last granules causes the fusion of their membranes with the extracellular membrane of the neutrophil, thus exposing those receptor on the cellular surface (*60*).

A final mechanism that neutrophils can use to eliminate pathogens is the release of "neutrophil extracellular traps" (NETs). NETs are composed of granules and cytosolic proteins aggregated with decondensed chromatin. NETs are released after the activation of a specific cell death program called NETosis (Figure 9). During NETosis, neutrophils disassemble the nuclear membrane allowing the binding of chromatin with several proteins of cytoplasmic and granular origin. Through the permeabilization of the plasma membrane, then, NETs are released in the extracellular environment where they trap, neutralize and kill pathogens. Among the proteins released during NETosis we can find proteases, such as neutrophil elastase, as well as myeloperoxidase and other antimicrobial peptides like defensins. NETs are usually released during infections with microorganisms that are too big to be phagocytosed, such as parasites and fungal hyphae. Despite their importance in an immune response to pathogens, NETs have been associated with several diseases and can cause damages to tissues. Occlusion of the vasculature, increase of atherosclerotic lesions as well as autoimmune diseases are some examples of disorders that have been associated with NETs release (*61*).

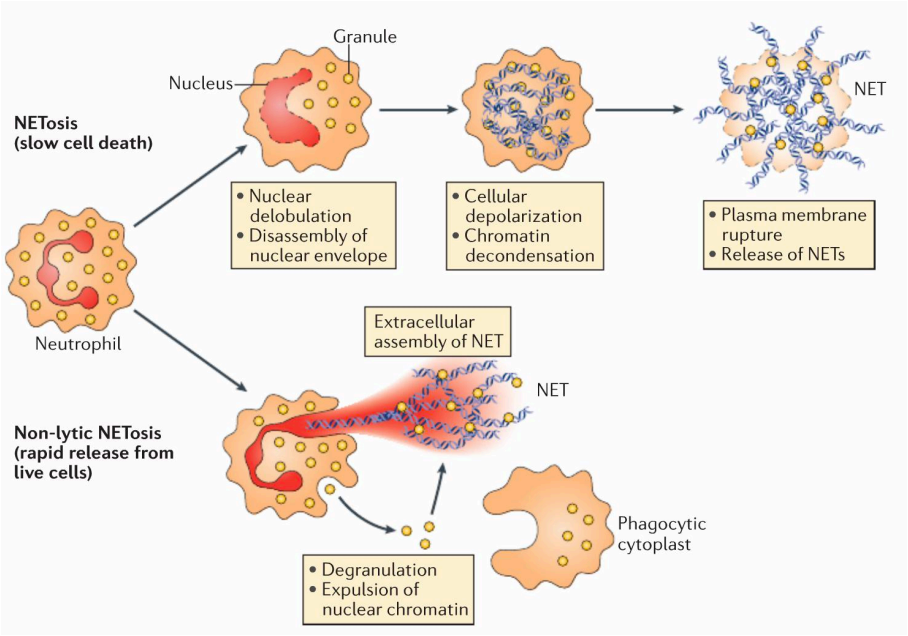

Figure 9. Schematic representation of the NETosis processes. Classical NETosis is a cell death program that causes decondensation of chromatin and the release of it, bound with several proteins of granular and cytoplasmic origin. Another NETosis process has been described that does not lead to neutrophil's death. This last process has been called non-lytic NETosis (*61*).

1.2.3. Natural Killer cells

Natural Killer (NK) cells have been originally identified in 1975 by Kiessling *et al*. (*11*). They described a population of cells in the mouse spleen with rapid cytolytic activity against leukemic cells. These cells were described as a third group of lymphocytes, different from both B cells and T cells (*62*).

From their discovery on, NK cells have been widely studied for their fundamental role in antiviral and anti-tumor immunity.

Back in 1990 it has been proposed a mechanism by which NK cells are able to distinguish healthy from unhealthy cells. This mechanism is based on the ability of NK cells to attack those cells that do not express MHC I molecules in a process that has been called “missing self” (*63*). This discrimination is based on the fact that, often, viral-infected cells downregulate the expression of MHC I in order to avoid a $CD8^+$ cytotoxic T cell response. It has also been reported that some malignant tumors downregulate the expression of MHC I. NK cells can exploit this mechanism through the expression of different receptors able to bind MHC I. The repertoire of NK cells receptors can be divided into two groups: activating receptors and inhibitory receptors.

The main difference between these two classes of receptors is due to the intracellular pathway activated downstream their binding with their ligands. Inhibitory receptors have, in their cytoplasmic tail, a sequence called “immunoreceptor tyrosine-based inhibitory motif” (ITIM) that is phosphorylated upon ligand recognition, finally leading to the inhibition of NK cells activation. On the other hand, most of the activating receptors signals through another motif, similar to the ITIM, called “immunoreceptor tyrosine-based activating motifs” (ITAM). This sequence is contained in the cytoplasmic tails of accessory molecules that are necessary for the signal transduction of these receptors (*64*). Since their first identification, both activating and inhibitory receptors’ number has increased over the years. Among the inhibitory receptors able to bind MHC I we can find the best

characterized killer cell immunoglobulin-like receptors (KIRs) in human, Ly49 in mice and the complexes CD94/NKG2A in both human and mice (*65*).

The role and activation of NK cells can be modulated by the interaction with other cells of the immune system. It has been demonstrated that DCs can directly activate NK cells through both cell to cell contact, as well as through cytokine production such as interleukin 12 (IL-12) and interleukin 18 (IL-18) (*66*–*68*).

After their activation, due to the unbalance of activating/inhibitory signals, NK cells release lytic granules that contain principally perforin and granzymes (Figure 10). Perforin binds to the plasma membrane of the target cell creating pores that enables granzymes to enter the cytoplasm thus inducing apoptosis due to the activation of apoptotic caspases (*69*).

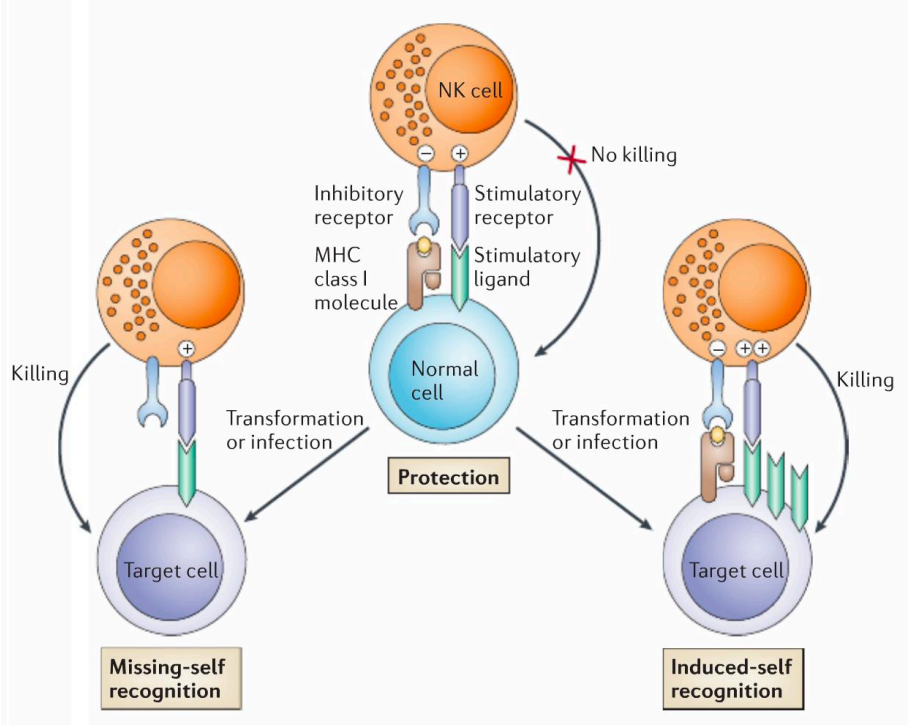

Figure 10. NK cell's activity is controlled by balanced signals coming from both activatory and inhibitory receptors. The absence of inhibitory signals leads to the NK cells activation ("missing-self recognition"). On the other hand, if inhibitory signals are present, but the activatory signals prevail on them, NK cells get activated and kill target cells (*70*).

NK cells are also able to release huge amount of pro-inflammatory cytokines. Among these cytokines, interferon-γ (IFN-γ) is important for the activation of several immune cellular types, such as type 1 macrophages and Th1 cells. This induction of type 1 immunity is mostly associated with a pro-inflammatory condition due to microorganisms' infections in order to eliminate the pathogens. IFN-γ can also interfere with the activation of the TGF-β pathway and profibrotic responses.

This interplay between IFN-γ and TGF-β has been studied for long time and the molecular mechanism of the effect of IFN-γ on the TGF-β pathway was disclosed just 20 years ago (*71*).

IFN-γ is primarily released by T cells and NK cells, but other sources, such as neutrophils, B cells and APCs, can be found within the tissue during an infection. Other than its role on the ECM deposition, IFN-γ is mostly known as an inflammatory cytokine with the ability to skew an immune response toward a type 1 immunity. Its best characterized ability is, in fact, to drive T cells fate into Th1. Moreover, this cytokine can play a variety of activities among which we can find the capacity to upregulate the expression of MHC class I and II molecules on the surfaces of APCs, thus prompting an immune response in the presence of a pathogen, as well as the induction of phagocytosis, oxidative burst and the intracellular killing of microbes (*72–74*).

1.3. Pattern Recognition Receptor

In 1989 Charles Janeway theorized what is now the basis of the innate immune system activation and the link between innate and adaptive immunity. He proposed that the most ancient form of immunity, *i.e.* innate immunity, would be able to recognize what he named pathogen-associated molecular patterns (PAMPs) that he thought could be general structures conserved in microorganisms but absent in the host. He also postulated the presence of specific, non-clonal, receptors able to recognize these structures that he named pattern recognition receptors (PRRs) (*75*).

This theory is now considered a milestone in immunology and, in the following decades, it has been confirmed by a countless number of laboratories. At present, a huge variety of these PRRs is known and they are divided into 4 classes: Toll-like receptors (TLRs), C-type Lectin receptors (CLRs), RIG-I helicase like receptors (RLRs), and NOD-like receptors (NLRs) (Figure 11).

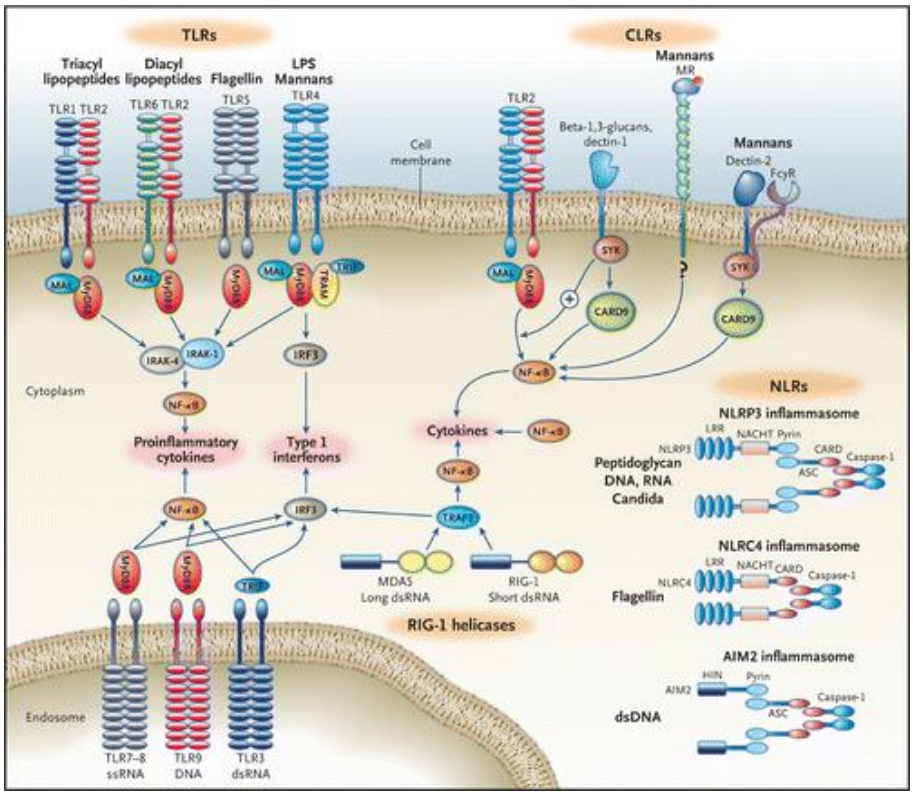

Figure 11. Since the discovery of the first PRR, TLR4, several PRRs have been identified. The 4 major classes, schematized in the figure, are TLRs, CLRs, NLRs and RLRs. Here only some of the PRRs part of these classes are depicted and their number is continuously increasing (*76*).

PRRs can be found in different subcellular locations: cell surfaces, endosomal compartments as well as in the cytosol. Their different localization dictates the type of ligands they can bind. PRRs located on the cell surfaces bind molecules present on the surface of microorganisms, PRRs located in the endosomal compartment bind nucleic acids, and cytosolic NLRs and RLRs recognize fragments of peptidoglycan and viral nucleic acids respectively (*77*).

In 1997 Medzhitov described the first human PRR, later named TLR4, identifying a human homologue of the Toll receptor in Drosophila (*78*) which was previously found to be important during fungal infection in these fruit flies (*79*). Since then, 12 TLRs have been identified in mice and 10 in humans. These receptors differ for their localization and for their ligands and each TLR can also recognize different PAMPs. The first identified TLR, the TLR4, is able to bind LPS through the formation of a complex with CD14, MD2 and LBP. TLR2 can recognize mannan, lipotechoic acid, as well as zymosan and other ligands, by dimerization with TLR1 or TLR6, while TLR5 is known for its ability to bind flagellin. On the other side, intracellular TLRs are mostly able to detect nucleic acids: TLR3 recognizes double-stranded RNA, TLR7 is important for single-stranded RNA detection, while TLR9 binds preferentially bacterial and viral DNA with unmethylated CpG motifs (*80*).

The other class of PRRs expressed on the cell surface is the CLR family. This class comprises 17 different receptors that either recognize carbohydrates or possess a structural similarity with the C-type lectin-like domains. Among the CLRs we can find receptors involved in the recognition of fungal components such as Dectin-1, able to recognize

β-glucans, Dectin-2, which detects α-mannans, and Mincle, able to bind α-mannose. Other receptors of the CLRs family are involved in the response to bacterial and viral infections, this is the case of DC-SIGN that is important in the immune response to Measles, *Lactobacillus* species and other pathogens (*81*).

Other than TLRs and CLRs, a peculiar mention is due to the NLRs receptor family. These receptors form a high molecular weight complex in the cytoplasm with the ability to activate pro-caspases into their active form, named caspases. The activation of the inflammasomes leads to distinct possible outcomes among which the most important and better described is the activation of the pro-inflammatory cytokine IL-1β. This cytokine is produced in an inactive form, pro-IL1β, and cleaved in its active form through the activation of the inflammasomes (*82*).

Even though PAMPs can be extremely diversified, they all share three characteristics. They are not components of the host but they are either part of microbes or released by them. They are shared among microorganisms that are part of a specific class, *i.e.* all Gram-negative bacteria express LPS on their surfaces. Finally, since recognition by the immune system of specific microbial components impose an evolutionary pressure that could give rise to mutants, to escape an immune response, these structures should be vital and necessary for either the survival or to address fundamental functions of the microorganisms (*83*).

The binding between PRRs and their ligands leads to the activation of several intracellular pathways, for the activation of transcription

factors including Nuclear Factor-κB (NF-κB), Activator Protein 1 (AP1) and the IFN regulatory factor (IRF) family. The activation of these three transcription factors leads to the production of pro-inflammatory cytokines as well as the release of interferons (*84*).

Another family of transcription factors activated upon PAMPs recognition is the Nuclear Factor of Activated T cells (NFATs) that will be the main focus of this thesis.

1.4. NFAT

NFAT was originally identified in T cells for its ability to induce interleukin-2 (IL-2) production (*85*) and for years its role has been studied in adaptive immunity (*86*). It was found that NFAT proteins not only induce IL-2 production, but also regulate T and B cell differentiation and activation. Therefore, its original identification as a transcription factor restricted to cytokine production was abandoned and its role gained more and more attention during the following years.

The origin of NFAT is dated back to 500 million years ago, in concomitance with the appearance of vertebrates and also for this reason, at the beginning its role was evaluated only in adaptive immune system (*86*). However, the activation of the NFAT transcription factor was later found to be important not just in the adaptive immunity, but also in the innate immune system. NFAT activation was, in fact, shown to be part of the immune response also

for cells of the innate immune system, such as DCs, neutrophils, mast cells and macrophages (*87*).

NFAT's family is composed of 5 members: NFATc1 (also known as NFAT2), NFATc2 (also known as NFAT1), NFATc3 (also known as NFAT4), NFATc4 (also known as NFAT3) and NFAT5 (Figure 12).

While NFATc1-4 are present only in vertebrates, the NFAT5 is the most ancient member of the NFAT family and it is shared between vertebrates and invertebrates (*88*).

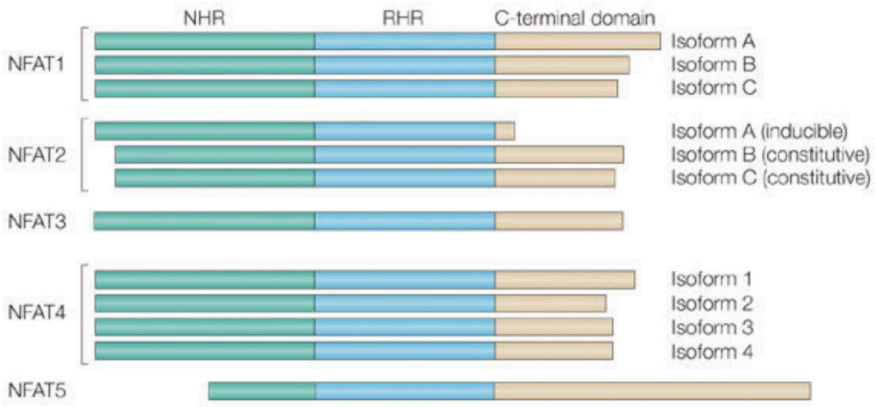

Figure 12. The transcription factor NFAT comprises 5 members. NFAT1-4 (part of the NFATc family) are present only in vertebrates while NFAT5 are more ancient and present both in vertebrates and invertebrates. The REL-homology domain (RLR) contains the DNA-binding motif while the NFAT-homology region (NHR) contains the calcineurin-docking site and the nuclear-localization signals. The regulatory domains inside the NHR are extremely conserved within the NFATc1-4 (*89*).

The NFATc1-4 are present in the cytoplasm in a phosphorylated and inactive form. The induction of an increase in intracellular Ca^{2+}

concentration, due to the opening of Ca^{2+} channels following PRRs activation, leads to the activation of the Ca^{2+}/calmodulin phosphatase Calcineurin (Cn) that de-phosphorylates the members of the NFATc family. This de-phosphorylation causes the exposition of nuclear import sequences thus inducing the translocation of NFATc in the nucleus. Here members of the NFATc family can form complexes with other proteins in order to start specific gene expression (*90*).

Activation of the NFAT pathway in innate immune cells can be induced by different stimuli and receptors. Our laboratory demonstrated, in the past years, that LPS is one of the stimuli able to activate NFATc2 with a mechanism driven by CD14 through the induction of an intracellular Ca^{2+} increase (*91*). Other than bacterial stimuli, fungal components have been described to be extremely potent activators of the NFATc pathways. The activation of Dectin-1, the β-glucans receptor, has been shown to activate NFAT in DCs and macrophages following the same mechanism of Ca^{2+} flux and Cn activation (*92*, *93*).

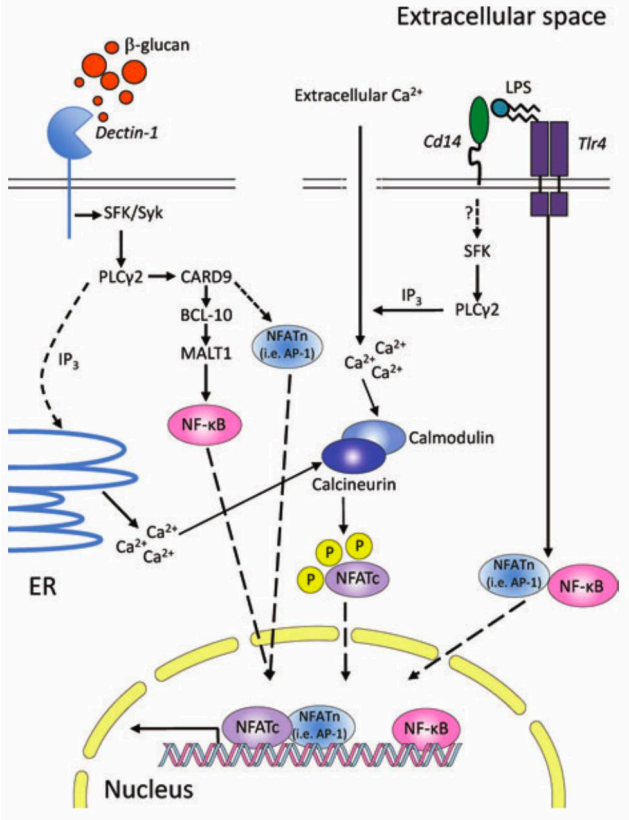

Figure 13. NFATc activation is driven by an increase in the intracellular Ca^{2+} concentration that follows the binding of different PRRs with their respective ligands. This process can be induced by different PAMPs recognition such as LPS and β-glucans (*87*).

Despite its first description in T cells, the role of NFAT in innate immune system is not secondary since, as we saw, it can be induced by several stimuli. The presence of these transcription factors in the innate immunity is necessary for the prompt response and pro-inflammatory cytokines production by those cells that are the first to encounter pathogens.

1.5. Inflammation

When an infection occurs, the organism responds with the process of inflammation. The inflammatory process was first described by Aulus Cornelius Celsus, a Roman doctor, in the 1st century. Celsus described the inflammation with 4 characteristic signs: rubor (redness), tumor (swelling), calor (heat) and dolor (pain). These four signs remained untouched until 1858 when Rudolph Virchow added a fifth sign: "loss of function" (*94*).

An acute inflammatory process is driven by inflammatory cytokines released at the site of infection by those cells that first detect the pathogen. These cytokines activate the endothelium, allowing the extravasation of neutrophils that can be then recruited at the inflammatory site. Here neutrophils release anti-microbial peptides as well as ROS and other inflammatory mediators thus boosting the inflammatory response. These processes are driven by particular cytokines and molecules among which we can find prostaglandins, in particular prostaglandin E_2 (PGE_2). PGE_2 is able, in fact, to induce vasodilation as well as microvascular permeability. This process causes the formation of the signs of inflammation such as redness, swelling and pain. The increase in vascular permeability, moreover, induces the release of liquids into the inflamed tissue, causing to the formation of an edema (swelling) (*95*). This edema formation is important in order to increase the interstitial pressure thus facilitating the ingress of free antigens into lymphatic vessels and the transport to the draining

lymph nodes, where they can be detected by APCs and activate an adaptive immune response (*96*).

PGE_2 is not the only mediator able to increase vascular permeability and edema formation. Among other mediators, vascular permeability can be induced also by IL-2. In pathological conditions, IL-2 induces the so-called Vascular Leak Syndrome (VLS). Treatment of patients with high dose of IL-2 can lead to VLS especially in the lung, thus causing edema formation and organ failure (*97*).

An acute inflammatory process terminates with the elimination of the infectious agent and the repair of the damaged tissue. If the acute inflammation does not come to an end, the inflammatory response become chronic (*98*). Typical chronic inflammatory diseases are obesity, fibrosis, type 2 diabetes, atherosclerosis as well as neurodegenerative diseases and cancers (*94*).

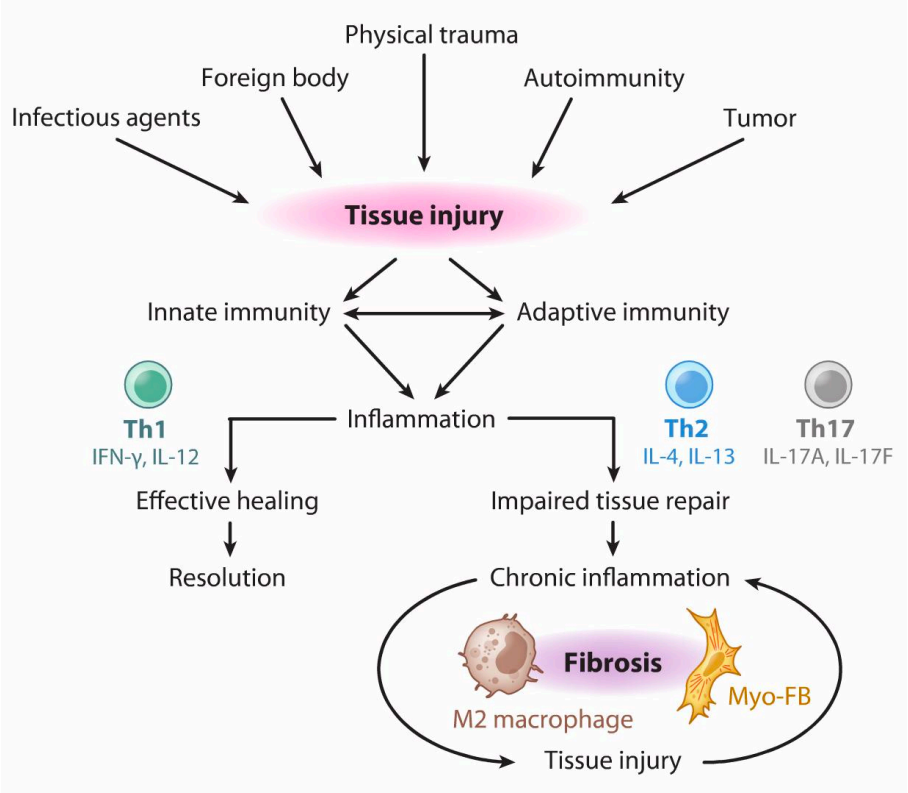

Figure 14. Several stimuli are able to induce inflammation and tissue injury. An impaired resolution of the inflammatory process, like the persistence of the infectious agent, can cause chronic inflammation as depicted in the figure. An example of chronic inflammation is the development of fibrosis that, finally, leads to further tissue injury, scar formation and alteration of the homeostasis (*99*).

Several events can lead to chronic inflammation: the persistence of the infectious agents, the formation of a positive feedback loop due to a continuous stimulation, the involvement of different immune cells that modify the homeostasis as well as the healing process that can lead to scar formation (*100*).

Not only microorganisms are capable of inducing inflammation. An inflammatory response can, in fact, be activated by non-microbial stimuli in a process called sterile inflammation. As for those caused by microorganisms, sterile inflammations are caused by the production of inflammatory cytokines and the recruitment of immune cells. Neutrophils, the first cells recruited, lead to an acute inflammatory response to restore a homeostatic condition (*101*). Sterile inflammation can be induced by trauma, ischaemia-reperfusion injury as well as inhalation or presence of chemical agents such as irritants or foreign substances and materials. As for the microbial-induced inflammation, the lack of the removal of the inflammatory stimulus can move a sterile acute inflammation toward a chronic status.

As for inflammation of microbial origin, sterile inflammation can become chronic. This is the case of the inhalation of asbestos. The presence of these minerals in the lungs causes the continue activation of alveolar macrophages (*101*). Since asbestos' fibers are not efficiently eliminated by phagocytes, the inflammatory stimulus persists in the lungs thus leading to a chronic inflammatory process which results, in this case, in a particular form of chronic inflammation named fibrosis.

1.6. Fibrosis

Fibrosis is defined as the excessive deposition of extracellular matrix (ECM) components mainly by fribroblasts (*99*). This increased and continuous deposition of ECM causes the so-called loss of function, the last marker of inflammation, thus ending in organ failure. Fibrosis can interest distinct organs such as kidney, lung and liver and it can also develop at the skin (scleroderma) (*102*).

During acute inflammation, innate immune cells are recruited at the site of infection where they produce pro-inflammatory cytokines and chemokines. These pro-inflammatory mediators can act directly on fibroblasts inducing their proliferation and differentiation into myofibroblasts, that are actually the cellular type responsible for the production of ECM (*102*). For the healing process to develop, this source of inflammatory mediators should be eliminated. In fact, if neutrophils and macrophages are not eliminated from the site of infection, there is a continuous production of pro-inflammatory mediators that can result in the development of a fibrotic response. Neutrophils, macrophages and other innate immune cells produce cytokines like TNF-α and IL-1β in order to create an inflammatory environment. These cytokines have been described to play an important role in the pathogenesis of fibrosis with a direct role on fibroblasts (*103*). They can, together with other mediators, cause hyperproliferation of fibroblasts and their differentiation into myofibroblasts. This process leads to an excessive deposition of ECM that cannot be eliminated by the activity of other cytokines and

inflammatory mediators, such as proteinases, that are usually necessary to dampen the inflammatory process and drive to the repair of the tissue (*99*).

One of the major players during fibrosis is TGF-β. This cytokine is produced by stromal cells as well as innate immune cells during inflammation. Its primarily role is to induce ECM synthesis by myofibroblasts in order to repair a wound and restore homeostasis. TGF-β is also present in the tissues in an inactive form and, during inflammation, it is activated by proteases, such as plasmin, metalloproteinases (MMPs) and ROS and can therefore increase ECM deposition (*104*).

TGF-β canonical signaling starts from the binding of this growth factor to its receptor, TGF-β receptor 1 (TGFR1). Activation of the TGFR1 leads to the phosphorylation of the complex Smad2/Smad3. Once phosphorylated, this complex translocates to the nucleus, together with Smad4, to drive gene transcription. Among the genes activated by the Smad2/Smad3 complex there are several profibrotic molecules such as α-smooth muscle actin (α-SMA), marker of myofibroblast differentiation, collagen genes and inhibitors of metalloproteinases. Given its role in fibrosis, TGF-β pathway is tightly regulated both by control of the availability of TGF-β within the tissue as well as direct regulation of the pathway inside the cells. Among Smad proteins, Smad7 is an inhibitor of the Smad2/Smad3 complex, thus adding one more checkpoint in the activation of a fibrotic response (*105*) (Figure 15).

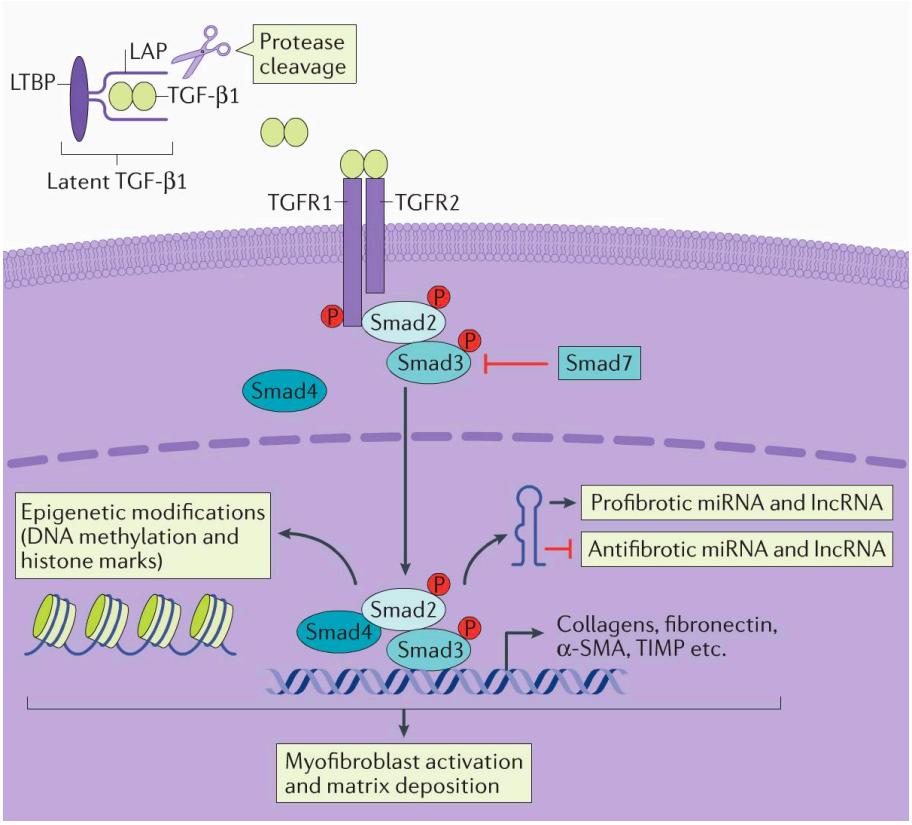

Figure 15. Activation of the TGF-β pathway requires the phosphorylation of the Smad2/Smad3 complex. Translocation of this complex to the nucleus causes the transcription of genes involved in fibrosis, like collagen and other ECM components (*105*).

Among ECM deposits, one of the main products released following TGF-β stimulation is collagen, a structural protein composed of three chains of protocollagen. Protocollagen chains form a triple helix that results in the superstructure, collagen, that is one of the main component of the ECM and is important for regulating chemotaxis, cell adhesion as well as tensile strength of the organ or tissue.

Myofibroblasts have been described to be able to produce huge amounts of Collagen I and III as well as IV, V and VI. Collagen deposition by myofibroblasts is an important event during wound repair. However, in chronic inflammation, this deposition continues over the time causing an accumulation of different types of collagen thus establishing a pathological condition. It has been demonstrated that, in a healthy tissue, myofibroblasts undergo apoptosis once the wound is repaired. If these cells survive the apoptotic event, the homeostasis of the organ is damaged because of the fibrotic effects these cells could have within the tissue (*106*).

In a healthy condition, pro- and anti-fibrotic events create a balance in order to maintain tissue homeostasis. Cells of innate and adaptive immune systems release inflammatory mediators such as IFN-γ, which is known to have an anti-fibrotic effect. IFN-γ can, in fact, suppress both fibroblasts proliferation and collagen deposition (*107*) through the inhibition of the TGF-β pathway. It has been demonstrated that, through the activation of the Jak1/Stat1 pathway, IFN-γ induces an upregulation of the negative regulator of the TGF-β pathway. Activation of Jak1/Stat1, in fact, causes an upregulation of Smad7 which, in turns, inhibits the TGF-β-mediated phosphorylation of Smad3 (*71*).

Fibrosis can develop only when there is an imbalance between these pro-fibrotic and anti-fibrotic signals.

Another mechanism used to impede fibrosis development is the release of proteinases during inflammation that degrade the ECM thus

creating a balance between newly synthesized proteins and protein degradation. Neutrophils are able to release MMPs within the tissue through granule exocytosis. As discussed above, neutrophils' granules contain several proteases whose main role is to degrade the ECM (*59*). Other than neutrophils, however, enzymes involved in ECM degradation can be released by stromal cells. This is the case of plasmin, which is released by the liver and kidney in an inactive form, plasminogen, and can be activated within the tissue where it can both cleave inactive tissue-TGF-β and degrade proteins of the ECM (*104*).

1.7. Fibrinolytic system

The goal of the fibrinolytic system is to convert the inactive proenzyme plasminogen into its active form, plasmin. Activation of plasminogen into plasmin usually happens through the activities of two key enzymes: urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). In the last decades, however, other roles have been described, assigning to the fibrinolytic system an extremely important function in modulating the extracellular environment both in physiological and pathological conditions (*108*).

Plasmin formation is tightly regulated through the activity of three main inhibitors: α2-antiplasmin and α2-macroglobulin, that directly inhibit plasmin, and plasminogen activator inhibitor type-1 (PAI-1), that acts by inhibiting the two activators, tPA and uPA. Actually, three types of PAIs have been identified, but the most important role in fibrinolytic system regulation is played by PAI-1 (*109*).

PAI-1 can be released by several cell types including vascular endothelial cells, fibroblasts and macrophages. Basal levels of PAI-1 are important for cell migration as well as tissue remodeling and the maintenance of homeostasis. Nevertheless, an excessive production of PAI-1 can be detrimental and cause fibrotic-like pathologies. It has been shown that blocking plasmin formation could cause matrix accumulation thus leading to fibrosis in several organs (*110*) due to plasmin proteolytic activity on proteins of the ECM. Furthermore, increasing evidences have shown that plasmin is important also in the activation of other proteinases, such as MMPs. MMPs play a crucial role in the maintenance of homeostasis and in remodeling the extracellular environment. The activation of the fibrinolytic system can have as outcome the activation of the pro-forms of several MMPs, including MMP-3 and MMP-9. These MMPs can, after being activated, cleave other MMPs thus enhancing the proteolytic activity during an inflammatory process (*111*). In addition to the activation of MMPs and the digestion of ECM, plasmin can also induce the activation of growth factors present in the matrix environment such as TGF-β. As described above, TGF-β can be found within tissues in a latent and inactive form. One of the proteinases able to activate the latent TGF-β is plasmin (*112*). Activation of TGF-β, together with the activation of proteinases, gives to the fibrinolytic system a central role in the maintenance of a homeostatic condition. It is easy to imagine how, an imbalance between activators and inhibitors of the fibrinolytic system could case pathological conditions.

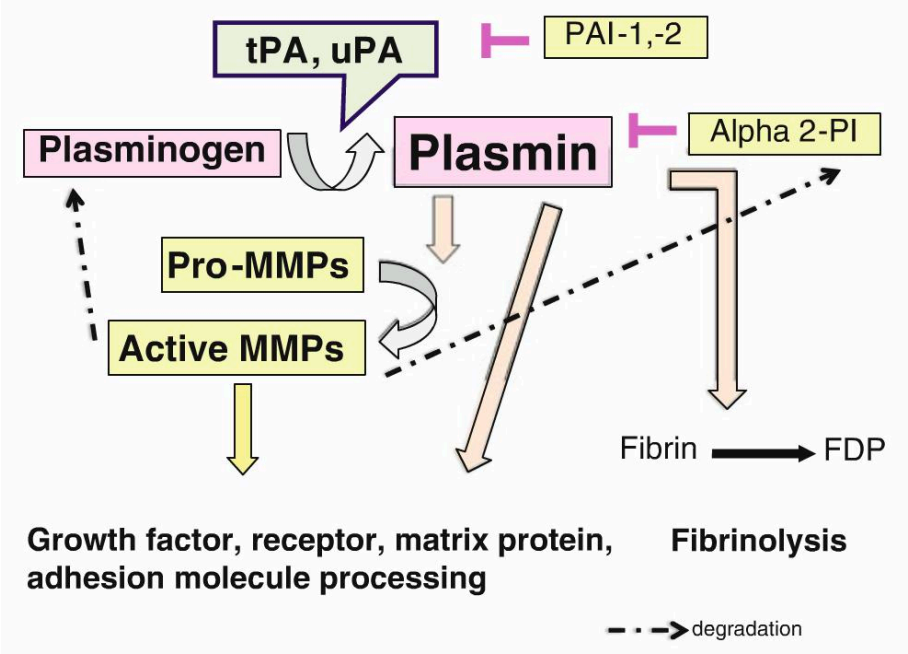

Figure 16. The fibrinolytic system has been discovered for its activity in degrading fibrin clots. Increasing evidences, however, demonstrated how the activation of plasmin can induce the formation of active MMPs, therefore causing a breakdown in the ECM. Furthermore, plasmin can degrade the ECM itself thus playing a principal role in tissue remodeling and matrix degradation (*109*).

It has also been discovered that some pathogens, both bacteria and fungi, take advantage of this ECM degradation ability of plasmin in order to facilitate their invasion within tissues. These pathogens exploit plasmin's functions through the expression of receptors on their surfaces with the ability to bind plasminogen as well as though their release in the extracellular environment. Plasminogen bound to these structures can be cleaved by both host factors and the receptors themselves (*113*). An example of these receptors is enolase, an

enzyme involved in the glycolytic pathways, that is expressed also on the surfaces of several pathogens, including *Candida albicans*, and has the ability to bind plasminogen thus increasing its cleavage and activation by uPA (*114*).

Increasing evidences have so far demonstrated how the fibrinolytic system is an actual player in the immune response to pathogen and its role is not restricted to degradation of fibrin clots.

1.8. Candida albicans

Candida (C.) albicans is a fungus that can grow in different morphological forms such as yeast, blastospores, pseudohyphae and hyphae. These different morphologies are dictated by environmental conditions (*115*).

Usually present as a commensal microorganism of the intestinal tract, the skin and the genital mucosa, *C. albicans* can cause severe infections once it either reaches the bloodstream or pass the epithelial barriers. The ability of this fungus to invade the epithelium requires its morphological change into hyphae. In its hyphal form, *C. albicans* is able to penetrate the epithelium thus invading the skin as well as the intestinal tract. In order to cross the epithelium, *C. albicans* releases proteases, such as aspartyl proteases, as well as toxins, like the recently discovered Candidalysin, a pore forming toxin able to damage epithelial cells (*116*).

Once penetrated inside the organism, *C. albicans* can be recognized by several immune cells due to the expression, on its surface, of different

PAMPs. Due to the high number and the different nature of the PAMPs expressed, the fungus can be recognized by CLRs, TLRs as well as NLRs and RLRs.

C. albicans cell wall is composed for the 90% of carbohydrate while the protein part stands only for 10% of the cell wall. The polysaccharide layer is made of three main elements: the outer part is composed of mannan proteins that undergo post-translational modification in order to form O-mannan and N-mannan. The inner part, that gives structural integrity to the fungus, is composed of β-1,3 glucans, β-1,6 glucans and chitin (Figure 17) (*117*).

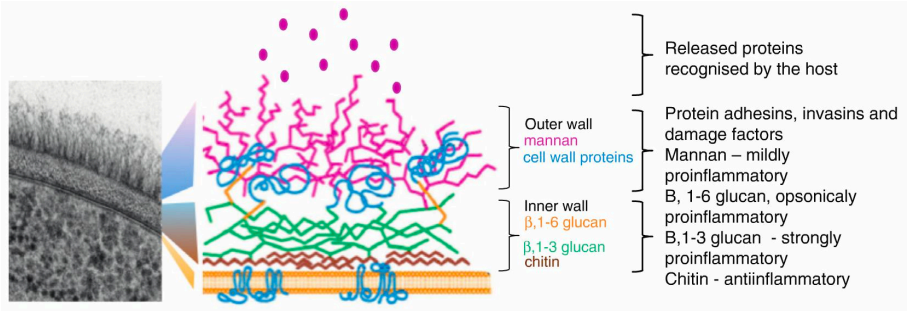

Figure 17. *C. albicans* cell wall is composed of three main elements. Mannans on the most external part, β-glucans and chitin in the inner part. These three components are important for *C. albicans*' functions as well as for its recognition by innate immune cells (*117*).

These structures can be recognized by different receptors of the immune system (Figure 18).

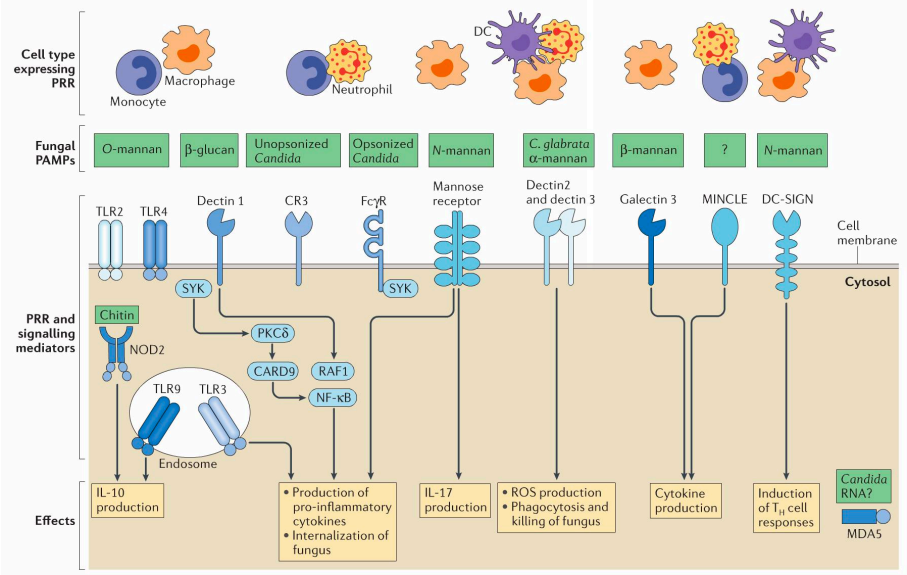

Figure 18. C. albicans can be recognized by different PRRs expressed by several immune cells. These receptors lead to the activation of an immune response through the production of cytokines and chemokines. Depending on the receptor and on the ligand, the transcription factor activated could be different and this correlates with a different outcome. Depending on the PRR, innate immune cells can respond to the fungus with the production of pro-inflammatory cytokines, phagocytosis, chemokines but also with the release of anti-inflammatory cytokines (*118*).

Among the PRRs involved in an immune response to *C. albicans*, the most studied one is Dectin-1 which can bind β-glucans. Dectin-1 is expressed by phagocytes, including DCs, macrophages and neutrophils, as well as on a subpopulation of splenic T cells (*119*). Binding of Dectin-1 with its ligands leads to the activation of different pathways. It has been shown that Dectin-1 can activate the NF-κB pathway both alone and in cooperation with TLR2. Dectin-1 activation

can also lead to ROS production as well as phagocytosis of the pathogen. Furthermore, recognition of pathogens through Dectin-1 causes, in DCs and macrophages, an increase in the intracellular Ca^{2+} concentration that leads to the activation of members of the NFATc family and the release of inflammatory cytokines such as IL-2 and IL-12 (*93*).

In addition to Dectin-1 and TLR2, *C. albicans* can be recognized by an elevated repertoire of PRRs including CLRs, TLRs as well as NLRs and RLRs. The mannan component of the *C. albicans* cell wall can be recognized by several CLRs such as the mannose receptor, Dectin-2, DC-SIGN, Galectin-3 and Mincle. These receptors are important in order to mount an inflammatory response through the production of ROS and pro-inflammatory cytokines such as IL-17.

For what concern the TLRs, TLR2 is the better studied receptors in immune response to *C. albicans* however, it has been reported that also TLR4 and TLR6 can recognize mannoproteins, while the intracellular receptors TLR3 and TLR9 seem to be also needed in an immune response to this fungus.

Another class of PRR is the NLR, important for the formation of the inflammasome. Several reports demonstrated the importance of the inflammasome NLRP3 in response to *C. albicans* hyphae infection. Finally, it has been recently reported that the RLR receptor MDA5 is associated with *C. albicans* recognition in both human and murine cells. This new finding reveals how the spectrum of the receptors involved in the recognition of this pathogen is actually wider than expected (*118*, *120*).

1.8.1. *C. albicans* in clinics

C. albicans is usually present in the healthy microbiota of most individuals colonizing the intestinal tract as well as the skin and the genital mucosa (*121*). However, under pathological conditions, especially when the immune system is compromised, *C. albicans* can lead to severe mycoses at the skin and mucosal level with the possibility to cause bloodstream infections. In the last decades, the incidence of fungal infections has increased dramatically due to the higher number of people with HIV-syndrome and people that are under chemotherapy or immunosuppressive drugs following transplantation (*122*). A surveillance study done in the United States showed how *C. albicans* is the causative agent of 9.5% of the nosocomial bloodstream infections (*123*).

One of the major problems of candidiasis is its diagnosis. Usually, in fact, *C. albicans* infections resemble bacterial infections and the diagnosis of candidiasis originates from the resistance of the infection to antibiotic therapy. This difficulty in diagnosis is a real problem since the percentage of mortality imputed directly to *C. albicans* in candidiasis is almost 40%. Moreover, if a patient gets rid of bloodstream infection, the fungus is able to create its own niche in several organs such as kidney and spleen thus causing severe pathological conditions (*124*).

Candidemia can develop in patients with neutropenia, caused by both specific pathological conditions, such as blood cancer, or immunosuppressive therapies. Another risk factor is the presence of

damages, like ulcers, along the gastrointestinal tract, that can allow the fungus to penetrate the organism thus causing the spread of the infection. Finally, an additional way that *C. albicans* can exploit in order to infect the organism is through medical devices such as catheters and artificial heart valves. The fungus can, in fact, form a biofilm on these solid surfaces thus increasing its ability to colonize the environment (*125*).

C. albicans can also cause skin abscess formation. This occurrence is, however, a rare event among the possibilities of infections (*126*). *Candida albicans* skin infections, in fact, usually occur following the formation of a cut or a wound. Therefore, in the last years, *C. albicans* skin infections have been widely studied using a transcutaneous model of infection where the mouse's skin is abraded with a sand paper and then the fungus is applied to the wound (*127*). With this model, different laboratories have identified the roles of several immune cells in the detection of the fungus and in their response to cutaneous infections. It has been demonstrated by different reports that the role of cytokines like IL-23 and IL-17, produced by both the innate and the adaptive immune branches in response to the pathogen, is fundamental in both human and mouse (*121*, *128*). Recently it has been demonstrated how also stromal cells and epithelial cells are involved in the host response to fungal infection. Their role is increasing in the last years in both the induction of innate immune response as well as in boosting and differentiation of an adaptive immune response (*129–131*). Taken together, what is now known is that the immune response to cutaneous fungal infections is an even

more complex scenario than expected. Since the importance of non-immune cells is now accepted and demonstrated, other pathways aside of the immune system should be assessed and analyzed in order to better describe and characterize how fungal infections can be eradicated.

1.9. Staphylococcus aureus

Staphylococci were identified for the first time in 1882 by Alexander Ogston while, two years later, Friedrich J. Rosenbach described a pigmented colony that he named *Staphylococcus* (*S.*) *aureus* (*132*). *S. aureus* is a Gram-positive bacterium that usually colonizes nares, skin and gastrointestinal tract of almost 30-50% of the population in an asymptomatic manner. *S. aureus* is also an opportunistic pathogen and staphylococcal infections mostly occur either when the skin or the mucosal barriers are altered or in patients with a compromised immune system. *S. aureus* infections can cause different diseases such as endocarditis, toxic-shock syndrome, bacteremia, soft-tissue infections, pneumonia as well as life-threatening diseases (*133*).

Most of the *S. aureus*' envelope is composed of a thick layer of peptidoglycan, a long polymer composed of polysaccharide subunits. Peptidoglycan can be recognized by the immune system as a PAMP thus leading to cytokines release, complement activation and platelets aggregation. TLR2 has been described to be the receptor able to recognize peptidoglycan for many years, however, its role has been questioned and it has been actually demonstrated that recognition of

peptidoglycan occurs through the activation of receptors of the NOD family. The misleading identification of TLR2 as receptor for peptidoglycan recognition was primarily due to contamination of peptidoglycan preparation with lipoteichoic acids (LTA) (*134*). Teichoic acids are long anionic polymers, component of the envelop of the bacterium together with peptidoglycan (Figure 19). Teichoic acids are divided into two classes: the wall teichoic acids, that are attached to peptidoglycan, and the lipoteichoic acids that are anchored directly to the membrane of the pathogen. These structures are those recognized by TLR2 leading to the production of inflammatory cytokines such as TNF-α and IL-1β (*135*, *136*).

S. aureus also expresses several surface proteins, called adhesins, that are linked to both peptidoglycan and teichoic acids and are able to recognize the ECM in order to mediate pathogen colonization (*137*).

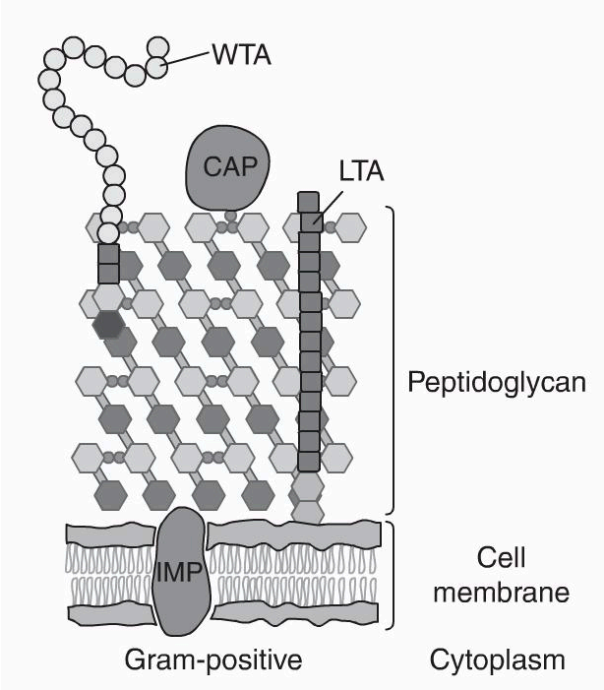

Figure 19. *S. aureus* envelope is composed of a thick layer of peptidoglycan which is a polymer made of polysaccharide units. Along with peptidoglycan there are lipoteichoic acids (LTA) and wall teichoic acids (WTA). These are long anionic polymers that can be attached either to peptidoglycan or directly to the cell membrane. Like other Gram-positive bacteria, *S. aureus'* envelope comprises also proteins that can be inside the cell membrane, integral membrane proteins (IMPs), or attached to the peptidoglycan layer and thus exposed on the surface of the pathogen, called covalently attached proteins (CAPs) (*136*).

Finally, *S. aureus* can release in the extracellular environment several products among which we can find both toxins and enzymes. Toxins derived from *S. aureus* are one of the principal causes of Staphylococcus associated diseases, such as toxic shock syndrome and

skin erythema and severe cutaneous infections. For what concerns the enzymes released by this pathogen, we can find proteases, lipases and hyaluronidases that confers the bacterium the ability to degrade the ECM thus increasing its spreading within the tissue (*137*).

1.9.1. *S. aureus* in clinics

S. aureus has always been a major cause of infection worldwide and for this reason this pathogen has been extensively studied. In the last decades, the number of cases associated with *S. aureus* infections has increased dramatically. In a surveillance study, *S. aureus* was identified as the cause of almost 20% of the cases of nosocomial bloodstream infections in the United States (*123*). The increase in *S. aureus* infections is mainly due to the exquisite ability of this pathogen in acquiring resistance to almost all antibiotic drugs. Penicillin, the first antibiotic drug discovered, was introduced in 1941 and only one year later a strain of *S. aureus* resistant to this drug was identified. During the rest of the XX century several other antibiotics have been introduced like erythromycin, streptomycin and tetracyclines. *S. aureus* was able to develop resistance to all these drugs. When, finally, methicillin was introduced, in 1959, the bacterium evolved resistance also to this last drug only two years later (*132*). β-lactam antibiotics act by inactivating a class of transpeptidases, called penicillin binding proteins, involved in the biosynthesis of peptidoglycan that thus are vital for the bacterium (*138*). The resistance to methicillin has been acquired through the expression of another protein, named PBP2a,

whose active site is located in a deeper pocket thus rending it inaccessible to β-lactam antibiotics (*139*). Other drugs have been introduced into clinics to overcome Methicillin-Resistant *Staphylococcus aureus* (MRSA). Probably the most successful one was Vancomycin, introduced in 1958, against which MRSA was unable to develop resistance for long time. However, like all other drugs, in 2002 a vancomycin-resistant *S. aureus* strain was identified (*132*).

This continuous evolution of *S. aureus* to newly developed drugs makes it one of the most important pathogens worldwide and the leading cause of pathogen-associated morbidity and mortality in the United States.

When *S. aureus* penetrates tissues it usually causes the formation of abscesses with a severe inflammation. Sepsis caused by *S. aureus* are usually consequences of the pathogen escaping those abscesses. When it reaches tissues, *S. aureus* induces the recruitment of immune cells such as neutrophils, that boost the inflammatory process. In order to confine the pathogen, the host respond to the infection through the formation of an abscess inside of which, though, the pathogen is able to proliferate. This continuous proliferation leads to the enlargement of the abscess that, in case of rupture, causes the spread of the pathogen to other sites. If the infection is not properly eliminated this process can end with a disseminated infection of the host and, finally, with the death of the patient (Figure 20) (*140*).

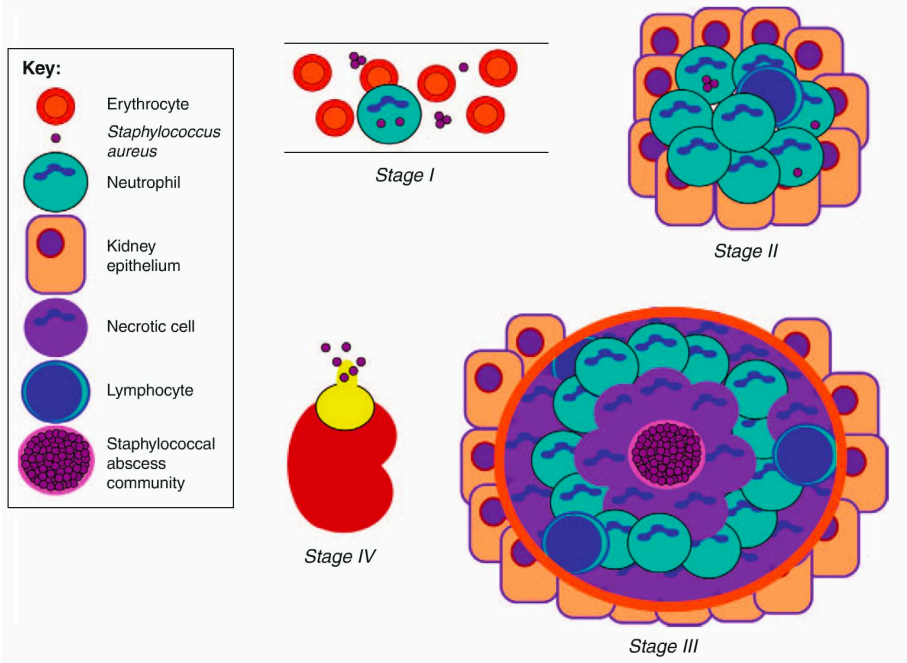

Figure 20. *S. aureus* infections can lead to the formation of abscesses. Particularly, *S. aureus* infect the host and, through the bloodstream (stage I) it reaches organs like kidney. Here, the formation of an inflammatory process induces the recruitment of neutrophils (stage II). *S. aureus* can escape killing by neutrophils and start proliferating, originating an abscess (stage III). When the abscess breaks, finally, *S. aureus* spreads across the body infecting the surrounding tissues and organs (stage IV) (*140*).

Understanding how the pathogen can be efficiently eliminated during these infections is therefore a must for biological research in order to find a way to overcome both drug resistance and persistence of infections due to other mechanisms like abscesses formation.

1.10. Scope of the Thesis

It is known that fungal ligands are able to strongly induce the activation of the transcription factor NFAT. Moreover, patients treated with NFAT inhibitors, such as cyclosporine A and tacrolimus, face increased susceptibility to both fungal and bacterial infection (*141*) and are more prone to developing cysts. Given this information, we attempted to determine the roles that NFAT plays in the eradication of fungal infections.

The aim of the thesis was, therefore, to understand the mechanisms that underlie the elimination of an invasive pathogen during skin infections with a particular focus on the activation of NFAT.

In order to characterize the molecular machinery activated during fungal infections, we used a model of intradermal *C. albicans* hyphae infection.

Finally, we wanted to understand if the mechanisms activated in response to *C. albicans* were also conserved and shared upon bacterial challenge, such as during *S. aureus* skin infections.

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Chapter 2: Skin infections are eliminated by cooperation of the fibrinolytic and innate immune systems

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2.1. Abstract

Nuclear factor of activated T cells (NFAT) is activated in innate immune cells downstream of pattern recognition receptors, but little is known about NFAT's functions in innate immunity compared with adaptive immunity. We show that early activation of NFAT balances the two

major phases of the innate response to *Candida albicans* skin infections: the protective containment (abscess) and the elimination (expulsion) phases. During the early containment phase, transforming growth factor-β (TGF-β) induces the deposit of collagen around newly recruited polymorphonuclear cells to prevent microbial spreading. During the elimination phase, interferon-γ (IFN-γ) blocks differentiation of fibroblasts into myofibroblasts by antagonizing TGF-β signaling. IFN-γ also induces the formation of plasmin that, in turn, promotes abscess capsule digestion and skin ulceration for microbial discharge. NFAT controls innate IFN-γ production and microbial expulsion. This cross-talk between the innate immune and the fibrinolytic systems also occurs during infection with *Staphylococcus aureus* and is a protective response to minimize tissue damage and optimize pathogen elimination.

2.2. Introduction

Nuclear factor of activated T cells (NFAT) proteins form a family of transcription factors that are estimated to have evolved about 500 million years ago, around the same time as the appearance of vertebrates (*1*). Given the relatively late appearance of NFAT proteins in evolution, their major roles were believed to center on regulation of the adaptive immunity, the more recent form of immunity that appeared with vertebrates. In contrast, innate immunity (the ancient form of immunity) was thought to be controlled primarily by signaling

pathways that are conserved during evolution (*2*), and the prevailing consensus was that NFATs were not involved in this form of immunity (*3*). However, we and others have shown that the NFAT family can be activated during innate immune responses to bacterial or fungal infections downstream of pattern recognition receptors (*4*–*6*).

The NFAT pathway in phagocytes is most effectively activated in response to β-glucan-bearing fungi (*7*–*9*). Fungal infections can develop at a variety of anatomical sites. Although these infections are readily controlled by healthy individuals, they may become systemic in immunocompromised individuals, hospitalized patients, or individuals with inherited mutations in immune genes. Calcineurin is a phosphatase involved in the activation of NFAT transcription factors, and calcineurin inhibitors, such as cyclosporine A (CsA) and tacrolimus, are commonly used as immunosuppressors for treating acute transplant rejection (*10*), autoimmune diseases (such as psoriasis) (*11*), and other immunological conditions (such as atopic dermatitis) (*12*). Although such inhibitors are highly efficient, they have potent side effects that include susceptibility to opportunistic infections from pathogens, such as *Candida albicans*, *Aspergillus fumigatus*, and many bacterial species, which are normally well con- trolled by innate and adaptive immunity (*13*, *14*). In principle, CsA and tacrolimus block interleukin-2 (IL-2) and other NFAT-dependent cytokine production by T cells; but increasing evidence supports the notion that susceptibility to microbial infections could mainly arise via inhibition of the NFAT pathway in innate immune cells (*6*), although the mechanisms that underlie this vulnerability remain elusive.

Here, we have investigated how NFAT activation during innate responses regulates the development and the progress of the inflammatory process induced in the skin by microorganisms and leads to the protection against microbial spreading and, eventually, to microbe elimination.

2.3. Results

NFATc2 is required for the elimination of *C. albicans* skin infections by innate immune cells.

We first investigated the role of NFATc2 in the innate control of *C. albicans* infections in the skin. We focused on the study of NFATc2, which is primarily activated in dendritic cells (DCs) and is not expressed in neutrophils, one of the major populations known to fight fungal infections [fig. S1A; (*6*)]. Thus, in this model, the neutrophil response is not altered [their phagocytic capacity remains unaltered compared to wild-type (WT) neutrophils; fig. S1B], whereas other phagocyte responses may be affected by the absence of NFATc2.

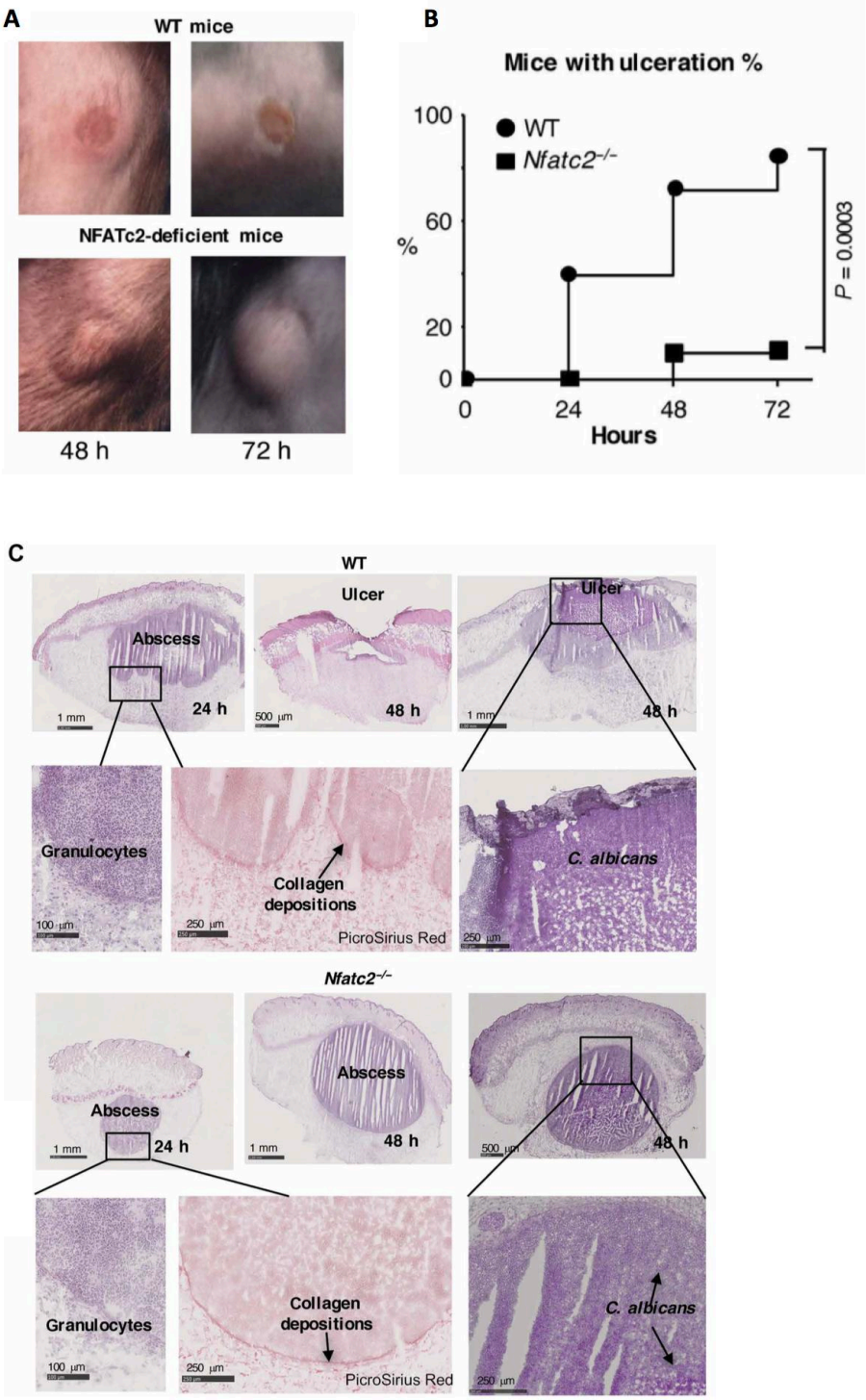
To investigate whether NFATc2 participates in the innate immune responses to fungal infections, we used NFATc2-deficient and WT mice in a model of *C. albicans* skin infection. Mice were infected in the deep dermis with *C. albicans* hyphae, and the temporal course of the lesions was evaluated. WT animals displayed skin ulceration at about 2 days after infection (Fig. 1, A and B, and figs. S2 and S3), whereas NFATc2-deficient mice exhibited a capsulated abscess containing *C. albicans*

with no ulceration (Fig. 1, A to C, and figs. S2 and S3). Although *C. albicans* could not be eliminated in NFATc2-deficient mice, the infection remained confined in the abscess with no further spreading (fig. S4). The phenotype of the NFATc2-deficient mice was not primarily due to the absence of NFATc2 expression in adaptive immune cells because *Rag2*$^{-/-}$ mice, which lack an adaptive immune system, had a response like that of WT mice (Fig. 1D).

Histological analyses revealed that the abscess was formed by granulocytes recruited to the site of the *C. albicans* infection, suggesting that recruitment was unchanged between WT and NFATc2-deficient mice (Fig. 1C and figs. S2 and S3). Use of quantitative reverse transcription polymerase chain reaction (qRT-PCR) to characterize other cell types recruited to the infection site showed that neutrophils, eosinophils, and basophils were present in both animal types (fig. S5). Monocytes were also recruited in both groups (fig. S5). Although a few differences in the composition of the infiltrate were measurable at specific time points (more eosinophils were recruited in WT mice, and more basophils were recruited in NFATc2-deficient mice), the capacity of WT and NFATc2-deficient animals to recruit immune cells was very similar.

Histological analyses of WT mice also showed that the abscess was surrounded by collagen deposits (Fig. 1, C and E, and figs. S2B and S3). Starting at 48 hours after infection, abscess formation was followed by skin ulceration and the discharge of dead cells and *C. albicans* from the skin (Fig. 1C and fig. S3). Complete elimination of *C. albicans* in WT mice occurred in about 6 to 7 days after infection (fig. S6), whereas *C.*

albicans persisted inside the abscess in NFATc2-deficient mice (fig. S6). However, infected skin in NFATc2-deficient mice showed the formation of an abscess surrounded by a well-organized thick collagen capsule (Fig. 1C and figs. S2B and S3), with *C. albicans* contained inside the abscess (Fig. 1C and figs. S3 and S6). A quantification of collagen deposits around the abscess 24 hours after infection showed significantly less collagen deposit in WT compared with NFATc2 mice (Fig. 1E). Fibroblasts surrounded the newly recruited granulocytes in both WT and NFATc2-deficient mice (Fig. 2A). Nevertheless, over time, in NFATc2-deficient but not WT mice, fibroblasts differentiated into myofibroblasts, as indicated by the expression of the α–smooth muscle actin (α-SMA) marker (Fig. 2B and fig. S7). These data indicate that WT animals contain and eliminate *C. albicans* skin infections, whereas NFATc2-deficient mice restrain the infection by forming a thick capsule around the abscess but are not able to eliminate the microorganism.


A
WT mice
NFATc2-deficient mice
48 h
72 h
B
Mice with ulceration %
100
60
20
0
%
WT
Nfatc2-/-
P = 0.0003
0
24
48
72
Hours
C
WT
Abscess
24 h
1 mm
Ulcer
48 h
500 µm
Ulcer
48 h
1 mm
Granulocytes
100 µm
Collagen depositions
PicroSirius Red
250 µm
C. albicans
250 µm
Nfatc2-/-
Abscess
24 h
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C. albicans
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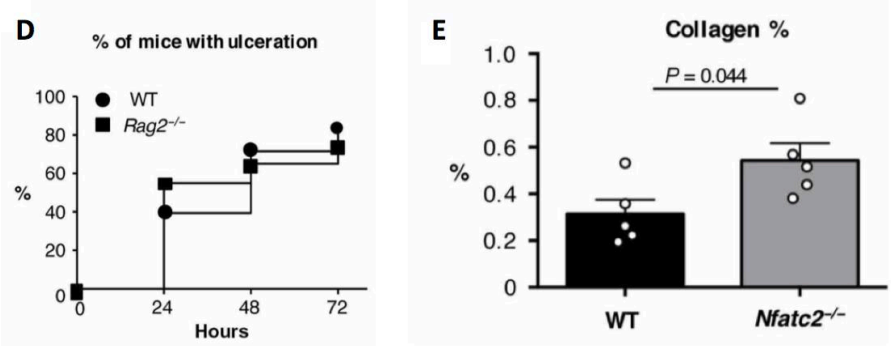
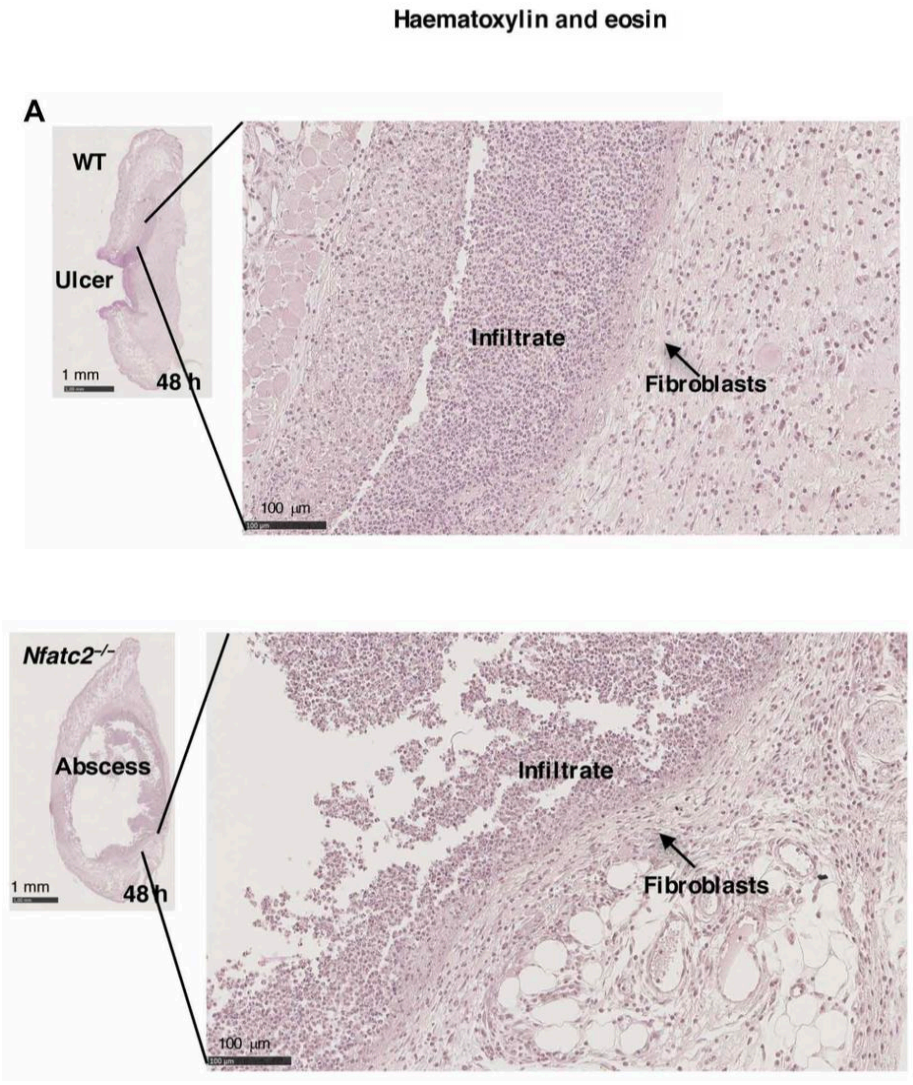


Fig. 1. NFATc2-deficient mice do not eliminate skin *C. albicans* infections. (**A**) Lesions in WT and NFATc2-deficient mice at the indicated time points after *C. albicans* hyphae injection in the deep derma. WT animals (top) undergo ulceration and *C. albicans* elimination, whereas NFATc2-deficient mice (bottom) develop a persistent abscess. (**B**) Kaplan-Meier curve showing the percentage of WT and NFATc2-deficient mice undergoing ulceration after *C. albicans* administration at the indicated time points; *n* = 10; log-rank test. Results are representative of at least six independent experiments. (**C**) Hematoxylin and eosin staining of WT (top) and NFATc2-deficient (bottom) mouse skin lesions at the indicated time points after *C. albicans* infections. Larger magnification of selected areas of the same section are shown to evidence granulocyte recruitment (left). PicroSirius Red staining is also shown to evidence collagen depositions (right, red deposits) and periodic acid-Schiff (PAS) staining to evidence *Candida*. See also fig. S2 for higher magnifications. Representative histological sections of four independent experiments are shown; see also fig. S3 (A and B). (**D**) WT and RAG-2-deficient mice respond similarly to primary skin infections with *C. albicans*. The Kaplan-Meier curve shows the percentage of WT and RAG-2–deficient mice undergoing ulceration after *C. albicans* administration at the indicated time points; *n* = 18 per group. (**E**) Digital image analysis quantification of

collagen staining. Five fields from two sections (24 hours after infection) of two independent experiments were analyzed. The analyzed fields covered the entire sections excluding the skin. Means and SEM are depicted. *n* = 5; statistical significance, two-tailed *t* test.



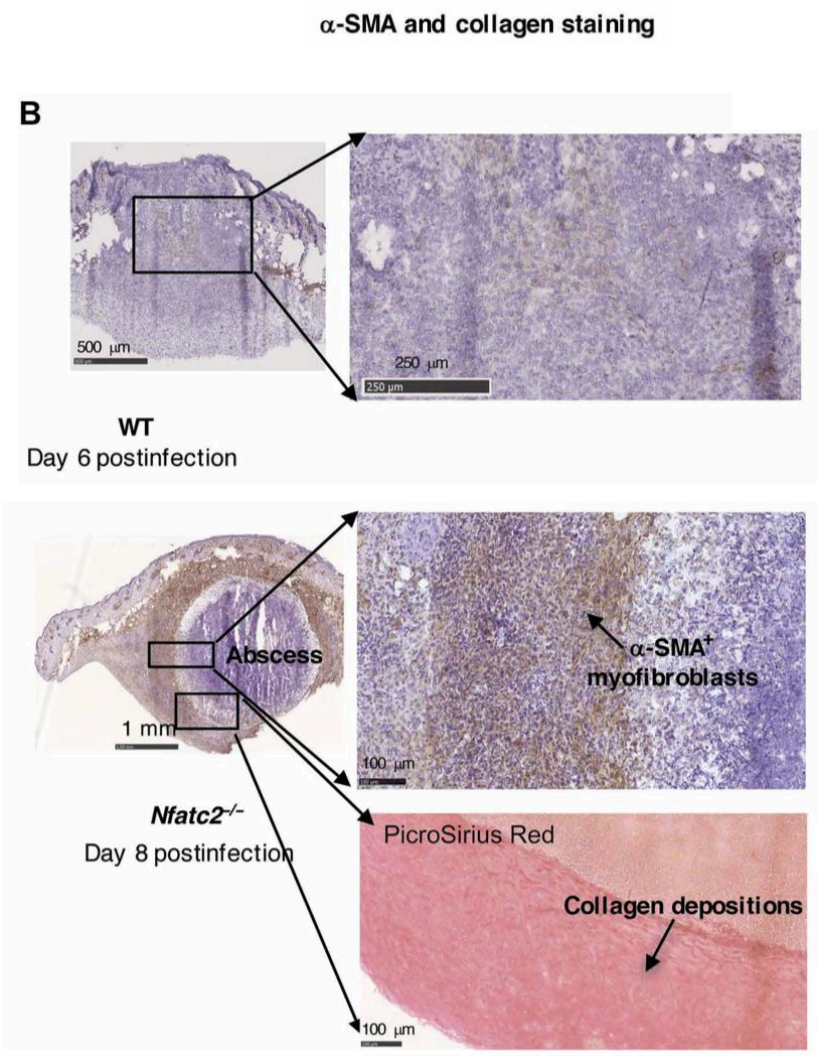


Fig. 2. Fibroblast activation in WT and NFATc2-deficient mice. (**A**) Histology of lesions induced by *C. albicans* hyphae in WT and NFATc2-deficient mice 48 hours after infections. Fibroblasts surrounding granulocytes are highlighted with black arrows. (**B**) Representative images of α-SMA immunohistochemical staining in skin sections of WT and NFATc2-deficient mice 6 and 8 days after *C. albicans* infection. PicroSirius Red staining is also

shown to evidence collagen capsule. Representative histological sections of three independent experiments are shown; see also fig. S7.

Transforming growth factor-β is required for the initial phase of infection containment by promoting fibrosis and is overactive in NFATc2-deficient mice.

The response that we observed in NFATc2-deficient mice had a prominent fibrotic component. Because the transforming growth factor-β (TGF-β) is a well-known profibrotic factor (*15*), we hypothesized that an overactivation of the TGF-β pathway was responsible for the behavior of mutant mice. To test this possibility, we examined the activation of the TGF-β transduction pathway in WT and NFATc2-deficient mice, evaluating the presence of phosphorylated SMAD2,3 proteins both histologically and by Western blot analysis. As shown in Fig. 3A, the TGF-β pathway was activated in both strains; however, the activation was three to four times more pronounced in NFATc2-deficient mice relative to WT mice. In addition, in the histologic evaluation, phosphorylated SMAD2,3 staining was more pronounced in sections from NFATc2-deficient mice (Fig. 3B and fig. S8).

Mice were next treated with an inhibitor of the TGF-β transduction pathway (SB-431542) to investigate whether TGF-β inhibition could restore the WT phenotype. Therefore, the effect of the inhibitor on the formation of the encapsulated abscess was analyzed after *C. albicans* infection. We observed that, in response to the reduced

activation of the TGF-β pathway, both WT and NFATc2-deficient mouse responses were affected. *C. albicans* was not contained and instead diffused into the subcutaneous space; this led to multiple granulocyte accumulations in both WT and NFATc2-deficient mice (Fig. 3C). Accordingly, the formation of nonorganized collagen deposits around the abscesses was observed in both mouse groups (Fig. 3D and figs. S9 and S10). Overall, these data suggest that the incapability to form adequate collagen capsules when the TGF-β pathway was down-modulated was responsible for the reduced containment of the infection.

These data demonstrate that the TGF-β pathway activation is required during the initial phase of the inflammatory process for the proper containment of the infection. However, the pathway is overactive in *Nfatc2*$^{-/-}$ animals and contributes to the formation of excessive collagen deposits around the abscess. The overactivation of the TGF-β pathway only partially explains the phenotype of NFATc2-deficient mice because ulceration was not restored in this group of mice even after TGF-β inhibition.

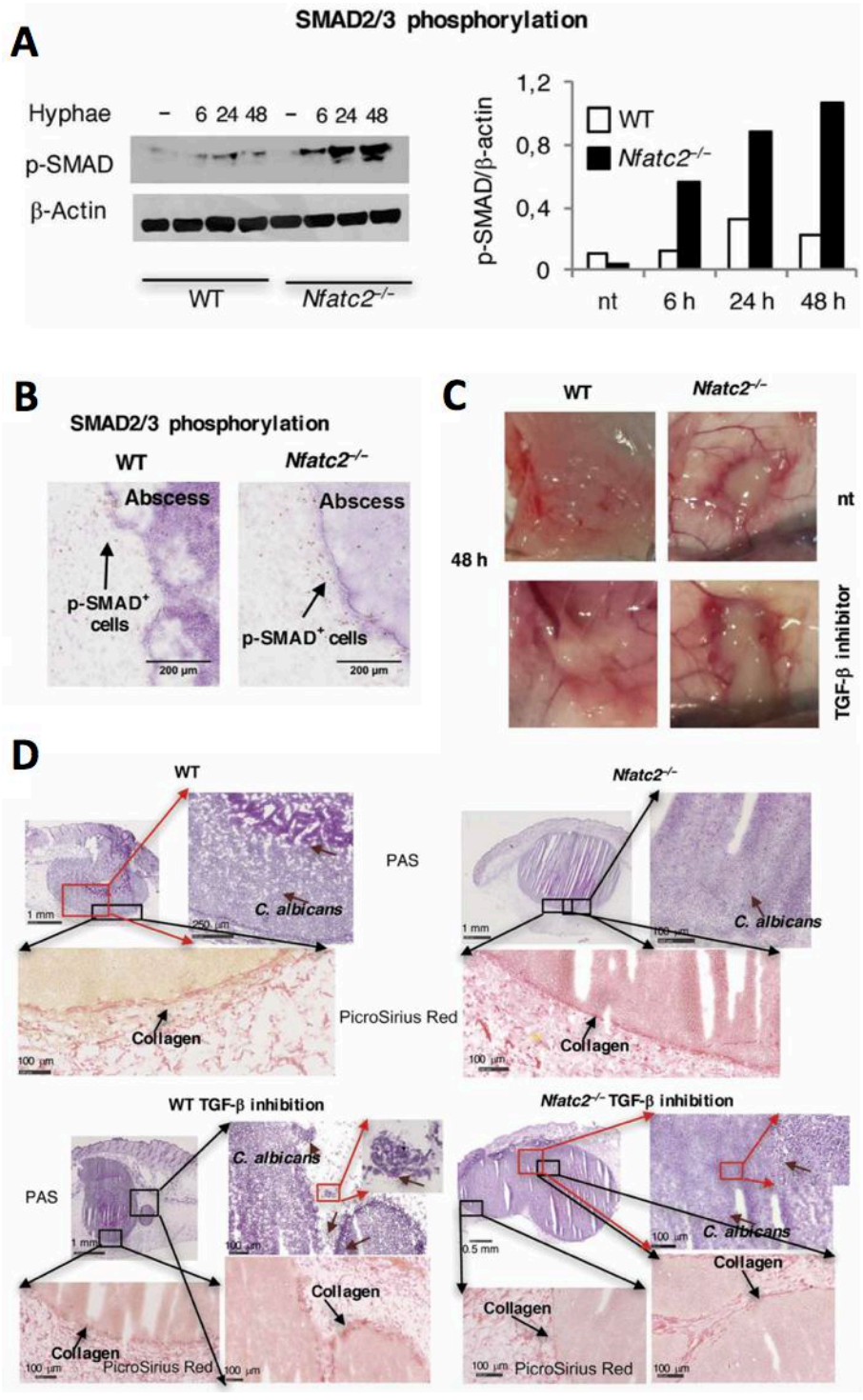


Fig. 3. The TGF-β pathway is activated in the skin after *C. albicans* infection

and is required to contain the infection. (**A**) Western analysis of SMAD2/3 phosphorylation at the indicated time points after infection of WT and NFATc2-deficient mice. Data were quantified and normalized on β-actin. Data are representative of three independent experiments. nt, untreated mice. (**B**) Representative images of p-SMAD2,3 immunohistochemical staining in skin sections 24 hours after *C. albicans* infection. p-SMAD2,3-positive cells are brown. The experiment was repeated twice with similar results; see also fig. S8. (**C**) Abscess formation in WT and NFATc2-deficient mice in the presence or not of the TGF-β inhibitor SB-431542. TGF-β inhibitor was administered ip (50 μg per mouse) for 3 days starting 1 day before *C. albicans* infection; the day of the infection was also coadministered locally with the hyphae. Note that the abscess is more diffused when the TGF-β pathway is inhibited. Two independent experiments with eight animals per group were performed. (**D**) Visualization of *C. albicans* (purple staining, brown arrows) by PAS staining in WT and NFATc2-deficient mouse skin lesions 24 to 48 hours after *C. albicans* infections in the presence of TGF-β inhibitor. PicroSirius Red staining of selected areas is also shown to evidence collagen depositions (arrows). Note that the collagen capsule is disorganized if the animals are treated with the TGF-β inhibitor and *C. albicans* can exit the abscess (see fig. S6 for higher magnifications). Representative histological sections of three independent experiments are shown; see also fig. S10.

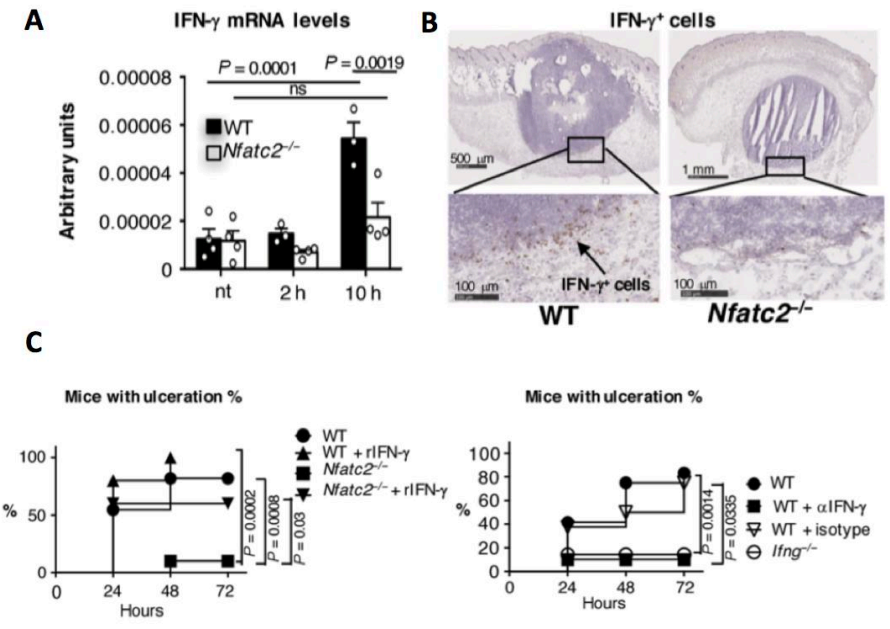
Interferon-γ antagonizes TGF-β signaling in WT animals

We next focused on the molecular mechanism that drives the overactivation of the TGF-β pathway in NFATc2-deficient mice. In the tissue, TGF-β is present in an inactive form and is associated with the

extracellular matrix. Various factors, including reactive oxygen species, pH, integrins, and proteases, can induce the release of the active cytokine (*16*–*19*). Because the TGF-β pathway was activated and required for infection containment in both animal groups (Fig. 3A) but was more active in NFATc2-deficient mice, we hypothesized that active TGF-β is released in WT and NFATc2-deficient mice but is then antagonized in WT animals by factors that are produced in an NFATc2-dependent manner. Interferon-γ (IFN-γ) is a very potent antagonist of TGF-β (*20*, *21*), and NFATc2-deficient mice were previously described to be prone to develop type 2 responses rather than type 1, IFN-γ-dependent responses (*22*). Therefore, we hypothesized that NFATc2-deficient mice showed a deficit in IFN-γ production that could explain the differences between the two animal groups. We compared the levels of IFN-γ in infected skins from WT and NFATc2-deficient mice. A significant up-regulation of IFN-γ mRNA was observed in WT controls but not in NFATc2- deficient mice (Fig. 4A), and more IFN-γ^+ cells were found in WT compared with NFATc2-deficient skins by immunohistochemical staining (Fig. 4B and fig. S11). Administration of recombinant IFN-γ in NFATc2-deficient mice at the time of *C. albicans* infection was sufficient to induce ulceration (Fig. 4, C and D), and this correlated with a reduction of the TGF-β pathway activation (Fig. 4E). In contrast, when IFN-γ was blocked in WT animals, they exhibited the same phenotype of NFATc2-deficient mice, namely, no ulceration (Fig. 4, C and D) and strong activation of the TGF-β pathway (Fig. 4F). In accordance with these observations, blocking IFN-γ in WT animals

favored the formation of a well-organized collagen capsule (Fig. 4D). On the other hand, the exogenous administration of IFN-γ to NFATc2-deficient mice hampered the formation of a thick collagen capsule (Fig. 4D). Accordingly, in vitro TGF-β promoted skin fibroblast proliferation that was antagonized by IFN-γ (Fig. 4G).

IFN-γ-deficient mice were also analyzed to investigate whether they could recapitulate the phenotype of NFATc2-deficient mice. As expected, IFN-γ-deficient mice did not undergo ulceration (Fig. 4C) and formed a thick fibroblast and collagen capsule, and fibroblasts differentiated into myofibroblasts (Fig. 4H). Overall, our data support a model in which IFN-γ controls abscess capsule formation at least in part by antagonizing TGF-β signaling.



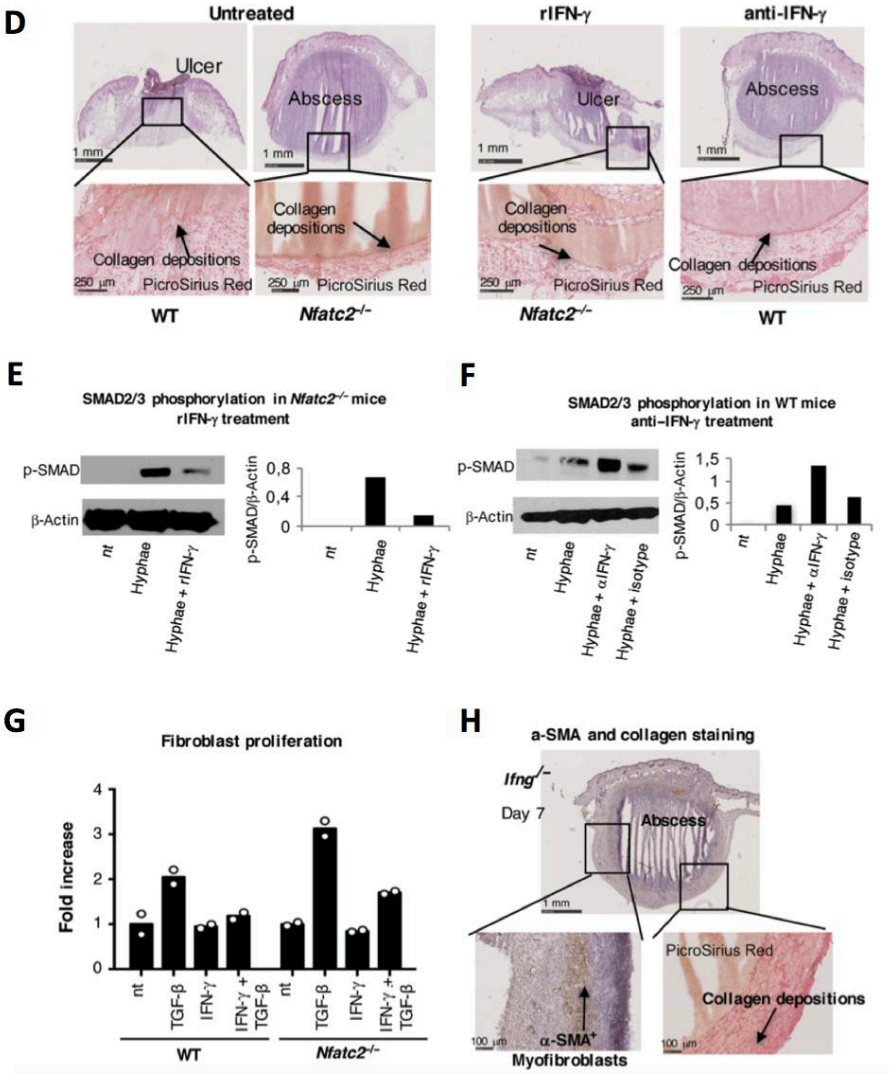


Fig. 4. IFN-γ antagonizes TGF-β in vivo. (**A**) qRT-PCR analysis of IFN-γ mRNA in *C. albicans*-infected tissues of WT and NFATc2-deficient mice at the indicated time points after infection; each dot represents a different mouse. Means and SEM are depicted. Statistical significance was determined with a two-way ANOVA. ns, not statistically significant. (**B**) IFN-γ

immunohistochemical staining (brown cells) in skin sections 24 hours after *C. albicans* infection. Representative histological sections from two independent experiments are shown; see also fig. S11. (**C**) Left: Kaplan-Meier curve showing the percentage of WT and NFATc2-deficient mice undergoing ulceration after *C. albicans* administration in the presence or not of IFN-γ at the indicated time points; *n* (WT) = 11, *n* (NFATc2) = 10, *n* (WT + rIFN-γ) = 5, *n* (NFATc2 + rIFN-γ) = 5. rIFN-γ, recombinant IFN-γ. Right: Kaplan-Meier curve showing the percentage of WT and IFN-γ-deficient mice undergoing ulceration after *C. albicans* administration in the presence or not of the indicated antibodies at the indicated time points; *n* (WT + isotype control) = 8, *n* (WT) = 12, *n* (WT + α-IFN-γ) = 10, *n* (IFN-γ-deficient) = 14; log-rank test. (**D**) Hematoxylin and eosin staining of WT and NFATc2-deficient mouse skin lesions 48 hours after *C. albicans* infections in the presence or not of the indicated stimuli. Larger magnification of PicroSirius Red staining of selected areas is also shown to evidence collagen depositions. The collagen capsule is loose in NFATc2-deficient mice if they are treated with rIFN-γ (1 μg per mouse). In contrast, the collagen capsule becomes thick in WT animals if IFN-γ is neutralized by an anti-IFN-γ antibody (50 μg per mouse). (**E**) Western blot analysis of SMAD2/3 phosphorylation in WT animals treated with *C. albicans* hyphae or *C. albicans* hyphae and rIFN-γ. Western blot analysis was performed 6 hours after *C. albicans* infection. Data were quantified and normalized on β-actin. Data are representative of two independent experiments. (**F**) Western blot analysis of SMAD2/3 phosphorylation from the infected skin of WT animals treated with *C. albicans* hyphae, *C. albicans* hyphae and the IFN-γ neutralizing antibody, or *C. albicans* hyphae and the isotype control antibody. Western blot analysis was performed 24 hours after *C. albicans* infection. Data were quantified and normalized on β-actin. Data are representative of three independent experiments. (**G**) In vitro WT

and NFATc2-deficient skin fibroblast proliferation in the presence or not of the indicated stimuli (TGF-β, 3 ng/ml; IFN-γ, 150 U/ml). Each dot represents a different sample. (**H**) α-SMA immunohistochemical staining in skin sections of IFN-γ-deficient mice 7 days after *C. albicans* infection. PicroSirius Red staining is also shown to evidence collagen capsule.

IFN-γ controls collagen capsule degradation by controlling the activation of the fibrinolytic system

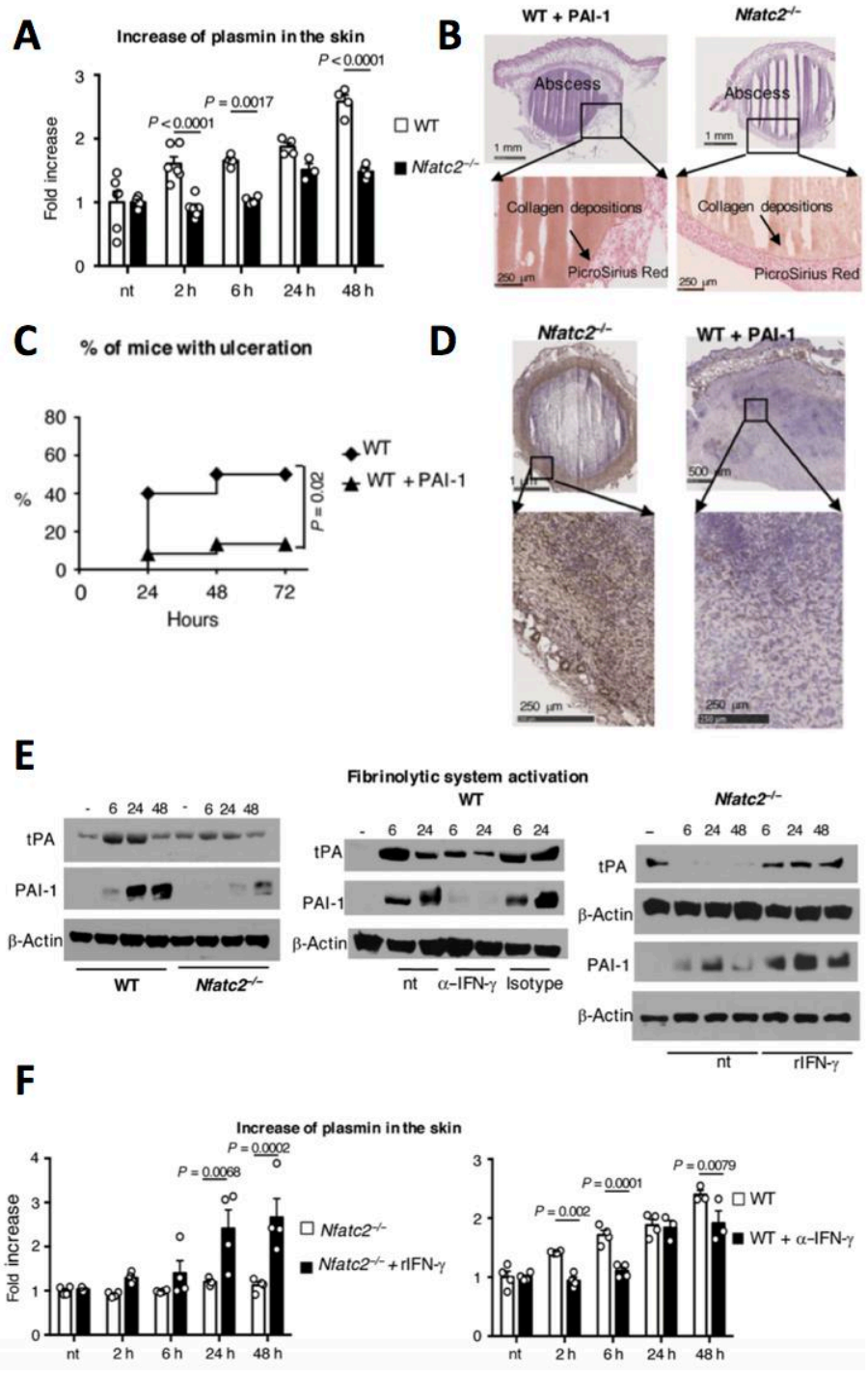
As evidenced above, the pharmacological treatment with IFN-γ, but not the inhibition of the TGF-β pathway, can fully restore the response to *C. albicans* skin infection in NFATc2-deficient mice. These observations imply that the effect of IFN-γ cannot be explained with only the antagonistic effect on TGF-β. Therefore, we predicted that, in addition to TGF-β inhibition, IFN-γ also contributed to the induction of factors required for capsule degradation and ulceration. This hypothesis was also supported by the observation that the process of capsule disruption in WT animals was further potentiated upon recombinant IFN-γ administration (fig. S12). We first focused on factors required for capsule degradation. Under several pathophysiological conditions, a collagen capsule is digested by metalloproteinases that are released by innate immune and stromal cells. Nevertheless, metalloproteinases are released in an inactive form and need to be activated by other proteases, such as plasmin (*23*–*25*). To investigate the potential role of plasmin in capsule digestion, we evaluated the levels of active plasmin in the tissue. These

were significantly higher in WT compared with NFATc2-deficient mice (Fig. 5A).

To confirm plasmin involvement in capsule digestion, we administered plasminogen activator inhibitor-1 (PAI-1) (*26*), an endogenous inhibitor of plasminogen-to-plasmin conversion, to WT animals to verify whether ulceration could be inhibited. PAI-1 administration initially led to the formation of an encapsulated abscess and no ulceration (Fig. 5, B and C, and fig. S13), confirming that plasmin is required for capsule digestion and ulceration. Nevertheless, at later time points, the abscess did not show the organization exhibited in NFATc2-deficient mice, and no differentiation of fibroblasts in myofibroblasts was observed (Fig. 5D). This was predictable given the presence of IFN-γ (which inhibits TGF-β) in the skin of WT animals. Plasminogen-to-plasmin conversion is induced by activators of plasminogen, such as tissue plasminogen activator (tPA). We therefore evaluated the presence of tPA in the tissue of infected mice. In line with our prediction, tPA levels were much higher in WT compared with NFATc2-deficient mice (Fig. 5E). We also analyzed the levels of endogenous PAI-1 that inhibits plasmin formation. PAI-1 was also more expressed in WT compared with NFATc2-deficient mice (Fig. 5E). In WT animals, tPA production was induced early, when PAI-1 was not present in the tissue (Fig. 5E). The counterregulation of tPA and PAI-1 observed in WT animals is presumably required to first allow plasmin generation and then avoid excessive plasmin accumulation. Given that plasmin is required for capsule digestion, we investigated whether IFN-γ influenced the activation of the fibrinolytic system. We

tested our hypothesis both by administering IFN-γ to NFATc2-deficient mice and by blocking IFN-γ in WT animals. As expected, adding IFN-γ restored tPA and PAI-1 production in NFATc2-deficient mice (Fig. 5E). On the contrary, blocking IFN-γ in WT animals inhibited the release of tPA and PAI-1 (Fig. 5E). This indicates that the entire pathway that leads to plasmin formation is altered by the absence of IFN-γ in NFATc2-deficient mice. To further support our conclusions, we also measured active plasmin in the tissue of NFATc2-deficient mice treated or not with IFN-γ and of WT animals treated or not with a blocking IFN-γ antibody at the time of *C. albicans* administration. As shown in Fig. 5F, IFN-γ restored plasmin generation in NFATc2-deficient mice, whereas the inhibition of IFN-γ down-modulated plasmin formation in WT animals.

Last, we evaluated the levels and function of activated matrix metalloproteinase-3 (MMP-3) during the infection. We focused on MMP-3 because it is efficiently activated by plasmin and is a master metalloproteinase that activates downstream metalloproteinases, such as MMP-9, and digests extracellular matrix components (*23*, *27*). As shown in fig. S14A, the increase of activated MMP-3 during the infection paralleled the increase of plasmin with the same relevant differences during abscess formation. In addition, treatment with an MMP-3 peptide inhibitor (MMP-3 inhibitor I) upon *C. albicans* administration inhibited ulceration (fig. S14B).


A
Increase of plasmin in the skin
Fold increase
P < 0.0001
P = 0.0017
P < 0.0001
WT
Nfatc2−/−
nt
2 h
6 h
24 h
48 h
B
WT + PAI-1
Nfatc2−/−
Abscess
1 mm
Collagen depositions
PicroSirius Red
250 µm
C
% of mice with ulceration
%
WT
WT + PAI-1
P = 0.02
Hours
D
Nfatc2−/−
WT + PAI-1
500 µm
250 µm
E
Fibrinolytic system activation
WT
Nfatc2−/−
tPA
PAI-1
β-Actin
WT
Nfatc2−/−
nt
α-IFN-γ
Isotype
rIFN-γ
F
Increase of plasmin in the skin
Fold increase
P = 0.0002
P = 0.0068
Nfatc2−/−
Nfatc2−/− + rIFN-γ
P = 0.0079
P = 0.0001
P = 0.002
WT
WT + α-IFN-γ

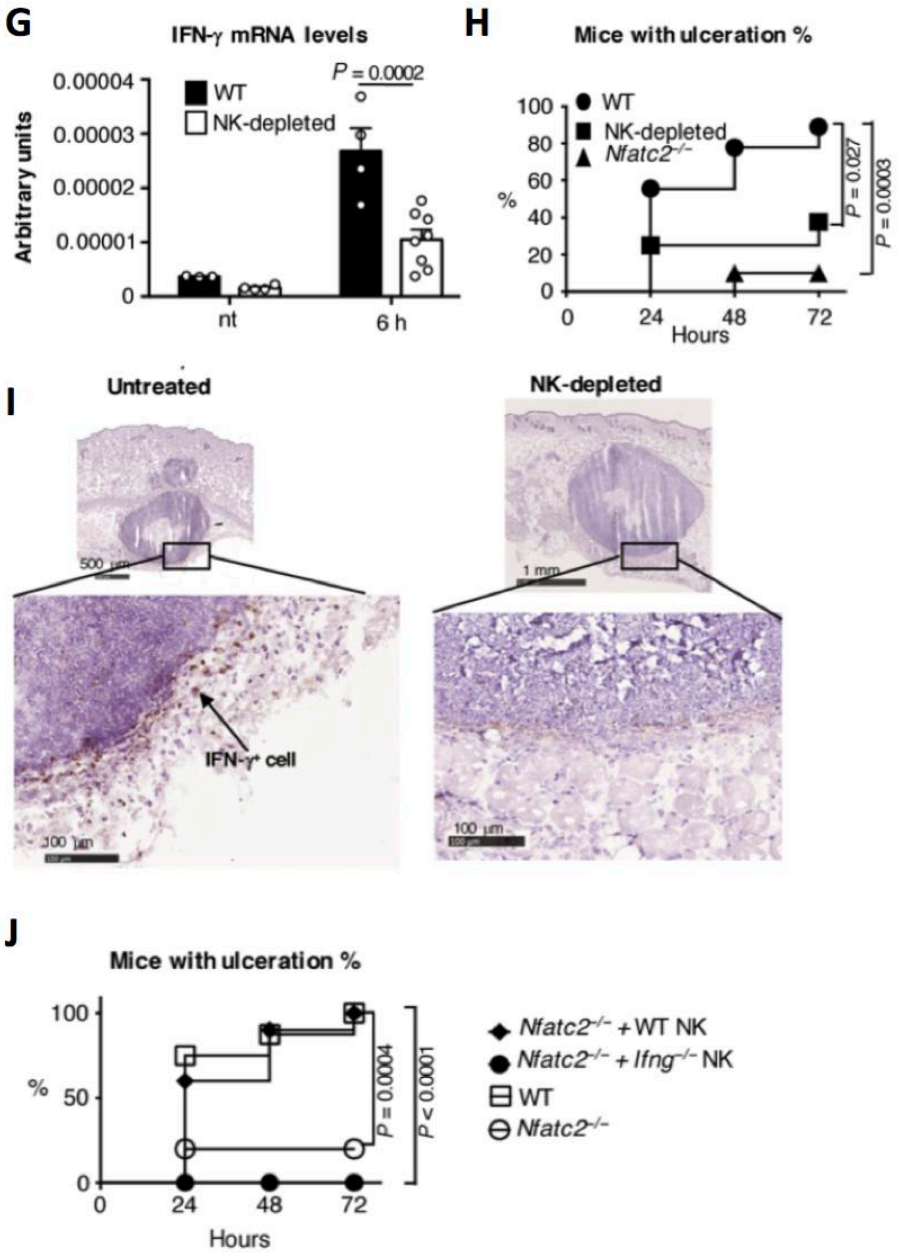


Fig. 5. IFN-γ activates the fibrinolytic system. (**A**) Increase of active plasmin levels in WT and NFATc2-deficient mice at the indicated time points after *C.*

albicans infection. Each dot represents a single mouse. Means and SEM are depicted; a two-way ANOVA was used for statistics. The experiment was repeated twice with similar results. (**B**) Hematoxylin and eosin staining of WT mouse skin lesions 48 hours after *C. albicans* infections in the presence of PAI-1 (0.65 μg per mouse) and NFATc2-deficient mice skin lesions. Larger magnification of PicroSirius Red staining of selected areas is also shown to evidence collagen depositions. Representative histological sections from two independent experiments are shown; see also fig. S13. (**C**) Kaplan-Meier curves showing the percentage of mice undergoing ulceration after *C. albicans* administration. Where indicated, mice were treated with PAI-1 (0.65 μg per mouse, coadministered with *C. albicans*). *n* (WT) = 10, *n* (WT + PAI-1) = 24; log-rank test. (**D**) Representative images of α-SMA immunohistochemical staining in skin sections of NFATc2-deficient and PAI-1–treated WT animals 8 and 7 days after *C. albicans* infection. (**E**) Left: Western blot analysis of tPA and PAI-1 levels measured in WT and NFATc2-deficient animals at the indicated hours after *C. albicans* infection. Data are representative of three independent experiments. Middle and right: Western blot analysis of tPA and PAI-1 levels measured in WT and NFATc2-deficient animals treated with an anti-IFN-γ blocking antibody and with rIFN-γ, respectively, at the time of *C. albicans* infection. The analysis was performed at the indicated time points. Data are representative of two independent experiments. (**F**) Left: Increase of active plasmin levels in NFATc2-deficient mice at the indicated time points after *C. albicans* infection in the presence or not of rIFN-γ. Right: Increase of active plasmin levels in WT mice at the indicated time points after *C. albicans* infection in the presence or not of anti-IFN-γ blocking antibody. Each dot represents a single mouse. Means and SEM are depicted; a two-way ANOVA was used for statistics. (**G**) qRT-PCR analysis of IFN-γ mRNA in *C. albicans*–infected tissues before and 6

hours after the infection in WT animals depleted or not of NK cells. Each dot represents a single mouse. Means and SEM are depicted; a two-way ANOVA was used for statistics. (**H**) Kaplan-Meier curve showing the percentage of WT (*n* = 9), NK cell–depleted WT (*n* = 8), and NFATc2-deficient mice (*n* = 10) undergoing ulceration after *C. albicans* administration at the indicated time points. Log-rank test was used. (**I**) IFN-γ immunohistochemical staining in skin sections (brown cells) of WT mice treated or not with anti-asialo GM to eliminate NK cells (NK-depleted) and infected with *C. albicans*. Note that IFN-γ^+ cells are strongly reduced in NK-depleted mice. (**J**) Kaplan-Meier curve showing the percentage of NFATc2-deficient mice (*n* = 10), NFATc2-deficient mice reconstituted with activated IFN-γ-sufficient NK cells (*n* = 10) or IFN-γ-deficient NK cells (*n* = 10), and WT animals (*n* = 8) undergoing ulceration at the indicated time points after *C. albicans* administration. Log-rank test was used.

Natural killer cells are the major source of IFN-γ in WT animals, and DCs are essential accessory cells for natural killer cell activation during fungal infections

The next step was to determine the source of IFN-γ. Because *Rag2*$^{-/-}$ mice behaved like WT animals and showed a potent up-regulation of IFN-γ mRNA in the skin after infection (Fig. 1D and fig. S15), we excluded a T cell origin for the IFN-γ and focused on innate immune cells. Elimination of natural killer (NK) cells by anti-asialo GM treatment strongly affected the levels of IFN-γ mRNA, as measured by qRT-PCR (Fig. 5G). Similarly, in the absence of NK cells, ulceration was strongly diminished after *C. albicans* infection of WT animals (Fig. 5H).

Upon NK cell depletion, no IFN-γ^+ cells were observed in the infected skins (Fig. 5I). These data confirm that NK cells are the major source of IFN-γ in our experimental system.

To formally demonstrate that NK cells are necessary and sufficient to provide IFN-γ under our experimental conditions, we first activated in vivo WT and IFN-γ-deficient NK cells and then adoptively transferred the activated NK cells into NFATc2-deficient mice infected with *C. albicans*. The course of the infection was then followed over time. After the adoptive transfer of activated WT but not IFN-γ-deficient NK cells, NFATc2-deficient mice showed ulceration (Fig. 5J). These data confirmed that IFN-γ provided by NK cells is necessary and sufficient to induce capsule degradation and ulceration.

Next, we unveiled the connection between NFATc2 and IFN-γ production by NK cells. In vitro, we observed that NFATc2 expression in accessory cells (*e.g.*, DCs), but not in NK cells themselves, was necessary for the production of IFN-γ by NK cells upon *C. albicans* encounter (fig. S16, A and B). We therefore predicted that eliminating DCs in vivo would influence IFN-γ production by NK cells after infection by *C. albicans*. We thus used B6.Cg-Tg(Itgax-DTR/OVA/ EGFP)1Gjh/Crl (DOG) mice [which express a diphtheria toxin (DT) receptor under the CD11c promoter to eliminate DCs (*28*)] and studied the course of the infection in the absence of DCs. As we previously showed, DT treatment eliminated DCs from the skin, lymph node, and spleen (fig. S17) (*29*–*31*). The recruitment of inflammatory cells was not altered by DC depletion (fig. S18). IFN-γ^+ cells were strongly diminished in DC-

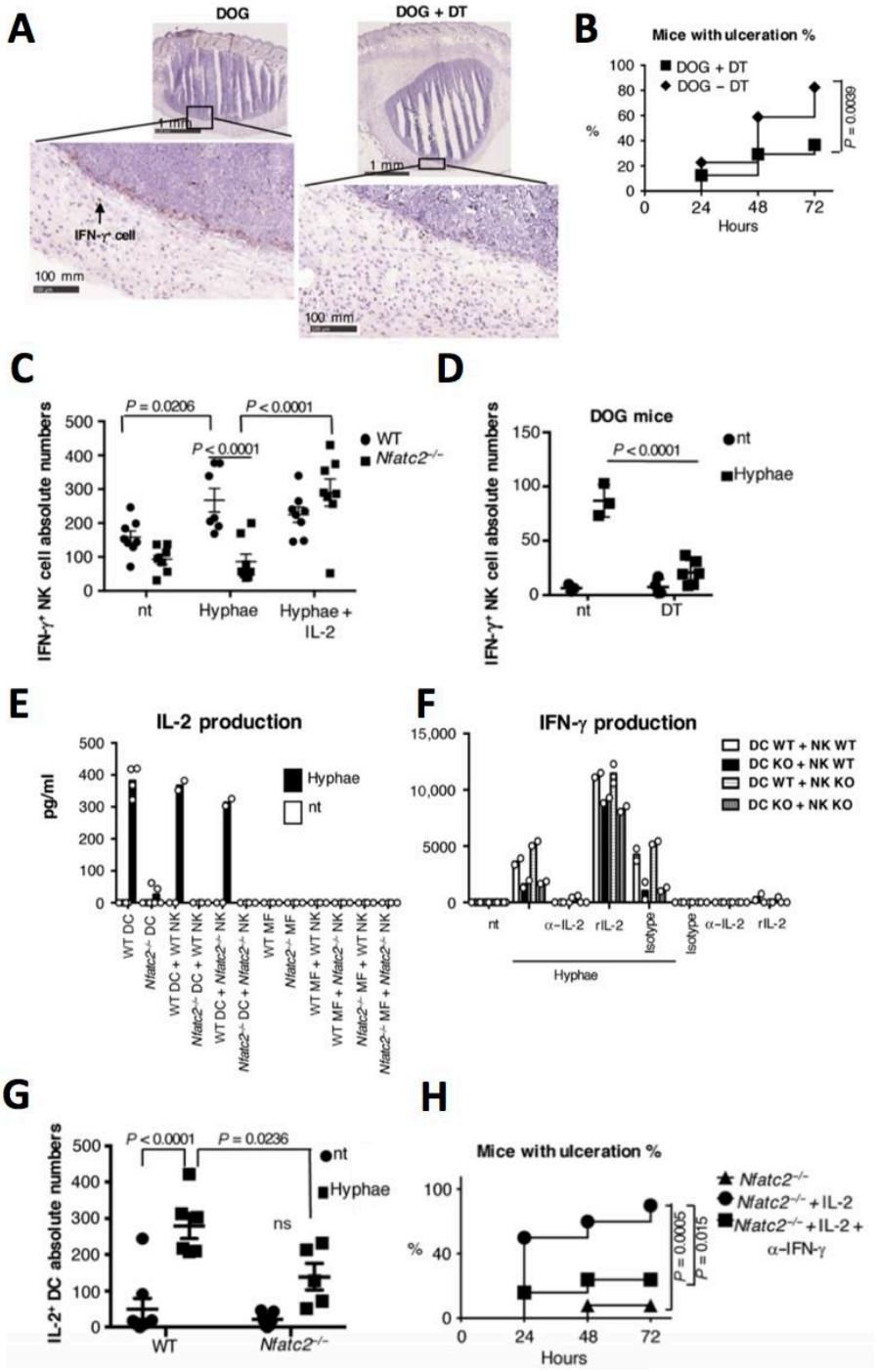
deficient mice infected skins compared with DC-sufficient mice, as revealed by immunohistochemistry (Fig. 6A and fig. S19). Accordingly, as depicted in Fig. 6B, the number of animals undergoing ulceration was significantly reduced. This finding suggests that DCs are required to elicit IFN-γ production by NK cells after *C. albicans* infection. In keeping with this observation, IFN-γ^+ NK cells were strongly diminished in the lymph nodes of NFATc2-deficient and DC-deficient mice after infection, compared with control animals (Fig. 6, C and D).

We then determined why NFATc2-deficient DCs are not able to activate NK cells. Two cytokines involved in NK cell activation, namely, IL-12 and IL-2, were described to be produced in an NFAT-dependent manner by DCs in response to zymosan (*5*). We first measured IL-12p70 production by DCs after *C. albicans* hyphae infection, but, although it was previously shown to be induced in DCs upon zymosan administration, we found no IL-12 production in response to the *C. albicans* hyphae challenge (fig. S20). We therefore focused on IL-2, described to be strongly induced in DCs during *C. albicans* hyphae infections as well (*32*–*34*) and also described to be necessary for eliciting IFN-γ production by NK cells during bacterial infections (*30*, *35*). Figure 6 (E and F) shows that DC-derived IL-2 is required for NK cell activation in the presence of *C. albicans* in vitro and that NFATc2-deficient DCs were unable to activate NK cells because they do not produce IL-2. We thus evaluated the presence of IL-2^+ DCs in the lymph nodes of WT and NFATc2-deficient mice after *C. albicans* administration. Whereas the numbers of DCs that produce IL-2 were strongly increased in WT animals after infection, no induction of IL-2

production by DCs was detected in NFATc2-deficient mice (Fig. 6G). Accordingly, in vivo IL-2 administration to NFATc2-deficient mice at the time of *C. albicans* infection restored NK cell activation (Fig. 6C). Moreover, after IL-2 administration, NFATc2-deficient mice presented ulceration (Fig. 6H). The restoration of ulcer formation in NFATc2-deficient mice upon IL-2 administration was inhibited when a blocking anti-IFN-γ antibody was also administered (Fig. 6H). These data demonstrate that after *C. albicans* infection, NFATc2-deficient DCs do not produce IL-2 and, consequently, do not induce IFN-γ release by NK cells.

To test whether DC-derived IL-2, produced in an NFATc2-depedent manner in response to *C. albicans* administration, was necessary and sufficient to induce ulceration, we performed the following experiment. NFATc2-deficient mice were adoptively transferred with WT, NFATc2-deficient, and IL-2-deficient DCs at the time of *C. albicans* administration, and the course of the infection was analyzed. As depicted in Fig. 6I, only mice that received WT DCs showed ulceration. Moreover, if NK cells were eliminated after the administration of WT DCs, ulceration was prevented (Fig. 6I).

Collectively, these data indicate that activation of NFATc2 in DCs during fungal infection regulates IL-2 production, which then elicits IFN-γ production by NK cells. In turn, IFN-γ is necessary for counteracting the TGF-β pathway and for allowing plasmin formation, collagen capsule digestion, and *C. albicans* expulsion.


A
DOG
DOG + DT
1 mm
IFN-γ+ cell
100 mm
B
Mice with ulceration %
DOG + DT
DOG – DT
P = 0.0039
%
Hours
C
P = 0.0206
P < 0.0001
P < 0.0001
WT
Nfatc2−/−
IFN-γ+ NK cell absolute numbers
nt
Hyphae
Hyphae + IL-2
D
DOG mice
P < 0.0001
nt
Hyphae
IFN-γ+ NK cell absolute numbers
nt
DT
E
IL-2 production
pg/ml
Hyphae
nt
WT DC
Nfatc2−/− DC
WT DC + WT NK
Nfatc2−/− DC + WT NK
WT DC + Nfatc2−/− NK
Nfatc2−/− DC + Nfatc2−/− NK
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F
IFN-γ production
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DC WT + NK KO
DC KO + NK KO
nt
α-IL-2
rIL-2
Isotype
Isotype
α-IL-2
rIL-2
Hyphae
G
P < 0.0001
P = 0.0236
nt
Hyphae
ns
IL-2+ DC absolute numbers
WT
Nfatc2−/−
H
Mice with ulceration %
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Nfatc2−/− + IL-2
Nfatc2−/− + IL-2 + α-IFN-γ
P = 0.0005
P = 0.015
%
Hours

I

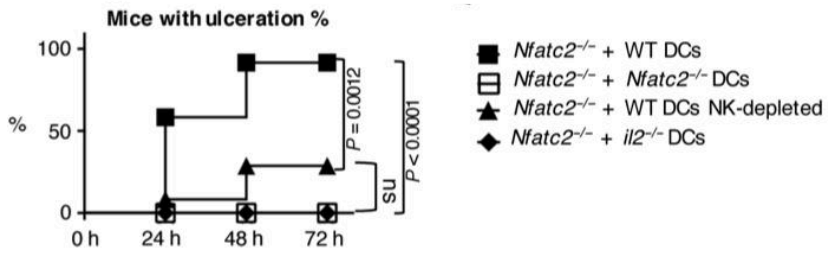


Fig. 6. DCs are required for NK cell activation. (**A**) IFN-γ immunohistochemical staining in skin sections (brown cells) of DOG mice and DC-depleted DOG mice (DOG + DT) after *C. albicans* infection. Representative histological sections from two independent experiments are shown; see also fig. S19. (**B**) Kaplan-Meier curve showing the percentage of DC-sufficient (*n* = 22) and DC-depleted (*n* = 32) DOG mice (DOG + DT) undergoing ulceration after *C. albicans* administration at the indicated time points; log-rank test. (**C**) Absolute numbers of IFN-γ^+ NK cells at the draining lymph nodes of WT and NFATc2-deficient mice 4 hours after *C. albicans* infections. Where indicated, mice were cotreated with *C. albicans* and rIL-2. Each symbol represents a different mouse. Means and SDM are depicted; a two-way ANOVA test was used for statistics. (**D**) Absolute numbers of IFN-γ^+ NK cells at the draining lymph nodes of DOG mice treated or not with DT 4 hours after *C. albicans* infections. Each symbol represents a different mouse. Means and SDM are depicted; a two-way ANOVA test was used for statistics. (**E**) IL-2 released in the supernatants by WT or NFATc2-deficient BMDCs and BM macrophages (MF) before and after *C. albicans* exposure (MOI, 0.05). IL-2 released by DCs-NK cell and macrophages-NK cell cocultures is also shown. Each dot represents a different sample. (**F**) Immature or *C. albicans*-activated WT and NFATc2-deficient BMDCs were cultured with NK cells for 18 hours.

Where indicated, IL-2 was blocked using the S4B6 anti-IL-2 antibody (α-IL-2), or rIL-2 was added to the cultures. Levels of IFN-γ in the supernatant were then quantified by ELISA. Each dot represents a different sample. Representative data of two independent experiments are shown. KO, knockout. (**G**) Absolute numbers of IL-2^+ DCs at the draining lymph nodes of WT and NFATc2-deficient mice 4 hours after *C. albicans* infections. Each symbol represents a different mouse. Means and SDM are depicted; a two-way ANOVA test was used for statistics. (**H**) Kaplan-Meier curves showing the percentage of NFATc2-deficient mice undergoing ulceration after *C. albicans* administration in the presence or not of the indicated stimuli and at the indicated time points; *n* (*Nfatc2*$^{-/-}$) = 10, *n* (*Nfatc2*$^{-/-}$ + IL-2) = 8, *n* (*Nfatc2*$^{-/-}$ + IL-2 + α-IFN-γ) = 10; log-rank test. (**I**) Kaplan-Meier curves showing the percentage of NFATc2-deficient mice reconstituted with DCs of the indicated genotype undergoing ulceration after *C. albicans* administration. Where indicated, NK cells were depleted. *n* = 12 per group; log-rank test.

IFN-γ antagonizes TGF-β signaling and allows abscess elimination through the activation of the fibrinolytic systems also during *Staphylococcus aureus* infections

Last, we investigated whether the cross-talk between the fibrinolytic and the innate immune systems, which regulates the persistence of the encapsulated abscess and the elimination of the microbes, applies not only to fungal but also to bacterial infections. Mice were infected with the Gram-positive bacterium *S. aureus*, which can infect the skin and form abscess. As shown in Fig. 7 (A and B), the infection induced skin ulceration in WT animals, whereas an encapsulated abscess formed in IFN-γ-deficient mice. As with *C. albicans* infections,

excessive activation of the TGF-β pathway and overproduction of tPA and PAI-1 in WT with respect to IFN-γ-deficient mice were observed (Fig. 7, C and D). This indicates that also during bacterial infections, IFN-γ can induce the activation of the fibrinolytic system to favor microbial elimination.

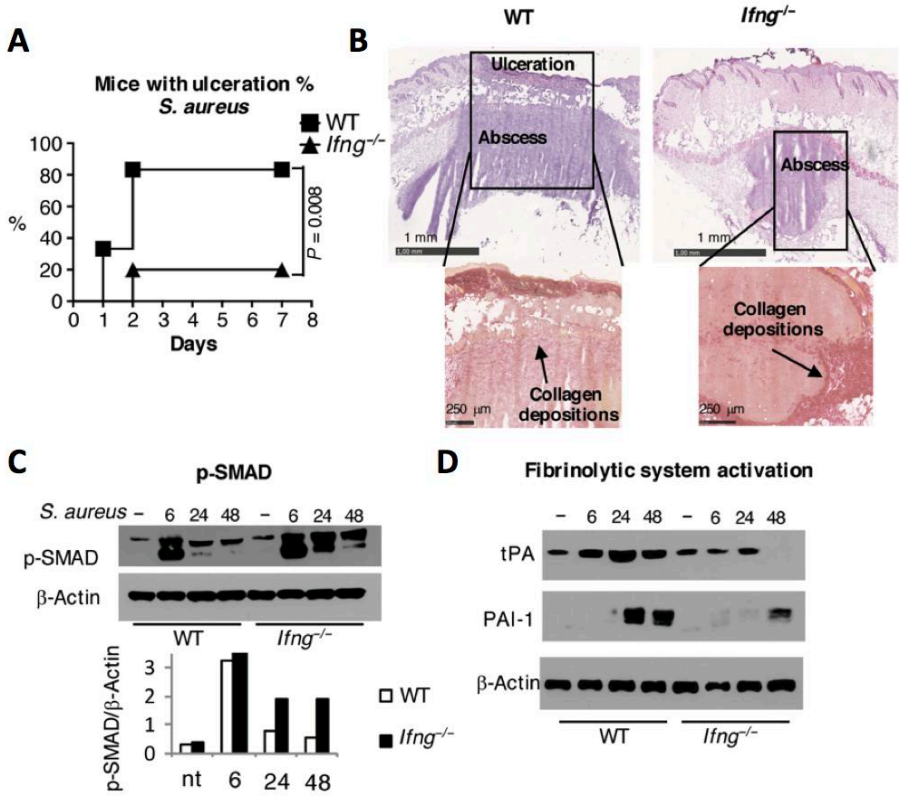


Fig. 7. Innate immune and fibrinolytic systems cooperate also during bacterial infections. (**A**) Kaplan-Meier curve showing the percentage of WT (*n* = 6) and IFN-γ-deficient mice (*n* = 10) undergoing ulceration after *S. aureus* administration at the indicated time points; log-rank test. Representative data of two independent experiments. (**B**) Hematoxylin and eosin staining of WT and IFN-γ-deficient mice skin lesions 48 hours after *S. aureus* infection.

PicroSirius Red staining is also shown to evidence collagen depositions. (**C**) Western blot analysis of SMAD2/3 phosphorylation at the indicated time points after *S. aureus* infection of WT and IFN-γ-deficient mice. Data were quantified and normalized on α-actin. Data are representative of three independent experiments. (**D**) Western blot analysis of tPA and PAI-1 levels measured in WT and IFN-γ-deficient animals at the indicated hours after *S. aureus* infection.

2.4. Discussion

In this study, we report two phases of the inflammatory response elicited by microbial infections of the skin. The first is the phase of infection containment. In this phase, granulocytes and fibroblasts form an organized abscess to control tissue damage and microbial spreading. The second phase leads to microbial elimination. This phase requires the activation of the fibrinolytic system that allows the discharge of microbes out of the skin. Upon infection, granulocytes are recruited to surround microbes, and active TGF-β promotes a profibrotic response that favors the deposit of collagen around the abscess to improve microbial containment. The release of IFN-γ in the skin by NK cells (previously activated in the lymph node by DCs) avoids excessive collagen deposition and the differentiation of fibroblasts into myofibroblasts, owing to the antagonistic effect of IFN-γ on the TGF-β pathway. IFN-γ also favors plasmin generation by inducing the production of tPA. Plasmin activates the process of collagen capsule digestion, skin ulceration, and microbe discharge (Fig. 8). NFATc2 regulates IL-2 production by DCs after fungal encounter, and DC-

derived IL-2 is required for eliciting IFN-γ secretion by NK cells. In the absence of NFATc2, IFN-γ is not produced; therefore, the continuous signaling of TGF-β and the consequent differentiation of fibroblasts into myofibroblasts, together with the limited activation of the fibrinolytic system, lead to the generation of a very thick capsule around the abscess. The thick capsule hampers skin ulceration and microbial elimination; thus, only the containment phase takes place.

The containment phase of the inflammatory process, although efficient, is an evolutionary primitive way to control the infections because a mechanical insult could be sufficient to break the containment and spread the infection. This type of infection control is probably reminiscent of ancient innate responses with microbial confinement via encapsulation and melanization (*2*). The appearance of the NFAT signaling pathway in evolution has eventually enabled the elimination phase favoring the interaction between the innate immune system and the fibrinolytic system.

From the pathogen side, the generation of plasmin has been described to favor microbial invasiveness by favoring extracellular matrix degradation (*36*). We show here that the fibrinolytic system can also help microbial elimination if activated in the correct time frame. The early activation of the TGF-β pathway is likely to take place to also counteract the effects of premature plasmin generation.

The requirement of IL-2 for IFN-γ production and the requirement of IFN-γ for plasmin activation and *Candida* eradication provide a molecular explanation for why some patients with chronic

mucocutaneous candidiasis show *C. albicans*-specific defects in IL-2 and/or IFN-γ production (*37*–*40*).

It is known that NFATc2 promotes T helper 1 (Th1) and suppresses Th2 responses (*22*). This has been attributed to the capacity of NFATc2 to regulate IFN-γ production by T cells (*41*). We show here that NK cells as well show a deficit of IFN-γ production in NFATc2-deficient mice. Diversely from T cells, the defect is not NK cell-intrinsic but affects DCs. A previous work demonstrates that human NK cells can be directly activated by *C. albicans* (*42*), whereas we show here that mouse NK cells do not respond directly to *Candida* but need the presence of DCs to acquire the effector functions necessary to fight the infection. Our data may seem to conflict with these findings or may suggest that human and mouse NK cells behave differently. Nevertheless, the discrepancy between the two works could only be ostensible. In the work by Hellwig *et al*. (*42*), NK cells are maintained in culture in the presence of IL-2, and we show that IL-2 is indeed fundamental to induce NK cell activation. Therefore, accessory cells could be required, also in humans, to provide an endogenous source of IL-2.

We showed that the absence of NFATc2 alters the levels of IFN-γ, and IFN-γ is required for capsule digestion and microbial expulsion from skin. Nevertheless, we cannot exclude the fact that other molecular events regulated by NFATc2 contribute to the formation of encapsulated abscesses during skin fungal infections. For instance, it is known that cardiac expression of α-SMA, which regulates the contractile activity of myofibroblasts, is induced by TGF-β via the

activation of the NFAT signaling pathway in both fibroblasts and mesangial cells (*43*, *44*). Therefore, it is also possible that the absence of NFATc2 could (directly or indirectly via deregulation of other NFAT member activation) generate a spontaneous tendency of fibroblasts to proliferate and differentiate into myofibroblasts. Although we found here that NK cells are activated at the draining lymph node, we cannot exclude the fact that NK cell activation can occur in the skin as well.

In conclusion, our study evidences the importance of the fibrinolytic system for the eradication of skin infections. Moreover, this work shows previously unknown roles for TGF-β and IFN-γ during the inflammatory process induced by microorganisms in the skin. TGF-β is mainly considered an anti-inflammatory cytokine that intervenes during the late phases of the inflammatory process to down-regulate inflammation and to start the resolution phase. In this study, it also emerges as a fundamental cytokine during the initial phases of the inflammatory response. By exerting its profibrotic functions, TGF-β increases the effectiveness of the inflammatory process to avoid excessive microbial spreading in the tissue. Moreover, IFN-γ contributes to microbial elimination not only by the induction of type 1 macrophages and neutrophils but also via the regulation of fibroblast functions (by antagonizing TGF-β) and the activation of the fibrinolytic system. IFN-γ-induced plasmin generation avoids excessive confinement of the infection (which obstructs microbial discharge) and allows microbial elimination that occurs not only via the

phagocytic and antimicrobial activities of macrophages and neutrophils but also through direct microorganism elimination out of the ulcerated skin.

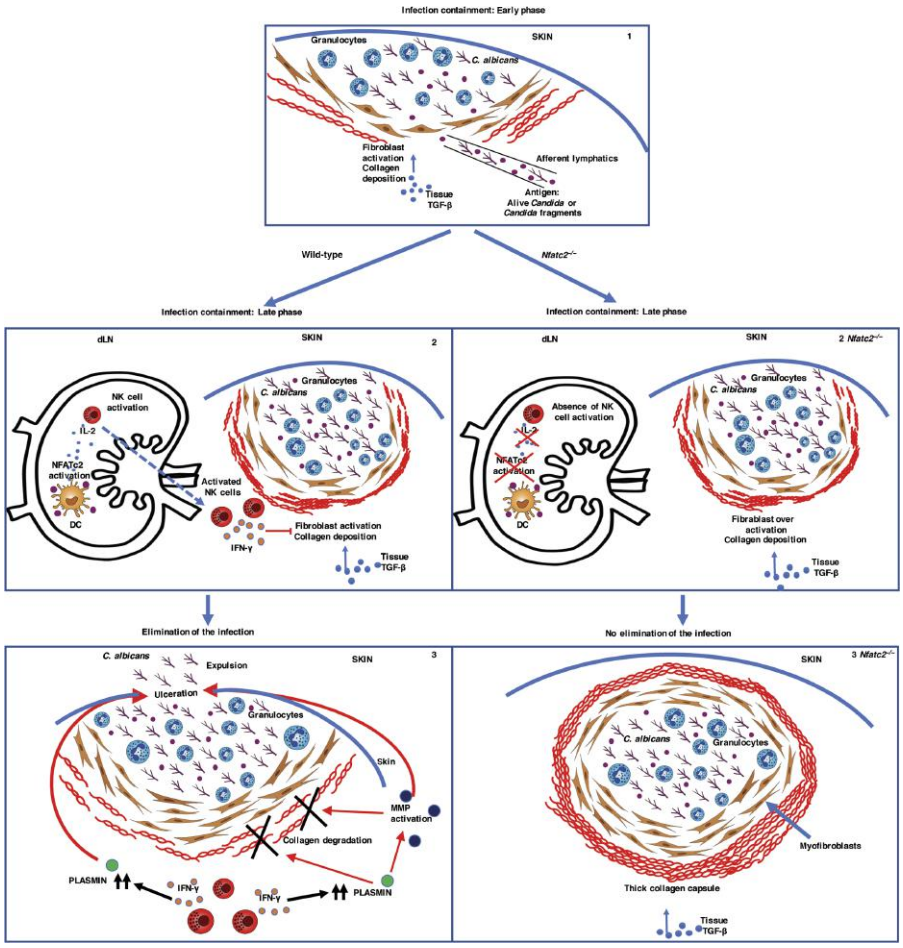


Fig. 8. NFAT activation in innate immune cells dictates the sterilization of *C. albicans* skin infection. Schematic of *C. albicans* skin infection containment and elimination. Distinct phases can be identified in the inflammatory process that takes place after *C. albicans* skin infections. During the very early phases (1), granulocytes are recruited and form an abscess around the invading microorganisms to avoid infection spreading. The containment of

the infection is ensured by fibroblasts that, once activated by TGF-β, proliferate and deposit collagen around the abscess to form a capsule. Later, IFN-γ, produced by NK cells activated in the draining lymph nodes (dLN), antagonizes TGF-β and thus avoids excessive fibroblast activation and excessive collagen deposition and also avoids the differentiation of fibroblasts into myofibroblasts (2). Last, IFN-γ ensures the activation of the fibrinolytic system and the consequent activation of metalloproteinases by plasmin (3). During the elimination phase, proteinases digest the collagen capsule and induce skin ulceration for the microbial expulsion out of the skin (3). NFATc2 activation in DCs after *C. albicans* exposure leads to IL-2 production. In turn, IL-2 is required to elicit IFN-γ release by NK cells (2). In the absence of NFATc2, IFN-γ is not produced in sufficient amount to counteract the TGF-β pathway (2 *Nfatc2*$^{-/-}$) and to induce the activation of the fibrinolytic system (3 *Nfatc2*$^{-/-}$); therefore, fibroblasts are hyperactive, deposit excessive collagen, and differentiate in myofibroblasts (3 *Nfatc2*$^{-/-}$). This leads to the formation of a thick capsule that prevents skin ulceration and microbial expulsion out of the skin.

2.5. Materials and methods

Study design

The overall objective of the study was to analyze the role of NFATc2 in the innate immune response to *C. albicans* and *S. aureus* infections of the skin. There was not a predefined study component. Mice were injected in the deep derma with *C. albicans* or *S. aureus*, and the

inflammatory response in the skin was investigated by histology, Western blot and cytofluorimetric analyses, and qRT-PCR. The study was not blind. For each experiment, the number of biological replicates is indicated in the figure legend.

Mouse strains

All mice, housed under specific pathogen-free conditions, had been on a B6 background for at least 12 generations and were used at 7 to 12 weeks of age. WT C57BL/6 mice were supplied by Envigo, Italy. IFN-γ-deficient mice were from the Jackson Laboratory. *Rag2*$^{-/-}$ mice were from CNRS Centre de Distribution, Typage et Archivage animal in Orleans, France. CD11c.DOG mice were provided by N. Garbi (Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany). In these mice, a specific DC ablation can be induced by DT injection. IL-2-deficient mice were provided by A. Schimpl (University of Würzburg, Würzburg, Germany) NFATc2-deficient mice were provided by E. Serfling (University of Würzburg, Germany).

***C. albicans* growth conditions and hyphal induction**

The *C. albicans* strain CAF3-1 (*ura3*Δ*::imm434/ura3*Δ*::imm434*), provided by W. A. Fonzi (Georgetown University), was routinely grown at 25°C in rich medium [YEPD (yeast extract, peptone, dextrose), 1% (w/v) yeast extract, 2% (w/v) Bacto Peptone, and 2% (w/v) glucose] supplemented with uridine (50 mg/liter) as described. In this growth condition, cells showed a typical yeast morphology, and growth was

monitored by counting the cell number using a Coulter Counter-Particle Count and Size Analyser. Once cells reached a concentration of about 8 × 10^6 cells/ml, the total culture was harvested by centrifugation and resuspended in an equivalent volume of YEPD-uridine medium buffered with Hepes (50 mM, pH 7.5). Cells were incubated at 37°C for hyphal induction. Formation of hyphae was evaluated under a microscope at different time points following induction until its amount was assessed at 95%.

S. aureus

S. aureus ATCC6538P cells were grown in LB medium (Difco) at 37°C. For subcutaneous infections, stationary phase cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 and then grown until they reached an OD_{600} of 0.25 that corresponded approximately to 10^6 colony-forming units (CFU)/ml. Cells were washed in phosphate-buffered saline, and appropriate dilutions were injected in mice.

In vivo infections

Mice were prepared by shaving the dorsal region at least 24 hours before injection. Mice were then injected in the deep derma with *C. albicans* hyphae (5 × 10^6 in a total volume of 50 μl) or 10^6 CFU of *S. aureus* in a final volume of 50 μl in the shaved dorsal regions and macroscopically analyzed 1, 2, 3, and 7 days later for skin ulceration. In addition, infected skin was collected at different time points for histological and biochemical analyses.

In some experiments, recombinant IFN-γ [1 μg per mouse subcutaneously (sc); catalog no. 315-05, PeproTech] or LEAF purified anti-mouse IFN-γ (50 μg per mouse sc; clone R4-6A2, catalog no. 505706; BioLegend) were co-injected together with *C. albicans* hyphae. LEAF purified rat immunoglobulin G1 κ (IgG1 κ) (clone RTK2071, BioLegend) was used as isotype control (35 μg per mouse sc); SB-431542 (catalog no. 13031, Cayman Chemical) was used as TGF-β inhibitor. It was injected daily intraperitoneally (ip) for 3 days, starting from day −1 (50 μg per mouse). For MMP-3 inhibition, MMP-3 inhibitor I was purchased from Calbiochem (catalog no. 444218) and injected sc together with *C. albicans* hyphae at a dosage of 125 μg per mouse. The inhibitor was then reinjected 6 hours after infection (250 μg per mouse) intravenously (iv). Plasmin activation was blocked by injecting human PAI-1 (0.65 μg per mouse) (25 μg; catalog no. A8111, Sigma-Aldrich) sc 6 hours before infection. Last, recombinant IL-2 (1 μg per mouse, 402-ML carrier-free, R&D Systems) was co-injected sc with *C. albicans*.

For NK cell depletion, mice received anti-asialo GM1 polyclonal antibodies (eBioscience, 30μg per mouse iv) at days −3, −1, and +1. For DC depletion, DOG mice were treated with DT (Sigma-Aldrich, 16 ng/g) sc and iv 4 hours before *C. albicans* infection. DT was readministered iv 48 hours later.

Histopathology

Immunohistochemistry

Explanted skins were embedded in optimal cutting temperature freezing media (Bio-Optica). Sections (5 μm) were cut on a cryostat, adhered to a Superfrost Plus slide (Thermo Scientific), fixed with acetone, and blocked with Normal Goat Serum (1:10) for 30 min at room temperature. Sections were then stained with primary antibody specific for α-SMA (ab5694, Abcam), p-SMAD2/3 (clone D27F4, Cell Signaling), or IFN-γ (XMG1.2, Thermo Scientific), 1 hour at room temperature. LEAF purified rat IgG1 κ (clone RTK2071, BioLegend) was used as isotype control for IFN-γ staining, whereas purified rabbit polyclonal IgG (BD Pharmingen) was used as isotype control for α-SMA and p-SMAD2/3 (see fig. S26). Sections were washed with tris-buffered saline buffer, then labeled 30 min at room temperature with the Dako EnVision Anti-Rabbit System-HRP according to the manufacturer's recommendations, and counterstained with Meyer's hematoxylin solution (Bio-Optica). After dehydration, stained slides were mounted with Eukitt, and images were acquired with the NanoZoomer (Hamamatsu).

Hematoxylin and eosin staining

Skin sections (5 μm) were stained with Meyer's hematoxylin solution for 8 min and then washed in warm running tap water for 5 min. Sections were stained with Eosin Y solution for 1 min, washed in warm running tap water for 5 min, rinsed in distilled water, and then dehydrated through passages in 95% and absolute alcohol. After dehydration, stained slides were cleared in xylene and mounted with

Eukitt. Images were acquired with the NanoZoomer (Hamamatsu).

PicroSirius Red staining

Sections were stained with Meyer's hematoxylin solution for 8 min and then washed for 10 min in running tap water. Sections were stained in PicroSirius Red for 1 hour and then washed in two changes of acidified water. After dehydration in three changes of 100% ethanol, slides were cleared in xylene and mounted in Eukitt. Images were acquired with the NanoZoomer (Hamamatsu).

For collagen quantification, five fields (20×) from two sections per group were analyzed by separation into a red, green, and blue (RGB) filter, and the red area was mathematically divided by the RGB area and multiplied by 100%. This calculation represents the percentage area staining positively for collagen fibers.

Periodic acid–Schiff staining

Sections were fixed with acetone for 1 min at room temperature and then washed for 1 min in slowly running tap water. Slides were rinsed in periodic acid solution for 5 min at room temperature. Slides were rinsed with several changes of distilled water and then with Schiff's reagent for 15 min at room temperature. After washing in running tap water, slides were counterstained in hematoxylin solution for 5 min. Last, slides were dehydrated in three changes of 100% ethanol, cleared in xylene, and mounted in Eukitt. Images were acquired with the NanoZoomer (Hamamatsu).

Flow cytometry

Intracellular staining was performed on lymph node single-cell suspension using Cytofix/Cytoperm reagents (BD Biosciences) according to the manufacturer's instructions. Single-cell suspensions were kept for 3 hours in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich) before staining.

The antibodies used were as follows: phycoerythrin (PE)-anti-mouse CD49b (clone DX5, catalog no. 108908; BioLegend); allophycocyanin (APC)-anti-mouse IFN-γ (clone XMG1.2, catalog no. 505810; BioLegend); fluorescein isothiocyanate (FITC)-anti-mouse CD3 (clone 17A2, catalog no. 100204; BioLegend); APC-anti- mouse CD11b (clone M1/70, catalog no. 101212; BioLegend); PE-anti-mouse CD11c (clone N418, catalog no. 117308; BioLegend); APC-anti-mouse CD11c (clone N418, catalog no. 117310; BioLegend); APC/Cy7-anti-mouse CD3 (clone 17A2, catalog no.100222; Bio-Legend); and peridinin chlorophyll protein-anti-mouse NK1.1 (clone PK136); Alexa 488-anti-mouse I-Ab antibody (clone AF&-120.1); and PE-anti-mouse IL-2 (clone JES6-5H4) (all from BioLegend).

The antibodies used as isotype controls were as follows: PE mouse IgG2a, κ (catalog no. 400211, BioLegend); FITC rat IgG2b, κ (catalog no. 400605, BioLegend); APC rat IgG1, κ (catalog number 400411); PE rat IgG2b, κ (catalog no. 400607, BioLegend); PE/Cy7 mouse IgG2a, κ (catalog no. 400253, BioLegend); and APC/Cy7 Armenian hamster IgG (catalog no. 400927, BioLegend). NK cells were identified as

$NK1.1^+CD3^-$ lymphocytes. DCs were identified as $CD11c^+MHCII^+$ cells. Samples were acquired with a Gallios flow cytometer (Beckman Coulter).

Cells

Bone marrow–derived dendritic cells (BMDCs) were generated by culturing bone marrow (BM) precursors, flushed from femurs, in Iscove's modified Dulbecco's medium (IMDM) (Euroclone) containing 10% heat-inactivated fetal bovine serum (Euroclone), 100 IU of penicillin, streptomycin (100 μg/ml), 2 mM l-glutamine (Euro- clone), and granulocyte-macrophage colony-stimulating factor (CSF) (10 to 20 ng/ml) for 8 days. BM-derived macrophages were cultured in IMDM containing 100 IU of penicillin, streptomycin (100 μg/ml), 2 mM l-glutamine (all from Euroclone), and macro- phage CSF (10 to 20 ng/ml) for 10 days.

DCs for adoptive transfer experiments were expanded in vivo by transplanting mice with B16 tumor cells transduced with Flt3 ligand (FLT3L). Ten days after in vivo expansion, $CD11c^+$ cells were purified from spleen by magnetic-activated cell sorting (MACS) by positive selection using CD11c microbeads (Miltenyi Biotec).

NK cells for in vitro experiments and for adoptive transfer experiments were purified from splenocytes (after red blood cell lysis) by MACS positive selection using CD49b (DX5) microbeads (Miltenyi Biotec). Purity was assessed by fluorescence-activated cell sorting (FACS)

analysis and was routinely between 93 and 96%.

Skin fibroblasts were isolated and differentiated from the ears of adult C57BL/6 WT and NFATc2-deficient mice. Mice were euthanized and ears were removed. The ears were then divided into two layers and cut into small pieces that were placed in a six-well tissue culture plate with 3 ml of Dulbecco's modified Eagle's medium (catalog no. ECB7501L, Euroclone) containing 10% heat-inactivated fetal bovine serum (catalog no. 10270, Gibco), 100 IU of penicillin, streptomycin (100 μg/ml), 2 mM l-glutamine (all from Euroclone), epidermal growth factor (1 μg/ml) (SRP3196, Sigma-Aldrich), and fibroblast growth factor 2 (1 ng/ml) (SRP4038, Sigma-Aldrich) for 1 week. After 1 week, adherent fibroblasts were detached with trypsin/EDTA (catalog no. ECB3001D, Euroclone) and grown in tissue culture plates. At 80% of confluence, cells were detached and divided by a ratio of 1:2 until passages 4 and 5 when they were used for experimental procedures.

NK-DC cocultures

BMDCs (1×10^5 per well) and NK cells (5×10^4 per well for IFN-γ release assays) were cocultured in flat-bottom 96-well plates in the presence or absence of *C. albicans* hyphae [multiplicity of infection (MOI), 0.005], and, where indicated, recombinant IL-2 (rIL-2) (7.5 ng/ml) (402-ML carrier-free, R&D Systems), or purified rat anti-mouse IL-2 (5 μg/ml) (BD Biosciences) or purified rat IgG2a (5 μg/ml) (BD Biosciences) as isotype control. Two hours after stimulation, amphotericin B (2.5 μg/ml) (Sigma-Aldrich) was added to the cultures.

TNF-α, IL-2, IFN-γ, and IL-12 measurement Concentrations of IL-2, TNF-α, IFN-γ, and IL-12 in supernatants were assessed by enzyme-linked immunosorbent assay (ELISA) kits purchased from BD OptEIA, eBioscience, and R&D Systems, respectively.

Quantitative reverse transcription polymerase chain reaction

Pieces of lateral skin were homogenized in TRIzol reagent, and then RNA was extracted using Qiagen RNeasy Mini Kit (catalog no. 74104). Single-strand complementary DNA (cDNA) was synthesized using High-Capacity cDNA Reverse Transcription Kits (catalog no. 4368814, Applied Biosystems). The NanoDrop (Thermo Scientific) was used to titer mRNA, and amplification was performed using either the TaqMan Gene Expression Master Mix (catalog no. 4369016, Applied Biosystems) and TaqMan probes (Ifng, Mm01168134_m1; Fcer1a, Mm00438867_m1; SiglecF, Mm00523987_m1; Ly6c, Mm03009946_m1; Cd11c Mm00498698_m1; Nfatc1, Mm00479445_m1; Nfatc2, Mm01240677_m1; Nfatc3, Mm01249200_m1; 18*S*, Mm03928990_ g1;Gapdh, Mm99999915_g1) or the Power SYBR Green PCR Master Mix (Applied Biosystems) (Ly-6G: forward, 5′-TGGACTCTCACAGAAGCAAAG-3′ and reverse, 5′-GCAGAGGTCTTCCTTC- CAACA-3′; Gapdh: forward, 5′-CTGGCCAAGGTCATCCATG-3′ and reverse, 5′-GCCATGCCAGTGAGCTTCC-3′). Relative mRNA expression was calculated using the Δct method, using either *Gapdh* or *18S* as a reference gene.

Western blot

Pieces of lateral skin were cut, put in Eppendorf tubes, and immersed in liquid nitrogen for snap freezing. Tissues were smashed and homogenized in 1 ml of lysis buffer [50 mM tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, and 1% NP-40 supplemented with pro- tease and phosphatase inhibitor cocktails; Roche] using a TissueLyser (full speed for 20 min, Qiagen). Samples were then maintained in constant agitation for 2 hours at 4°C and centrifuged for 20 min at 13,000*g* at 4°C. The supernatants were collected into a new Falcon tube. Proteins were quantified using a bicinchoninic acid assay (Euroclone). Cell lysates (150 μg) were run on a 10% polyacrylamide gel, and SDS–polyacrylamide gel electrophoresis was performed following standard procedures. After protein transfer, nitrocellulose membranes (Thermo Scientific) were incubated with the antibodies specific for phosphorylated SMAD2/3 (clone D27F4, Cell Signaling), mSerpin E1 (goat polyclonal IgG, R&D Systems), tPA (rabbit poly- clonal, NOVUS Biologicals), and β-actin (rabbit polyclonal, Cell Signaling) and developed using an enhanced chemiluminesence substrate reagent (Thermo Scientific).

Plasmin and MMP-3 activity assays

Pieces of lateral skin were cut, put in Eppendorf tubes, and immersed in liquid nitrogen for snap freezing. Tissues were smashed and homogenized in 1 ml of an optimized buffer [150 nM NaCl, 1% NP-40, and 50 mM tris-HCl (pH 8.0)] with a TissueLyser (Qiagen) (full speed

for 20 min at 4°C). Samples were then maintained in constant agitation for 2 hours at 4°C and centrifuged for 20 min at 13,000*g* at 4°C. Samples (50 μl) were then used for plasmin measurement using the Plasmin Activity Assay Kit (Abcam) and for active MMP-3 measurement using the Activity Assay Kit (Abcam).

Adoptive transfer experiments

For the adoptive transfer of NK cells, WT or IFN-γ–deficient mice were injected iv with lipopolysaccharide (LPS) (2 mg LPS/gbw) to activate NK cells. Two hours and 30 min after activation, NK cells were purified from spleen and administered iv to NFATc2-deficient mice (2×10^6 per mouse) 4 hours after *C. albicans* infection. For the experiments of DC adoptive transfer, DCs were purified form the spleen of WT, NFATc2-deficient, and IL-2–deficient mice. Purified DCs were then co-injected with *C. albicans* (2 × 10 per mouse). The same mice also received purified DCs by intravenous administration (10^6 per mouse) after *C. albicans* infection.

Statistical analysis

Means were compared by either unpaired parametric *t* tests or two-way analysis of variance (ANOVA). Data are expressed and plotted as means ± squared deviations from the mean (SDM) or ± SEM values. Sample sizes for each experimental condition are provided in the figure legends. *P* values for Kaplan-Meier curves were calculated with log-rank test. All *P* values were calculated using Prism (GraphPad).

Differences were considered significant if $P \leq 0.05$.

Acknowledgments: CD11c.DOG mice were provided by N. Garbi (Institute of Molecular Medicine and Experimental Immunology, Bonn, Germany). IL-2–deficient mice were provided by A. Schimpl (University of Würzburg, Würzburg, Germany), and NFATc2-deficient mice were provided by E. Serfling (University of Würzburg, Germany). **Funding:** I.Z. is supported by NIH grants 1R01AI121066-01A1 and 1R01DK115217, HDDC (Harvard Digestive Diseases Center) P30 DK034854 grant, the Harvard Medical School Milton Fund, the CCFA (Crohn's & Colitis Foundation) Senior Research Award, the Eleanor and Miles Shore 50th Anniversary Fellowship Program, and the Cariplo Foundation. F.G. is supported by the Associazione Italiana per la Ricerca sul Cancro (IG 2016Id.18842), the Cariplo Foundation (grant 2014-0655), and the Fondazione Regionale per la Ricerca Biomedica. G.S. is supported by fellowships from SYSBIONET, the Italian roadmap of the European Strategy Forum on Research Infrastructures.

2.6. Supplementary materials

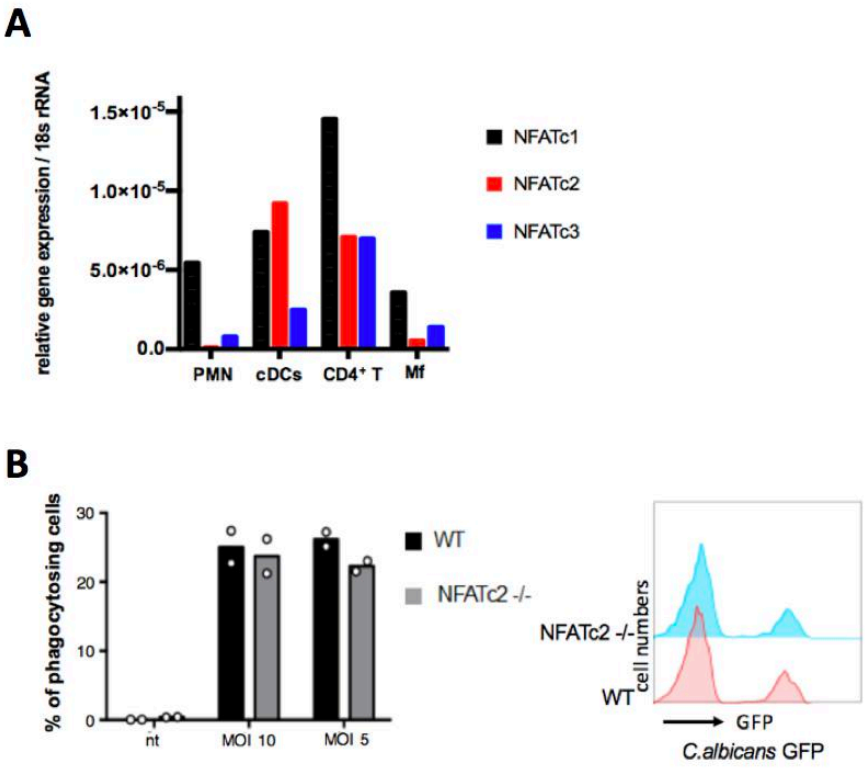


Fig. S1. Expression of NFATc1, NFATc2, and NFATc3 in immune cells. (**A**) Quantitative Real- Time PCR analysis of NFATc1-c3 expression by granulocytes from bone marrow (PMN); conventional DCs from spleen (cDCs), $CD4^+$ T cells ($CD4^+$ T) from spleen and peritoneal macrophages (Mf). (**B**) Phagocytic capacity of WT and NFATc2-deficient neutrophils. Neutrophils were incubated with GFP expressing *C. albicans* at the indicated MOI and the efficiency of microbe internalization evaluated by cytofluorimetric analysis.

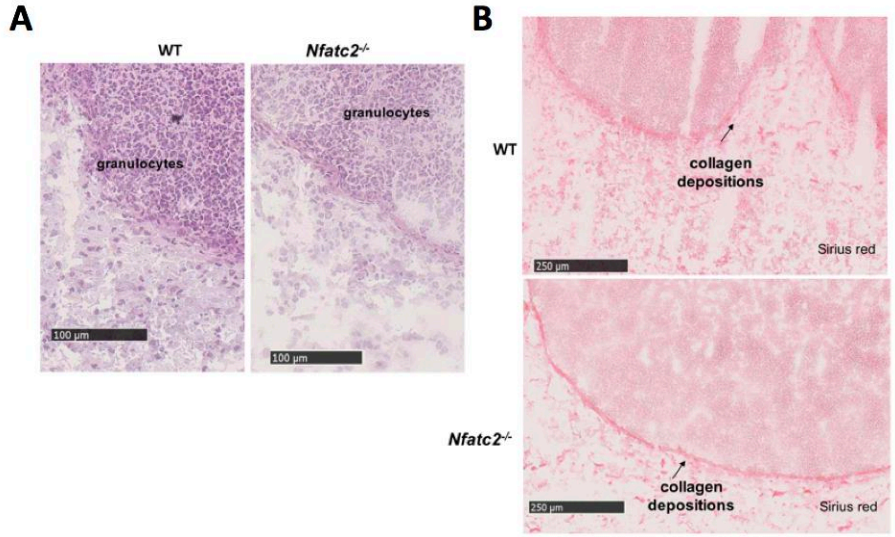


Fig. S2. Magnifications of the selected areas shown in Fig. 1C to evidence similarities and differences between WT and NFATc2-deficient mice after *C. albicans* infection. (**A**) Haematoxylin and eosin staining showing granulocyte recruitment at sites of infection in WT and NFATc2-deficient mice. (**B**) PicroSirius Red stain to evidence collagen depositions (red deposits). Notice that the collagen capsule is loose in WT animals and well defined and organized in NFATc2-deficient mice.

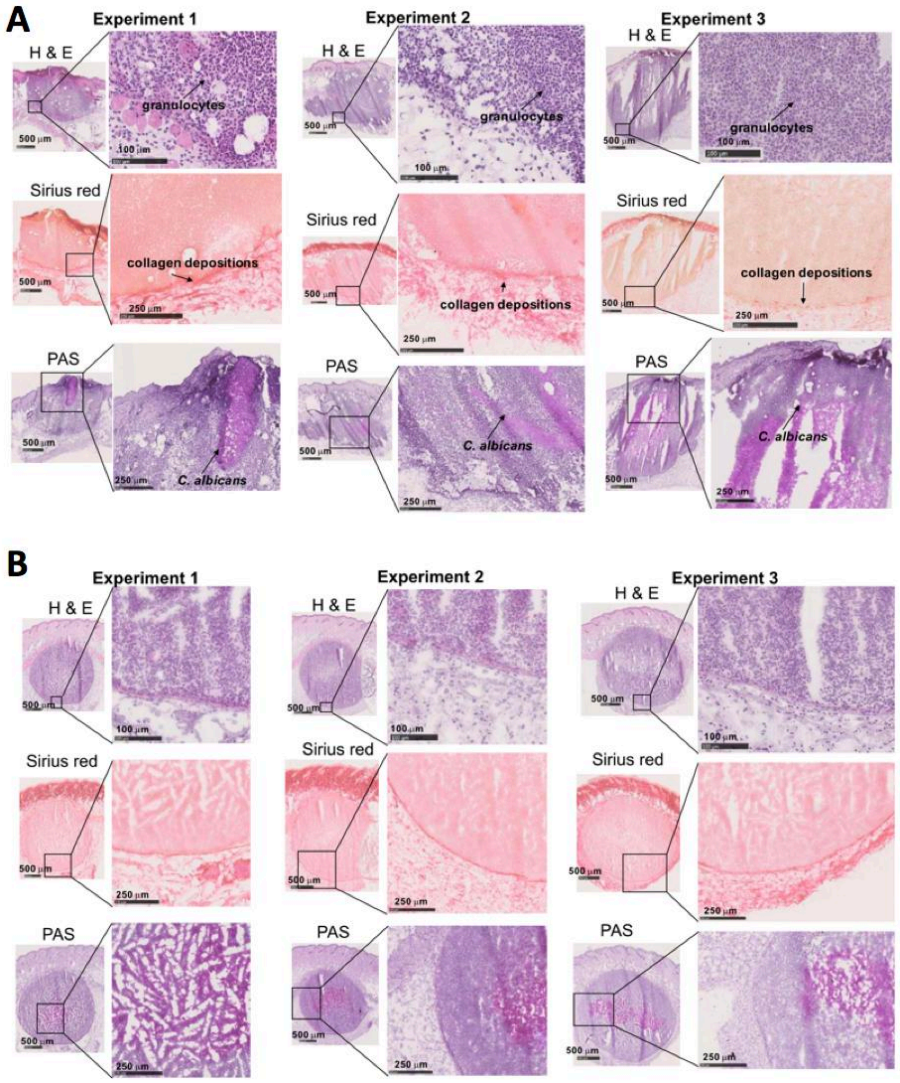


Fig. S3. Additional histological images of the abscess after *C. albicans* infection. Hematoxylin and eosin (H&E), PicroSirius Red and PAS stainings of WT (**A**) and NFATc2-deficient (**B**) mouse skin lesions after *C. albicans* infections, larger magnifications of selected areas are shown to evidence granulocyte recruitment, collagen depositions and *C. albicans*. Images from three different experiments are shown.

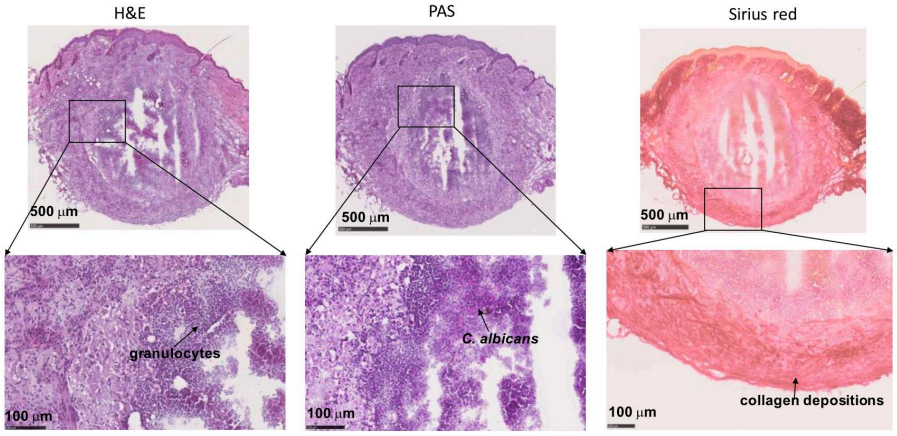


Fig. S4. Histology of the abscess 1 month after infection of NFATc2-deficient mice. Haematoxylin and eosin staining showing granulocyte recruitment at sites of infection, PicroSirius Red stain to evidence collagen depositions (red deposits) and PAS staining (purple) to evidence *C. albicans* are shown.

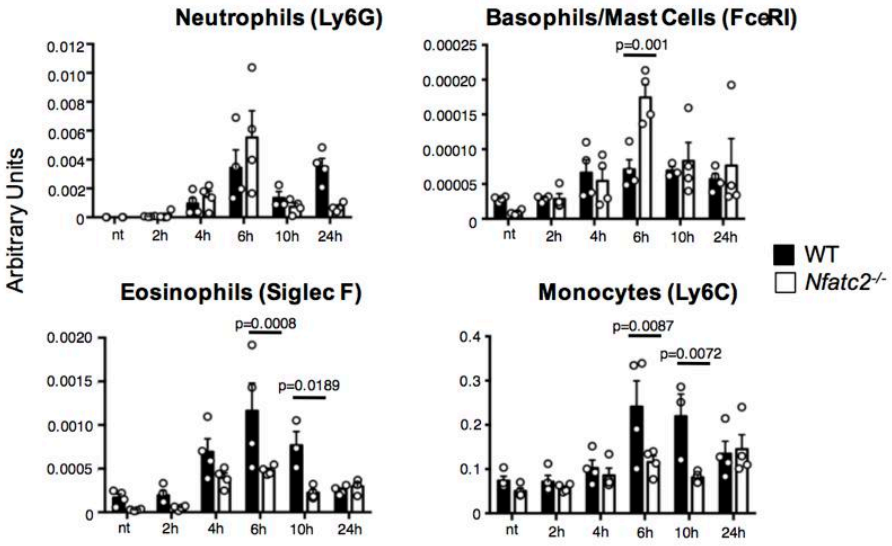


Fig. S5. Granulocyte and monocyte recruitment at the infection site of WT and NFATc2-deficient mice. Quantitative Real-Time PCR analysis of Ly6G,

Ly6C, FcεRI and Siglec F mRNAs in *C. albicans* infected tissues at the indicated time points after infection, each dot represents a different mouse. Mean and SEM are depicted. Statistical significance was determined with a two- way ANOVA test. nt, non-infected mice.

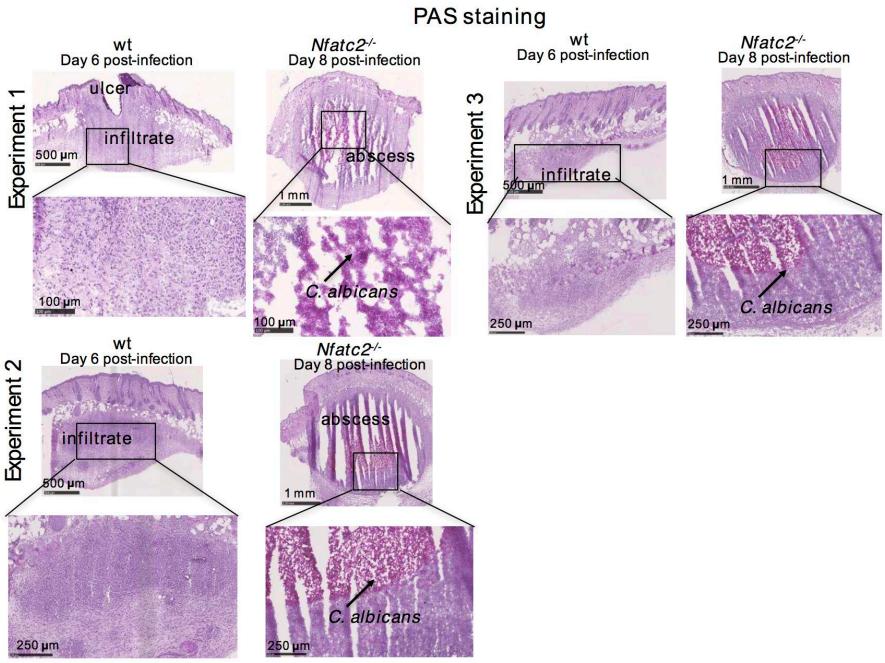


Fig. S6. Visualization of *C. albicans* at the infection site 6 to 8 days after infection. PAS staining (purple) at the indicated time points after infection of WT and NFATc2-defcient mice. Notice that in WT animals *C. albicans* is eliminated 6 days after infection while it persists inside the abscess in NFATc2-deficient mice. Histology is representative of 3 per group. Images from three different experiments are shown.

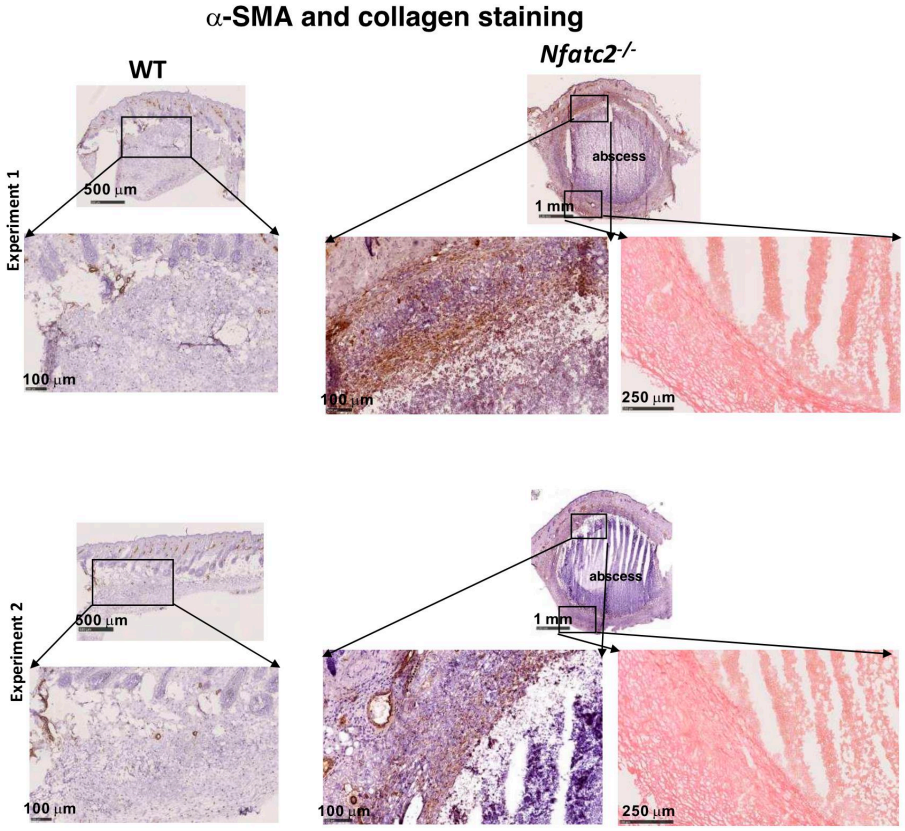


Fig. S7. Additional histological images of α-SMA staining in skin sections of WT and NFATc2-deficient mice. PicroSirius Red staining is also shown to evidence collagen capsule. Images from two independent experiments are shown.

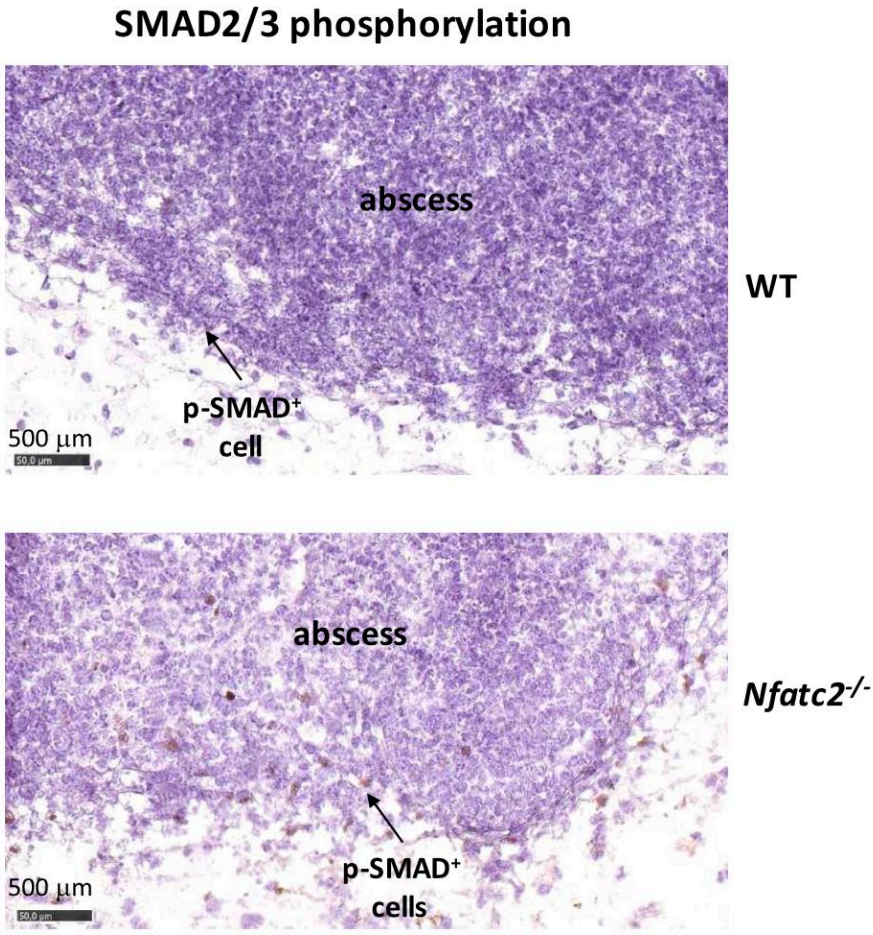


Fig. S8. Additional histological images of p-SMAD2,3 staining. p-SMAD2,3 immunohistochemical staining in skin sections of WT and NFATc2-deficient mice after *C. albicans* infection. p-SMAD2,3 positive cells are brown.

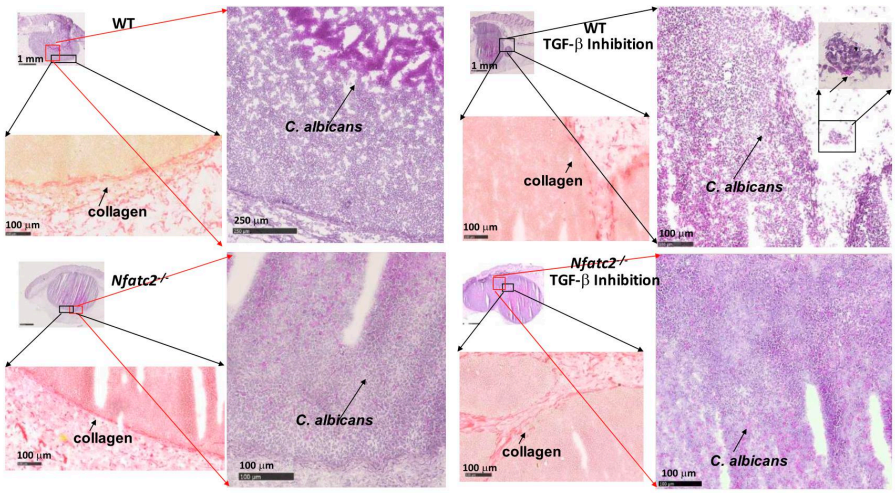


Fig. S9. Magnifications of the selected areas shown in Fig. 3D to evidence the behavior of WT and NFATc2-deficient mice infected with *C. albicans* in the presence or not of the TGF-β inhibitor. (Left panels) *C. albicans* staining (purple) and collagen depositions in untreated mice, (right panels), *C. albicans* staining (purple) and collagen depositions in mice infected with *Candida* during the inhibition of the TGF-β pathway. Notice that collagen depositions are disorganized if the animals are treated with the inhibitor compared to untreated mice. Moreover, in the presence of the inhibitor *C. albicans* is not properly contained, presumably due to the inefficient formation of the capsule, and can exit the abscess.

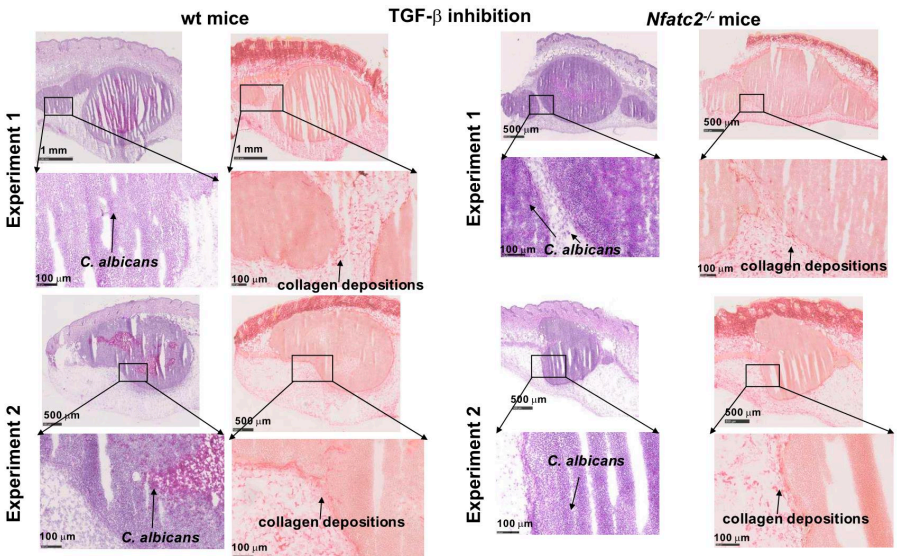


Fig. S10. Additional histological images of the abscesses after *C. albicans* infection in the presence of a TGF-β inhibitor. Visualization of *C. albicans* (purple staining) by PAS staining and collagen deposition by PicroSirius Red staining in WT and NFATc2-deficient mouse skin lesions 24-48h after *C. albicans* infections in the presence of TGF-β inhibitor. Notice that the collagen capsule is disorganized if the animals are treated with the TGF-β inhibitor and multiple abscesses are formed. Images from two independent experiments are shown.

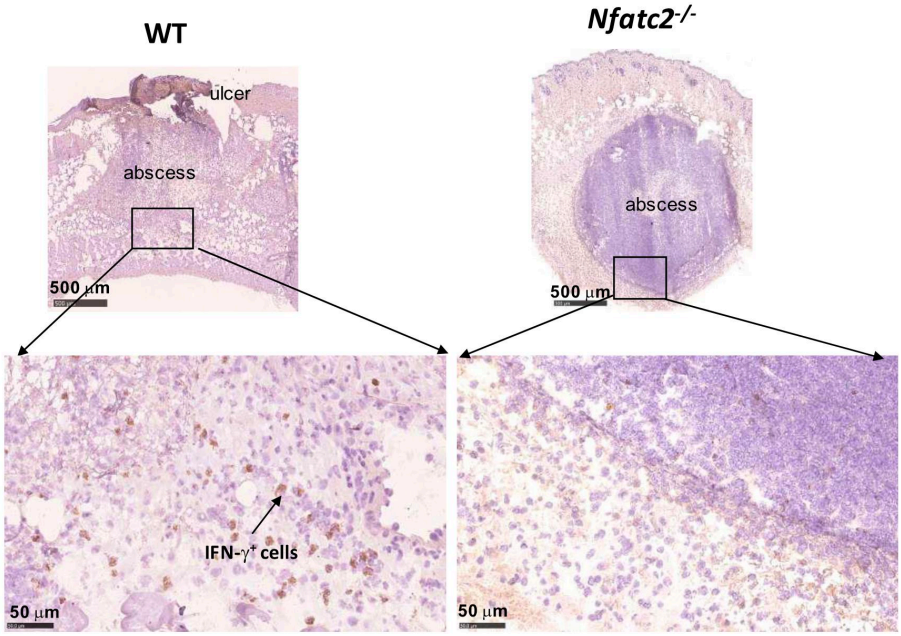
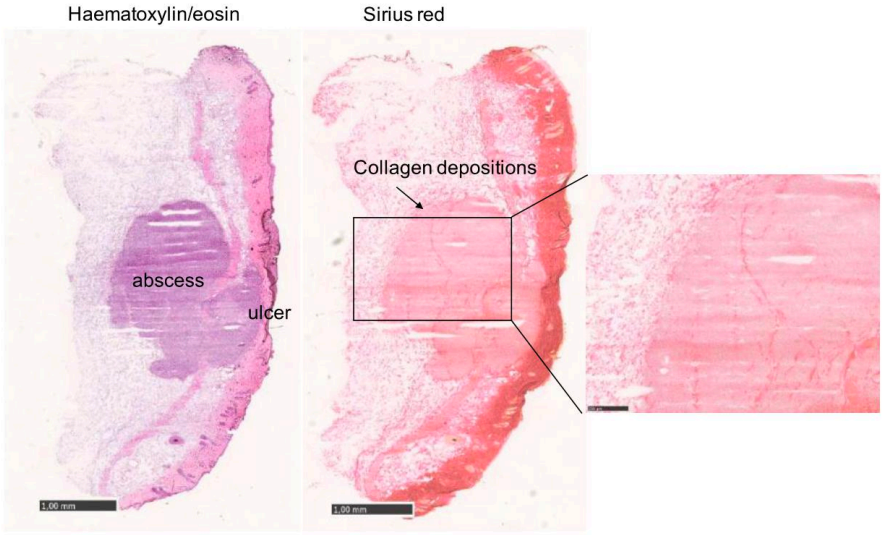


Fig. S11. Additional histological images of IFN-γ staining. Immunohistochemical staining of IFN-γ (brown cells are IFN-γ^+ cells) in skin sections of WT and NFATc2-deficient mice after *C. albicans* infection.



WT 24 hours after C. albicans infection and rIFN-γ treatment

Fig. S12. IFN-γ induces capsule digestion. Hematoxylin/eosin and PicroSirius Red (collagen) staining of a skin section of a WT animal treated with rIFN-γ at the time of *C. albicans* infection. Notice that the collagen capsule is already almost completely digested only after 24 hours, as highlighted in the higher magnification.

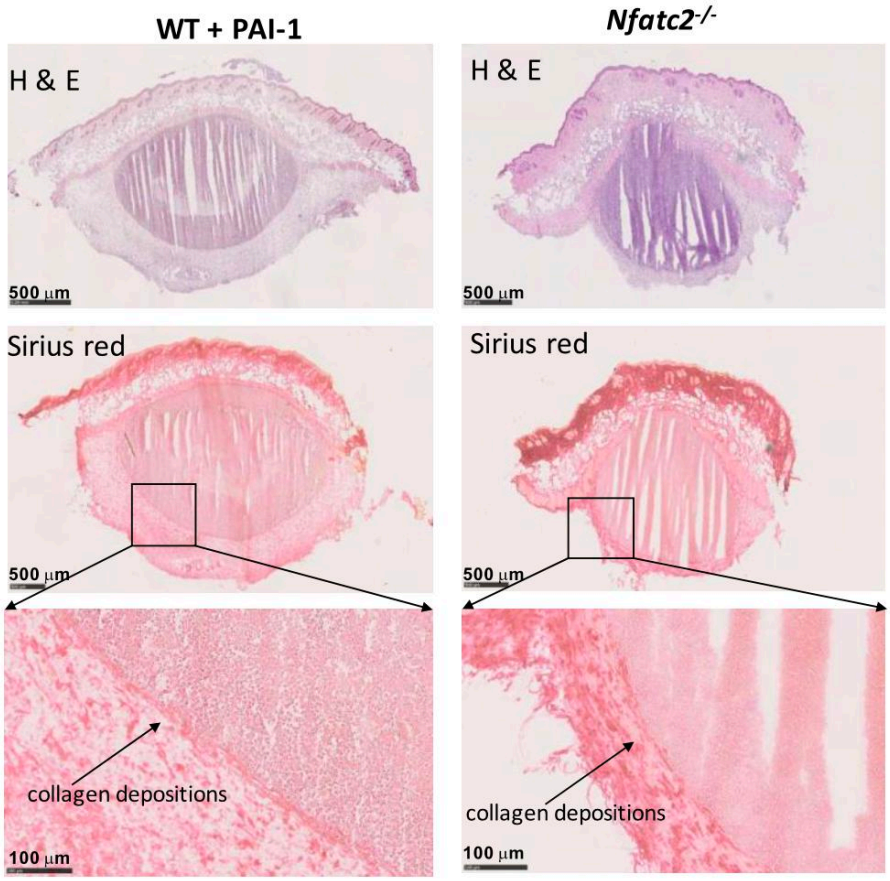


Fig. S13. Additional histological images of the abscesses after *C. albicans* infection in the presence of PAI-1. Hematoxylin and eosin and PicroSirius Red staining of WT mouse skin lesions 48h after *C. albicans* infections in the presence of PAI-1 (0.65 μg/mouse). NFATc2-deficient mice skin lesions are also shown. Larger magnification of PicroSirius Red staining of selected areas

is shown to evidence collagen depositions.

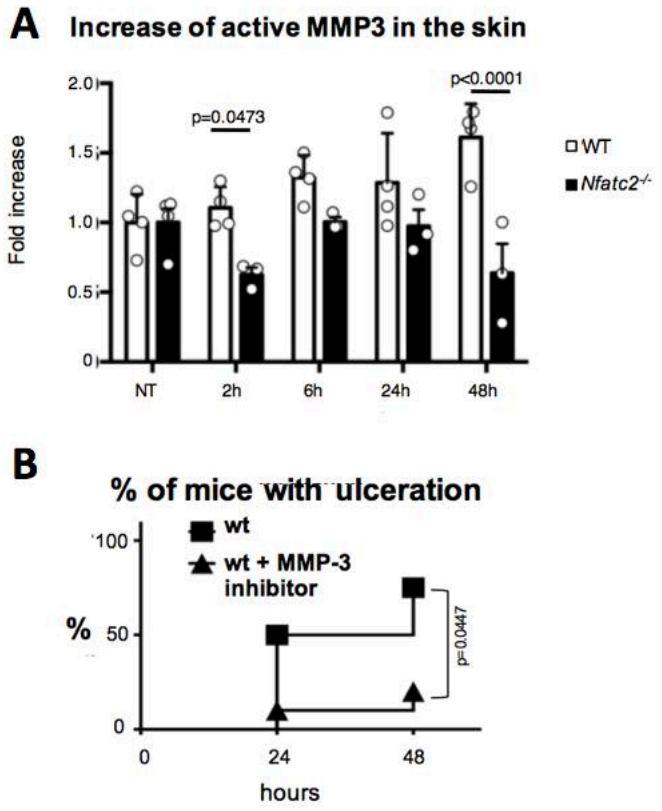


Fig. S14. Inhibition of plasmin or MMP-3 interferes with *C. albicans* elimination. (**A**) Increase of active MMP-3 levels in WT and NFATc2-deficient mice at the indicated time points after *C. albicans* infection. Mean and SDM are depicted. Statistical significance was determined with a two-way ANOVA test. (**B**) Kaplan-Meier curves showing the percent of mice undergoing ulceration after *C. albicans* administration. Where indicated, mice were treated with an MMP-3 inhibitor (MMP-3 inhibitor I, co-administered with *C. albicans* sc, 125 μg/mouse, and re-administered ip 8 hours later, 250 μg/mouse). N(WT)=4; N(WT + inhibitor)=10; log-rank test.

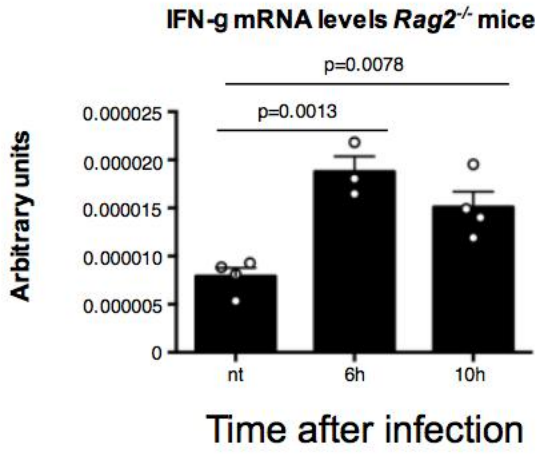


Fig. S15. IFN-γ mRNA is up-regulated in the infected skin of RAG-2–deficient mice. Quantitative Real-Time PCR analysis of IFN-γ mRNA in *C. albicans* infected tissues of RAG-2– deficient mice at the indicated time points after infection; each dot represents a different mouse. Mean and SEM are depicted. Statistical significance was determined with a two-tailed t test. Representative data of two independent experiments are shown. nt, untreated mice.

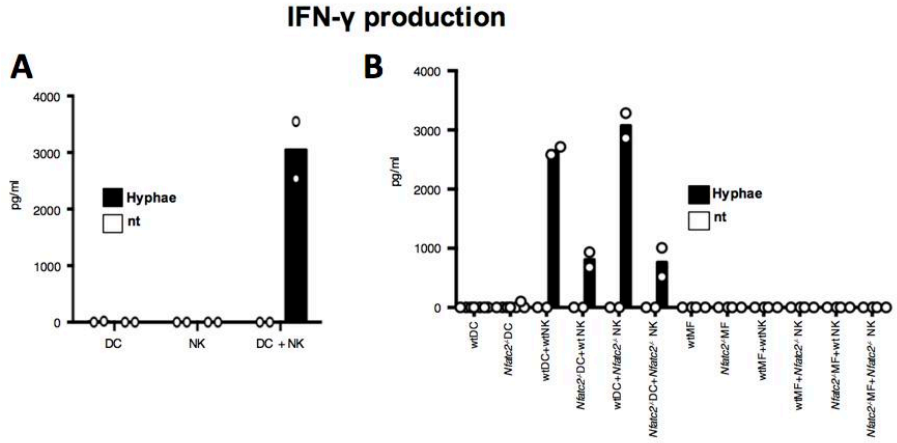


Fig. S16. DCs are necessary for the activation of NK cells in the presence of *C. albicans* in vitro. (**A**) IFN-γ secretion by NK cells alone, BMDCs alone or BMDCs and NK cell co-cultures in the presence of or absence of *C. albicans* hyphae. In the cocultures immature or *C. albicans*- activated BMDCs (DCs, MOI 0.05) were cocultured with NK cells for 18 hours. IFN-γ released in the supernatant by NK cells was measured by ELISA. (**B**) Immature or *C. albicans*-activated BMDCs and unstimulated or *C. albicans*-stimulated macrophages (MF) of the indicated genotype were cultured with NK cells of the indicated genotypes, and NK cell released IFN-γ measured 18 hours later by ELISA. Notice that macrophages and NFATc2-deficient DCs are not able to elicit IFN-γ production from NK cells. Each dot represents a biological replicate.

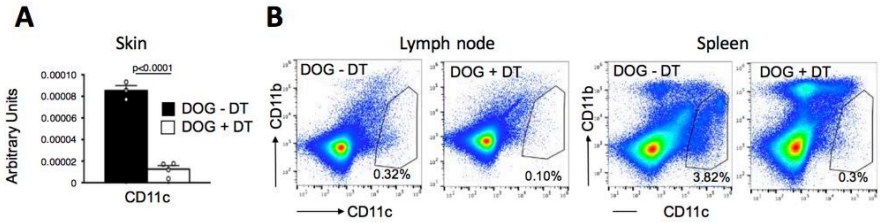


Fig. S17. DC depletion after DT injection in DOG mice. (**A**) Quantitative Real-Time PCR analysis of CD11c expression in the skin of DOG mice before and after DT administration. Mean and SEM are depicted. Statistical significance was determined with a two-tailed t test. (**B**) Cytofluorimetric analysis showing $CD11c^{high}$ cell depletion in the lymph node and spleen of DOG mice after DT treatment.

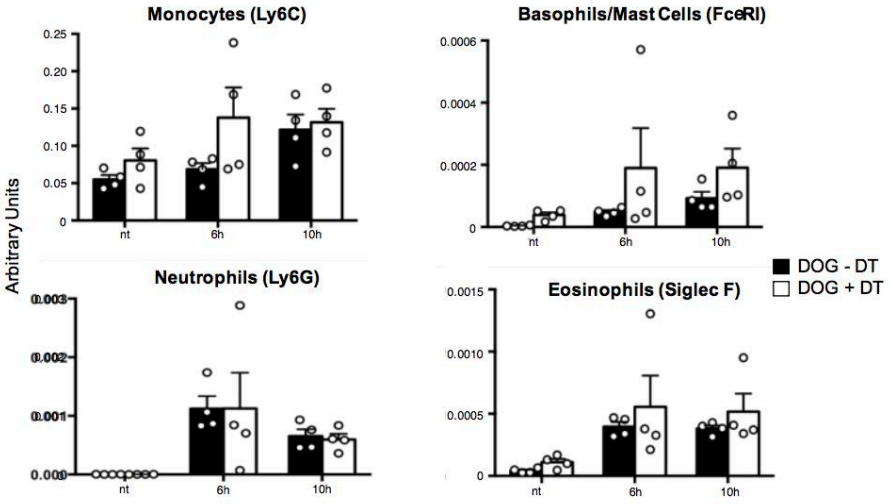


Fig. S18. Granulocyte and monocyte recruitment at the infection site of DOG mice treated or not with DT to deplete DCs. Quantitative Real-Time PCR analysis of Ly6C, FcεRI, Ly6G, and Siglec F mRNAs in *C. albicans* infected tissues of DOG mice treated or not with DT at the indicated time points after infection. Mean and SDM are depicted. Each dot represents a single mouse. Statistical significance was determined with a two-way ANOVA test. ns, not statistically significant; nt, non-infected mice.

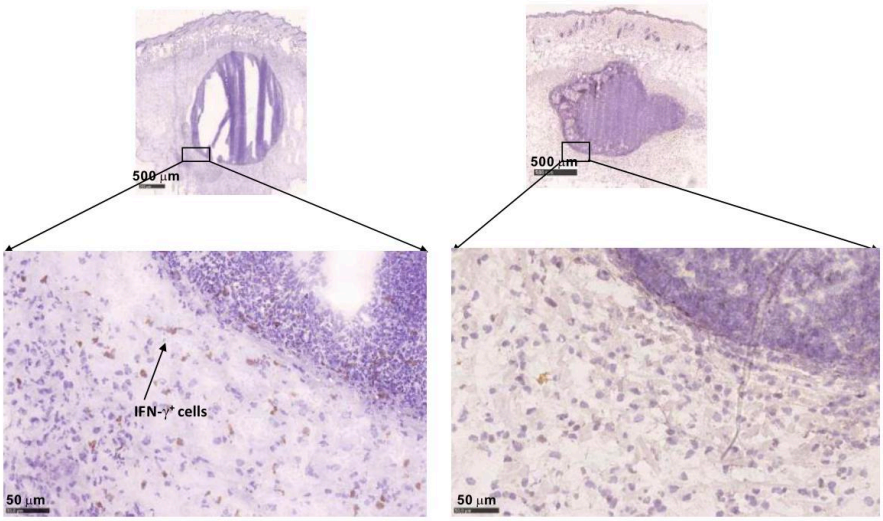


Fig. S19. Additional histological images of IFN-γ staining in the infected skin of DOG mice. IFN-γ immunohistochemical staining in skin sections (brown cells) of DOG mice and DC-depleted DOG mice (DOG+DT) after *C. albicans* infection

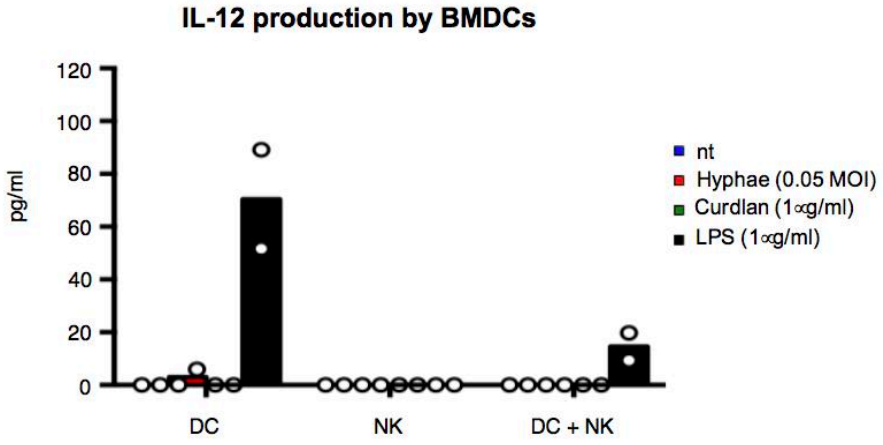


Fig. S20. IL-12 production by BMDCs after *C. albicans* stimulation. IL-12p70 released in the supernatants by WT BMDCs in the presence of absence of the indicated stimuli; *C. albicans* (MOI 0.05); Curdlan (1µg/ml); LPS (1µg/ml).

Amounts of IL-12p70 released in the supernatant have been measured by ELISA after 18h of stimulus exposure. IL-12 released by DCs-NK cell cocultures is also shown. nt, untreated cells. Each dot represents a different sample.

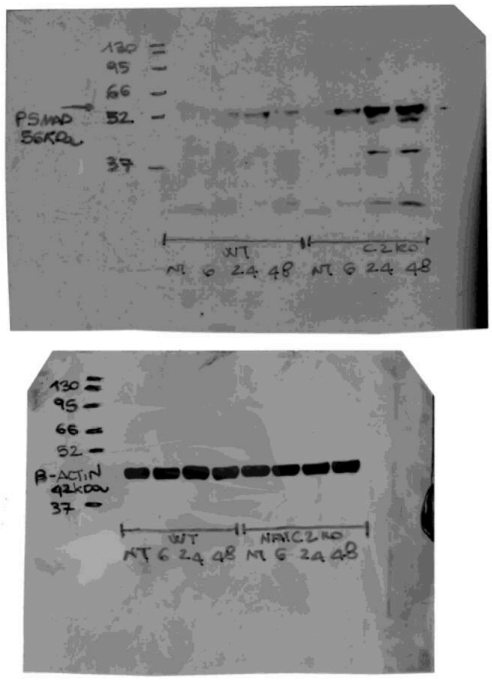


Fig. S21. Original Western blots shown in Fig. 3A. Complete Western blots for p-SMAD2/3 and β-actin shown in Fig. 3A.

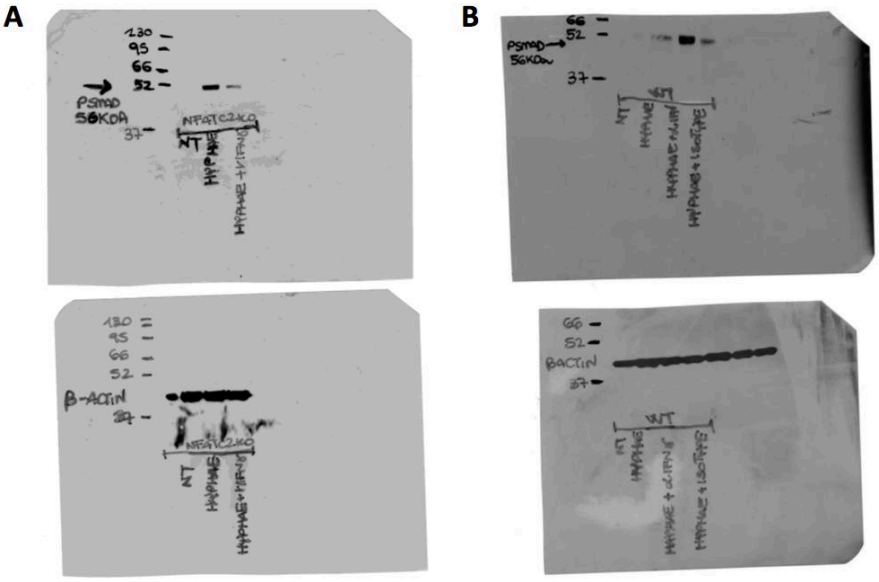


Fig. S22. Original Western blots shown in Fig. 4 (E and F). (**A**) Complete Western blots for p-SMAD2/3 and β-actin shown in Fig. 4E. (**B**) Complete Western blots for p-SMAD2/3 and β-actin shown in Fig. 4F.

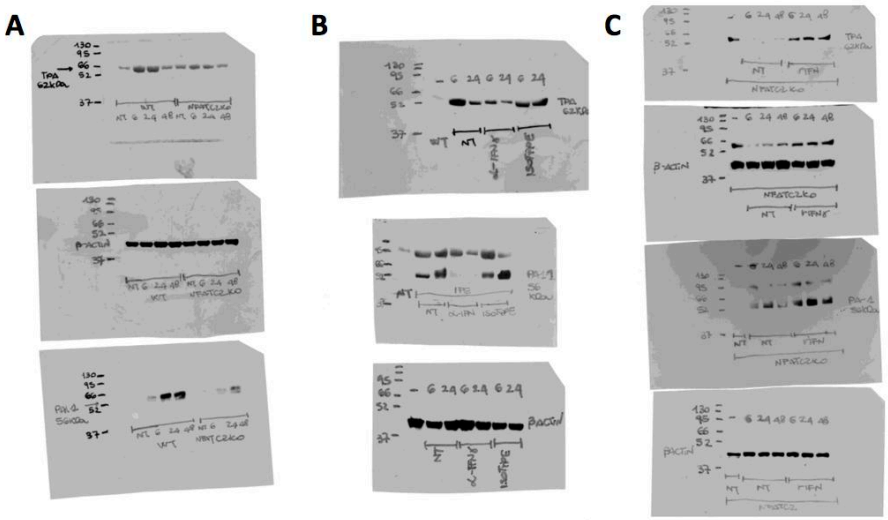


Fig. S23. Original Western blots shown in Fig. 5E. (**A**) Complete Western

blots for tPA, PAI-1 and β-actin shown in Fig. 5E (left panels). (**B**) Complete Western blots for tPA, PAI-1 and β-actin shown in Fig. 5E (middle panels). (C) Complete Western blots for tPA, PAI-1 and β-actin shown in Fig. 5E (right panels).

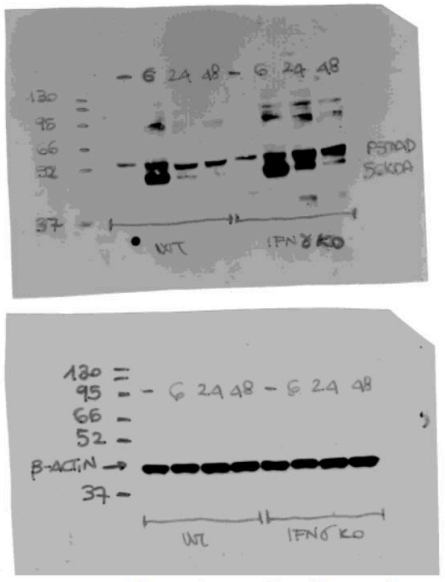


Fig. S24. Original Western blots shown in Fig. 7C. Complete Western blots for p-SMAD2/3 and β-actin shown in Fig. 7C.

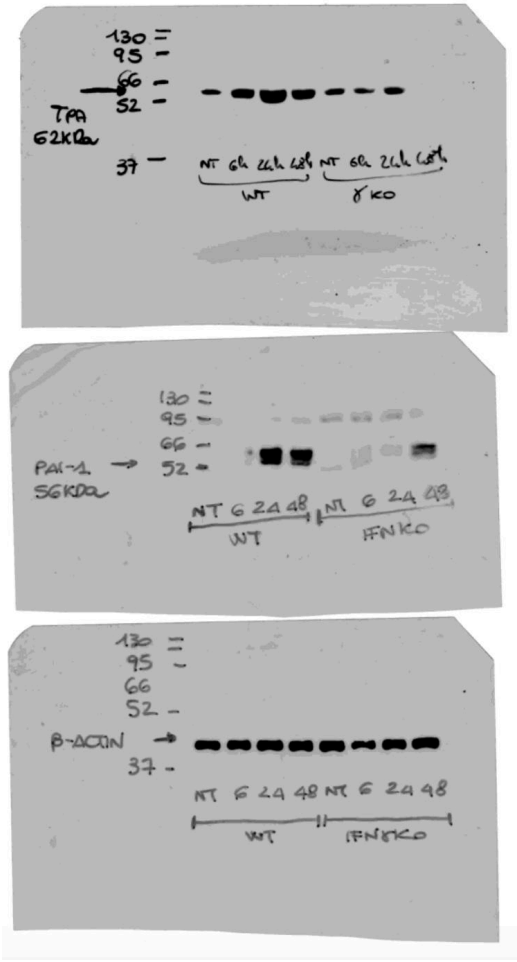


Fig. S25. Original Western blots shown in Fig. 7D. Complete Western blots for tPA, PAI-1 and β-actin shown in Fig. 7D.

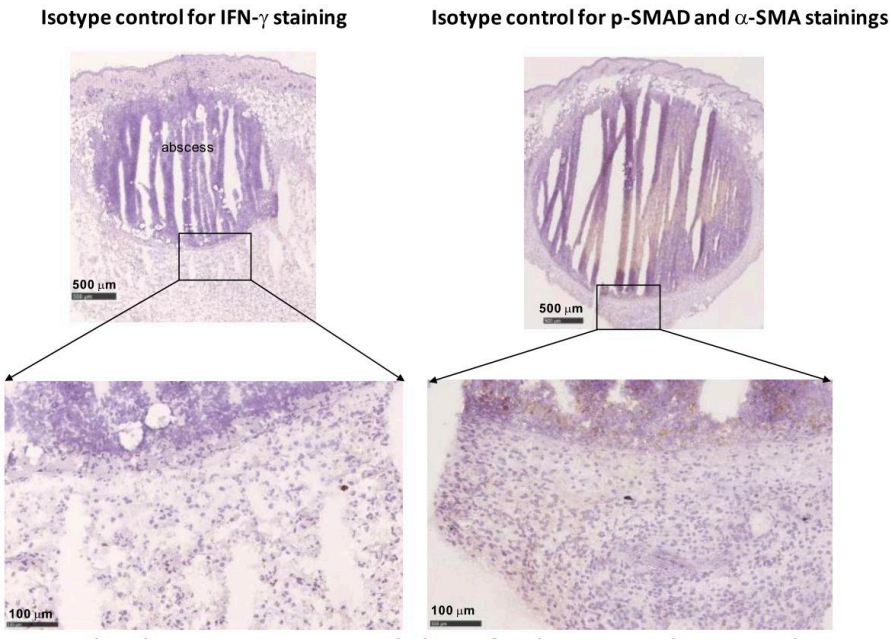


Fig. S26. Representative isotype control stainings for immunohistochemical analyses.

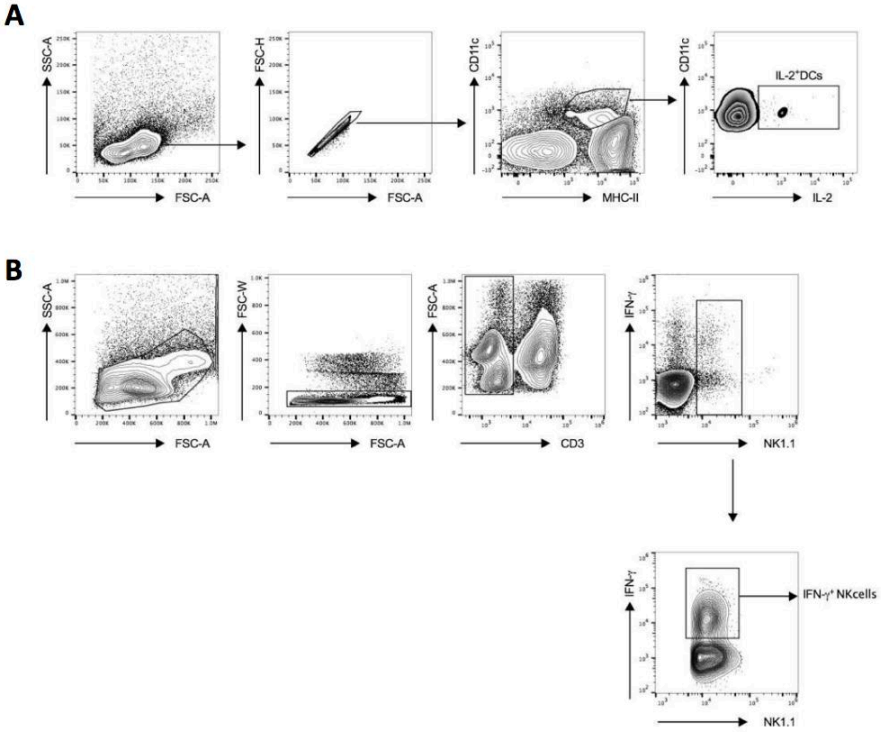


Fig. S27. Gating strategies used in cytofluorimetric analyses. (**A**) Gating strategy used in Fig. 6G. (**B**) Gating strategy used in Fig. 6 C and D.

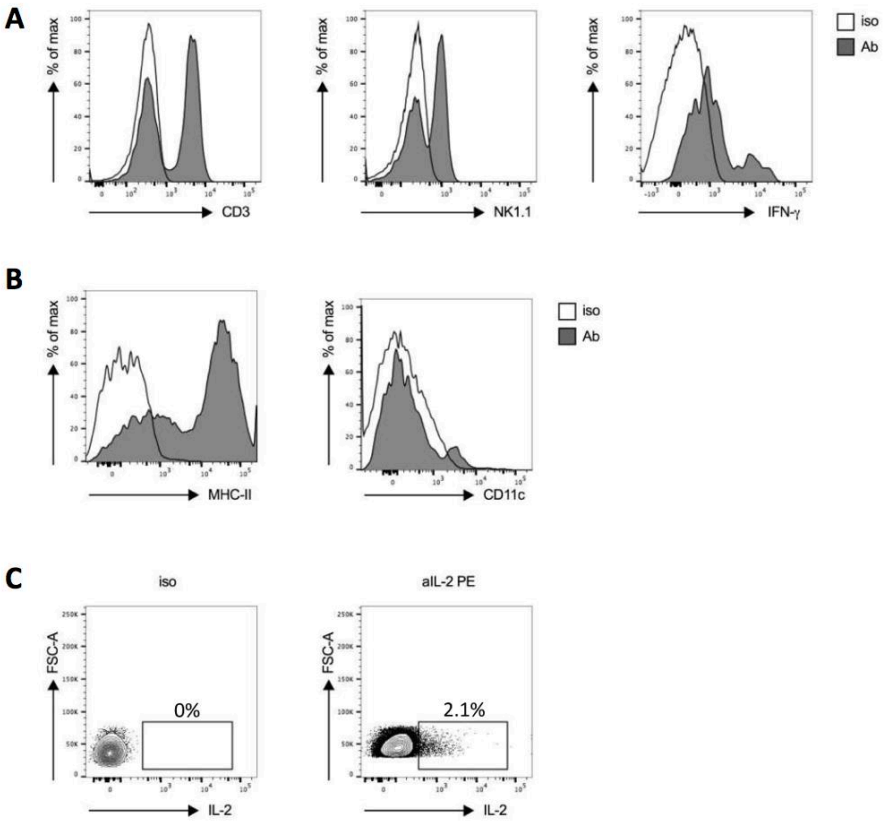


Fig. S28. Representative isotype control stainings for cytofluorimetric analyses. (**A**) Samples treated as in Fig. 6 C and D have been stained with the indicated antibodies (full histogram) and the corresponding isotype control antibodies (black line). (**B**) Samples treated as in Fig. 6G were stained with the indicated antibodies (full histogram) or with the corresponding isotype control antibodies (black line). (**C**) Samples treated as in Fig. 6G were stained with an anti-IL-2 antibody (right panel) or with the corresponding isotype control antibody (left panel).

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Chapter 3: Final conclusions

3.1. Summary

C. albicans is a fungus that can grow in different morphological forms and, in its hyphal form, it has its highest invasiveness and its associated to several pathological conditions (*1*). *C. albicans* is usually present in healthy individuals as a commensal microorganism but, in conditions of altered homeostasis, it can cause infections of the host leading to several pathologies. These are the cases of patients with a compromised immune system, *i.e*. HIV-patients, and individuals that have received transplantation and are therefore under immunosuppressive drugs (*2*).

Here we investigated how the activation of Nuclear Factor of Activated T cells (NFAT) in innate immune cells controls skin microbial infections. *Candida albicans* was used to unravel the innate immune pathways that control skin infections. All the main conclusions were confirmed using *Staphylococcus aureus*.

These microbial infections can develop in different anatomic sites, including the skin. While they are controlled by healthy individuals, they may become systemic in immuno-compromised individuals, nosocomial patients, or those with inherited mutations in immune genes.

Fungal ligands are among the most potent stimuli able to induce the activation of NFAT signaling pathway in innate immune cells downstream Pattern Recognition Receptors (PRRs). The NFAT pathway functions have been best characterized in adaptive immunity while very little is known in innate myeloid cells (*3*).

In the present work, by investigating the role of NFAT activation in innate immune cells in the inflammatory process during microbial infection, we defined an important molecular mechanism regulating two major phases of the innate response to *C. albicans* and *S. aureus*: the initial containment phase, and the elimination phase via skin ulceration and microbial discharge out of the skin.

During the containment phase, granulocytes are recruited to surround microbes and form an abscess. We show that TGF-β plays a fundamental role in this very early phase of anti-microbial responses. TGF-β activates fibroblasts that deposit collagen around granulocytes to form a protective capsule thus preventing infection spreading. If the TGF-β pathway is blocked, the protective collagen deposits are not formed, microbial infections are not appropriately contained and diffuse inside the subcutaneous space where multiple granulocyte accumulation and larger lesions can be observed.

In the elimination phase of the inflammatory process the activation of the fibrinolytic system is necessary for collagen capsule digestion and the discharge of microbes out of the skin. For this phase to occur, IFN-γ must be released in the tissue by activated NK cells. By antagonizing

TGF-β, IFN-γ down-modulates PAI-1 production and allows plasmin formation.

In the absence of IFN-γ, the continuous signaling of TGF-β not only impedes plasmin formation but also induces the differentiation of fibroblasts into myofibroblasts with the generation of a very thick capsule around the abscess that hampers skin ulceration and microbial elimination. Therefore, microbes persist alive inside the abscess.

During the early infection phase, microbe-exposed dendritic cells acquire the ability to elicit IFN-γ production from NK cells. DC-derived cytokines required for NK cells activation are produced in NFATc2-dependent manner. In the absence of NFATc2, IFN-γ is not produced and only the containment phase takes place with the formation of an encapsulated abscess and infection persistence. Interestingly IFN-γ-deficient and NFATc2-deficient mice show the same behavior in response to these infections.

This study significantly advances the inflammation field because:

- It shows that TGF-β is fundamental during the inflammatory process to contain the infection, reduce tissue damage, and favor microbial discharge. TGF-β is mainly considered a cytokine that negatively regulates the inflammatory process. Here we show that, by exerting its profibrotic functions (fibroblast activation, collagen deposition, inhibition of the

fibrinolytic system), TGF-β is fundamental to increase the effectiveness of the inflammatory process.

- It describes a new role for IFN-γ during the inflammatory process elicited by infections, that is the activation of the plasminogen/plasmin system.
- It is shows that, from the host point of view, the plasminogen/plasmin system is fundamental for microbial elimination because it counteracts the formation of a too thick collagen and myofibroblast capsule and allows microbial discharge out of the skin.
- It shows that NK cells are the source of early IFN-γ release in the inflamed skin during *C. albicans* infection and discloses the mechanism through which NFATc2 regulates IFN-γ release.

3.2. Discussion

In our study, we show how the immune response to microbial skin infections, both of fungal and bacterial origin, can be divided in two phases: an early containment phase and the late elimination phase. For the proper evolution of the inflammatory process and the discharge of the pathogen, the host must activate different pathways, belonging to different systems, in an extremely regulated time line.

We found that the immune system, in order to eliminate pathogens from the skin, acts not only by directly fighting the microorganisms but also through the activation of the fibrinolytic system. This activation is

mandatory for the elimination of the pathogens from the skin through the formation of an ulcerative process.

The mechanism, by which the host fights and eliminates the pathogen, is tightly regulated by two main cytokines. First of all, for the containment phase, TGF-β, that has always been described as an anti-inflammatory cytokine involved in the healing process, is required for fibroblasts proliferation and collagen deposition thus allowing the confinement of the pathogen at the site of the infection. However, if this process is not counteracted, only the containment phase takes place. Therefore, another cytokine plays a fundamental role in avoiding the persistence of the pathogen at the site of the infection. This cytokine is IFN-γ, produced by NK cells, that acts by blocking the TGF-β activity on fibroblasts.

Furthermore, we demonstrate that IFN-γ is not only important for its action on fibroblasts proliferation. We show here that IFN-γ production is necessary for the degradation of the ECM as well as for the ulcerative process to take place. To perform these functions IFN-γ activates the fibrinolytic system with the formation of plasmin.

IFN-γ controls the process of plasmin formation by regulating the expression of the activator, tPA, and the inhibitor, PAI-1, of the plasminogen-to-plasmin conversion. We thus demonstrated a link between innate immune system and fibrinolytic system in order to fight and eliminate skin infections.

Moreover, the production of IFN-γ by NK cells is induced by the release of IL-2 by DCs in a NFATc2-dependent manner. Thus, by blocking the activity of this transcription factor, IL-2 release is inhibited, causing the lack of NK cells activation and IFN-γ production that provokes a decrease in plasmin formation, finally leading to the formation of a fibrotic response due to an imbalance between pro- and anti-fibrotic pathways.
This key role of NFATc2 in regulating the immune system response and the activation of the fibrinolytic system during skin infection could explain what has been shown in clinics, where inhibition of NFAT causes increased susceptibility to form cysts, especially in the presence of infections.

Since we were able to observe this phenomenon following both *C. albicans* and *S. aureus* infections, we hypothesize that it is a conserved mechanism of response that the host puts in action during skin infections of different origins.

We demonstrated that a properly activation of the TGF-β pathway is necessary at the beginning of an infection but can be detrimental if it persists over the time. This is also true for the activation of the fibrinolytic system.

During the last years the pathogenicity of fungal and bacterial skin infections has been widely studied. The scenario of an immune response to cutaneous infections has become more and more

complex. Laboratories around the World have demonstrated important roles for neutrophils in the early response to *C. albicans* at the skin level. However other cells have been associated with an immune response to skin infections, like NK cells, Langerhans cells, T cells and, recently, an important role was associated with γδ T cells (*4*, *5*).

Furthermore, it is known that epithelial and stromal cells are important in both the recognition and the reaction to pathogen invasion. We have demonstrated here that also fibroblasts are necessary for a proper containment of the infection, by avoiding the spreading of the pathogen that could lead to a systemic infection, and that their proliferation and deposition of ECM is controlled by the action of the innate immune system.

Here we have expanded the scheme of the host response to pathogen infection adding a clear role to the fibrinolytic system which has never been associated with the immune response to skin infection before. We have therefore disclosed a new connection between immune and non-immune responses, necessary for a proper eradication of cutaneous infections.

3.3. Future Perspectives

In our work, we demonstrate the importance of the transcription factor NFAT in the elimination of skin infections. However, *C. albicans*

expresses different PAMPs on its surface, each of which can be recognized by different receptors of the innate immune system (*6*). In the future, our laboratory will try to disclose the importance of each ligand in the activation of innate immunity in order to understand which fungal component is involved in the formation of the ulcerative process. The isolation of the different component of the fungal cell wall, *i.e.* mannans, glucans and chitins, will allow the study of the different pathways activated by each stimulus.

β-glucans are recognized by Dectin-1, already described to be able to induce NFAT activation, while Dectin-2 is one of the receptors involved in the detection of mannans (*7*, *8*). Using purified components will allow the identification of the specific receptor involved in the early activation of the immune response during *C. albicans* skin infections.

In addition to CLRs, *C. albicans*' PAMPs can also be recognized by TLRs (*6*). TLRs can signal via MyD88 for NF-κB activation, resulting in the production of several pro-inflammatory cytokines among which we can find TNF-α, one of the major pro-inflammatory cytokines. Signaling through MyD88 has been described to be important, in response to fungal infections, also in Langerhans cells (*9*). Therefore, in our model of infections, MyD88-dependent signals could play a role in the early phase of the inflammatory process through the production of TNF-α, which can induce increased neutrophils recruitment as well as mast cells activation. Further analysis of the involvement of this transcription factor will be needed.

The process we described of cross-talk between innate and fibrinolytic systems follows the formation of an abscess due to the recruitment of granulocytes and their subsequent accumulation at the site of infection. Another question that should be answered is if the presence of a particulate antigen is required for this early process to develop. Once we can determine which component is necessary to induce immune system activation, it is essential to understand its physical form in order to develop drugs that can be either soluble or particulate in order to better induce the discharge of the pathogen.

We demonstrate that skin infections are counteracted with a process divided in two phases: an early containment phase and a late elimination phase. During the containment phase, there is the formation of a capsulated abscess that confines the pathogen at the site of infection, impeding its spreading within tissues. This process of capsule formation has also been found to be active in some tumors and, sometimes, around artificial prosthesis (*10*–*12*). Once understood, the mechanisms that induce capsule formation, evaluation of differences and similarities between its development during microbial infections and tumorigenesis could be an important tool in order to better act on tumor progression.

3.4. References

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3.5. Publications

Rusconi M, Gerardi F, Santus W, Lizio A, Sansone VA, Lunetta C, Zanoni I, Granucci F. Inflammatory role of dendritic cells in Amyotrophic Lateral Sclerosis revealed by an analysis of patients' peripheral blood. *Sci Rep.* 2017 Aug 10;7(1):7853. doi: 10.1038/s41598-017-08233-1. PMID: 28798369

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