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Immunohistochemical analysis of infection-induced long-term resident group 1 ILC populations in the skin

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Summary

Innate lymphoid cells (ILCs) are a heterogeneous group of recently discovered innate lymphocytes that have critical functions for tissue homeostasis and in the local responses to infection. Group 1 ILCs, which consist of Natural Killer (NK) cells and ILC1, contribute to the control of viral infections. NK cells are considered circulatory cells that develop continuously from the bone marrow, whereas ILC1s have been shown to be tissue-resident, and to develop early during ontogeny. We wanted to test whether NKs and ILC1s can also differentiate into tissue-resident cells in the adult organism during infection. To this end, we analysed via microscopy cryosections of murine tissues, the skin, in which these cells are not generated during ontogeny, as well as the skin-draining lymph node and we looked at early and late time points post infection. Vaccinia virus (VV) was used to infect the ears skin of C57BL/6 mice and skin and lymph nodes were analysed by immunofluorescence analysis on day 3 and at week 5 post infection. Our analyses show that g1 ILCs are recruited to the skin upon VV infection and are able to persist long-term in the tissue. We also present evidence that local infection can dramatically alter the skin immune compartment and induce long-lasting changes in the tissue, in which g1ILCs may have an important role in it.

1. Introduction

The immune system is a complex network of cells and proteins responsible for protecting the host from disease-causing germs – like bacteria, parasites, viruses and fungi – foreign cells and tumors. Its main task is to detect a wide variety of “foreign” pathogens and orchestrate effective immune responses in order to supress pathogen replication and distribution.

The immune system can be classified into two subsystems, the innate immune system and the adaptive immune system, which cooperate in a harmonized manner to achieve immunity against infectious agents. The innate immune system provides a preconfigured immediate response against pathogens or other external stimuli, as the defence mechanisms are encoded in the host germline. In contrast, the adaptive immune system creates a customized response by generating antigen receptor diversity in order to provide a big pool of cells with a higher degree of molecular specificity to a diverse range of pathogens. Therefore, adaptive immunity can more effectively focus its resources to overcome pathogens that have evaded or overwhelmed innate immunity.

The innate immune system is composed of different evolutionarily ancient hematopoietic cell types, including dendritic cells, monocytes, macrophages and granulocytes. Recently identified innate lymphoid cells (ILCs), including natural killer cells (NK), act as part of the innate immune system, mainly exerting key functions in coordinating local immune responses.

1.1. ILCs

Until recently, only two subpopulations of lymphocytes were characterised without antigen receptor, being those known as NK cells and lymphoid tissue-inducing (LTI) cells. Nowadays these two cell types are considered part of the innate lymphoid cell (ILC) population.

ILCs are a growing heterogeneous family of immune cells involved in immunity and regulation of tissue homeostasis that can contribute to pathology in mice and humans. Due to the lack of adaptive antigen receptors, ILCs react to the microenvironment mainly through cytokine or germ-line encoded receptors (1).

ILCs are rapid producers of both pro-inflammatory and regulatory cytokines in response to local injury, inflammatory stimuli, pathogen infection or commensal microbiota perturbation. Recruitment of ILC to barrier tissues occurs during embryonic development and further migration of ILCs is would occur in the context of ongoing inflammation (2).

Like T and B-lymphocytes, all ILC subsets can be generated from a hematopoietic stem cell-derived common lymphoid precursor (CLP) cell in the bone marrow. Thus, CLP lead to an early innate lymphoid precursor (EILP). Moreover, EILP can bring on natural killer cells via a NK precursor (NKp) or all the remaining ILC subtypes through an innate lymphoid cell precursor (ILCP), which in turn originates from a common helper-like ILC progenitor (CHILP) (1,3) (Fig. 1).

Once generated, mature ILCs exit these sites, circulate through the blood and enter tissues following “codes” based on adhesion molecules and chemokines, similar to the ones used by T cells. However, ILC precursors (NKP and CHILP) may leave the bone marrow and complete their maturation in response to local signals, many of which also play a role in the differentiation of T cells into distinct effector subsets during inflammation (1).

ILCs can be further classified into group 1, 2 and 3 ILCs, each of which contain other subsets, based on differential expression of transcription factors, cell surface markers, and effector cytokines. All members of the ILC family are characterized by a classic

lymphoid cell morphology, but lack the expression of cell-surface molecules that identify other immune cell lineages, and are therefore defined as cell lineage marker-negative (Lin−) cells (4).

G1ILCs comprise NK cells and ILC1s. These cells require the T-box transcription factor T-bet for their development and function, and they produce interferon-gamma (IFN-γ) as their hallmark cytokine (5). Group 2 ILCs consist of one major subset, ILC2s, which are dependent on GATA3 and RORα (6), and produce type 2 cytokines, predominantly IL-5 and IL-13 (7). Group 3 ILCs include natural cytotoxicity receptor $(NCR)^-$ ILC3s, NCR^+ ILC3s, and LTi cells, all of which can produce IL-17 and/or IL-22 (8) and are dependent on the transcription factor RORγt (9). In this regard it has recently been suggested to classify ILCs into five subsets instead of three, based on their development and function, which are NK cells, ILC1s, ILC2s, ILC3s and LTi cells (Fig. 1 and 2) (3,10).

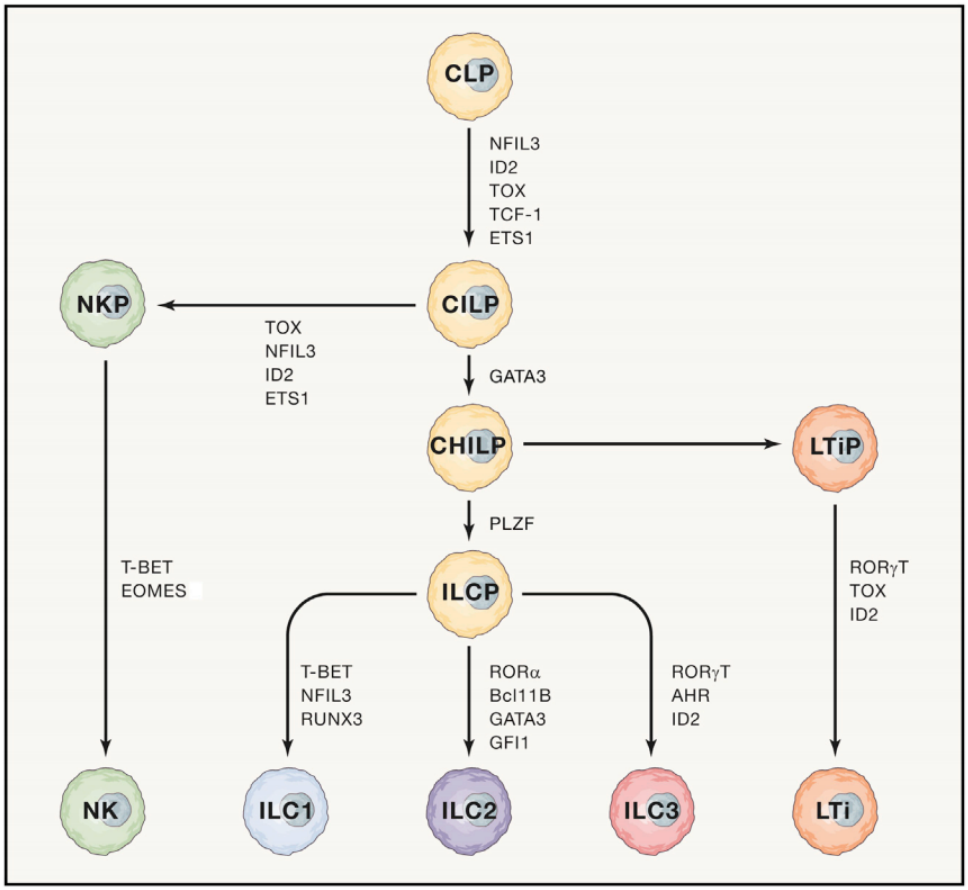

Figure 1. Differentiation process of ILCs. Drawing of the different developmental pathways of ILCs and the transcription factors required for each stage. All ILCs subsets differentiate from common innate lymphoid progenitors (CILPs), which in turn develops from common lymphoid progenitors (CLPs). CILP can differentiate into NK precursors (NKP) and thus to NK cells or into common helper innate lymphoid progenitors (CHILP). CHILP can further give rise to ILC1s, ILC2s and ILC3s through innate lymphoid cell precursors (ILCP) or to LTi cells via lymphoid tissue inducer precursors (LTiP). (3)

It has been proposed that ILC precursors represent the innate homologs of naïve T cells, as they mirror the phenotypes and functions of T cells. ILC1s, ILC2s, and ILC3s mirror $CD4^+$ T helper (Th)1, Th2, and Th17 cells, respectively, in terms of function, whereas natural killer (NK) cells mirror the functions of $CD8^+$ cytotoxic T cells. ILCs and T cells play key roles in orchestrating the most appropriate immune response to the threat faced by the individual. ILC1s and Th1 cells fight intracellular pathogens, such as viruses, as well as tumours; ILC2s and Th2 cells respond to large extracellular parasites and allergens; and ILC3s and Th17 cells combat extracellular microbes, such as bacteria and fungi (Fig. 2) (3).

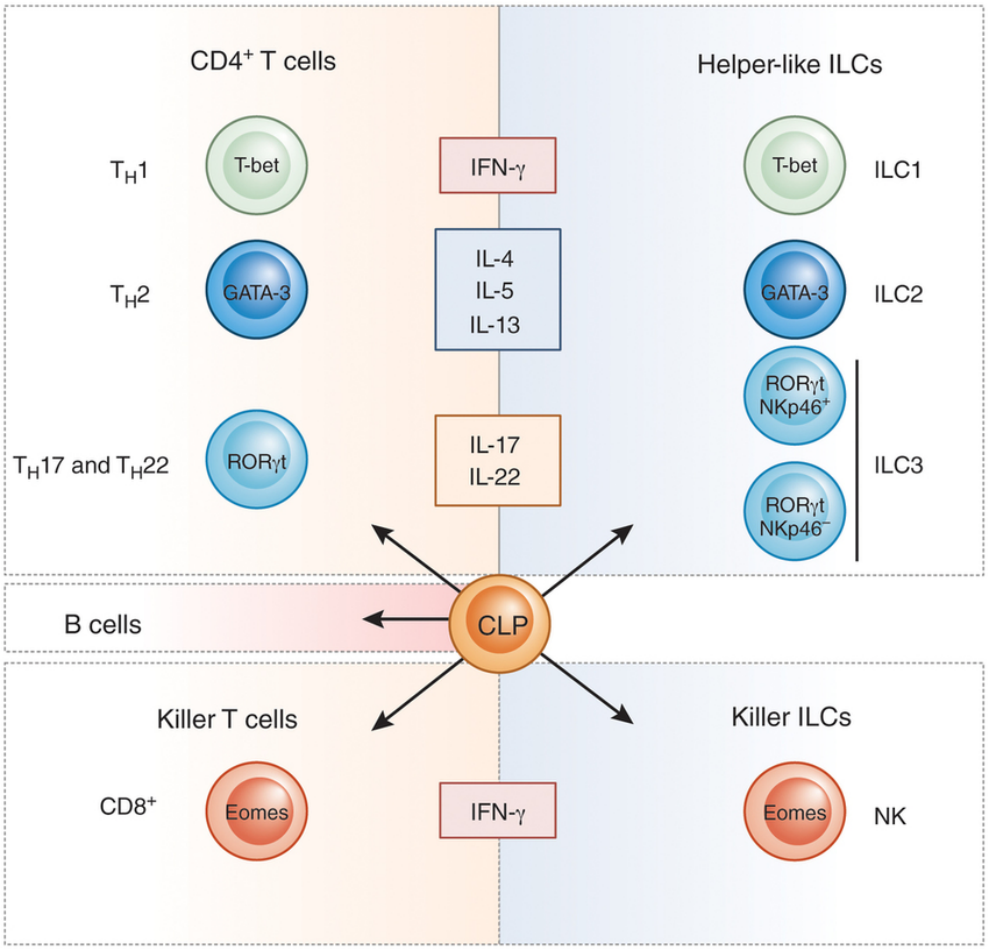

Figure 2. The diversity of ILCs mirrors the diversity of T cells. The main transcription factors that drive the function and differentiation of the different cells are labelled inside each type. Labels in the middle indicate the main cytokine produced by each subset. (11)

Indeed, in contrast to T cells, these lymphocytes have a thymus-independent development and lack antigen receptors encoded by rearranged genes, so do not undergo clonal selection and expansion when stimulated. For this, their response is quicker (1,3). Differing to naïve T cell activation, which requires T cell receptor–MHC

interactions in the lymph nodes, ILCs are activated in the tissue by sensing epithelial-cell-, stromal-cell- or myeloid-cell-derived signals such as alarmins, cytokines, and other inflammatory mediators expressed by tissue-resident cells, which enable rapid immune responses to environmental cues (12).

At early time points after infection, both ILCs and T cells are active. ILCs can express major histocompatibility complex (MHC) class II molecules and process antigens, thereby regulating the activity of antigen-specific T. In turn, these T cells produce interleukin-2 (IL-2), promoting ILC activities. Both types of cells generate positive-feedback loops, amplifying and regulating their responses, but they also compete for the same inducer cytokines and survival factors. Thus, these two types of lymphoid cells mirror one another's activities partially, thereby ensuring the timely orchestration of the immune response (3,13).

1.2. ILC1s and NKs

G1ILCs are defined based on their ability to produce interferon-γ (IFN-γ), the expression of the T-box transcription factor T-bet and co-expression of activating receptors NKp46 and NK1.1. Both NKs and ILC1s play a key role in protecting the organisms against pathogens, viruses and tumors.

Despite the similarities, ILC1 and NK cells differ in several aspects in terms of differentiation, localization, phenotype and function (Figure 1). Still, they are difficult to discriminate, particularly under conditions of infection and inflammation, as the markers used to identify them are modulated during immune responses. Indeed, recent data suggest that ILC1s and NK cells might have different phenotypes and functions in different tissues (14–16).

NK cells are considered a counterpart of memory CD8+ T cells, as both play an important role in cytotoxicity and INF-γ production and both, but not all CD8+ T cells (such as TRMs), express the transcription factor Eomes and T-bet. On the other hand, ILC1s can be comparable to Th1 cells due to their expression and dependence on T-bet but lack of Eomes and due to their production of INF-γ. By contrast, NK cells develop in a strictly Eomes-dependent manner, but only partially require T-bet, at least for terminal NK cell maturation (5,14,17,18).

NK cells are dedicated cytotoxic cells that circulate in the bloodstream whereas ILC1s are generally non-cytotoxic or weakly cytotoxic and function as a first line of defence against infections with viruses and certain bacteria (3,15). In line with this, tissue

resident-ILC1s respond to infection through the production of INF-γ, but have not yet been shown to directly kill infected cells.

Some parabiosis studies have shown that, in absence of infection, NK cells circulate through vasculature while on the contrary ILC1s are mostly tissue resident in almost all organs in mice (19). During inflammation, ILC1 residency is maintained, and NK cells may also be recruited into the tissue (19,20). This underpins their ability to respond quickly to any tissue perturbation and so their critical role in regulating tissue homeostasis and repairing (21).

1.2.1. NK cells

NK cells were first described over 40 years ago and derive their name from their ability to spontaneously lyse tumor cell lines in vitro. They recirculate through both lymphoid (spleen and lymph nodes) and nonlymphoid (liver, lung and blood) tissues, scanning them for infected or malignant cells (16).

NK cells are lymphocytes that participate in different facets of immunity, such as immunosurveillance, immunoregulation or instructing and regulating immune responses by producing pro-inflammatory cytokines. They can also produce granzyme, perforin, and granulysin and thereby participate in the direct destruction of microorganisms and neoplastic cells. Besides, they play key roles in the pathogenesis of inflammatory and infectious skin diseases (16).

NK cells can be activated in response to interferons or certain dendritic cells- and macrophage-derived cytokines. However, unlike T cells, killing by NK cells is triggered by germline-encoded receptors that recognise molecules on the surface of infected or malignantly transformed cells, which involves changes in the expression of MHC-I molecules. The interaction between NK cell receptors and their ligands delivers either inhibitory or activating signals. Activating receptors trigger the NK cell to kill its target, whereas inhibitory receptors inhibit signals from activating receptors (16,22).

NK cells participate via antibody-dependent cellular cytotoxicity (ADCC), as they carry receptors for the constant region of immunoglobulins (Fc receptors), which entails NK cell's activation (16,23). An important finding about the regulation of the quick response of NK cells was the identification of MHC-I-binding receptors on its surface, such as killer lectin-like receptors (KLRs), like Ly49 receptors (only in mice), (24) or killer cell immunoglobulin-like receptors (KIR) (25). The interaction of these inhibitory receptors with MHC-I-expressing cells prevents NK cells' activation and maintain self-tolerance, while cells lacking MHC-I are not protected from NK cells-damage (23).

In addition to the KIR and KLR receptors, NK cells also express activating receptors that sense the present of infection or other perturbations more directly, called natural cytotoxicity receptors (NCRs), such as NKp30,NKp44, NKp46 and specifically NKG2D (23).

Because of their invariant receptors, NK cells are classified as part of the innate immune system. Activated NK cells serve to contain virus infections while the adaptive immune response is generating antigen-specific cytotoxic T cells and neutralizing antibodies that can clear the infection. Stimulated NK cells can secrete large amounts of the cytokine INF-γ, what is crucial in controlling some infections before the INF-γ produced by activated CD8 cytotoxic T cells becomes available. Furthermore, the early production of INF-γ by NK cells may influence CD4-T cells to respond against infectious agents (22).

1.2.2. ILC1 cells

ILCs can be found in both lymphoid and non-lymphoid tissues in both mice and humans, but typically reside in barrier tissues that are at the interface between the host and the environment. ILC1s have been predominantly studied in the mouse liver, a common site of viral dissemination and replication. However, the list of tissues in which ILC1s have been identified is readily growing, and currently includes liver, spleen, salivary gland, small intestine, lung, adipose, skin, kidney, uterus, and peritoneal cavity (5,19,20,26–28).

Studies using parabiotic mice evidenced that ILC1s maintain long-term residency and appear to be maintained predominantly via local self-renewal rather than through replenishment from blood-derived ILC1s or their precursors. This is in contrast to NK cells, which recirculate throughout the host during both homeostasis and inflammation (19,29–31).

The prototypic function of g1ILCs is potent expression of IFN-γ upon activation with cytokines or surface receptor crosslinking. IFN-γ plays important roles in the immune defence to intracellular pathogens, and cNK cells have been recognized for their critical functions in the immune defences against a variety of viral and bacterial pathogens (5,15). In most of these studies, all cells with NK-like surface phenotype and ability for IFN-γ production were regarded as cNK cells. However, it appears likely that in some of the earlier studies, ILC1s may have contributed to IFN-γ production but were not recognized as a separate lineage. Thus, specific roles of ILC1s, as compared to cNK cells, during immune defences to pathogens are only since recently being investigated. The identification of specific markers for ILC1s, such as CD49a expression in liver or

expression of CD127 and lack of Eomes in lamina propria (intestine) ILC1s, has made it possible to more accurately assess specific host protective roles of ILC1s compared to cNK cells (32).

1.3. NKs and viral infection

Innate immune responses partly are crucial to control and eliminate viral infections. Thus, it has been shown that NK cells play a critical role in innate immune defence against various viral infections in vivo, such as herpes virus, cytomegalovirus or vaccinia virus (33–36).

NK cells are among the first cells recruited to a site of viral infection (37) reason why they are an obvious target for viruses to modulate by direct infection. After encountering a potential target cell, NK cells make intimate contact with the target cell and then release their cytolytic granules. At a site of infection, NK cells intimately contacting an infected target cell are likely exposed to the virus possibly making them a target for infection (38).

NK cells possess innate cytolytic activity and can be further stimulated by cytokines such as IFN-a and b, IL-12 and IL-18 that are produced mainly by dendritic cells in response to viral infection. NK cells are also stimulated by direct interaction with dendritic cells and aid in stimulation of dendritic cells to instigate an adaptive immune response (39). Accordingly, NK cells act by lysing infected cells and impeding viral replication through release of cytolytic granules and antiviral cytokines such as INF-γ (40). Despite NK cells respond grounded on integration of positive and negative inputs they received, it has been shown there are NK activating receptors that specifically recognise infected cells (34,35,38).

It has been shown that infection with Vaccinia Virus (VACV or VV) provokes a significant increase in NK lysing target cells and that the recognition of VV-infected cells by human NK cells is, in part, mediated by NKp30, NKp44 and NKp46 (41). Those, represent the major receptors driving activation of NK cells, despite the different activating receptors for NK cells to recognise different types of target cells varies depending on the ligand expression by target cells (42).

Vaccinia virus belongs to the poxvirus family, and it was the virus used as a live vaccine against smallpox, also known as variola virus, which was finally eradicated in 1980. Since then, VACV has been used as an advantageous research tool as well as an application for live vaccines because of its facility as a foreign-genes-expression vector. The search for the identification of VACV virulence genes with the aim of creating stable attenuated vaccines resulted in the discovery of some of its proteins that inhibit the innate response

against its infection (43). Upon poxviral infection, NK cells are activated, expand, and accumulate at the site of infection, and the activated NK cells are important for recovery of the infection (34,42). It has also been shown that NK activation and its effector function in response to VV is directly regulated by type I INF (44).

1.4. Memory

The concept immunological memory refers to the ability of the immune system to respond rapidly and provide enhanced protection of the host against a previously encountered pathogen. Long-lived memory cells are generated after initial infection and display heightened responses upon secondary challenge with the same pathogen (45).

Because cells of the innate immune system lack the ability to undergo somatic rearrangement of their receptor genes, it was hypothesized that these cells, including NK cells and ILC1s, lack antigen specificity and therefore cannot develop classical immunologic memory. In this way, natural killer (NK) cells have historically been considered short-lived cytolytic cells that can rapidly respond against pathogens and tumors in an antigen-independent manner and then undergo cell death (46). Nevertheless, O'Leary JG et al. stablished a model of chemical hapten-induced contact hypersensitivity and showed NKs can acquire immunological memory in a manner similar to that of T and B cells (47).

Indeed, in C57BL/6 mice, the activating receptor Ly49H is expressed on ~50% of NK cells and binds with exquisite specificity to the mouse cytomegalovirus (MCMV)-encoded glycoprotein m157 expressed on infected cells to drive the expansion of virus-specific NK cells during the acute phase of MCMV infection (35,48,49). Sun et al. made use of the cognate recognition of m157 by Ly49H+ to establish the specificity of the enhanced host protection provided by memory Ly49H+ NK cells upon re-exposure to MCMV (50).

1.5. Aim of the thesis

NK cells are generally considered as circulatory cells (19,20), however it has recently been suggested that in some tissues, such as female genital track (51) and salivary gland (52), these cells can exist as tissue-resident NK cells (trNK). It has been proposed that these tissue resident-NK cells are generated early during ontogeny. It remained unclear, however, whether trNK cells and ILC1s could also be generated in adult organisms a consequence of infection, reminiscent of the differentiation of tissue-resident memory T cells (53).

The aim of this study is therefore to test whether it is possible to generate a pool of tissue-resident NKs and ILC1s during infection in tissues where these cells are not generated during ontogeny, and whether this group 1 ILCs can persist in the tissue after infection and in which location. To this end, we chose the skin as target tissue as it is an organ almost devoid of g1 ILCs. In order to generate a potent infection, and thus a large pool of these cells, we used fully replicating virus, vaccinia virus (VACV), engineered to express the NK-specific glycoprotein m157 (35,54).

Lymph nodes are classified as peripheral or secondary lymphoid organs, which maintain mature naïve lymphocytes and where adaptive immune responses are initiated after an innate immune response to the infection has occurred. If a pathogen is detected in a tissue, immune cells in the local draining lymph node will expand and induce long-lasting changes in it (55). Dutton, Emma E et al. provided evidence of ILCs residing in lymph nodes and, in particular, they showed that ILC1s are able to migrate from tissues to lymph nodes, and can therefore contribute to the initiation of adaptive immunity (56). Indeed, TRMs from tissue also were shown to migrate and form resident populations (57).

For these reasons, we also found it interesting to analyse the cervical lymph nodes, which drain the ear skin and thus look at how this innate cells alert the rest of the immune system to the presence of a pathogen.

2. Materials and Methods

Mice infection

Seven C57BL/6 mice used in this project were bred in the pathogen-free animal facility of the Institute for Systems Immunology Würzburg. Five of these mice were infected epicutaneously on the ear skin using Vaccinia Virus (VACV). In brief, mice were anaesthetised prior to the infection with 100µl/10g body weight of 70 mg/kg Ketamin 25 mg/ml and 10mg/kg Xylazin 20mg/ml. Then each ear was infected with $2*10^6$ pfu VACV in 5µl PBS, which was spread over the entire tissue with the aid of a 23G needle. After applying the virus, ears were delicately scratched 25-30 times with the tip of the needle in order to facilitate the entry of the virus. The infection process was conducted in the animal facility of the Institute, following all hygiene and sterility requirements.

Tissue harvest and preparation

Two mice were sacrificed on day 3 and three at week 5 post infection using CO_2. Ears and draining lymph nodes were harvested and fixed overnight at 4°C in 700µl of 1% FA (BD Cytofix/Cytoperm diluted 1 in 4 in PBS). After fixation, tissues were washed in PBS and dehydrated overnight at 4°C 500-1000 uL of sucrose 30% in order to preserve tissue integrity. Eventually, the excess sucrose was removed and the dehydrated tissues were placed into cryomoulds containing Tissue-Tek® O.C.T.™, quickly frozen on dry ice and stored at -80°C.

Tissues were cut at -17°C using cryostat and passed onto microscope slides. Superfrost Plus slides were used for lymph nodes, whereas, in order to minimise tissue detachment during immunostaining, Superfrost Plus Gold slides were instead used for ears.

Staining and mounting

For the immunofluorescence staining, tissue sections were first re-hydrated with PBS for 10 minutes at room temperature and, after removing the excess solution by decanting, blocked with Immunofluorescence (IF) Blocking buffer, composed of 1%BSA, 1% gelatine from cold water fish, 0.3% Triton X-100 and supplemented with 4% horse serum, 2% normal mouse serum and 0,01% sodium azide. Each slide (which contained 3-6 tissue sections) was blocked with 140 µl of IF blocking buffer, covered with parafilm to prevent sections from drying out and incubated 40 minutes at room temperature in a dark humidified chamber.

After blocking, slides were stained with 140 µl/slide of antibody cocktail solution. In brief, antibodies were diluted in IF blocking buffer and centrifuged for 10 minutes at 13,3 rpm at 4°C. After removing the blocking buffer from the slides (using a pipette or by decanting), the antibody cocktail was added onto the slides, which were then covered with parafilm and incubated at 4°C in a dark humidified chamber.

For primary antibodies, slides were incubated overnight whereas for secondary antibodies slides were instead incubated 4 hours at 4°C. After each staining step, slides were washed for 10 minutes in PBS. After staining and washing, Fluoromount Aqueous Mounting Medium was used to fix the coverslips on the support glasses for immunofluorescence microscopy. Mounted slides were then kept at room temperature for at least 30 minutes to let the mounting medium dry before analyzing. Stained mounted slides were then stored in the dark at 4°C.

Antibodies

The used antibodies (Ab) are summarized in the tables below.

Table 1. Description of the primary antibodies (Ab) used for the immunofluorescence staining.

Primary Ab	Conjugate	Isotype	Clone	Manifacturer	Catalog n.	Dilution
CD3ε	BV421	Arm. Ham. IgG	145-2C11	BioLegend	100341	300
CD3	AF594	Rat IgG2b, κ	17A2	BioLegend	100240	300
Keratine 14	-	Chicken polyclonal	-	BioLegend	906004	200
MHC-II (I-A/I-E)	AF700	Rat IgG2b, κ	M5/114.15.2	BioLegend	107621	100
Eomes	-	Rabbit Monoclonal	EPR19012	Abcam	EPR19012	800
NKp46/NCR1 (CD335)	-	Goat Polyclonal	-	R&D systems	AF2225	300
CD8a	PE	Rat IgG2b, κ	5H10	Invitrogen	MCD08047	200

Table 2. Description of the secondary antibodies (Ab) used for the immunofluorescence staining.

Secondary Ab	Conjugate	Manifacturer	Catalog n.	Dilution
Donkey anti Goat	AF488	Invitrogen	A32814	2000
Donkey anti Rabbit	AF647	Invitrogen	A32795	2000
Donkey anti Chicken	AF405	Jackson ImmunoResearch	703-475-155	1000

Microscopy

Immunofluorescence microscopy was performed mainly with Virtual Slide Microscopy (model VS200; Olympus), which allows manual loading of one to six standard slides, along with any associated meta-data, and enables the visualization of 5 different parameters. It can create high-resolution brightfield images and can also scan in full multi-fluorescence mode. Image processing was then performed using its own software.

Confocal microscopy (Leica DMi8) was performed for certain skin sections. This microscope is based on a point excitation mechanism on the sample and point detection of the fluorescent signal in a way that only the light produced by the fluorescence very close to the focal plane is detected, so that the optical resolution of the image is much

better than that of wide-field microscopes. Images were processed using Leica Application Suite X software and Imaris 9.2 Bitplane.

3. Results

Skin

Different tissue sections were imaged through Virtual Slide Microscopy and the most representative ones were chosen for the discussion. Our confocal microscopy approach allows for the visualization of 5 different parameters, so we stained the skin using the following markers: CD3, NKp46, MHCII, Eomes and K14.

CD3 was used to identify T cells, NKp46 to identify g1 ILCs, MHCII to identify antigen-presenting cells (DCs and macrophages) and Eomes to identify NKs and a small subset of CD3+ T cells. In order to discriminate between dermis and epidermis we used keratin 14, a marker for keratinocytes, which are the principal and most prominent cells of the epidermis.

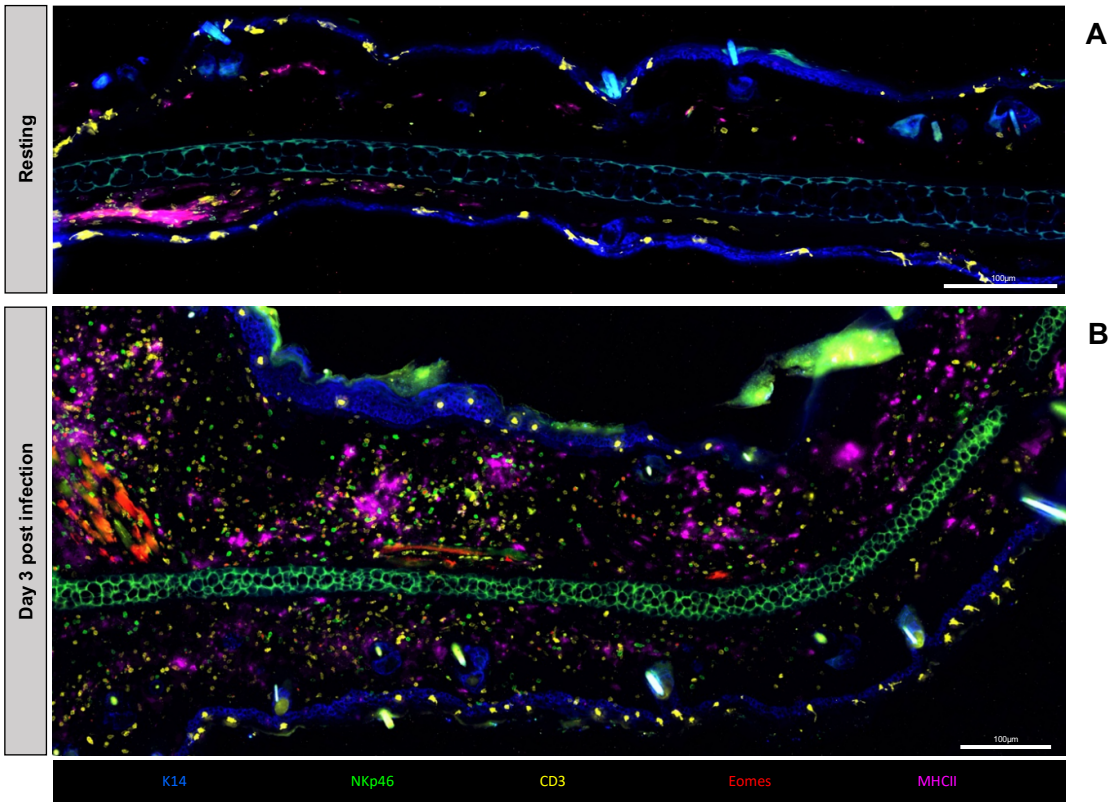

Figure 3. Virtual Slide Microscopy images of skin-sections of resting (A) and day 3 post Vaccinia Virus infection (B) ears. All skin sections were stained for K14 (blue) to identify the epidermis, NKp46 (green) to identify g1ILCs, CD3 (yellow) for T cells, Eomes (red) for NKs and subsets of T cells and MHCII (magenta) for APC cells. During the acute phase of the infection (B) immune cells infiltrate the skin.

The skin of humans and mice is known to be populated by a wide range of immune cells, including T cells and myeloid cells. When we analysed resting ear skin we confirmed that T lymphocytes (CD3+) and antigen-presenting cells (MHCII+) were present throughout the tissue (Figure 3A). However, unlike human skin, resting mouse skin was almost devoid of g1 ILCs. Most CD3+ T cells were located in the epidermis and despite their morphology resembled that of tissue-resident memory T cells (TRMs), we assumed they were dendritic epidermal T cells (DETC), a subset of epidermis-confined γδ T cells (58), since TRMs are very rare in resting skin.

Upon acute infection, corresponding to day 3 post VV infection, the whole tissue and its cellular niche have changed dramatically (Figure 3B). The tissue itself appears visibly thicker due to inflammation and infiltration of immune cell, in particular T cells (CD3+) and g1ILCs (Nkp46+). MHCII+ APCs also appear increased in infected skins, but to a lesser degree than T cells and g1ILCs.

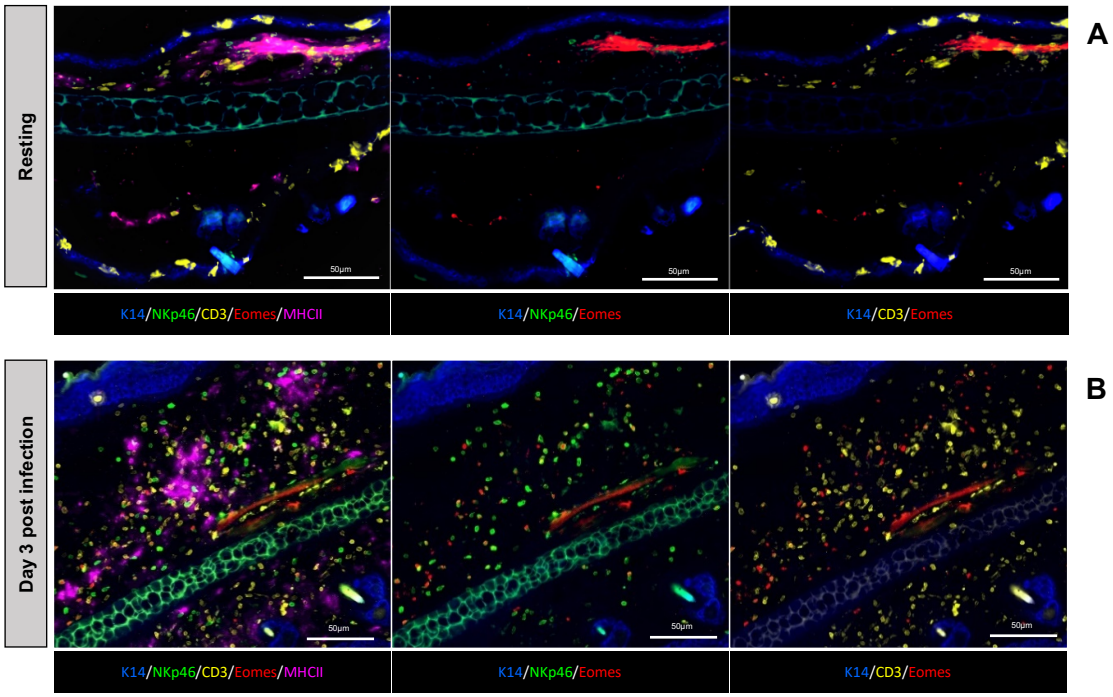

Figure 4. Zoom in of skin-sections from Figure 1. (A) Resting skin is almost devoid of NKp46+ g1ILCs, and the immune compartment is dominated by myeloid and T cells, which are the main populations without infection. (B) A large innate immune cell infiltration all along the tissue can be seen at day 3 post infection. NKp46+ cells, most Eomes-, have been prominently recruited to the infected tissue, as well as CD3+ cells.

A close-up view of the infected ear (Figure 4) reveals the presence of two different subsets of CD3+ cells in the dermis; CD3+Eomes- and CD3+Eomes+ cells. It is now well known that both T-bet and Eomes transcription factors play critical roles in the formation of CD8+ T cell effector and memory subsets (59) and it has been suggested that Eomes

is necessary for full effector differentiation of CD8+ T cells (60). Thus, Eomes is expressed in activated CD8+ T cells as well as resting and activated NK cells. Based on the images shown, on day 3 post infection CD3+Eomes+ cells represent only a small percentage of all CD3+ T cells.

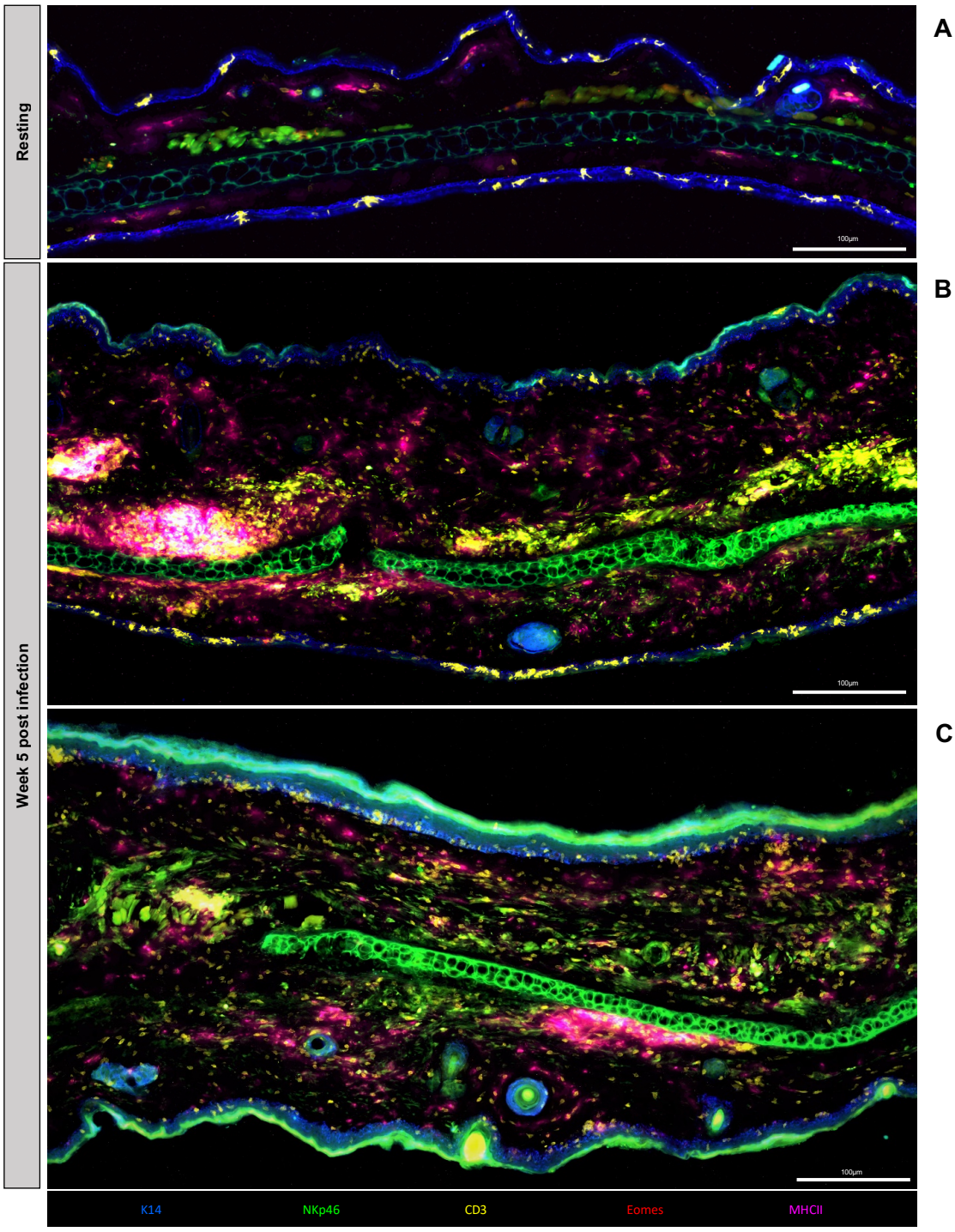

Figure 5. Virtual Slide Microscopy images of skin sections of resting (A) and week 5 post Vaccinia Virus infection (B and C) ears. All skin sections were stained for K14 (blue) to identify the epidermis, NKp46 (green) to identify g1ILCs, CD3 (yellow) for T cells, Eomes (red) for NKs and subsets of T cells and MHCII (magenta) for APCs. B and C are taken from the same skin cut, and show long-lasting changes in the immune skin niche.

Both NK and ILC1 cells express NKp46+ and can be discriminated by Eomes expression, since NK cells express this transcription factor but ILC1s do not. Similar to T cells, g1ILCs appear distributed randomly throughout the dermis, but, unlike T cells, none could be found within the epidermis.

Based on microscopy results, it seems that, at this early time point post infection, ILC1s outnumber NKs. This is in contrast with flow cytometric analysis (unpublished observation, data not shown) which reveals that over 85% of g1ILCs in the skin on d3 post infection are Eomes+ NKs, making them by far the most abundant g1ILC subset.

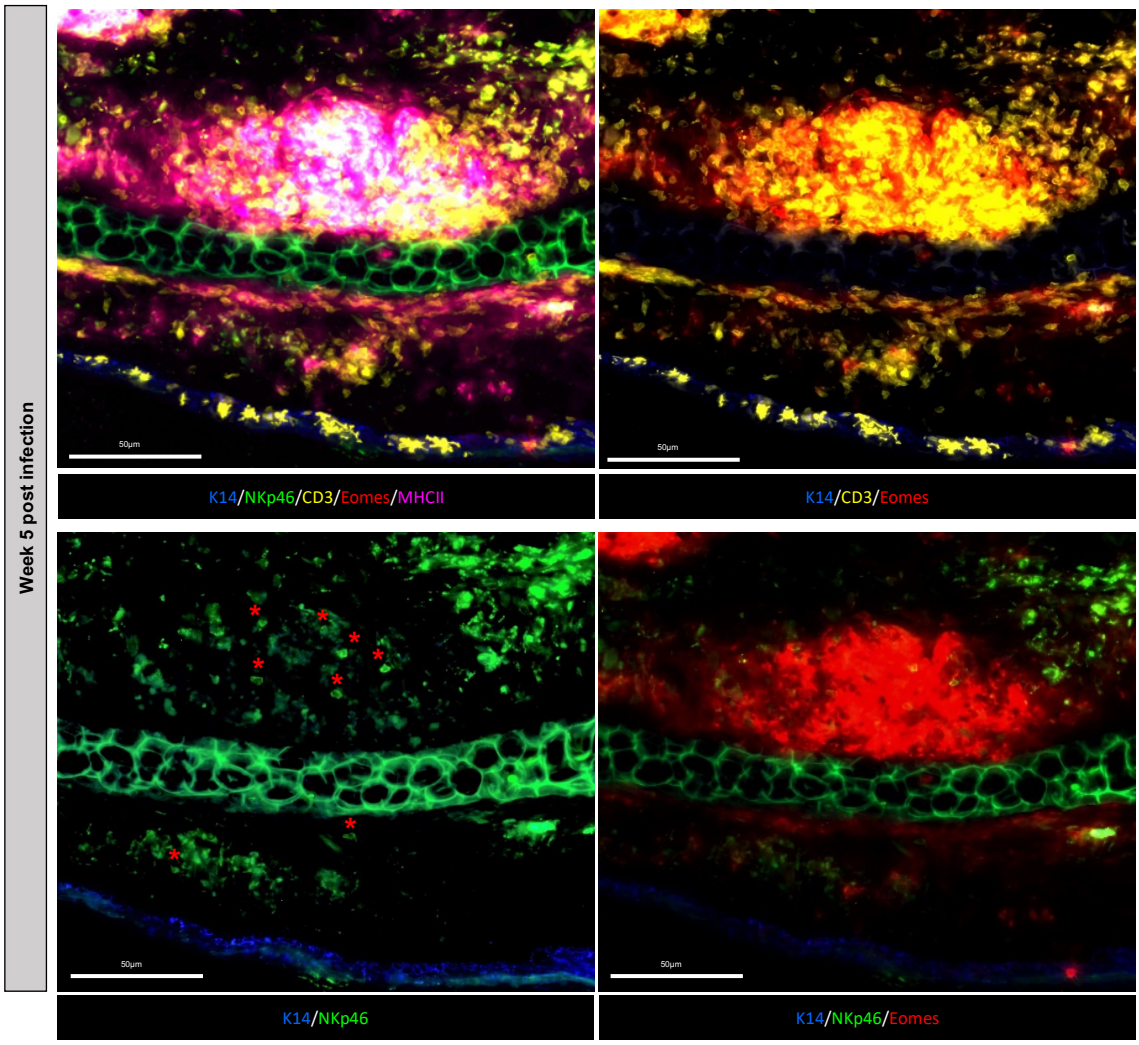

Figure 6. Zoom in of the skin sections in Figure 5B. Different single markers are shown in order to characterize better the different cells. Images show a leukocyte cluster with high presence of myeloid and T cells and few NKp46+ cells within it.

At memory time points (week 5) post infection, despite the fact that the infection should have been long cleared and the tissue should have returned to homeostatic conditions, the skin immune niche is far from resembling resting ears. As we can see in Figure 5,

changes in the quality and quantity of immune cells that start occurring in the early phases of infection appear to be long term, as the memory skin harbours greater numbers of T and antigen-presenting cells compared to resting ears, as well as g1ILCs.

In comparison with the tissue on d3 post infection (Figure 3B and 4B), where cells were disseminated throughout the tissue, at w5 post infection we can appreciate how most of the cells are accumulating in different clusters deep in the tissue (Figure 5,7 and 9). The number of MHCII+ and CD3+ cells has noticeably increased, and that is also true for epidermal T cells. Apparently, the quantity of T cells in the dermis appear similar on d3 and w5 post infection, although Eomes+ T cells are rarer at memory time points.

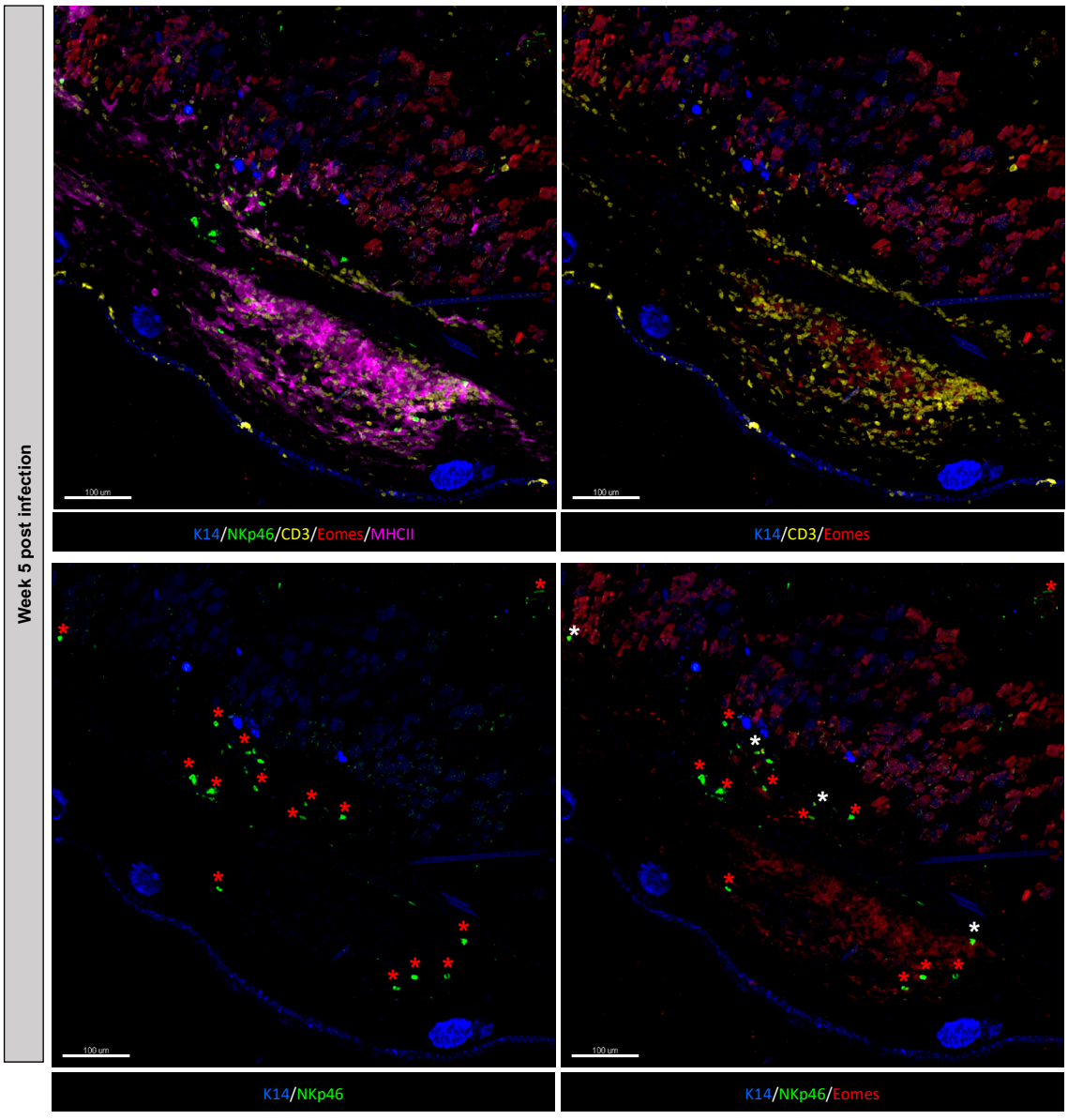

Figure 7. Confocal microscopy images of ears 5 weeks post infection. Images show a leukocyte cluster composed of mainly myeloid (MHCII+) and T cells (CD3+) with several NKp46+ cells within and around it.

A remarkable difference from d3 post infection is that wk5 epidermis is richer in CD3+ cells, many of which are likely TRMs, a T cell subset generally absent in resting and d3 post infection ears. T cells in general are distributed throughout the tissue, but are more often organised in cell clusters.

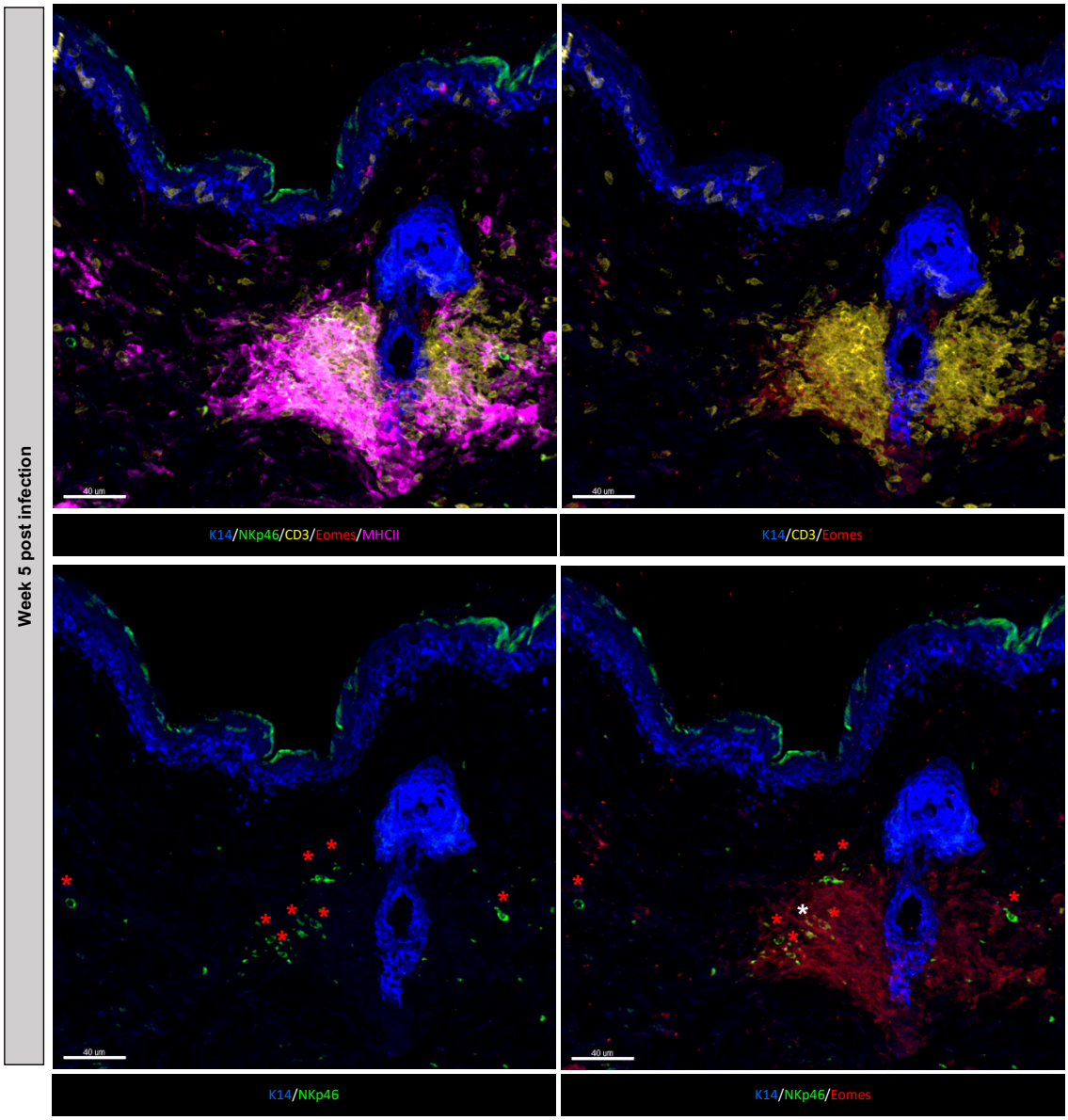

Figure 8. Confocal microscopy images of ears 5 weeks post infection. Images show immune cell cluster around a hair follicle.

As expected, g1ILCs were not very abundant in most of the sections analysed. Nevertheless, the ear skin is a large tissue and their presence in it at a memory time point can be appreciated in the images (Figure 5, 6, 7, 8 and 9). To make it easier to visualize them, NKp46+Eomes-cells are highlighted with red asterisks and NKp46+Eomes+ with white ones. The amount of g1 ILCs at week 5 post infection has

decreased dramatically from d3 post infection, this notwithstanding g1ILCs are still more abundant in memory ears than in resting ones.

As NKp46+ cells were difficult to identify and visualize clearly with the Virtual Slide Microscopy because of high auto fluorescence and background, we decided to further analyse our skin sections using Confocal microscopy (Figure 7, 8 and 9).

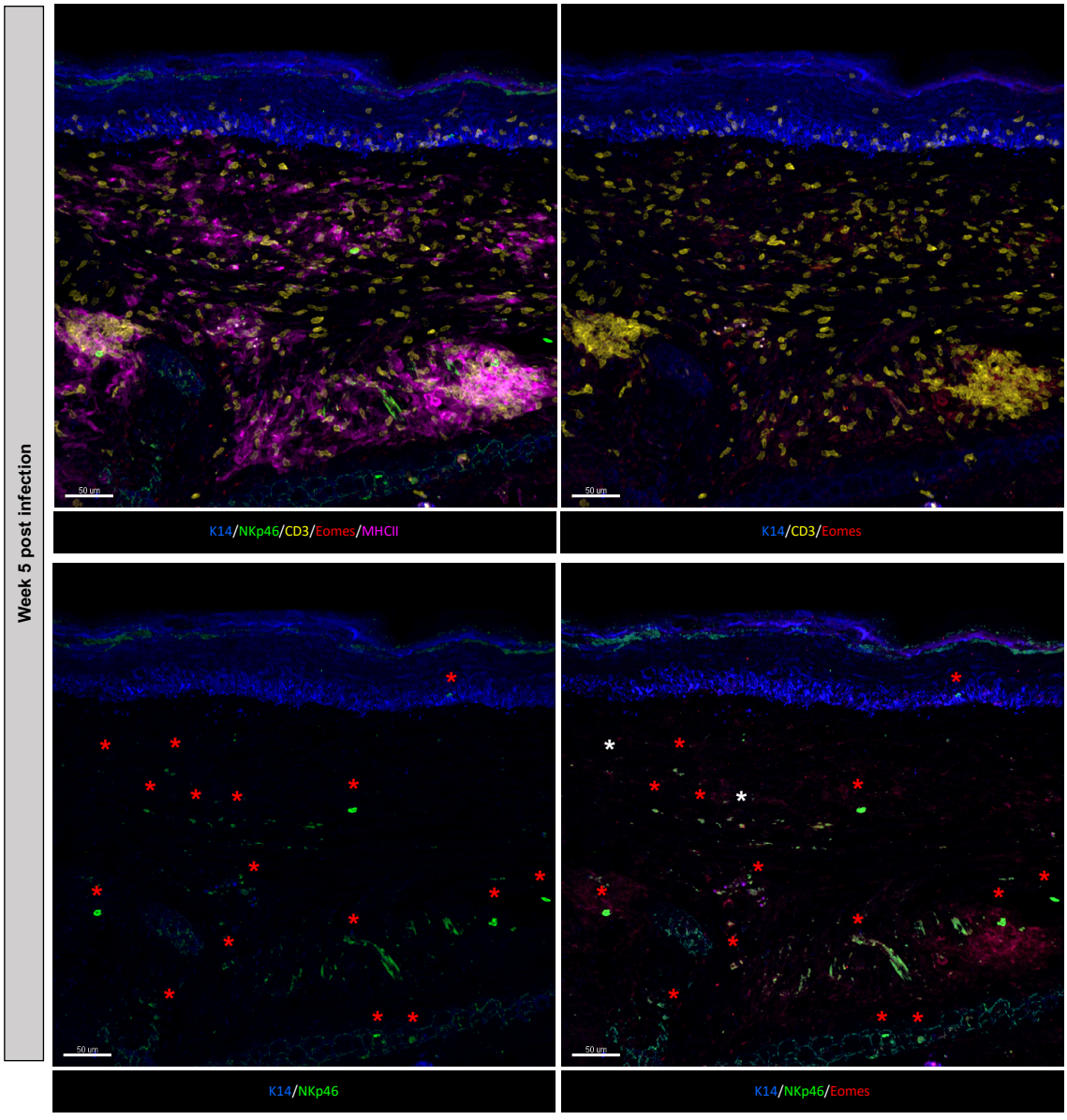

Figure 9. Confocal microscopy images of ears 5 weeks post infection. NKp46+ cells appear randomly distributed throughout the dermis, with some localizing within and near an immune cells cluster (bottom right). One NKp46+Eomes- cell can be detected in the epidermis.

At late time points, the g1ILCs population is reassembled dramatically. Eomes- are still the dominant g1ILC population, since just few of the NKp46+ cells were Eomes+ NKs. Most g1ILCs are found within immune cell clusters (Figure 6, 7 and 9), although some

isolated ones can be also observed throughout the dermis. Interestingly, at week 5 post infection, NKp46+ cells (largely Eomes-) were also found within immune cell clusters around hair follicles (Figure 8). Of note, we have also been able to spot one NKp46+ cell in the epidermis (Figure 9).

Lymph nodes

Extracellular fluid from tissues is drained by lymphatic vessels through the lymph nodes, and back into the blood, so lymphocytes circulate through the blood and the lymph and are abundant in lymphoid tissues and organs. Lymph nodes are classified as peripheral or secondary lymphoid organs, which maintain mature naïve lymphocytes and where adaptive immune responses are initiated after an innate immune response to the infection has occurred. For that reason, we decided to analyse the skin-draining lymph nodes (cervical lymph nodes), to investigate were g1ILCs were localize early and late after infection.

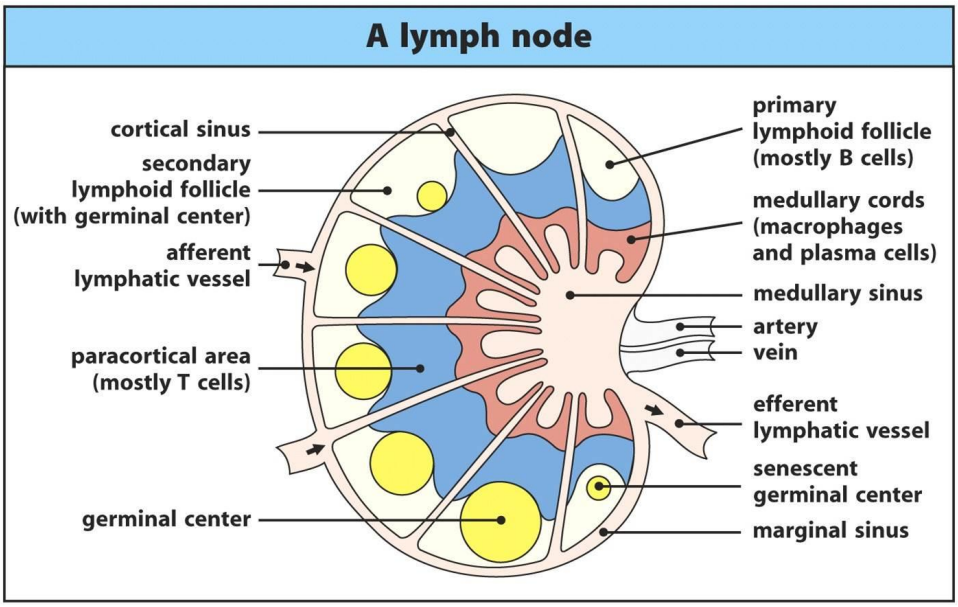

Figure 10. Organization of a lymph node. The cortex is composed of an external cortex of lymphoid follicles made up mainly of B cells and of paracortical areas where T cells and dendritic cells reside. Secondary follicles contain what is denominated germinal centers, where B cells proliferate when an immune response is under way. Antigens are carried mainly by phagocytic dendritic cells and macrophages from the tissues to the lymph node by lymph draining from the body via the afferent lymphatics. Lymph leaves via the efferent lymphatics in the medulla. The medulla consists of medullary cords with macrophages and plasma cells Naive lymphocytes enter the node from the bloodstream through afferent lymphatic vessel and leave with the lymph through the efferent lymphatic (22).

For lymph node staining, we used a similar panel to the one we used for the skin. CD3 was used to identify T cells, NKp46 to identify g1 ILCs, MHCII to identify antigen-presenting cells (DCs and macrophages) and Eomes to identify NKs and a small subset of CD3+ T cells. Since keratinocytes cannot be found in lymph nodes, we decided to use

CD8 marker instead and so we could verify the reliability of Eomes marker not only in lymph nodes but also in skin sections.

For the correct understanding of our results images is important to have in mind lymph node anatomy and the distribution of the lymphoid cells in it (Figure 10). A lymph node involves an external cortex, which is composed of different follicles containing mostly B cells, and a paracortical area made up mainly of T cells and dendritic cells. Lymphocytes migrating from the blood enter the paracortical areas first via High Endothelial Venules, and that is a reason why antigen-presenting dendritic cells and macrophages localize there (22).

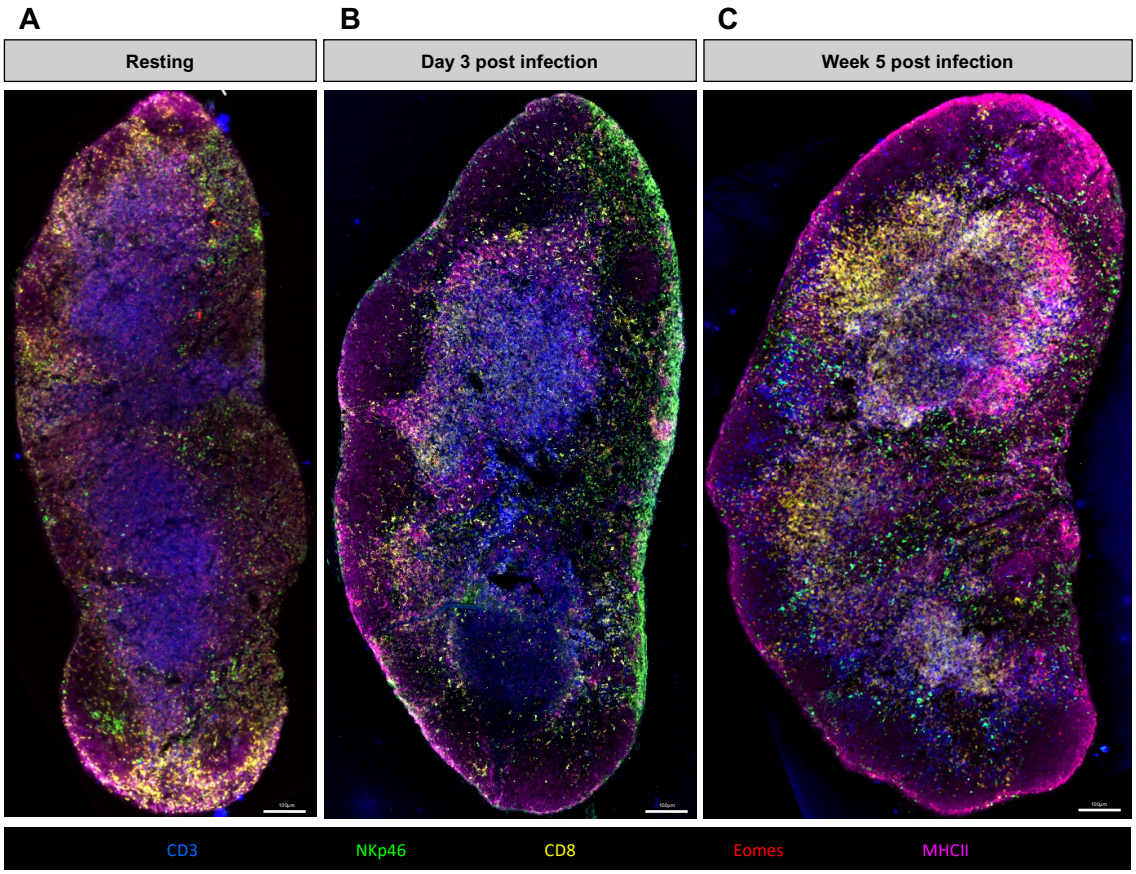

Figure 11. Virtual Slide Microscopy of longitudinal resting (A), day 3 post infection (B) and week 5 post infection (C) lymph node sections. All lymph node sections were stained for CD3 (blue) for T cells, NKp46 (green) to identify g1ILCs, CD8 (yellow) for CD8+ T cells, Eomes (red) for NKs and subsets of T cells and MHCII (magenta) for APC cells. (A) Without infection, NKp46+ cells can be appreciated mostly in inter-follicular areas in the paracortex around the T cell zone. (B) NKp46+ have been recruited to the medullary and marginal sinus and the amount of CD8+ cells in the paracortical area seems to have decreased.

Despite the images shown for resting lymph node are not exactly a longitudinal section, the paracortical area is observed well defined by T cells, with some of them being Eomes+ (Figure 11 and 14). NKp46+ cells do not seem to have a specific compartment in which to localize, but they appear to be found mostly in inter-follicular areas and in the paracortex around the T cell zone (Figure 12).

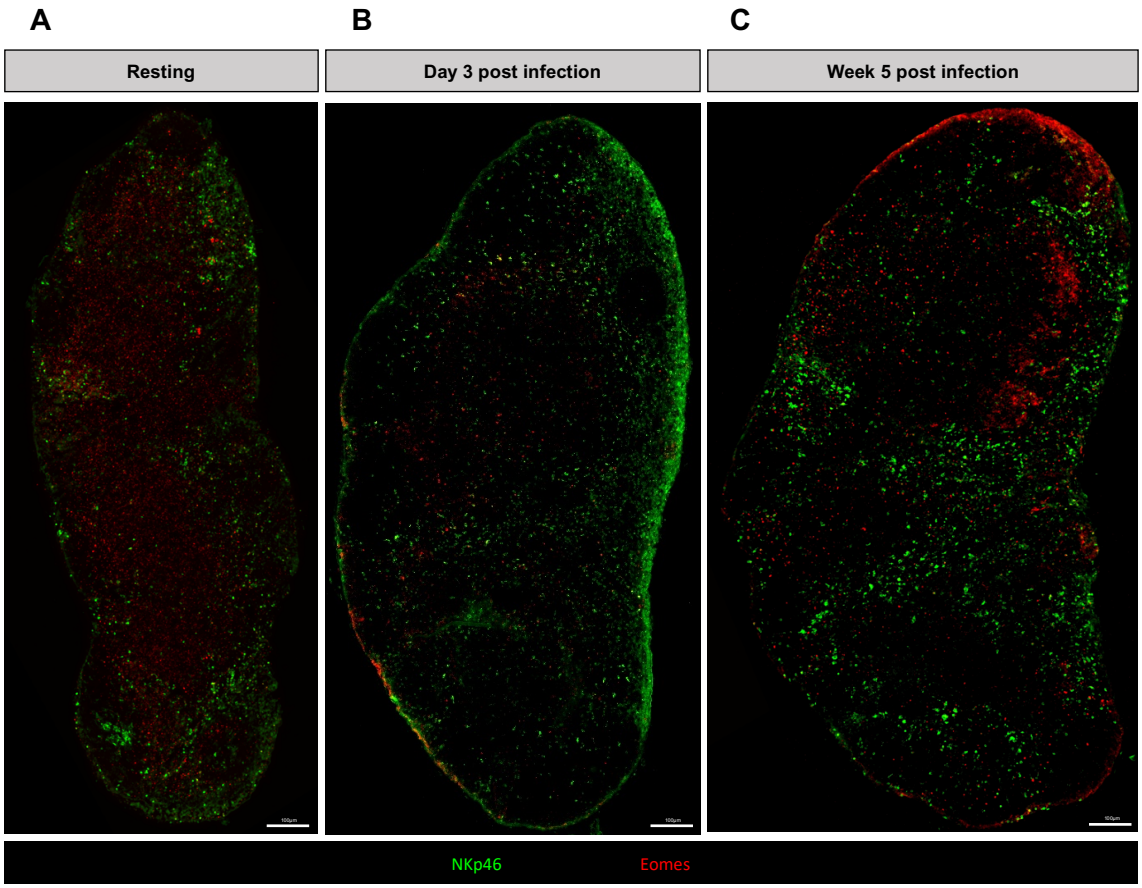

Figure 12. Virtual Slide Microscopy of longitudinal resting (A), day 3 post infection (B) and week 5 post infection (C) lymph node sections. Images show the distribution and location of NKp46+ cells on each time point.

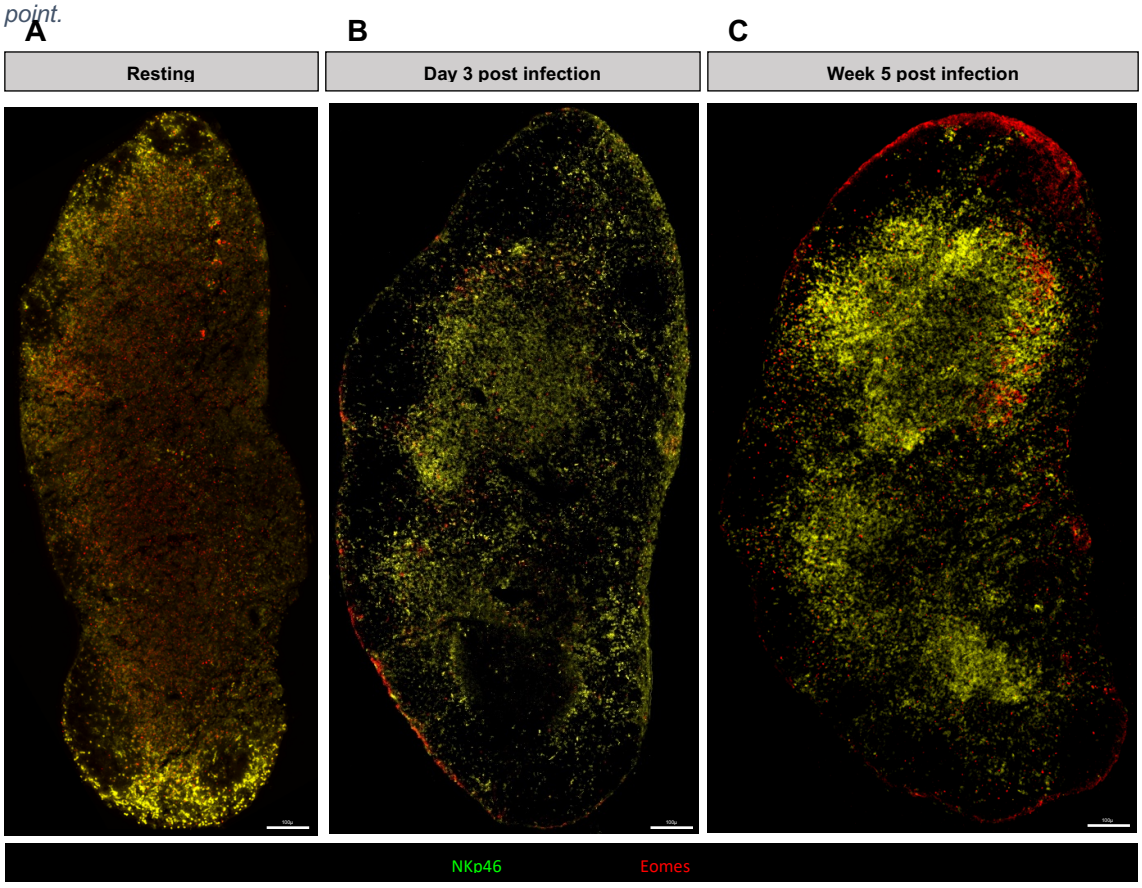

Figure 13. Virtual Slide Microscopy of longitudinal resting (A), day 3 post infection (B) and week 5 post infection (C) lymph node sections. Images show the distribution and location of CD8+ cells on each time point.

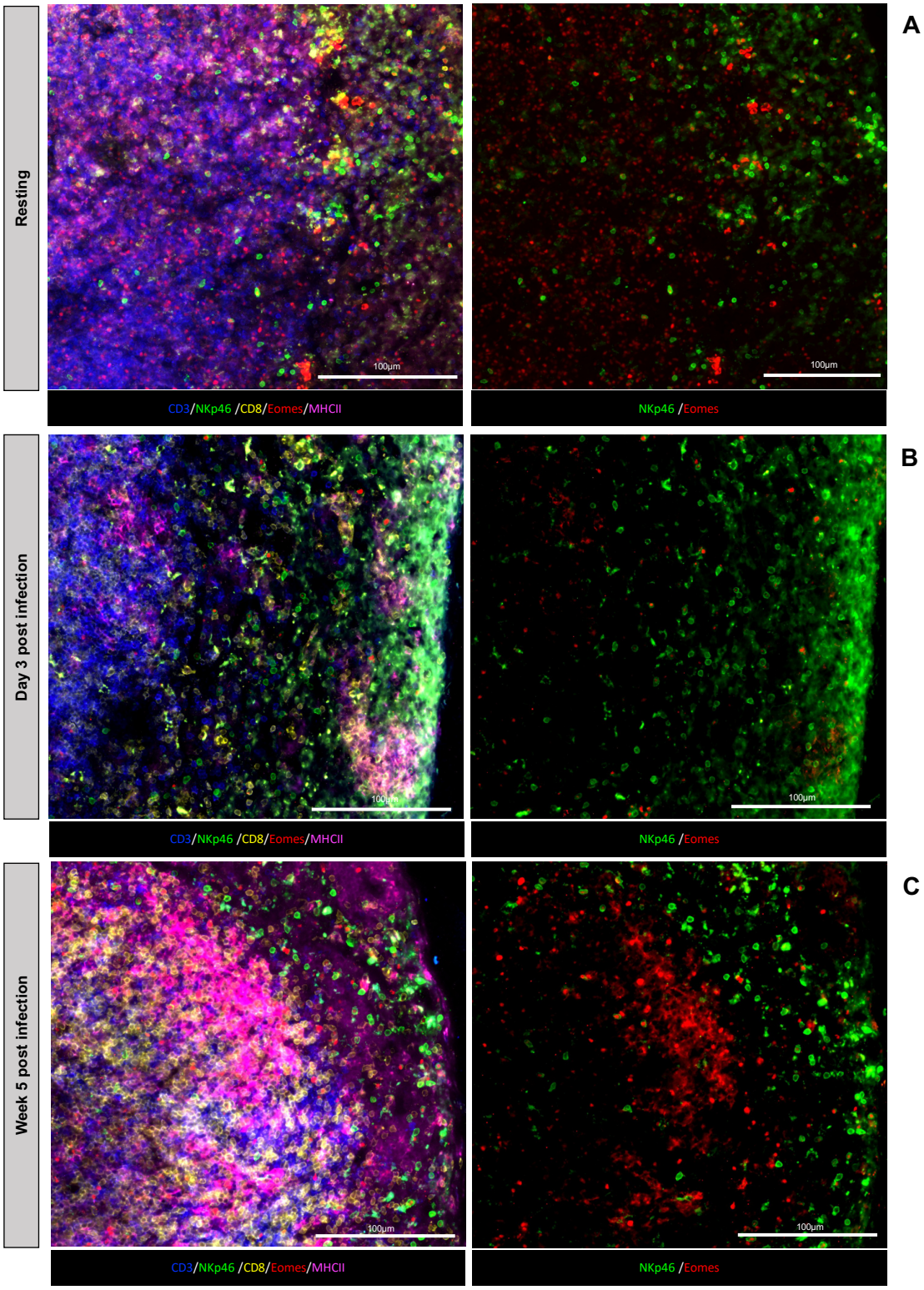

Figure 14. Zoom in of the lymph node sections resting (A), day 3 post infection (B) and week 5 post infection (C). Depicts show the distribution and localization of NKp46+ cells around the medullary area of the lymph node.

At d3 post infection the presence of MHCII+ cells is more intense around the outer part of T cell zone and the amount of CD8 cells in the paracortical area seems to have

decreased (Figure 13). The amount of NKp46+ cells allocated to the paracortical area do not seem to decrease in comparison with the resting tissue despite there is a clear accumulation of g1ILCs in the medullary sinus and marginal sinus, most of which seem to be Eomes- (Figure 12 and 14).

At w5 post infection, the anatomy of the lymph node resembles more the resting tissue rather than the lymph node on d3 post infection. Some of the T cells localize now in the secondary lymphoid follicle and the intensity of MHC-II marker has increased within the medullary cords, the region for macrophages and plasma cells, meaning there is a higher expression of MHC-II or more APCs cells (Figure 11). There is no longer appreciable recruitment of NKp46+ cells into the medullary and marginal sinus, observed on day 3 post infection, however the amount of g1ILCs in the lymph node seems to be a bit higher compared to resting lymph nodes (Figure 12).

4. Discussion

As the largest organ of the human body and the primary interface between the body and the environment, the skin provides a first line of defence, harbouring several types of immune innate and adaptive cells that provide essential protection of the body from injury and infection. Thus, its immune system has long been studied and described both health and disease (61–64). In this study, we have presented evidence that local infection can dramatically alter the skin immune compartment and induce long-lasting changes in the tissue.

At the steady state, the mouse ear skin appears like a rather empty organ from the immunological point of view, with only few myeloid cells scattered through the tissue and some DETCs lining the epidermis. During the acute phase of the infection, as a result of the inflammation the skin appears thicker than the resting one, and infiltrated by a number of T cells, myeloid cells and g1ILCs. The latter, interestingly, were almost absent in resting skins, suggesting that these ILCs could be recruited to the skin specifically during infections.

Interestingly, these changes that affect the skin at early time points post infection appear to persist to some degree also late after infection, once the pathogen has been neutralised and the tissue should have gone back to homeostasis. Five weeks post infection the skin is still rich in leukocytes compared to resting skins, in particular myeloid cells and T cells, the latter being abundant in both the dermis and the epidermis. At this time point these dermal T cells form often clusters with other immune cells either deep

into the tissue or around hair follicles, where they likely receive trophic factors and other cues that promote their presence in that specific location. As for epidermal T cells, which at the steady state are composed mostly of DETCs, at this late time point they are likely a mix of DETCs and tissue-resident T cells. Surprisingly, g1ILCs also appear to persist in the skin, and they can be found either alone or in clusters with T cells and myeloid cells, both deep into the tissue or around hair follicles, but almost exclusively in the dermis. This finding is remarkable since the infection is able to generate a persisting population of ILC1s and NKs that are almost absent at the steady state, suggesting that the skin immune niche can be reorganised in a way that it includes also cells that are new to the tissue. This has important clinical implications since the generation ex novo of persisting immune cells in a barrier tissue, such as the skin, suggest that these cells could synergise with TRMs and provide enhanced protection against pathogens.

Different subsets of immune cells have been described to shown memory phenotypes and functions within the skin but, as innate immune effectors, ILC1s and NK have long been considered to lack immunological memory. Due to their heterogeneity (65), g1ILCs is the most difficult ILC group to define and little is known about its physiological role in tissues. In the last decade it was shown that most ILC members are tissue resident (19) and recent studies reported an adaptive role for NKs either during recall responses with viruses (50,66,67), haptens (68) or induced by cytokines (69). Distinct subsets of tissue-resident NK cells (trNKs) and ILC1 cells have been described in diverse organs and tissues, including the liver, uterus, thymus, adipose tissue, and skin, among others. Indeed, many of these trNK populations exhibit distinct tissue-specific phenotypes, functions, and developmental requirements (5,29,31,52,65).

In our study, infection-driven skin ILC1s appear to be more long lived than NKs, fact expected due to the literature already mentioned and cytometric analysis (data not shown), and both subsets are mostly surrounded by other immune cells or within cell clusters, but are also found alone along the tissue. During the analysis of different sections, we always observed g1ILCs in the dermis, so the finding of an ILC at the end of the epidermis was peculiar and unique. The localization of g1ILCs around the air follicles is important because hair follicles are known to be an immune privileged site due to the production of different cytokines, such as TGF-b, IL-7 or IL-15, which are known to mediate skin-memory T cell homeostasis, but that can also sustain ILC1s and NK cells. Thus, it would make sense to think that hair follicles are an ideal niche for T cells and g1ILCs (70). Furthermore, there are some studies showing that NKs cells and ILC1s can regulating T cell responses (71–73), and that could explain the frequent presence of g1ILCs within T-cell-rich areas in memory skins.

When an infection occurs in a tissue such as the skin, primarily free antigen and antigen-bearing dendritic cells are attracted by chemokines and travel from the site of infection through the afferent lymphatic vessels into the draining lymph nodes, where they activate antigen-specific lymphocytes. The latter, proliferate, differentiate and finally return to the bloodstream via the efferent lymphatic vessel to be carried to the tissues where they will act.

Despite ILCs were initially discovered in developing lymph nodes as lymphoid tissue inducer (LTi) cells, it was not until recently that it was shown whether ILCs migrate between lymphoid and nonlymphoid tissues and in what context. Conventional natural killer (cNK) cells are highly migratory (19) and are known to enter to lymph nodes directly from the circulation via high endothelial venules (74). cNK and ILC1s in lymph nodes rapidly produce effector cytokines, mainly INF-g, and modulate the microenvironment of the LN shaping the T cells responses generated (75,76). Studies have shown that DCs activate NK cells and that NK cells can influence DC maturation in vivo (77,78) and its production of INF-g is important for CD4+T cell Th1 differentiation (76,79).

It is known that at the steady state g1ILCs are distributed in nearly equal numbers in both the paracortex and medulla. Upon day 3 post infection, we were expecting NKs and ILC1s to localize around the afferent vessel and accumulate under the B cell follicles due to the arrival of infected and antigen-bearing cells and other studies about localization and behaviour of NKs in lymph nodes (74,75). Instead, we can appreciate they mostly localize in the medulla. A possible reason for the accumulation of g1ILCs at this site is the potential deployment of these cells to skin, the site of infection. Further experiments need to be performed to investigate more in detail the fate of these medullary NKs and ILC1s.

One of the fundamental characteristics of a classical memory response is that memory cells exhibit enhanced activation upon secondary stimulation. Therefore, some interesting future experiments would implicate a secondary infection, both with the same virus and another different pathogen. Flow cytometry analysis are needed for an accurate quantification of g1ILCs and to investigate whether these ILCs can modulate the skin immune niche.

Despite the generally good quality of most images, memory skin proved quite hard to cut and stain and often g1ILCs resulted poorly defined and not easily identifiable. The background and autofluorescence of the images at this time point was higher than for other time points and tissues and the NKp46 signal was dim, making it hard to identify g1ILCs. Thanks to flow cytometric analysis we are certain about presence of numerous

g1ILCs at late time points post infection in the skin, so one potential explanation for the difficult identification of g1ILCs could be because of the used markers or the staining protocol, despite those variables work good for the other time points. Another possibility would be that at memory time points skin g1ILCs downregulate the expression of the activating receptor NKp46.

Indeed, flow cytometric analysis (data not shown) reveal a larger amount of Eomes+ g1ILCs, both day 3 post infection and memory time point, that those regarded with microscopy. The possible explanations for these conflicting results are 2: either the Eomes staining on skin sections is not sensitive enough to detect all NKs, even the ones that express lower levels of the transcription factor, or there is an isolation bias when skins are digested in order to obtain immune cells suspensions for FACS analysis. Those both issues makes us reconsider the immunofluorescence staining protocol or even think about the possibility of using some Eomes reporter mice instead.

5. Conclusion

In this study we have shown through immunohistochemical analysis that g1ILCs are recruited to the skin following Vaccinia Virus infection, and are able to persist in the tissue. Local infection causes re-arrangement of the whole skin immune compartment and g1ILCs may have an important role in it.

Demonstrating the presence of g1ILCs at late time points following Vaccinia Virus infection through imaging and the possibility to distinguish NKs from ILCs by Eomes marker it is just the really beginning of the process of characterizing and understanding these recently discovered cells. Although the knowledge of these newly identified cells is increasing, many questions remain to be addressed from now on: what would be the role of these resident NKs and ILC1s? How do they phenotypically change, if so? Do they resemble to the trNKs already described in other tissues? Can these cells respond faster to skin re-infection compared to newly recruited g1ILCs? Would these memory cells respond faster to other pathogens different from Vaccinia virus? Answering these questions would enable a better understanding of not only group 1 ILCs but innate immunity in general, and provide insights into potential therapeutic strategies for human diseases.

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