Reinforcing involvement of NK cells in psoriasiform dermatitis animal model

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Abstract. Psoriasis (Ps) is a chronic inflammatory immune-mediated disease with skin and joint manifestations, characterized by abnormal and rapid proliferation of keratinocytes and infiltration of psoriatic lesions with immune cells. Extensive literature suggests that Ps is a T-cell mediated disease its pathogenesis being highly related to innate and adaptative immune cells. Although natural killer (NK) cells are involved in the inflammatory process of Ps through pro-inflammatory cytokine secretion (tumor necrosis factor α, interferon γ), their role in this pathology is not yet fully eluci-dated. In order to study the involvement of NK subpopulations in the pathogenesis of Ps we used the imiquimod-based mouse model of psoriasiform dermatitis and NK cells complex phenotype patterns from peripheral blood (PB) and spleen were investigated. Skin inflammation and the disease severity were assessed using *in vivo* measurements (erythema, desquamation and induration parameters, PASI modified score), splenomegaly assessment and histopathological evalu-ation. Phenotypic characterization of NK cells in imiquimod (IMQ)-treated mice was performed by flow cytometry, for both PB and spleen cell suspension. A large panel of surface markers was used: maturation and activation markers [cluster of differentiation (CD)49b, CD11b, CD43, CD27, KLRG1, CD335, CD69, CD28, gp49R, CD45R, CD11c] and markers for cytokine receptors (CD25, CD122, CD132). Our experimental data showed important differences in IMQ-treated mouse NK cell phenotype as compared to control group. The maturation markers (CD11b, CD43, CD27, KLRG1) were found increased on NK cells, in periphery and spleen, while CD49b$^+$NK1.1$^+$ was significantly lower, and the alterations correlated with the severity of the disease. Our findings reflect the immune engagement toward activatory profile of NK cells and draw attention to evaluating Ps intensity correlated with the mature profile of circulating NK cells.

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Abbreviations: APC, allophycocyanin; BD, Becton Dickinson; CD, cluster of differentiation; DC, dendritic cell; EDI, erythema, desquamation and induration; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; H&E, hematoxylin and eosin; FITC, fluorescein isothiocyanate; IFN, interferon; Ig, immunoglobulin; IL, interleukin; IMQ, imiquimod; K2-EDTA, kalium 2 ethylenediaminetetraacetate; KGF, keratinocyte growth factor; KLRG1, killer cell lectin-like receptor G1; LC, langerhans cell; NK, natural killer cells; PASI, psoriasis area severity index; PB, peripheral blood; PE, phycoerythrin; PE/Cy, phycoerythrin complex with cyanine; PerCP/Cy, peridinin chlorophyll protein complex with cyanine; R, receptor; RBC, red blood cell; RPMI, Roswell Park Memorial Institute; SD, standard deviation; SW/BW, spleen weight/body weight; Th, helper T cells; TLR, Toll-like receptor; TNF, tumor necrosis factor; UV, ultraviolet; VEGF, vascular endothelial growth factor

Key words: NK cells, imiquimod, psoriasiform dermatitis, inflammation

Introduction

Psoriasis (Ps) is a chronic inflammatory immune-mediated disease with skin and joint manifestations (1). Histologically it is characterized by abnormal and rapid proliferation of keratinocytes and infiltration of psoriatic lesions with immune cells, especially T cells and dendritic cells (DC) (2). Globally, Ps affects 2-4% of the adult population, especially Caucasians, women and men equally, and 0.1-1% of children; prevalence is dependent on age, ethnicity, geographic area,

and environmental factors (3). It can start at any age, from childhood to old age, but 75% of those affected developed the disease before the age of 40 years. Psoriatic arthritis affects up to 30% of patients diagnosed with Ps (children and adults) and approximately 50% of people who develop Ps observe changes in the nails (from fingers and/or toes). Ps is associated with several co-morbidities which include diabetes mellitus (4), obesity (5), hypertension (6), and cardiovascular diseases (7). Due to visible lesions, Ps reduces the self-esteem of patients and leads to a decrease in quality of life, depression and suicidal ideations (8).

Ps has a multi-factorial aetiology with important immunologic, genetic and environmental components (9). The main risk factors are ultraviolet (UV) exposure (10), medications (11,12), smoking (13), diet and obesity (14), alcohol intake (15), infections (16), and stress (17). It may occur in mild, moderate or severe forms, with lesions varying in appearance depending on the type of Ps: plaque (90%), guttate, pustular, inverse, and erythrodermic. Ps is unique to each patient, the severity of Ps lesions varies from person to person and the treatment depends on the type of Ps, severity, the area where Ps is located, patient age and medical history, and the effect that Ps has on the patient (physically and emotionally). Besides classic Ps therapies, such as topical treatment (for mild forms), UV light therapy (for moderate to severe forms) and systemic treatments (for moderate to severe Ps which has not successfully responded to topical treatments or UV therapy) (18) biological therapies (19) have entered the panel of therapeutical approaches. Biologic treatments such as etanercept, adalimumab, infliximab [anti-tumor necrosis factor (TNF)-α], ustekinumab [anti-interleukin (IL)-12/-23], secukinumab (anti-IL-17) are used for patients with severe Ps who have not responded to systemic treatments such as methotrexate, ciclosporin and acitretin. Recent studies suggest the role of complementary or alternative medicine products on the psoriatic lesions (20,21). Despite these various treatments, Ps remains incurable but clinically manageable.

Extensive literature suggests that Ps is a T-cell mediated disease and in its pathogenesis the innate and adaptative immune cells are highly involved; an important role is played by different T helper lymphocyte (Th) subsets accompanied by several pro-inflammatory cytokines which maintain the chronic inflammatory status (22). Although it is considered a T cell mediated inflammatory disease, several cell types from the adaptive and innate immunity arm, as well as non-immune cells are highly involved. DC, natural killer (NK) cells and macrophages from the innate immunity arm are involved in the pathogenesis of Ps, establishing intense interactions with keratinocytes and endothelial cells (9). The interactions between immune cells [T cells, DC, NK cells, Langerhans cells (LC), macrophages] and non-immune cells (hyperproliferative keratinocytes) are mediated by immune-related molecules (cytokines, chemokines) and non-immune-related molecules [vascular endothelial growth factor (VEGF), keratinocyte growth factor (KGF)], all these interactions lead to development of psoriatic lesions (23). Initiation of psoriatic events occurs when, under the action of triggering factors (genetics, environmental, skin injury, infections), non-specific immune cells (NK cells, macrophage, plasmacytoid DC) and keratinocytes secrete TNFα, IFNγ, IL-1β, IL-6 which will activate myeloid DC. The activated myeloid DC are able to

secrete IL-12 and IL-23, which will further cause differentiation of resident T cells into Th1, Th17 and Th22 cells. These effector Th subsets will release TNFα, IFNγ, IL-17A/F and IL-22 therefore activating the keratinocytes which will produce mainly pro-inflammatory cytokines (TNFα, IL-1β, IL-6), chemokines (CXCL9, 10, 11) and LL-37 (an antimicrobial peptide considered to be a possible autoantigen in Ps). Activated keratinocytes, by releasing a panel of chemokines, will promote the recruitment and activation of neutrophils and macrophages, thus propagating and maintaining the skin inflammation (24).

In humans, NK cells are defined as cluster of differentiation (CD)56+CD3- cells, and can be divided into CD56bright NK cells (with predominantly immunoregulatory properties) and CD56dim NK cells (with marked cytotoxic function) (25). NK cells were found in the inflammatory infiltrate in psoriatic skin lesions and many cytokines known to be crucial of Ps are related to NK biology and are either released by NK cells (IFNγ, TNF-α, and IL-22) or are important in their activation (IL-15, IL-18, IL-12, and IL-23). Although NK cells are involved in the inflammatory process of Ps through these pro-inflammatory cytokines, their role in this pathology is not yet fully elucidated (26). Especially known for their ability to recognise and kill viral or cancer cells, NK cells are also involved in some other cutaneous pathologies such as atopic dermatitis (27), pemphigus vulgaris (28) and *alopecia areata* (29).

In order to aid information to the pathogenesis of Ps and to identify potential therapeutic targets, numerous experimental models performed *in vitro* and/or *in vivo* were developed, each of them heaving advantages and disadvantages. *In vivo* mouse models of Ps can be grouped into spontaneous (chronic proliferative dermatitis cpdm/cpdm, flaky skin *Ttcf^{sn}/Ttcf^{sn}*, homozygous asebia *Scd1^{ab}/Scd1^{ab}*), genetically engineered (Involucrin/IFNγ, K14-KGF, K14-VEGF, and K5-Stat3C), xenotransplantation (human skin on SCID mice, on athymic nude mice or on AGR129 mice), and directly induced [intradermal injection of IL-23, 5% imiquimod (IMQ)] models (30).

In recent years, one of the most used experimental models of Ps, is IMQ-based mouse model of psoriasiform dermatitis. This model has not only reduced the costs and high reproducibility but also can furnish relevant results. IMQ has a nucleoside [1-isobutyl-1H-imidazo(4,5-c)quinolin-4-amine] analogue of imidazoquinoline family, is used in clinics for its anti-viral and anti-neoplastic properties in the treatment of human papilloma virus-derived genital warts (31), squamous cell carcinoma (32) and actinic keratosis (33).

Our prior published results using IMQ animal model showed that there is a clear alteration of lymphocyte percentages in peripheral blood (PB) and in secondary organs, pinpointing towards NK lymphocyte deregulation (34). As NK cells are one of the main immune populations that balance innate and adaptive immunity we enlarged the evaluated subtypes of NK subpopulations identified in PB and in secondary lymph organs seeking to establish the best pattern of NK phenotype related to the evolution of psoriatic lesions.

Acknowledging the importance of NK lymphocyte population in developing psoriatic lesions we used an IMQ-based mouse model of psoriasiform dermatitis to study the NK cell phenotype from PB and spleen cell suspensions detecting the phenotypes described in Table I.

Materials and methods

IMQ-based mouse experimental model of psoriatic dermatitis. C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were provided by the Animal Husbandry from 'Victor Babeș' National Institute of Pathology. They were accommodated in individual cages in an open cage system, in a temperature-controlled, air-conditioned animal house (20±4°C, 55±10% humidity) with a 12/12-light /dark cycle, and received food and water *ad libitum*. The animals were monitored daily. The study design was approved by the Ethics Committee from 'Victor Babeș' Institute, and the experiments were done in accordance with recognized principles of Laboratory Animal Care in the framework of EU Directive 2010/63/EU for animal experiments (35).

IMQ-based mouse model of psoriasiform dermatitis was replicated according to the protocols described in literature (36). Two groups of C57BL/6 mice were considered (1:1 sex ratio, 8-11 weeks old): i) IMQ group: received a daily topical dose of 62.5 mg IMQ cream (5% Aldara Cream; MEDA AB Sweden) on the shaved back region for 5 consecutive days (6 mice); and ii) Control group: no topical treatment (5 mice).

Erythema, skin scaling and thickening were monitored daily on a 0-4 scale (0, none; 1, slight; 2, moderate; 3, marked; and 4, very marked) and a modified PASI score (erythema + skin scaling + thickening) was calculated daily in order to score the inflammation due to IMQ treatment. Body weight was measured on the first day of experiment and prior to sacrifice. At the end of IMQ treatment (day 6) the mice were anesthetized (ketamine / acepromazine, 100/5 mg/kg, Ketaset; Wyeth/Fort Dodge Animal Health, Overland Park, KS, USA; Vedco, St. Joseph, MO, USA) and weighed. Blood was collected in K2-EDTA coated tubes (Microvette, Sarstedt AG & Co.) by retro-orbital veni-puncture and then the mice were sacrificed for spleen and skin sampling. The spleens were removed and weighed immediately (Balance AEP-1500 A; Adam Equipment Co., Ltd.) in order to assess the splenomegaly. Further spleens were harvested in RPMI-1640 media with 5% FBS (Biochrom GmbH) and passed through a 70 μm cell strainer (BD Falcon-BD Biosciences) to isolate the entire spleen cell populations. The spleen cell suspensions were centrifuged for 5 min at 350 x g (20°C), resuspended in RBC Lysis Buffer (BioLegend), and incubated 5 min on ice. The lysis was stopped by adding 10 ml Cell Staining Buffer (BioLegend). Cell suspensions were centrifuged for 5 min at 350 x g (20°C) and the cell pellet was resuspended twice in Cell Staining Buffer (BioLegend). Viable cells were counted and resuspended in Cell Staining Buffer (BioLegend) at 1x10^6 cells/ml. Skin samples were processed for histopathological assessment (fixed in 10% buffered formalin, embedded in paraffin, sectioned in 5 μm thick sections, stained with hematoxylin and eosin and examined by pathologists).

Flow cytometry analysis. NK cell phenotype was performed by flow cytometry, with an 8-color system setup, based on the expression of surface markers. EDTA-anticoagulated whole blood samples and spleen cell suspensions were incubated with TruStain fcX (anti-mouse CD16/32, isotype Rat IgG2a, λ) antibody (BioLegend) in order to block non-specific antibody binding. After blocking, all samples

Table I. NK cell phenotype maturation, activation and cytokine receptor markers.

Maturation markers	
Precursors	
Pre-NK	CD27^{+}; CD122^{-}
R-NK	CD27^{+}; CD122^{+}
Immature NK cells	
Stage A	CD27^{+}; CD122+
Stage B	CD27^{+}; CD122^{+}; NK1.1^{+}; CD43^{+}
Stage C	CD27^{+}; CD122^{+}; NK1.1^{+}; CD43^{+}; NKp46^{+}
Mature NK cells	
Stage D	CD27^{+}; CD122^{+}; NK1.1^{+}; CD43^{+}; NKp46^{+}; CD49b^{+}
Stage E	CD27^{+}; CD122^{+}; NK1.1^{+}; CD43^{-}; NKp46^{+}; CD49b^{+}; CD11b^{+}
Stage F	CD27^{-}; CD122^{+}; NK1.1^{+}; CD43^{-}; NKp46^{+}; CD49b^{+}; CD11b^{+}; KLRG1^{+}

Activation markers
CD335 (NKp46, NCR1); CD69; CD28; gp49R, CD45R (B220); CD11c

Markers for cytokine receptors
CD25 (IL-2Rα); CD122 (IL-2R/IL-15Rβ); CD132 (common γ chain)

NK, natural killer cells; IL, interleukin; CD, cluster of differentiation.

were incubated for 20 min at room temperature in the dark with the following monoclonal antibodies conjugated with fluorochromes (in the quantities indicated by the producers): FITC anti-mouse CD3ε (clone 145-2C11, isotype Armenian Hamster IgG), PerCP/Cy5.5 anti-mouse CD11c (clone N418, isotype Armenian Hamster IgG), APC/Cy7 anti-mouse CD45R (B220) (clone RA3-6B2, isotype Rat IgG2a, κ), PE/Cy7 anti-mouse CD69 (clone H1.2F3, isotype Armenian Hamster IgG), PE anti-mouse KLRG1 (clone 2F1, isotype Syrian Hamster IgG), PerCP/Cy5.5 anti-mouse/rat/human CD27 (clone LG.3A10, isotype Armenian Hamster IgG), APC anti-mouse CD11b (clone M1/70, isotype Rat IgG2b, κ), APC/Cy7 anti-mouse CD43 (clone RA3-6B2, isotype Rat IgG2a, κ), PE/Cy7 anti-mouse CD335 (NKp46) (clone 29A1.4, isotype Rat IgG2a, κ), PE anti-mouse CD132 (common γ chain) (clone TUGm2, isotype Rat IgG2b, κ), PerCP/Cy5.5 anti-mouse CD122 (IL-2R/IL-15Rβ) (clone TM-β1, isotype Rat IgG2b, κ), APC/Cy7 anti-mouse CD25 (IL-2Rα) (clone PC61, isotype Rat IgG1, λ), PE anti-mouse CD28 (clone 37.51, isotype Syrian Hamster IgG) (BioLegend), Brilliant Violet 510 anti-mouse NK1.1 (clone PK136, isotype Mouse IgG2a, κ) (BD Horizon, BD Biosciences), eFluor 450 anti-mouse CD49b (DX5) (clone DX5, isotype Rat IgM, κ), eFluor 660 anti-mouse gp49R (clone H1.1) (eBioscience Inc.). After surface staining, red

Day 1 **Day 2** **Day 3**

Day 4 **Day 5** **Day 6**

Figure 1. The evolution of back skin inflammation during the IMQ-treatment. On day 1, the IMQ-based treatment was initiated, and on day 6 the mice were sacrificed. IMQ, imiquimod.

blood cells were lysed with RBC Lysis Buffer (BioLegend) for 10 min at room temperature in the dark, followed by centrifugation for 5 min at 350 x g at 20°C. Cells were washed twice with Cell Staining Buffer (BioLegend) and analysed by flow cytometry. Non-specific fluorescence signals obtained due to spectral overlap were automatically compensated (UltraComp eBeads, Invitrogen by Thermo Fischer Scientific, Inc.) and unlabeled cells were used as negative controls. Data acquisition and analysis were performed on a BD FACSCanto II cytometer with BD FACSDiva v.6.1 software (BD Biosciences). Cytometer performances were checked using CST beads (BD Cytometer Setup & Tracking Beads kit; BD Biosciences).

Statistical analysis. Statistical analysis was performed using Microsoft Excel. The results for spleen weight and body weight are presented as mean spleen weight ± SD, respectively mean ratio SW:BW ± SD. The flow cytometry data were expressed as percentages of NK1.1+ cells (mean values ± SD), gated from CD3ε- lymphocytes. Student's t-test (two-tailed, assuming equal variance) was used to assess the differences between the experimental groups, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Psoriasiform dermatitis mouse model induced by IMQ. IMQ-based mouse model of psoriasiform dermatitis was replicated according to the protocols described by us (34), by applying a daily topical dose of Aldara cream on the shaved back skin of C57BL/6 mice for 5 consecutive days. Skin inflammation and the disease severity were assessed using *in vivo* measurements (erythema, desquamation and induration parameters, PASI modified score), splenomegaly assessment and histopathological evaluation.

In vivo measurements of skin inflammatory parameters. Erythema, desquamation and induration parameters (EDI) were assessed to study the progress of skin inflammation and hence the disease severity by daily monitoring. EDI were scored daily on a scale from 0 to 4: 0, none; 1, slight; 2, moderate; 3,
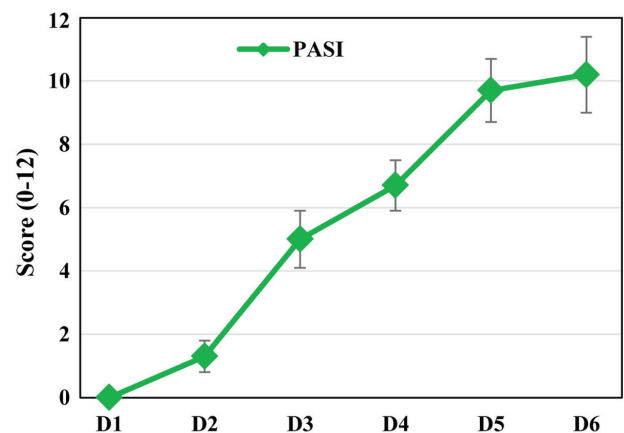

Figure 2. *In vivo* measurements of PASI cumulative score. PASI cumulative score was calculated daily by adding the scores obtained for erythema, skin scaling and thickening: D1: 0; D2: 1.3±0.5; D3: 5±0.9; D4: 6.7±0.8; D5: 9.7±1; D6: 10.2±1.2. The results are presented as mean score ± SD; n=6. n, number of mice; D, day; PASI, psoriasis area severity index.

marked; and 4, very marked. Fig. 1 presents a representative case of inflammation induced by IMQ and the EDI scoring for all the mice in the IMQ group matched the pattern previously published by us for this experimental model (34). As previously shown, all EDI parameters, erythema is the first parameter that can be scored after one day of IMQ applications, followed after another day by the subsequent registered parameters, thus starting from day 2, all EDI parameters are registered in all the animals subjected to IMQ (34).

As in psoriatic patients, in our animal model, the severity of inflammation was estimated based on a modified PASI score (0-12 scale), calculated daily by adding the independent daily scores obtained for EDI (the affected area was not taken into account). The PASI score had a progressive evolution during the IMQ-treatment (Fig. 2) matching the increased severity of the psoriatic lesions.

As the PASI score clearly depicted the evolution of the psoriatic lesions we evaluated the histopathology of psoriatic-like skin in our model.
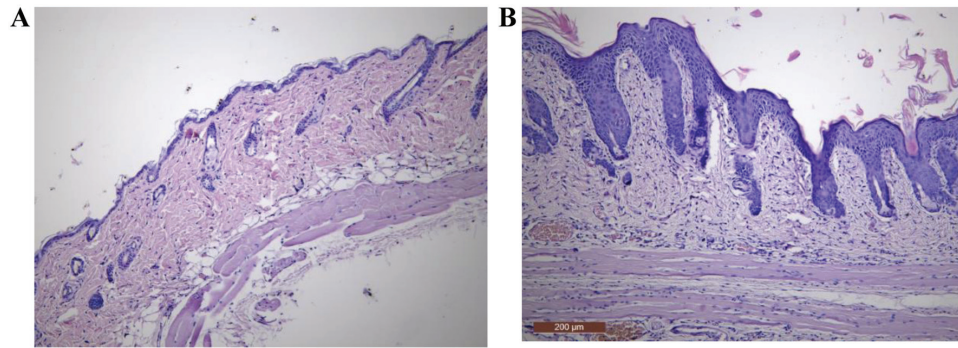
Figure 3. Histopathological evaluation of mouse skin samples (H&E staining). (A) Normal skin. (B) IMQ-treated skin. IMQ treatment induced hyperkeratosis, parakeratosis, acanthosis and elongation of rete ridges (B). IMQ, imiquimod; H&E, hematoxylin and eosin.
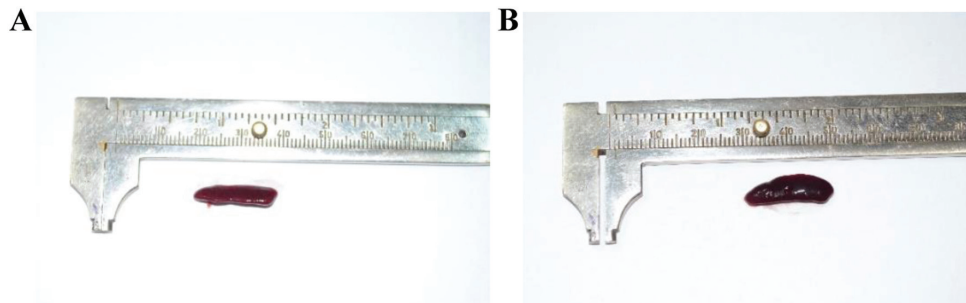

Figure 4. Representative images of splenomegaly evaluation. (A) Spleen harvested from a control mouse. (B) Spleen harvested from a mouse treated with IMQ-based cream. IMQ, imiquimod.
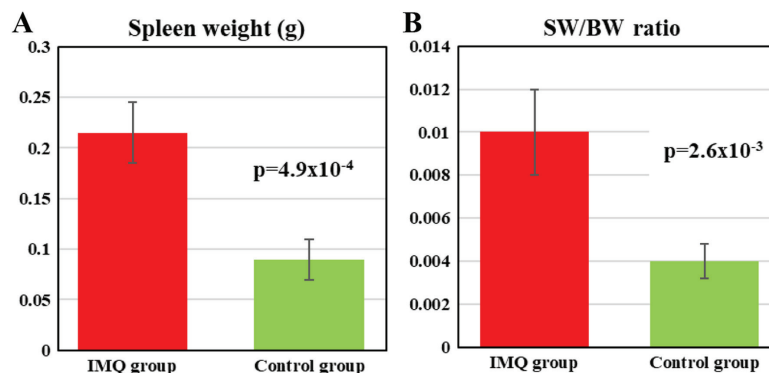

Figure 5. Splenomegaly assessment. (A) Weight of the spleens. The weight of the spleens for IMQ-treated mice (0.215±0.03) and the control group (0.09±0.02) is statistically different (P=4.9x10^{-4}). (B) SW:BW ratio. The SW:BW ratio for IMQ-treated mice (0.010±0.002) and the control group (0.004±0.0008) is statistically different (P=2.6x10^{-3}). The results are presented as mean spleen weight ± SD, respectively, mean ratio SW:BW ± SD. IMQ, imiquimod; SD, standard deviation; SW/BW, spleen weight/body weight.

Histopathological evaluation. After 5 days of treatment, IMQ-based cream induced pathological alterations in the epidermis, by compromising its integrity. Several histopathological features that are typical for human Ps, such as hyperkeratosis, parakeratosis, acanthosis and elongation of rete ridges were observed (Fig. 3B).

Splenomegaly assessment. At the end of the experiment (day 6) the mice were weighed and sacrificed; spleens from all the animals were removed and weighed in order to assess the splenomegaly, SW/BW ratio (spleen weight/total body weight) was calculated. The IMQ-treated mice SW was significantly higher compared to healthy mice (control group) (0.215±0.03 vs. 0.09±0.02, P=4.9x10^{-4}) (representative measurement is presented in Figs. 4 and 5A). When assessing SW/BW ratio we found that in IMQ-treated mice this ratio was 2.5 times greater compared to control mice (0.010±0.002 vs. 0.004±0.0008, P=2.6x10^{-3}; Fig. 5B).

NK cell phenotype in PB and spleen samples. To evaluate the immune populations that are circulating in the PB and that are resident in the secondary lymph organ such as the spleen, we have extensively characterized the NK cell phenotype from IMQ-psoriatic mice compared to control animals. Besides lineage markers such as CD161 (NK1.1) and CD3ε, a large panel of surface markers was used as follows: i) maturation markers: CD49b (DX5), CD11b, CD43, CD27, KLRG1;

Table II. Distribution of maturation markers on NK1.1^{+} cells in peripheral blood and spleen suspension.

Markers	Peripheral blood			Spleen suspension		
	Control (mean ± SD)	IMQ group (mean ± SD)	P-value	Control (mean ± SD)	IMQ group (mean ± SD)	P-value
CD49b	70±14.1	57±5.8	NS	69±4.2	47±10.2	P=0.002
CD11b	92±1.8	95±1.2	P=0.01	74±1.4	80±3.1	P=0.004
CD43	93±2.6	96±1.6	P=0.04	82±3	81±3.4	NS
CD27	15±3.7	17±1.9	NS	30±3.7	42±3.9	P=0.001
KLRG1	63±7.6	77±4.8	P=0.007	40±5.4	52±3.5	P=0.002

NK, natural killer cells; NS, not statistically significant; SD, standard deviation; IMQ, imiquimod; CD, cluster of differentiation; KLRG1, killer cell lectin-like receptor G1.

ii) activation markers: CD335 (NKp46), CD69, CD28, gp49R, CD45R (B220), CD11c; and iii) markers for cytokine receptors: CD25 (IL-2Rα), CD122 (IL-2R / IL-15Rβ), CD132 (common γ chain).

Analysis of maturation markers (CD11b, CD43, CD27, KLRG1) revealed a significant tendency to increase their expression in NK cells, in both PB and spleen cell suspension, the only exception being CD49b (Fig. 6A and B). The percentages of CD49b^{+}NK1.1^{+} cells in IMQ-treated mice is lower compared to controls in both compartments, PB and spleen, the difference being statistically significant in the spleen (P=0.002; Table II).

Depending on the presence or absence of CD11b and CD27, four stages of maturation of NK cells can be distinguished: immature NK cells (CD27^{-}CD11b^{-}), early mature NK cells (CD27^{+}CD11b^{-}), mature NK cells (CD27^{+}CD11b^{+}) and late mature cells (CD27^{-}CD11b^{+}). In our model, in PB, we found significantly decreased values for the immature stages and early mature stages, while the percentages for mature subsets (CD27^{+}CD11b^{+} and CD27^{-}CD11b^{+}) were higher in IMQ-treated mice compared to the control group, but without statistical significance (Table III). In spleen cells the level of immature NK cells in IMQ-treated mice was significantly decreased, while the values for early mature and completely matured NK cells were increased. The late mature NK population had low values (52±4.1 vs. 56±3) but not statistically significant (Fig. 7A and B).

NKp46 expression on NK cells showed decreased values for IMQ-treated mice as compared to the control group, in both PB (91±13.1 vs. 98±0.2) and spleen cells (64±2.7 vs. 74±6.9). Significant differences for this activating receptor were observed in spleen cells (P=0.008; Table IV, Fig. 8).

Analysis of NK cell activation markers (CD69, B220, CD11c, gp49R, CD28) revealed significantly increased values in spleen cells in IMQ-treated mice as compared to control group (Table IV, Fig. 9B). In PB, of all the tested activation markers, only CD69, CD11c and gp49R showed significantly increased values as compared to the control group (Fig. 9A).

When studying B220^{+}CD11c^{+}NK1.1^{+} cell population, a DC subset overlapping functionally with NK cells (37) we found in both PB and spleen cells that their percentage was higher in IMQ-treated mice (5±2 and 11±2.2 vs. 3±1.3 and 4±0.5).
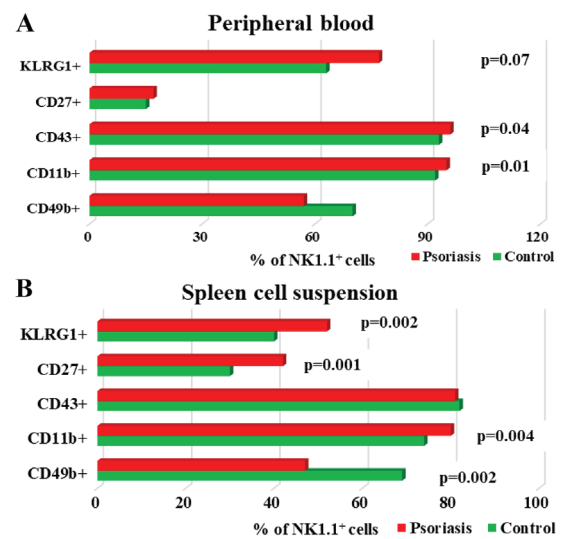

Figure 6. Expression of CD49b, CD11b, CD43, CD27 and KLRG1 levels on NK1.1^{+} cells. (A) PB. CD49b^{+}, CD11b^{+}, CD43^{+}, CD27^{+} and KLRG1^{+} cells in IMQ-treated mice (n=6) (57±5.8; 95±1.2, P=0.01; 96±1.6, P=0.04; 17±1.9 and 77±4.8, P=0.007) compared to control group (n=5) (70±14.1, 92±1.8, 93±2.6, 15±3.7 and 63±7.6) in PB. (B) Spleen cell suspension. CD49b^{+}, CD11b^{+}, CD43^{+}, CD27^{+} and KLRG1^{+} cells in IMQ-treated mice (n=6) (47±10.2, P=0.002; 80±3.1, P=0.004; 81±3.4; 42±3.9, P=0.001 and 52±3.5, P=0.002) compared to control group (n=5) (69±4.2, 74±1.4, 82±3, 30±3.7 and 40±5.4) in spleen cell suspension. The results are presented as a percentage from NK1.1^{+} cells (mean ± SD); n, number of mice; IMQ, imiquimod; SD, standard deviation; NK, natural killer cells; CD, cluster of differentiation; KLRG1, killer cell lectin-like receptor G1.

Significant differences between the experimental groups were observed only in spleen cell suspension (P=0.0001; Fig. 10).

Regarding the distribution of cytokine receptors, the main changes observed in our experimental data for IMQ-treated mice was the significant increase of the receptor for IL-2. This finding was consistent for both of the receptor units (CD132 and CD122) (73±8.3, P=5x10^{-7} vs. 7±4.3 and 95±1, P=2x10^{-5} vs. 89±1.3) expressed on NK cells from PB. This finding is somewhat to be expected as it comprises the functional units of the same cytokine receptor. CD25 is the α chain of the high-affinity IL-2 receptor and its expression was significantly low in IMQ-treated mice (0.5±0.2, P=5x10^{-9}) as compared with the control mice (8±0.6) (Fig. 11A). Contrary to PB, in spleen cells, the levels of CD132 and CD122 are lower in

Table III. Distribution of NK1.1$^+$ subsets in peripheral blood and spleen suspension.

	Peripheral blood			Spleen suspension		
	Control (mean ± SD)	IMQ (mean ± SD)	P-value	Control (mean ± SD)	IMQ (mean ± SD)	P-value
Immature (CD27$^-$CD11b$^-$)	6±1.6	3±0.7	P=0.001	14±1.4	7±0.8	P=3x10^{-6}
Early mature (CD27$^+$CD11b$^-$)	4±1.5	2±0.5	P=0.004	10±1.5	13±2.4	NS
Mature (CD27$^+$CD11b$^+$)	11±2.4	14±1.7	NS	20±3.4	29±3.2	P=0.002
Late mature (CD27$^-$CD11b$^+$)	78±5.3	82±1.5	NS	56±3	52±4.1	NS

NK, natural killer cells; NS, not statistically significant; SD, standard deviation; IMQ, imiquimod; CD, cluster of differentiation.
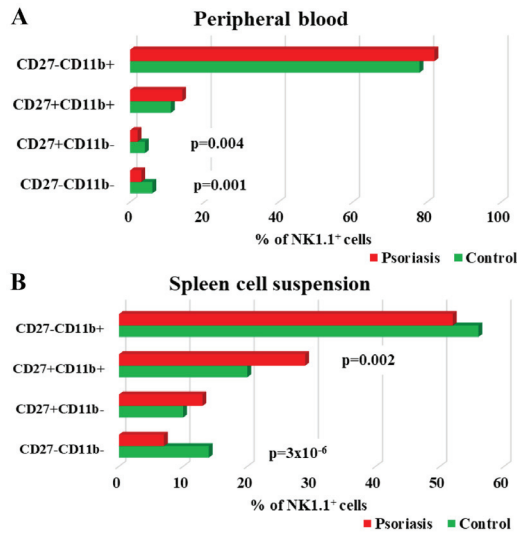

Figure 7. CD27CD11bNK1.1$^+$ subpopulations in PB and spleen cell suspension. (A) PB. Distribution of CD27$^-$CD11b$^-$, CD27$^+$CD11b$^-$, CD27$^+$CD11b$^+$ and CD27$^-$CD11b$^+$ NK1.1$^+$ cells in IMQ-treated mice (n=6) (3±0.7, P=0.001; 2±0.5, P=0.004; 14±1.7 and 82±1.5) compared to control group (n=5) (6±1.6, 4±1.5, 11±2.4 and 78±5.3) in PB. (B) Spleen cell suspension. Distribution of CD27$^-$CD11b$^-$, CD27$^+$CD11b$^-$, CD27$^+$CD11b$^+$ and CD27$^-$CD11b$^+$ NK1.1$^+$ cells in IMQ-treated mice (n=6) (7±0.8, P=3x10^{-6}; 13±2.4; 29±3.2, P=0.002 and 52±4.1) compared to control group (n=5) (14±1.4, 10±1.5, 20±3.4 and 56±3) in spleen cell suspension. The results are presented as a percentage from NK1.1$^+$ cells (mean ± SD). n, number of mice; PB, peripheral blood; IMQ, imiquimod; SD, standard deviation; NK, natural killer cells; CD, cluster of differentiation.

IMQ-treated mice (16±4.4; 75±8, P=0.004) than in the control group (18±3.6; 91±2.6), as well as in PB. The level of CD25 was found increased (6±1.5 vs. 2±0.6) but not statistically significant (Fig. 11B).

Discussion

IMQ-based mice model of psoriasiform dermatitis replicates human Ps in terms of skin inflammation and disease severity assessed by erythema, desquamation, induration parameters, PASI modified score and histopathological evaluation (hyperkeratosis, parakeratosis, acanthosis and elongation of rete ridges). Moreover, the mouse model displays splenomegaly and altered NK populations related to the severity of the disease.

In our previous study regarding psoriasiform dermatitis in a mouse model, we investigated immunological changes induced by IMQ topical application in lymphocyte populations from PB and spleen (34). One of the main observed changes was the significant decrease of NK1.1$^+$ cell percentages, which led to the present study to investigate complex phenotypic pattern of circulating and spleen resident NK. Using flow-cytometry, we investigated a large panel of surface markers: maturation and activation markers (CD49b, CD11b, CD43, CD27, KLRG1, NKp46, CD69, CD28, gp49R, CD45R, CD11c) and markers for cytokine receptors (CD25, CD122, CD132). To our knowledge there is no similar study regarding NK cells in blood and spleen cells in an IMQ-based mouse model. We have previously used this panel to characterize NK cells in another mouse model of skin cancer, namely in cutaneous melanoma bearing mice (38).

Murine NK cells develop in specialized bone marrow niches and derive from common lymphoid progenitor, going through three important stages: NK cell progenitors (pre-NK cell precursors and refined-NK cell precursors), immature NK cells (stage A-C) and mature NK cells (stage D-F). Early stages are characterized by the expression of IL-7Rα (CD127), CD27, CD244 and CD122, while the acquisition of NKG2D, CD27 (stage A), NK1.1, CD43, CD62L, CD226 (stage B) and NCR1 (stage C) marks the conversion to immature NK cells. Mature NK cells further express CD49b, Ly49 (stage D), loose CD43 and acquire CD11b (stage E) and in the final stage of maturation (stage F) downregulate CD27 and acquire KLRG1 (39). KLRG1 is a C-type lectin inhibitory receptor (also expressed on subsets of T cells) allowing identification of NK terminal development stages and is associated with diminished proliferation and effector functions (40).

In our model, analysis of maturation markers (CD11b, CD43, CD27, KLRG1) revealed a significant tendency to increase their expression on NK cells, in both PB and spleen cell suspension with the exception of CD49b. The percentage of CD49b$^+$NK1.1$^+$ cells in IMQ-treated mice is significantly lower than in the controls in spleen cell suspension. This finding agrees with another recently reported study where

Table IV. Distribution of activation markers on NK1.1$^+$ cells in peripheral blood and spleen suspension.

	Peripheral blood			Spleen suspension		
	Control (mean ± SD)	IMQ (mean ± SD)	P-value	Control (mean ± SD)	IMQ (mean ± SD)	P-value
NKp46	98±0.2	91±13.1	NS	74±6.9	64±2.7	P=0.008
CD69	12±1.4	60±13	P=0.005	2±0.2	62±3	P=1x10^{-10}
B220	7±2.6	6±2	NS	9±0.8	14±2.2	P=0.003
CD11c	25±2.4	66±11	P=6x10^{-5}	21±0.6	76±6.2	P=1x10^{-7}
gp49R	1±0.3	11±7	P=0.02	1±0.5	23±5.7	P=5x10^{-5}
CD28	0.4±0	3±2	NS	0.5±0.2	33±6.3	P=8x10^{-6}

NK, natural killer cells; NS, not statistically significant; SD, standard deviation; IMQ, imiquimod; CD, cluster of differentiation.
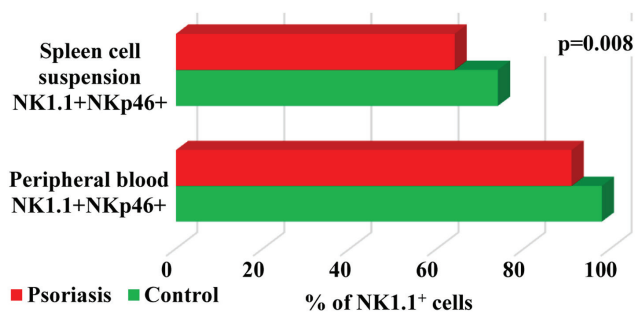

Figure 8. NK1.1$^+$NKp46$^+$ distribution in PB and spleen cell suspension. NK1.1$^+$NKp46$^+$ cells in IMQ-treated mice (n=6) in PB (91±13.1) and spleen cell suspension (64±2.7, P=0.008) compared to control group (n=5) (98±0.2 in PB; 74±6.9 in spleen cell suspensions). The results are presented as percentage from NK1.1$^+$ cells (mean ± SD); n, number of mice; PB, peripheral blood; IMQ, imiquimod; SD, standard deviation; NK, natural killer cells.

Figure 9. Expression of CD69, B220, CD11c, gp49R and CD28 levels on NK1.1$^+$ cells; (A) PB. CD69$^+$, B220$^+$, CD11c$^+$, gp49R$^+$ and CD28$^+$ cells in IMQ-treated mice (n=6) (60±13, P=0.005; 6±2; 66±11, P=6x10^{-5}; 11±7, P=0.02 and 3±2) compared to control group (n=5) (12±1.4; 7±2.6; 25±2.4; 1±0.3 and 0.4±0) in PB. (B) Spleen cell suspension. CD69$^+$, B220$^+$, CD11c$^+$, gp49R$^+$ and CD28$^+$ cells in IMQ-treated mice (n=6) (62±3, P=1x10^{-10}; 14±2.2, P=0.003; 76±6.2, P=1x10^{-7}; 23±5.7, P=5x10^{-5} and 33±6.3, P=8x10^{-6}) compared to control group (n=5) (2±0.2; 9±0.8; 21±0.6; 1±0.5 and 0.5±0.2) in spleen cell suspension. The results are presented as a percentage from NK1.1$^+$ cells (mean ± SD); n, number of mice; PB, peripheral blood; IMQ, imiquimod; SD, standard deviation; NK, natural killer cells; CD, cluster of differentiation.

CD49b$^+$ was found low on T cells in psoriatic patients, this decrease being correlated with the severity of the disease (41).

Depending on the presence or absence of CD11b and CD27, there are several maturation stages of NK cells. CD27$^+$ NK cell subsets display a greater effector function and responsiveness to chemokines then CD27$^-$ NK cell subsets. CD27$^-$CD11b$^+$NK cell subsets represent the final stage of maturation (42). In PB, our experimental data revealed significant decreased values for the immature stages, while the percentages for mature subsets were higher in IMQ-treated mice than in control group, but without statistical significance. In spleen cell suspension the level of immature NK cells in IMQ-treated mice was significantly decreased (P=3x10^{-6}), while the values increased for early mature and mature (P=0.002) NK cells, and in the final stage of maturation, the values were low but not statistically significant. This finding reflects the immune engagement toward activatory profile of NK cells and draws attention to evaluate Ps intensity correlated with the mature profile of circulating NK cells.

NKp46 (similar NCR1 or CD335), a major activating receptor, is an NK cell specific surface marker and is found on all mature NK cells. It is part of natural cytotoxicity receptor group together with NKp44 and NKp30 (43). It is involved in human NK cell activation, in tumor cell recognition and plays an important role 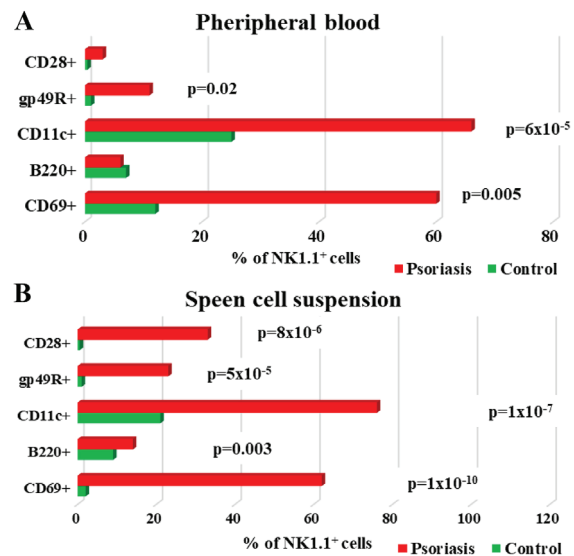in natural cytotoxicity against different tumor target cells (44). Our data showed a decreased NKp46 expression on NK cells for IMQ-treated mice as compared to the control group, in both PB and spleen cell suspensions. The finding is not surprising because, as already mentioned, the maturation and activation of NK cells are characteristics of our psoriatic mouse model.

In addition to NKp46, our study also included other relevant markers to NK cell activation such as CD69, B220, CD11c, gp49R and CD28. Leukocyte activation receptor CD69, also called very early antigen, was found on human NK cells from psoriatic lesions inflammatory infiltrate. Most of these NK cells had a CD56^{bright} phenotype and are known to produce *in vitro* large quantities of IFNγ as a response to IL-2 stimulation (45).
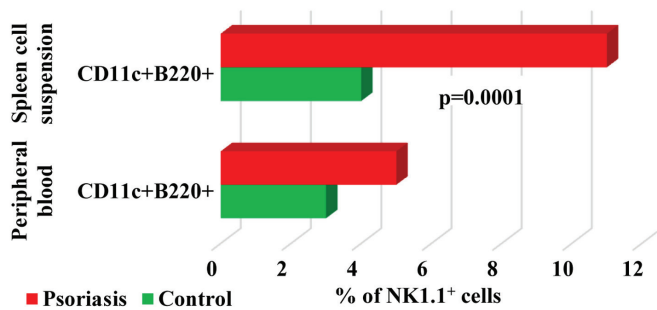
Figure 10. NK1.1$^+$CD11c$^+$B220$^+$ cells in PB and spleen cell suspension. Distribution of NK1.1$^+$CD11c$^+$B220$^+$ cells in IMQ-treated mice (n=6) in PB (5±2) and spleen cell suspension (11±2.2, P=0.0001) compared with the control group (n=5) (3±1.3 in PB and 4±0.5 in spleen cell suspensions). The results are presented as percentage from NK1.1$^+$ cells (mean ± SD); n, number of mice; PB, peripheral blood; IMQ, imiquimod; SD, standard deviation; NK, natural killer cells.
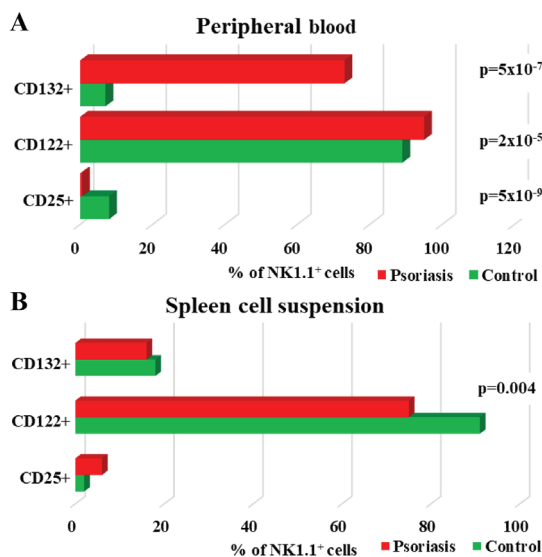

Figure 11. Expression of CD25, CD122 and CD132 levels for NK1.1$^+$ cells. (A) PB. CD25$^+$, CD122$^+$ and CD132$^+$ NK1.1$^+$ cells in IMQ-treated mice (n=6) (0.5±0.2, P=5x10^{-9}; 95±1, P=2x10^{-5} and 73±8.3, P=5x10^{-7}) compared to control group (n=5) (8±0.6, 89±1.3 and 7±4.3) in PB. (B) Spleen cell suspension. CD25$^+$, CD122$^+$ and CD132$^+$ NK1.1$^+$ cells in IMQ-treated mice (n=6) (6±1.5; 75±8, P=0.004 and 16±4.4) compared to control group (n=5) (2±0.6, 91±2.6 and 18±3.6) in spleen cell suspension. The results are presented as a percentage from NK1.1$^+$ cells (mean ± SD); n, number of mice; PB, peripheral blood; IMQ, imiquimod; SD, standard deviation; NK, natural killer cells; CD, cluster of differentiation.

Murine NK cells also express inhibitory receptors belonging to Ig superfamily-related (gp49) receptors. Activated NK cells express gp49B receptor which displays structural homology with human killer inhibitory receptors (46). CD28, a cell surface molecule with a critical role in T cell activation is also expressed by mouse NK cells, and its triggering NK cell proliferation, cytotoxicity, and cytokine secretion (47). Analysis of activation markers of NK cells (CD69, B220, CD11c, gp49R, CD28) revealed significant increased values in spleen cell suspension in IMQ-treated mice as compared to control group. In PB, only CD69, CD11c and gp49R showed significantly increased values as compared to the control group. The finding reveals that psoriatic lesions can induce high activation in secondary lymph organs. When evaluated in periphery, only CD69, CD11c

and gp49R showed significant increased values as compared to control group. In an attempt to obtain a panel of peripheral immune cells that can be applied further to Ps patients a thorough selection of significant activation should be done because although lymphoid organs display a high activation pattern, in periphery only selected activated populations can be identified.

B220$^+$CD11c$^+$NK1.1$^+$ cells appear to be the equivalent of human CD56^{bright} NK cells due to their ability to produce higher levels of IFNγ (37). The percentages of B220$^+$CD11c$^+$NK1.1$^+$ subset in both PB and spleen cell suspension was higher in IMQ-treated mice than in control mice, finding that emphasizes once more the activation of this lymphocyte subset. Moreover the cytokine network that is triggered by NK activation can act on the cells by themselves in an autocrine manner and/or can influence other important players in the immune response. Thus, the cytokine network is particularly important for the proliferation, activation and functional capacity of NK cells. In our study, we investigated the distribution of three cytokine receptors: CD25 (IL-2Rα), CD122 (IL-2R/IL-15Rβ), CD132 (common γ chain-IL-2, IL-4, IL-7, IL-9, IL-15, IL-21). The main changes observed in our experimental data for IMQ-treated mice were the significant increase of CD132 (P=5x10^{-7}) and CD122 (P=2x10^{-5}) expression on NK cells in PB. The expression of CD25 is significantly low in IMQ-treated mice as compared with the control mice. In spleen cells, the levels of CD132 and CD122 are lower in IMQ-treated mice than in the control group, as well as in PB. The level of CD25 increased but was not statistically significant. As we have an inverse correlation of these cytokine receptors we can postulate that there is a flux of activated cells toward periphery from the secondary lymphoid organs that are drawn toward the induced psoriatic lesions. IL-17 plays a leading role in the pathogenesis of Ps and in the concert of cells that secrete this regulatory cytokine, NK cells are one of the main cells (48). IL-17 has a heterodimeric receptor IL-17RA/IL-17RC located on non-immune cells such as keratinocytes, endothelial cells and fibroblasts and if IL-17 increases the Ps prognosis is not favorable as it is sustaining the development of psoriatic lesions. This finding correlates with the decrease of IL-2 circulatory level, recently reported in psoriatic patients (49). We can again postulate that there is a negative regulation in psoriatic disease where NK cells balance this cytokine axes IL-17-IL-2 and if this balance is deregulated activation of non-immune cells, e.g., keratinocytes, is induced and the psoriatic lesions appear.

Taking into account that there is a continuous search for developing new therapy targets in Ps (50) and that NK cells can be future modulatory targets (26,27) finding new markers that can aid the dermatologists in clinical management (51-53) would lead to novel cellular pattern for monitoring this auto-immune disease.

In conclusion, imiquimod-based murine model of psoriasiform dermatitis was analysed in order to evaluate the involvement of NK cells in the pathogenesis of this autoimmune disease. Evaluating a large panel of NK surface markers from the maturation and activation sets along with markers for cytokine receptors we obtained important differences in experimentally induced mouse NK cell phenotypes as compared to the control group, reflecting high activation in correlation with the degree of the psoriatic lesions. Our evaluation was intended to shed light on the involvement of

NK functionality in Ps development and draw some outlines regarding NK as disease evolution cellular marker.

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Availability of data and materials

The data sets used and/or analysed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MS, RIH, GI, MN and MC: research creation and design, data acquisition, analysis and interpretation of data, statistical analysis, manuscript drafting, critical revision of the manuscript for important intellectual content. ANM, IRP, OB, CarC, LS, IZ and ConC: interpretation of data, manuscript drafting, critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was approved by the Ethics Committee of 'Victor Babeș' National Institute of Pathology.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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