

Nickel challenge up regulates CD69 expression on T lymphocyte sub-sets from patients with nickel induced contact dermatitis

Shakoor Zahid¹, Adam Mustafa², Assiri Dina², Bakry Sawsan², AlMarshad Felwa², GadElRab Mohammed³, Abdulaziz Hasan⁴

1. Department of Pathology, College of Medicine and University Hospitals, King Saud University, Riyadh.
2. King Khalid University Hospital, King Saud University, Riyadh, Kingdom of Saudi Arabia.
3. University of Khartoum, Republic of Sudan.
4. Alrebat University, Republic of Sudan.

Abstract:

Background: Persistent antigenic stimulation due to repeated exposure to nickel may lead to chronic inflammation resulting in allergic contact dermatitis (ACD).

Objectives: This study was performed to assess nickel induced immune activation among patients sensitized against nickel.

Patients and Methods: A total of 35 patients (29 females and 6 males; mean age 36 ± 9 years) with nickel contact dermatitis and 20 patch test negative healthy individuals (14 females and 6 males; mean age 29 ± 7 years) were included in this study. Peripheral blood of patients and controls was incubated with nickel sulfate for 24 hours. Immune activation was assessed by CD69 up-regulation on T lymphocyte sub-sets by flow cytometry.

Results: Base line expression of CD69 on CD8⁺ lymphocytes was higher among patients compared to controls ($4.1 \pm 1.3\%$ vs $2.8 \pm 1.1\%$; $p < 0.009$). There was no difference in proportions of CD[±]CD69⁺ cells between patients and controls ($3.2 \pm 0.9\%$ vs $2.3 \pm 0.8\%$). Exposure to nickel induced expression of CD69 on a significantly higher proportion of CD4⁺ lymphocytes ($22.1 \pm 6.2\%$) of the ACD patients compared to controls ($2.8 \pm 2.5\%$; $p < 0.0001$). Similarly nickel induced CD69 expression on a higher proportion of CD8⁺ lymphocytes ($18.2 \pm 5.3\%$) from ACD patients compared to the controls ($1.9 \pm 1.8\%$; $p < 0.0006$).

Conclusion: CD69 molecule appears to be an important regulator of immune response in nickel contact dermatitis.

Keywords: Nickel, CD4⁺, CD8⁺, CD69, contact dermatitis.

DOI: <https://dx.doi.org/10.4314/ahs.v19i1.19>

Cite as: Zahid S, Mustafa A, Dina A, Sawsan B, Felwa A, Mohammed G, et al. Nickel challenge up regulates CD69 expression on T lymphocyte sub-sets from patients with nickel induced contact dermatitis. *Afri Health Sci.* 2019;19(1). 1460-1466. <https://dx.doi.org/10.4314/ahs.v19i1.19>

Introduction

Contact sensitization due to nickel is common and about 15% population globally is believed to have allergic contact dermatitis (ACD) due nickel.¹ Inflammation associated with nickel induced ACD is driven by delayed type

hypersensitivity reaction mediated by T cell responses.² Experimental studies have shown that nickel ions serving as haptens are processed and transported to the draining lymph nodes by dendritic cells where these are presented to T lymphocytes eventually leading to priming of T lymphocytes.³ Persistent exposure to nickel results in activation and proliferation of the primed T cells in situ⁴ generating a complex inflammatory milieu clinically manifesting as ACD.⁵ Evidence suggests that CD8⁺ cells perform as effector cells whereas CD4⁺ are endowed with down-regulatory functions⁶ by limiting the development of effector CD8⁺ cells in ACD.⁷ Massive infiltration of IL-17, IL-22 and interferon gamma (IFN- γ) producing

Corresponding author:

Shakoor Zahid,
College of Medicine and University Hospitals,
King Saud University, Riyadh, Kingdom of
Saudi Arabi.
Email: shakoor_zahid@yahoo.com

CD4⁺ cells at the site of nickel challenged skin indicates important role of CD4⁺ T cells as effector cells in ACD⁸ most likely by CD69 mediated regulation of Th17 cells.⁹

CD69 is an early cell activation marker expressed on stimulated cells and is a reliable predictor of cell proliferation.¹⁰ Monocytes, platelets and Langerhans cells normally express CD39 whereas the resting lymphocytes in the peripheral blood do not express this molecule.¹¹⁻¹³ CD69 is rapidly induced on human T and B lymphocytes, natural killer cells, neutrophils and eosinophils following activation.^{11,12,14} More over type 1 interferons have been shown to up-regulate the expression of CD69.¹⁵ Following in vitro stimulation CD69 can be detected on the surface of lymphocytes after two hours, peaks between 18 to 24 hours and gradually decreases after 24 hours.¹⁶ Rapid induction of CD69 is due to the presence of this molecule in the cytoplasm and does not require RNA or protein synthesis.¹⁴ It is primarily due to this property that CD69 is widely used as an early marker for cell activation. This in vitro study was performed to assess the expression of CD69 on T cell sub-sets following exposure to nickel among patients with nickel induced ACD.

Methods

Study population

This study was performed at King Khalid University Hospital between January 2012 and June 2014 among patients with clinical suspicion of ACD. Out of the 55 patients testing positive to nickel as single allergen 35 consented to participate in the study. This group of patients included 29 female and 6 males (mean age 36±9 years). All patients were suffering from clinically active disease and among them 23 had lesions on hands, 7 on feet and 5 had lesions on ears. A control group comprising of twenty otherwise normal individuals with negative patch test reactivity was also included in the study. This group of healthy individuals included 14 females and 6 males with the mean age of 29±7 years. Patients with either negative patch test reactivity or reacting to allergens other than nickel or with positive reactions against nickel along with other allergens were not included in the study. None of the patients were receiving oral corticosteroids at the time of sample collection or at least one month prior to inclusion in the study. Patients with chronic inflammatory disorders or with the history of transient viral or bacte-

rial infection one month before were also excluded from the study. This study was approved by institutional review board.

Patch test

Patch test was performed using TRUE Test (Mekos Laboratories AS, Denmark) comprising of a panel of 24 allergens/allergen mixes. The test panels of 12 allergens each were applied on the upper back of the patient on the healthy skin free of acne, scars, dermatitis or any other skin condition that might interfere with the interpretation of the results. Patients were instructed to wear the patches for 48 hours, avoid strenuous exercise and contact with water. Interpretation of the results was performed initially after 48 hours followed by a second evaluation 72-96 hours after the application. Patients were instructed to report back to the clinic in case of delayed reactions. The interpretation of the results was performed by Allergologist in accordance with the recommendations of the International Contact Dermatitis Research Group and the North American Contact Dermatitis Group. The intensity of the skin reactivity was graded as +, ++ and +++.

Nickel sulfate challenge assay

After obtaining the informed consent of each patient and control 5ml of venous blood was withdrawn from antecubital fossa in a vacutainer containing anti-coagulant. Assessment of CD69 expression was performed at the time of sample collection as the baseline CD69 expression and after nickel sulfate challenge. The optimal concentration of nickel sulfate was determined by checker board titration assay. Prior experiments had demonstrated that the whole blood incubation with nickel sulfate at a concentration of 100 µM/ml for 24 hours was adequate for maximum expression of CD69 marker. To a 200 µl aliquot of heparinized whole blood in 5ml test tube both from the patients and controls equal volume of nickel sulfate at a concentration of 100 µM/ml was added. For the negative control tube 200 µl of 10% RPMI-1640 was dispensed in 200 µl of heparinized whole blood. The cultures were incubated in 5% CO₂ at 37°C and 95% humidity for 24 hours in CO₂ incubator (Thermo Scientific, Heraeus, HERA cell 150). After the incubation the cells were labeled with relevant monoclonal antibodies for flow cytometry.

Cell labeling and flowcytometry

To the contents of each 5ml test tube a cocktail of antibodies comprising of 20 μ l of each fluorochromes conjugated mouse anti-human CD69, anti-CD4, anti-CD8 and anti-CD3 monoclonal antibody (Becton Dickinson Immunocytometry system, San Joes Calif) was added. Each tube was vortexed gently and incubated for 15 minutes in dark at room temperature. After the incubation 2ml of 1xFACS lysing solution (B.D Biosciences, San Jose, CA 59131 USA) was dispensed into each tube and incubated for 10 minutes in dark at room temperature. After the incubation 2 ml of wash buffer was added (Optimized PBS for cell preparation and cell washing B.D Biosciences, Cat No. 349524) and each tube was centrifuged at 500 x g for 5 minutes at room temperature. The supernatant was decanted and the cell pallet was finally re-suspended in 500 μ l of 1% paraformaldehyde (B.D Biosciences pharmingen 10975 Torreyana Rd. San Diego, CA 92121) in calcium and magnesium free PBS (Dulbecco's Phosphate-Buffered Saline PAA Laboratories GmbH PAA-Strasse 1 Austria) prior to flow cytometric analysis. Flow cytometry was performed using FACSCalibur (Becton Dickinson, San Jose, CA, USA). BD CaliBRITE beads were used for setting photomultiplier tube voltages, fluorescence compensation and for checking instrument sensitivity for lymphocyte surface markers. In acquisition

mode a total of 10000 events were acquired and the analysis was performed using Cell Quest software (Becton Dickinson). The negative thresholds were set using isotype-control-labeled cells from both patients and normal controls. Data were collected as percentage of each cell type expressing the relevant marker.

Statistical analysis

Data analysis was performed using Statistical Package For Social Sciences (SPSS) version 20 computer software. Categorical data were summarized as number and percentages and numeric data were summarized as mean and standard deviation. Comparison between the groups was performed using t test and a $p < 0.05$ was considered statistically significant.

Results

Figure 1 shows comparison of the baseline expression of CD69 on CD4⁺ T lymphocytes and CD8⁺ T lymphocytes among the patients with ACD and the control group. The mean percentage of CD4⁺ T lymphocytes expressing CD69 molecule among the control group was $2.3 \pm 0.8\%$ and among the patients was $3.2 \pm 0.9\%$. Similarly the mean percentages of CD69 expressing CD8⁺ T lymphocytes among the controls and patients were $2.8 \pm 1.1\%$ and $4.1 \pm 1.3\%$ respectively.

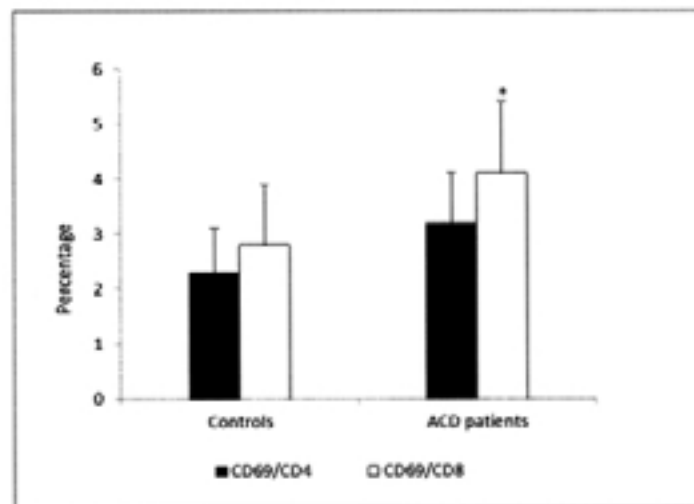


Fig 1. Base line expression of CD69 on CD4⁺ and CD8⁺ T lymphocyte subsets of nickel induced allergic contact dermatitis and controls.

*= $p < 0.009$

Whereas the percentages of CD69/CD4⁺ were no different between the patients and the controls the proportion of CD69/CD8⁺ among ACD patients were significant-

ly higher than the controls ($p < 0.009$). Nickel challenge among 24 controls failed to induce CD69 on CD4⁺ and CD8⁺ T lymphocytes (Fig. 2).

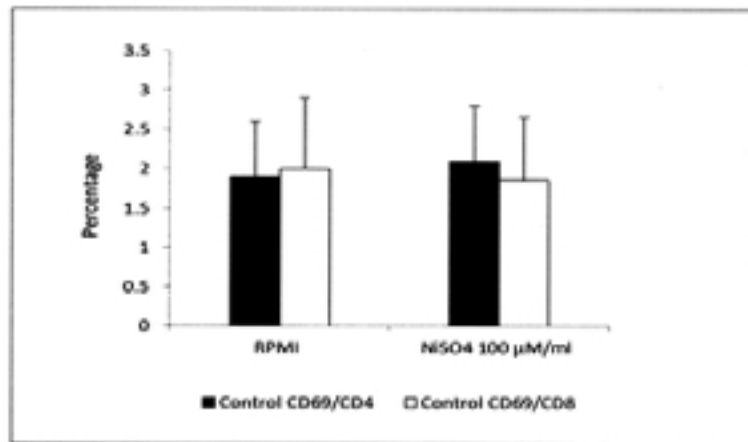


Fig. 2. CD69 expression on CD4⁺ and CD8⁺ T lymphocyte subsets of controls following 24 hour incubation with either RPMI or nickel sulfate (100 µM/ml).

Nickel challenge for 24 hours however was associated with significant up-regulation of CD69 expression on both CD4⁺ and CD8⁺ T lymphocytes. Figure 3 shows data for nickel induced CD69 expression on both the T cell sub-sets. After 24 hours of incubation of whole blood from patients with positive patch test for nickel the

percentage of CD69/CD4⁺ cells ($22.1 \pm 6.2\%$) was significantly higher ($p < 0.0001$) than the controls ($2.8 \pm 2.5\%$). Similarly the percentage of CD69/CD8⁺ T lymphocyte following 24 hours incubation with nickel sulfate ($18.2 \pm 5.3\%$) was significantly higher ($p < 0.0006$) than the controls ($1.9 \pm 1.8\%$).

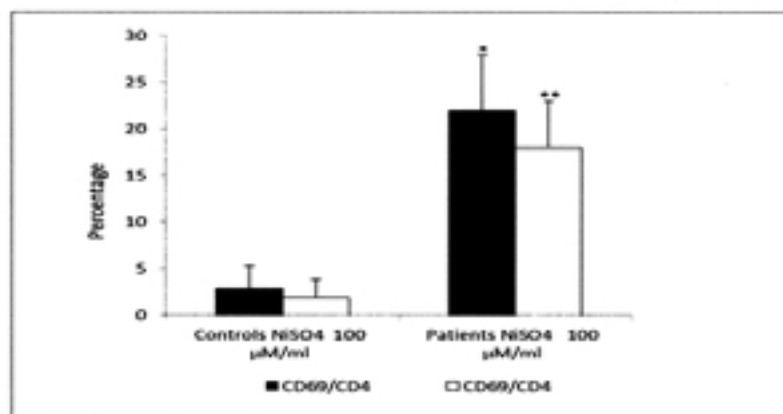


Fig. 3. Comparison of CD69 induction on CD4⁺ and CD8⁺ T lymphocyte subsets from patients with nickel induced contact dermatitis and controls following 24 hour incubation with nickel sulfate. (100 µM/ml).

*= $p < 0.0001$
 **= $p < 0.0006$

Discussion

Base line expression of CD69 on CD8⁺ T lymphocytes was higher in patch test positive patients with ACD. Enhanced expression of CD69 on a variety of cells has been associated with allergic disorders. Increased number of CD69⁺ eosinophils has been reported in the bronchoalveolar lavage fluid of patients with allergic asthma following segmental allergen provocation.¹⁷ Presence of higher proportion of circulating CD69⁺ eosinophils among infants with atopic dermatitis has not only been linked with high levels of total IgE but has also been associated with active allergic inflammation.¹⁸ Elevated numbers of CD69⁺ lymphocytes found in the sputa of patients with asthma are believed to be due to enhanced antigenic challenge.¹⁹ Similarly exposure of peripheral blood mononuclear cells to allergens has been shown to increase the surface expression of CD69 on natural killer cells.²⁰ Induction of CD69 expression on a wide variety of hematopoietic cells as a result of allergenic stimulation could possibly be a function of allergen specific clones releasing high levels of IFN- γ particularly in nickel allergy.²¹ Collectively these data suggest that increased expression of CD69 in allergic disorders may serve as marker of disease activity.

Nickel was able to induce CD69 molecule on both CD4⁺ and CD8⁺ T cells after 24 hours in the present study. A study investigating the effect of monosodium urate-trinitrochlorobenzene-induced contact hypersensitivity responses in mice has revealed increased expression of CD69 on both CD4⁺ and CD8⁺ T lymphocytes indicating activation of both cell types.²² Interaction between activated T cell subsets particularly in contact dermatitis appears to be complex. Hapten-stimulated CD8⁺ effector cells mainly produce IFN- γ promoting an inflammatory milieu whereas CD4⁺ lymphocytes on the other hand exert a negative regulatory effect by producing IL-4, IL-5 and IL-10.²³ Because of the cytokine profile it may be inferred that ACD is CD8⁺ lymphocyte induced disorder regulated by CD4⁺ lymphocytes. However in some mouse models CD4⁺ lymphocytes have also been shown to behave as effector cells.²⁴ A recent study has demonstrated nickel dependent IL-9 release from the peripheral blood mononuclear cells of nickel sensitized individuals. IL-9 by promoting IL-4 production may also perform a regulating cytokine in nickel induced dermatitis.²⁵ In addition naïve CD4 T lymphocytes from CD69 deficient animals fail to differentiate in Foxp3⁺ and induction of

CD69 promotes generation of Foxp3⁺ regulatory cell population.²⁶ The pathogenesis of contact dermatitis in general and nickel induced ACD in particular remains obscure and could possibly be a mixed Th1/Th2 response.

The ability of nickel sulfate to consistently induce CD69 on CD4⁺ and CD8⁺ T lymphocytes offers a useful opportunity for development of in vitro test for detection of nickel sensitization as an alternate test for in vivo, cumbersome and time consuming patch test. In addition the existence of a strong correlation between the induction of CD69 and cell proliferation¹⁰ may obviate the need for using potentially hazardous radioactive materials in lymphoproliferation assays. Moreover flowcytometric assessment of CD69 up-regulation on CD4⁺ T lymphocytes in vitro has been successfully applied in detection of sensitization due to *Mycobacterium* TB antigen.²⁷ Metal ions released from orthopedic implants have been shown to induce CD69 molecule and activate CD3⁺ lymphocytes that can be detected by flow cytometry with a relative ease.²⁸ In vitro stimulation of CD4⁺ T lymphocytes with drugs and induction of CD69 surface expression has been successfully evaluated in discriminating individuals with drug allergy from those with no drug allergy.²⁹ As opposed to other metals nickel sulfate has non-specific stimulatory properties that may interfere with in vitro testing for nickel sensitization.³⁰ The inherent stimulatory properties of nickel probably did not interfere with the results in the present study as the expression of CD69 on control cells whether challenged with nickel sulfate or RPIM was no different. Development of in vitro testing for nickel and other contact allergens based on induction of CD69 may be a useful in avoiding intense local inflammatory reactions often associated with patch test. The observations of the present study require validation in immune-compromised individuals particularly HIV infected patients where anergic delayed type hypersensitivity skin reactions are frequently observed that tend to normalize following successful anti-retroviral treatment.³¹

Conclusion

Peripheral blood cells from patients with ACD sensitized against nickel responded to in vitro nickel challenge by enhanced expression of CD69 activation marker. Although antigenic stimulation and cytokine release may have induced CD69 on T cell sub-sets the possibility of CD69 up-regulation on nickel specific T cell clones could

not be excluded. Sensitization due to nickel is common and development of in vitro test based on nickel induced CD69 up-regulation may be a useful alternative to the gold standard patch test.

Declaration

Authors declare no conflict of interest.

References

1. Mahler V, Geier J, Schnuch A. Current trends in patch testing – new data from the German Contact Dermatitis Research Group (DKG) and the Information Network of Departments of Dermatology (IVDK). *J Dtsch Dermatol Ges.* 2014; 12: 583–92. doi: 10.1111/ddg.12371.
2. Martin SF, Esser PR, Weber FC, Jakob T, Freudenberg MA, Schmidt M, et al. Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy.* 2011; 66: 1152–63. doi: 10.1111/j.1398-9995.2011.02652.x. PubMed.
3. Kaplan DH, Igyarto BZ, Gaspari AA. Early immune events in the induction of allergic contact dermatitis. *Nat Rev Immunol.* 2012; 12: 114–24. doi: 10.1038/nri3150. PubMed.
4. Natsuaki Y, Egawa G, Nakamizo S, Ono S, Hanakawa S, Okada T et al. Perivascular leukocyte clusters are essential for efficient activation of effector T cells in the skin. *Nat Immunol.* 2014; 15: 1064–9. doi: 10.1038/ni.2992. PubMed.
5. Kerstan A, Leverkus M, Trautmann A. Effector pathways during eczematous dermatitis: where inflammation meets cell death. *Exp Dermatol.* 2009; 18: 893–9. doi: 10.1111/j.1600-0625.2009.00919.x. PubMed.
6. Saint-Mezard P, Berard F, Dubois B, Kaiserlian D, Nicolas JF. The role of CD4+ and CD8+ T cells in contact hypersensitivity and allergic contact dermatitis. *Eur J Dermatol.* 2004;14(3):131-8. PubMed.
7. Gautam S, Matriano J, Chikkala M, Edinger M, Tubbs R. L3T4 (CD4) T cells that mediated contact sensitivity to trinitrochlorobenzene express I-A determinants. *Cell Immunol.* 1991;135:27.
8. Dyring-Andersen B, Skov L, Løvendorf MB, Bzorek M, Søndergaard K, Lauritsen JP, et al. CD4(+) T cells producing interleukin (IL)-17, IL-22 and interferon- γ are major effector T cells in nickel allergy. *Contact Dermatitis.* 2013;68(6):339-47. PubMed. doi: 10.1111/cod.12043.
9. Martín P, Sánchez-Madrid F. CD69: an unexpected regulator of TH17 cell-driven inflammatory responses. *Sci Signal.* 2011;22;4(165):pe14. doi: 10.1126/scisignal.2001825. PubMed.
10. Caruso A, Licenziati S, Corulli M, Canaris AD, De Francesco MA, Fiorentini S, et al. Flow cytometric analysis of activation markers on stimulated T cells and their correlation with cell proliferation. *Cytometry.* 1997;27(1):71-6. PubMed.
11. Testi R, Phillips JH, Lanier LL. Constitutive expression of A phosphorylated activation antigen (Leu 23) by CD3(bright)human thymocytes. *J Immunol.* 1988;141(8):2557–2563.
12. Sanchez-Mateos P, Cebrian M, Acevedo A, Lopez-Boitet M, Landazuri MO, Sanchez-Madrid F. Expression of agp33/27,000Mw activation inducer molecule (AIM) on human lymphoid tissues. Induction of cell proliferation on thymocytes and B lymphocytes by anti-AIM antibodies. *Immunology.* 1989;68(1):72–79.
13. Testi R, Pulcinelli F, Frati L, Gazzaniga PP, Santoni A. CD69 is expressed on platelets and mediates platelet activation and aggregation. *J Exp Med.* 1990; 172 (3): 701–707.
14. Risso A, Smilovich D, Capra MC, Baldissarro I, Yan G, Bargellesi A, et al. CD69 in resting and activated T lymphocytes: its association with a GTP binding protein and biochemical requirements for its expression. *J Immunol.* 1991;146(12):4105–4114.
15. Kamphuis E, Junt T, Waibler Z, Forster R, Kalinke U. Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia. *Blood.* 2006;108(10): 3253–3261.
16. Testi R, Ambrosio DD, deMaria R, Santoni A. The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. *Immunology Today.* 1994;15(10):479–483.
17. Julius P, Luttmann W, Knoechel B, Kroegel C, Matthys H, Virchow JC Jr. CD69 surface expression on human lung eosinophils after segmental allergen provocation. *Eur Respir J.* 1999; 13:1253–1259. PubMed. [PubMed:10445598]
18. Toma T, Mizuno K, Okamoto H, Kanegane C, Ohta K, Ikawa Y et al. Expansion of activated eosinophils in infants with severe atopic dermatitis. *Pediatr Int.* 2005; 47:32–38. PubMed.
19. Lourenco O, Mafalda Fonseca A, Taborda-Barata L. T cells in sputum of asthmatic patients are activated

- independently of disease severity or control. *Allergol Immunopathol (Madr)*. 2009; 37:285–292. doi: 10.1016/j.aller.2009.05.004.
20. Werfel T, Boeker M, Kapp A. Rapid expression of the CD69 antigen on T cells and natural killer cells upon antigenic stimulation of peripheral blood mononuclear cell suspensions. *Allergy*. 1997; 52:465–469. PubMed.
21. Kapsenberg ML, Wierenga EA, Stiekema FE, Tiggleman AM, Bos JD. Th1 lymphokine production profiles of nickel-specific CD4⁺ T lymphocyte clones from nickel contact allergic and non allergic individuals. *J Invest Dermatol*. 1992; 98: 59–63. PubMed.
22. Liu L, Inoue H, Nakayama H, Kanno R, Kanno M. The endogenous danger signal uric Acid augments contact hypersensitivity responses in mice. *Pathobiology*. 2007;74(3):177-85. PubMed.
23. Xu H, DiJulio NA, Fairchild RL. T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: interferon gamma-producing (Tc1) effector CD8⁺ T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4⁺ T cells. *J Exp Med*. 1996; 183: 1001–12. PubMed.
24. Honda T, Egawa G, Grabbe S, Kabashima K. Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis. *J Invest Dermatol*. 2013; 133: 303–15. PubMed. doi: 10.1038/jid.2012.284.
25. Liu J, Harberts E, Tammaro A, Girardi N, Filler RB, Fischelevich R. IL-9 regulates allergen specific Th1 responses in allergic contact dermatitis. *J Invest Dermatol*. 2014; 134: 1903–11. PubMed. doi: 10.1038/jid.2014.61.
26. Radulovic K, Manta C, Rossini V, Holzmann K, Kestler HA, Wegenka UM, et al. CD69 regulates type I IFN-induced tolerogenic signals to mucosal CD4 T cells that attenuate their colitogenic potential,” *J Immunol*. 2012;188(4):2001–2013. PubMed. doi: 10.4049/jimmunol.1100765.
27. Avgustin B, Kotnik V, Skoberne M, Malovrh T, Skralovnik-Stern A, Tercej M. CD69 expression on CD4⁺ T lymphocytes after in vitro stimulation with tuberculin is an indicator of immune sensitization against Mycobacterium tuberculosis antigens. *Clin Diagn Lab Immunol*. 2005;12: 101-106.
28. Granchi D, Ciapetti G, Savarino I, Stea S, Filippini F, Sudanese A, et al. Expression of the CD69 activation antigen on lymphocytes of patients with hip prosthesis. *Biomaterials*. 2000; 21: 2059-2065 PubMed.
29. Beeler A, Zaccaria L, Kawabata T, Gerber BO, Pichler WJ. CD69 upregulation on T cells as an in vitro marker for delayed-type drug hypersensitivity. *Allergy*. 2008; 63:181-188 PubMed.
30. Cederbrant K, Anderson C, Andersson T, Marcussonstahl M, Hultman P. Cytokine production, lymphocyte proliferation and t-cell receptor Vbeta expression in primary peripheral blood mononuclear cell cultures from nickel-allergic individuals. *Int Arch Allergy Immunol*. 2003; 132: 373-379.
31. Minidis NM, Mesner O, Agan BK, Okulicz JF. Delayed type hypersensitivity (DTH) test anergy does not impact CD4 reconstitution or normalization of DTH responses during antiretroviral therapy. *J Int AIDS Soc*. 2014 Feb 4;17:18799. doi: 10.7448/IAS.17.1.18799.