

Strain differences in contact hypersensitivity reaction to dinitrochlorobenzene (DNCB) in rats



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ARTICLE INFO

Article history:

Received 16 May 2014

Accepted 11 November 2014

Available online 20 November 2014

Keywords:

AO and DA rats

Contact hypersensitivity

Dinitrochlorobenzene

Draining lymph node

Proliferation

Cytokines

ABSTRACT

Genetic factors are among the most important determinants of susceptibility to induction of allergic contact dermatitis. A limited number of studies of experimental contact hypersensitivity (CHS) in animals has shown differences in the severity of CHS; however, the underlying mechanisms are unknown. In this study comparative analysis of CHS to low and high dinitrochlorobenzene (DNCB) doses regimen of sensitization/challenge in inbred Dark Agouti (DA) and Albino Oxford (AO) rats was examined. Basic aspects of draining lymph node (dLN) activity (cellularity, proliferation), proinflammatory (IFN- γ , IL-17) and anti-inflammatory (IL-10) cytokine gene expression and production, as well as IL-12 and IL-23 subunits mRNA expression, were examined in challenge and sensitization phase of CHS reaction. Lower (compared to DA) intensity of CHS in AO rats was associated with lack of (or negligible) dLN responses in challenge phase (*ex vivo*, hapten- or IL-2-stimulated cell proliferation and proinflammatory cytokine mRNA and production levels) but with lack of changes in IL-10 response. Less pronounced dLN activity of sensitized animals of this strain was observed as well. Higher proliferative activity and more pronounced proinflammatory cytokine response during challenge and sensitization phase suggest these activities as underlying mechanisms of higher susceptibility of DA rats to CHS response to DNCB.

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1. Introduction

Allergic contact dermatitis (ACD) is a common T-cell mediated skin inflammatory disease in humans, that develops at the site of skin re-exposure to contact allergens in individuals previously sensitized with the same agent (Saint-Mezard et al., 2004). Contact hypersensitivity (CHS) to haptens, animal model of ACD, has provided a great contribution to understanding pathophysiology of this disease. Two distinct phases are necessary to achieve optimal CHS reaction: the sensitization phase (known as afferent or induction phase) starting with the first contact of skin with hapten and challenge phase (known as efferent or elicitation phase) which occurs following second hapten encounter (Grabbe and Schwarz, 1998; Kish et al., 2009). While sensitization phase has no clinical manifestations in the majority of cases, challenge of skin of sensitized individuals leads to local inflammatory reaction. CHS is

considered usually as Th1/type1 immune reaction, with IFN- γ as main effector cytokine (Gocinski and Tigelaar, 1990; Xu et al., 1996). Recent studies of ACD in humans and CHS in mice have stressed importance of both Th1/type1 and Th17/type17 cells for full development of reaction (He et al., 2009; Pennino et al., 2010).

Despite the fact that limited studies exist in humans, genetic factors (along with dermal integrity), beside environmental factors (Schnuch and Carlsen, 2011), are considered as most important determinants of susceptibility to induction of ACD (Felter et al., 2002). Concerning CHS, there are only few studies that have examined genetically determined differences of susceptibility to haptens in inbred strains of experimental animals. It was shown that more severe and mild intensity of CHS reaction to dinitrofluorobenzene (DNFB) in C57BL/J and BALB/C mice, respectively, correlated with proinflammatory effects of the hapten in challenged skin of these strains (Bonneville et al., 2007). Studies in rats with different propensity of development of Th1/Th2 cytokine responses showed that Th1-prone Lewis (Lew) (RT-1^M MHC haplotype) rats were more sensitive to dinitrohalobenzenes, compared to Th-2 prone Brown Norway (BN) rats (RT-1ⁿ MHC haplotype), based on microscopic and macroscopic evaluation of challenged skin (Peszowski et al., 1994). Cellular and molecular mechanisms underlying such differences in CHS expression, however, remained largely unknown.

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Studies of genetic factors that are involved in differences related to development of inflammatory/immune mediated diseases in these rats showed both MHC-dependent (due to a deficit in CD8⁺ cells in BN rats) as well as MHC-non-dependent differences (predominance of type 2 and regulatory cytokines, higher representation of CD45RC^{low} cells) (Fournié et al., 2001).

Using Dark Agouti (DA, Rt1^{av1}) rats the proinflammatory character (IFN- γ and IL-17 response by draining lymph node cells) of sensitization phase of CHS to low DNCB dose (0.4% w/v) was previously shown (Popov et al., 2011). Lower intensity of proinflammatory cytokine production resulted from preliminary experiments in that study in Albino Oxford (AO RT^u) rats indicated differences in CHS between these two rat strains. Studies of immune-inflammatory disease such as experimental autoimmune encephalomyelitis (EAE) in DA and AO rats showed that major differences which caused susceptibility to disease were mainly related to type 1 (Lukic et al., 2001; Mostarica-Stojković et al., 1982) and Th17 cytokines (Markovic et al., 2009). In the light of these reports, the aim of this study was comparative analysis of immune/inflammatory responses in CHS to DNCB in these two strains. Low and high DNCB doses were used and cardinal aspects of activity of lymph nodes that drain challenged as well as sensitized skin were analyzed. It was shown that lower intensity of CHS in AO rats was associated with lack (or low levels) of reactivity of draining lymph nodes to challenge (proliferative activity and inflammatory cytokine gene expression and production). Less pronounced draining lymph node response to sensitization was observed in this rat strain.

2. Materials and methods

2.1. Chemicals and reagents

One-chloro-2,4-dinitrobenzene (DNCB) was obtained from BDH Chemicals Ltd., Hull, UK and dissolved in 4:1 acetone:olive oil (vehicle). RPMI-1640 culture medium (PAA Laboratories, Pasching, Austria) supplemented with 25 mM HEPES, 2 mM glutamine, 20 mg/ml gentamycin, 5 μ g/ml antifungal agent voriconazole and 5% (v/v) heat-inactivated fetal calf serum (FCS) (PAA Laboratories) was used in cell culture experiments. Two, four-Dinitrobenzenesulfonic acid (DNBS) (Aldrich Chemical Company Inc., Milwaukee, USA), radiolabeled [³H]-thymidine (GE Healthcare, UK), recombinant human IL-2 (rIL-2, R&D Systems, Minneapolis, USA) and Concanavalin A (ConA) (Pharmacia, Uppsala, Sweden) were dissolved in culture medium, and added in final concentration 10 μ g/ml, 50 μ Ci/ml, 50 i.u./ml and 1 μ g/ml, respectively. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (ICN Biomedicals Inc, Aurora, USA) was dissolved in phosphate-buffered saline (PBS, pH 7.2). FITC-labeled mouse antibodies to rat CD4, PE-labeled mouse antibodies to rat CD8 and PE-labeled mouse antibodies to rat CD25 (IgG1) were purchased from eBioscience Inc. (San Diego, CA, USA). Propidium iodide and RNase were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals

Ten to twelve week old male Dark Agouti (DA) and Albino Oxford (AO) rats, bred and conventionally housed at the Institute for Biological Research "Sinisa Stankovic", Belgrade, Serbia, were used. They were maintained at 12 h photoperiod, 21/24 °C temperature control and 60% relative humidity. All rats had *ad libitum* access to standard rodent chow and water throughout the study. Four to six animals were assigned to each treatment group per experiment, in at least two independent experiments. After treatment, animals were caged individually. All animal procedures were complied with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee of the Institute for Biological Research "Sinisa Stankovic".

2.3. Contact hypersensitivity

Animals were sensitized by applying 100 μ l of solution of 0.4% (w/v) or 4% (w/v) DNCB dissolved in acetone/olive oil (vehicle) for two consecutive days to the upper part (~16 cm²) of the dorsal skin clipped of hair. Five days following the last application of DNCB, rats were challenged to the ventral and dorsal surfaces of the right ear by an application of 50 μ l of three times lesser dose of DNCB. Rats sensitized with 0.4% DNCB were challenged with 0.13% DNCB (low dose sensitization/challenge regimen), while rats that were sensitized with 4% DNCB were challenged with 1.3% DNCB (high dose sensitization/challenge regimen). Control rats received vehicle (acetone/olive oil) both to the dorsal skin area and to the ears. One group

of non-sensitized animals were challenged with 0.13% DNCB (0%/0.13%) or 1.3% DNCB (0%/1.3%), as additional controls. The ear swelling, an *in vivo* measure of contact hypersensitivity (CHS) response, was assessed in a blinded fashion by measuring pinnal thickness 24 hours following ear challenge, by a hand-held engineer's micrometer (six measurements per ear). In separate experiment, ear thickness was measured 48 hours following ear challenge. An increase in ear swelling was defined as a difference between ear thickness 24 hours or 48 hours after challenge and ear thickness before challenge.

2.4. Histology

Ears skin samples were taken 24 hours or 48 hours following challenge. Dorsal skin was taken three and five days following sensitization. Tissue samples were fixed in 4% buffered formalin (pH 6.9), embedded in paraffin wax for sectioning at 5 μ m, and stained with hematoxylin and eosin (H&E). Pathohistological analyses were made (by a certified specialist) using a Coolscope digital light microscope (Nikon, Tokyo, Japan). Epidermal and dermal infiltrate of mononuclear cells (MNC) and polymorphonuclear cells (PMNC) was counted in at least 30 non-overlapping microscopic fields under immersion, covering the entire area of the section. Area of one field of view corresponded to 0.196 mm², at 1000 $\times$ magnification (10 $\times$ ocular/100 $\times$ objective). The average cell number per field, determined for every animal, was used for statistical analysis, and the results were presented as median and range.

2.5. Draining lymph node (dLN) cell preparation

Auricular lymph nodes (that drain the challenged ear skin) were harvested 24 hours or 48 hours following challenge. Suprascapular and axillary lymph nodes (that drain sensitized skin) were isolated five days following sensitization. Lymph nodes were also harvested from control animals. Cell suspensions were prepared by mechanical teasing of dLN over nylon mesh (70 μ m nylon, BD Bioscience, Bedford, USA) and cells were counted by the improved Neubauer hemacytometer. The number of viable cells, determined by using a trypan blue exclusion assay, always exceeded 95%.

2.6. Draining lymph node cell proliferative activity

For proliferation measurements, freshly isolated dLN cells (0.6×10^6 /well) were cultured in 96-well microplates (Sarstedt Inc., Newton, NC, USA) for 16 hours with 0.5 μ Ci [³H]-thymidine/well added at the onset of culture (*ex vivo* proliferation). For IL-2-stimulated proliferative activity dLN cells (0.6×10^6 cells/well) were cultured in the culture medium solely or in the presence of human rIL-2 (50 i.u./ml), with 0.5 μ Ci [³H]-thymidine/well added following 24 hours of culture and incubated for the next 16 hours. For hapten-stimulated proliferative activity dLN cells (0.6×10^6 cells/well) were cultured in the culture medium solely or in the presence of DNBS (10 μ g/ml) for 96 hours, with 0.5 μ Ci [³H]-thymidine/well added during the last 16 hours of culture. Incorporation of [³H]-thymidine was measured by liquid scintillation counting (1219 RackBeta, LKB Wallac, Turku, Finland) and proliferation expressed as counts per minute (c.p.m.).

2.7. Analysis by flow cytometry

Draining lymph node cells (1×10^6) were incubated on ice with FITC- and PE-conjugated antibodies to rat CD4 and CD8, respectively, or PE-conjugated antibodies to rat CD25 for 30 minutes. After washing, the cells were fixed with 1% paraformaldehyde and assayed for fluorescence intensity on a FACSCalibur cytometer (BD Biosciences, Heidelberg, Germany).

2.8. MTT assay for draining lymph node cell metabolic viability

For assessing the dLN cell viability, MTT reduction assay described for lymphocyte cell lines (Denizot and Lang, 1986) was employed. Cells (1×10^5 cells/well of 96-well plate) were placed in wells with culture medium. MTT (final concentration 0.5 mg/ml) was added immediately and incubated with cells for 3 hours at 37 °C in a humidified atmosphere with 5% CO₂. Produced formazan was dissolved by overnight incubation in acidified SDS (10% SDS–0.01 N HCl). Extracted chromogen was quantitated spectrophotometrically at 540 nm using ELISA 96-well plate reader (Shimadzu Corporation, Lakewood, USA).

2.9. Analysis of hypodiploid cells in draining lymph node cell suspensions by propidium iodide and flow cytometry

For measuring dLN cell apoptosis, the presence of hypodiploid cells was analyzed by staining with propidium iodide and flow cytometry analysis as described (Riccardi and Nicoletti, 2006). Cells (2×10^6) were washed in PBS, and then fixed by adding 70% (v/v) cold ethanol (in PBS), for at least 30 minutes on ice. Cells were then washed twice in PBS, at 900 $\times$ g, re-suspended in 1 ml of staining solution (10 μ g/ml of propidium iodide and 51 μ g/ml RNase, all in PBS) and incubated for 45 minutes at room temperature in the dark. Cells were analyzed on FACSCalibur cytometer (BD Biosciences), collecting 20,000 events. Apoptotic nuclei appeared as a broad

hypodiploid DNA peak which was discriminable from the peak of normal (diploid) DNA content in the red fluorescence channels.

2.10. Draining lymph node cell culture for cytokine production

For cytokine production, dLN cells (1.2×10^6 /well) were cultured in 96-well plates (Sarstedt Inc.) for 72 hours solely (spontaneous production) or in the presence of $10 \mu\text{g/ml}$ of DNBS (hapten-specific production).

2.11. ELISA

Cytokine production was determined in media conditioned by dLN cells using commercially available ELISA sets for rat IFN- γ and IL-10 (purchased from R&D), and mouse IL-17 (cross-reactive with rat IL-17) (purchased from eBioscience). Cytokine titers were calculated by reference to a standard curve constructed using known amounts of respective set-provided recombinant cytokines.

2.12. Reverse transcription – real time polymerase chain reaction (RT-PCR)

Total RNA was isolated from dLN cells immediately after isolation with an RNA Isolator (Metabion, Martinsried, Germany) according to the manufacturer instructions. Reverse transcription of isolated RNA was conducted using random hexamer primers and MMLV (Moloney Murine Leukemia Virus) reverse transcriptase following the manufacturer instructions (Fermentas, Vilnius, Lithuania). Amplification of prepared cDNA was done in a total volume of $20 \mu\text{l}$ using Power SYBR[®] Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Thermocycler conditions were: an initial step at 50°C for 5 min, followed by a step at 95°C for 10 min and subsequent 2-step PCR program at 95°C for 15 s and 60°C for 60 s for 40 cycles. PCR primers (forward/reverse) used in the study were:

IFN- γ : 5'-AACAGTAAAGCAAAAAGGATGCA-3'/5'-TGTGCTGGATCTGTGGTGTG-3';

IL-17: 5'-CTACCTCAACCGTTCACCTTCAC-3'/5'-CCTCCAGATCAGAGAAGGATATC-3';

p35: 5'-CCGGTCCAGCATGTGTCA-3'/3'-GCCGAAGTGAGGTGGTTAGG-5'

p40: 5'-ACGGACTTGAAGTTTAAACATCAAGAG-3'/3'-AGAGATGCTCGTCCACATGTCA-5';

p19: 5'-GACCAGCTTCATACCTCCCTACT-3'/3'-TCAGGCGAGGCATCTGTTG-5';

β actin: 5'-CCCTGGCTCTAGCACCAT-3'/5'-GAGCCACCAATCCACACAGA-3'.

PCR products were detected in real-time and results were analyzed with 7500 System Software (Applied Biosystems) and calculated as $2^{-\Delta\text{Ct}}$ where ΔCt was the difference between Ct values of specific gene and the endogenous control (β -actin). Results were expressed as arbitrary units, relative to control group.

2.13. Statistics

Results were pooled from at least two experiments and expressed as median values and range (min–max). Significance was defined by Mann–Whitney U test, and

p -values lower than 0.05 were considered significant. Correlations were analyzed by linear regression.

3. Results

3.1. Ear swelling response after challenge of CHS in DA and AO rats

Animals were sensitized by 0.4% or 4% DNCB for two consecutive days and five days following the last application of DNCB, ears were challenged with the respective three times lesser dose of DNCB, i.e. 0.13% for rats sensitized with 0.4% DNCB or 1.3% for those individuals sensitized with 4% DNCB. Such treatments were assigned as low-dose and high-dose sensitization/challenge regimens of treatment with DNCB, respectively. Non-sensitized animals were challenged as well with low DNCB dose (0%/0.13%) or higher dose (0%/1.3%).

Measurements of ear thickness in rats treated according to low DNCB dose regimen 24 hours following challenge revealed the response that was numerically ($p = 0.070$) higher compared to non-sensitized/non-challenged (control) animals (Fig. 1, Panel A). High DNCB dose treatment resulted in significantly more intense ear swelling response in both DA and AO rats, but it was lower in AO compared to DA rats. Challenge of non-sensitized animals of both strains resulted in increase (compared to vehicle treated individuals) in ear thickness only at higher challenge dose (0%/1.3%), but it was significantly lower compared to sensitized/challenged animals (4%/1.3%).

Histological analysis of ear skin of animals challenged 24 hours earlier with low DNCB dose revealed signs of inflammation (dermal edema, vascular congestion, low-grade leukocyte cell infiltration) (not shown). These changes were not seen in all animals. High DNCB dose treatment revealed pronounced inflammation with characteristic tissue edema, and cell infiltration in all animals treated, but it was more intense in DA compared to AO rats. Number of mononuclear (MNC) and polymorphonuclear cells (PMNC) that infiltrated ear skin 24 hours following challenge was increased only after high DNCB dose treatment compared to controls, in both DA and AO rats; however, significantly lower number of MNC was detected in AO compared to DA rats (Fig. 1, Panel A). Increased (compared to vehicle-treated controls) numbers of MNC and PMNC were noted only in samples of non-sensitized DA animals challenged with 1.3% DNCB dose, but mononuclear cell counts were lower compared to the

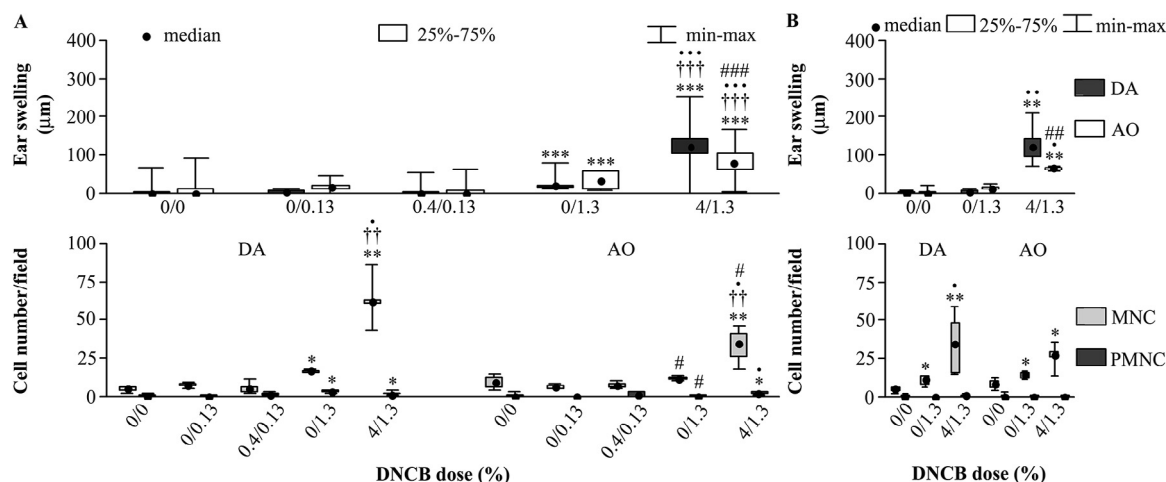


Fig. 1. Ear swelling response after challenge of CHS reaction in DA and AO rats. Increase in ear thickness and number of cells that infiltrated ear skin 24 hours (Panel A) and 48 hours (Panel B) following challenge. Number of mononuclear cells (MNC) and polymorphonuclear cells (PMNC) that infiltrated ear skin were counted at $1000\times$ magnification (at the area of one field corresponding to 0.196 mm^2). Results were pooled from at least three experiments with 4–6 animals per group (for 24 h) and presented as median and range (min–max). Results for 48 h were obtained from separate experiment. Significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs 0/0% DNCB; †† $p < 0.05$, ††† $p < 0.001$ vs 0.4/0.13% DNCB; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs non-sensitized/challenged controls; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DA rats.

individuals treated with high DNCB dose (4%/1.3%) protocol (though higher compared to AO rats).

Measurements conducted 48 hours following challenge in a separate experiment (Fig. 1, Panel B) showed significantly lower ($p < 0.05$ compared to 24 hours) values of ear thickness in non-sensitized DA rats challenged with 1.3% that became similar to values noted in non-sensitized non-challenged (control) animals. Challenge of sensitized animals resulted in similar (vs 24 h) ear swelling, but with lower values in AO compared to DA rats. Lower numbers ($p < 0.05$ compared to 24 hours) of PMNC were present in skin of non-sensitized DA and AO rats challenged with 1.3% DNCB and were similar to numbers noted in individuals treated with high DNCB dose (4%/1.3%). Numbers of PMNC were similar in sensitized and challenged DA and AO rats, and comparable to strain-respective controls. Lower numbers ($p < 0.05$ vs 24 hours) of MNC were noted in DA rats and were comparable to MNC numbers in AO rats. Similar numbers of MNC were observed in sensitized and challenged DA and AO rats, but were higher compared to controls.

Although high challenge dose induced elevated ear swelling of non-sensitized rats 24 hours later in both strains compared to non-sensitized/non-challenged animals (however significantly lower compared to sensitized animals), this treatment (0%/1.3%) did not affect the activity of dLN nodes (measured for *ex vivo* proliferation, and IFN- γ and IL-17 production) (data not shown).

3.2. Draining lymph node cellularity and basic T cell subset composition 24 hours following challenge

To examine the activity of lymph nodes which drain challenged skin, cellularity and basic T cell subset composition were measured first (Table 1). Both low and high DNCB dose sensitization/challenge regimens resulted in significantly higher (compared to controls) dLN cell numbers in DA rats, while numerical increase ($p = 0.067$) was observed solely at high DNCB dose regimen in AO rats, however significantly lower compared to DA rats. Although unchanged, cellularity of dLN of AO rats treated with 0.4%/0.13% DNCB was lower compared to similarly treated DA rats. Analysis of dLN T cell subset composition revealed decreased (compared to controls) relative numbers of CD4⁺ cells and increased numbers of CD8⁺ cells at both regimens of DNCB treatment in DA rats. A decrease of CD4⁺ cells was noted in AO rats as well (significant at low-dose and numerical, $p = 0.077$, at high-dose regimen of DNCB treatment), while only high-dose treatment increased CD8⁺ cells. Due to gain in dLN cellularity lack of changes of total numbers of CD4⁺ cells were found in DA rats, while even an increase was noted at high-dose regime

of DNCB treatment in AO rats. Higher numbers of CD8⁺ T cells were found at both low-dose and high-dose of DNCB exposure in DA rats, while only at the latter regimen in AO rats, but were lower compared to DA rats. Although no changes were observed at low DNCB regime of sensitization/elicitation in dLN of AO rats, cell numbers were lower than in DA rats. Calculation of CD4⁺ to CD8⁺ cell ratio revealed significantly lower values at both treatments and in both strains.

3.3. Draining lymph node cell proliferative activity 24 hours following challenge

Dose-dependently increased *ex vivo* (Fig. 2A), as well as IL-2 stimulated (Fig. 2C), proliferation of dLN cells was noted in DA rats, while both these activities remained unchanged in AO rats (compared to controls), but were lower compared to DA rats at high dose for *ex vivo* proliferation and at both doses for IL-2-stimulated proliferation. Increased hapten-stimulated proliferation was detected in DA rats only at high DNCB dose regimen, compared to controls and low DNCB dose regimen, while in AO rats it was unchanged compared to controls, and significantly lower at high dose compared to DA rats (Fig. 2B). Measurements of membrane expression of CD25 molecule (Fig. 2D) showed lower percentages of CD25⁺ cells in control (non-sensitized/non-challenged) AO compared to DA rats and numbers stayed unchanged (compared to controls) after low-dose and high dose treatment regimens. In contrast, higher numbers of cells that express CD25 molecules were noted in DA rats (though only numerically, $p = 0.076$ at low-dose DNCB treatment). Lower ($p < 0.05$) density (Mean Fluorescence Intensity, MFI) of CD25 expression was observed in control AO (median 20.3, range 18.2–20.4) compared to DA rats (median 25.9, range 23.2–26.4). MFI increased ($p < 0.05$) in DA rats following challenge (median 32.6, range 28.4–35.5, and median 36.0, range 32.4–37.9, for low and high DNCB dose treatments, respectively, compared to controls). Increase ($p < 0.05$ compared to controls) was observed at high dose regimen solely in AO rats (median 23.6, range 22.2–28.0) but was lower ($p < 0.05$) compared to values obtained at respective DNCB dose treatment in DA rats.

To obtain the information if there were alterations in cell viability (a change that might have contributed to proliferative inactivity of dLN cells of AO rats), MTT assay was employed for metabolic viability determination and percentage of apoptotic cells was determined by propidium iodide staining and flow cytometry analysis. Results showed no compromise in MTT reduction by dLN cells of challenged as compared to control animals in both strains (Fig. 3A)

Table 1
Draining lymph node cellularity and basic T cell subset composition during challenge phase.

Parameters	DA			AO		
	DNCB dose (%)			DNCB dose (%)		
	0/0	0.4/0.13	4/1.3	0/0	0.4/0.13	4/1.3
Cell number ($\times 10^6$)	6.7 (0.6–15.4)	13.1* (5.2–20.0)	12.3* (5.7–20.0)	4.6 (2.0–7.8)	3.5* (1.5–7.3)	5.6* (4.4–8.8)
CD4 ⁺ cells	49.1 (42.7–58.2)	45.1* (40.4–48.9)	44.3* (38.7–53.9)	47.3 (42.3–51.3)	40.9* (35.7–50.3)	43.8 (39.4–48.3)
	$\times 10^6$	2.9 (1.0–7.5)	5.91 (2.4–8.8)	4.2 (1.8–8.9)	2.0 (0.6–3.2)	2.4* (1.9–4.2)
CD8 ⁺ cells	21.5 (15.3–28.8)	27.5* (22.4–29.7)	25.6* (18.8–29.1)	22.2 (16.4–31.9)	23.6 (19.9–28.0)	25.8* (21.6–36.5)
	$\times 10^6$	1.3 (0.3–4.0)	3.7* (1.4–5.6)	0.9 (0.2–1.5)	0.4 (0.2–2.1)	2.0* (1.3–4.1)
CD4 ⁺ /CD8 ⁺	2.2 (1.6–2.7)	1.7* (1.5–1.9)	1.7* (1.5–2.0)	2.4 (1.7–2.6)	1.9* (1.4–2.1)	1.7* (1.3–2.1)

Auricular draining lymph node (dLN) cells were isolated 24 h after challenge of sensitized rats.

Results are presented as median and range (min–max).

Significantly different at: * $p < 0.05$ vs 0/0% DNCB; # $p < 0.05$ and ## $p < 0.01$ vs DA rats.

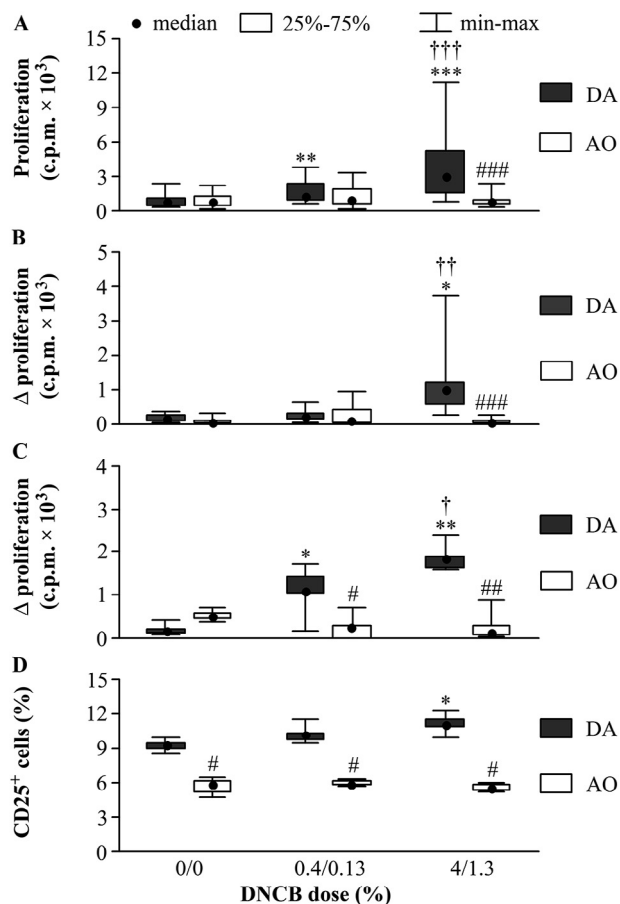


Fig. 2. Draining lymph node cell proliferative activity in challenge phase of CHS. Auricular draining lymph node (dLN) cells were isolated 24 h after challenge of sensitized rats. *Ex vivo* proliferative activity (A). Hapten-stimulated proliferative activity (expressed as differences between hapten-stimulated and non-stimulated proliferation) (B); IL-2 stimulated proliferative activity (expressed as differences between IL-2-stimulated and non-stimulated proliferation) (C). Number of CD25⁺ cells (D). Results were pooled from at least two experiments with 4–6 animals per group and presented as median and range (min–max). Significantly different at **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs 0/0% DNCB; †*p* < 0.05, ††*p* < 0.01, †††*p* < 0.001 vs 0.4/0.13% DNCB and #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 vs DA rats.

and even increased values were observed in DA rats (significantly at high- and a tendency, *p* = 0.060, at low-dose treatment with DNCB). However, increased percentages of cells with hypodiploid DNA content were observed in dLN cells of AO rats after high dose DNCB sensitization/challenge regimen (compared to controls and DA rats), while no changes could be observed in DA rats (Fig. 3B).

3.4. Cytokine mRNA expression and production by draining lymph node cells 24 hours following challenge

Measurements of IFN- γ gene expression and cytokine production by dLN cells (Fig. 4A) revealed an increase of mRNA expression in dLN cells at both low or high DNCB dose treatments in DA rats, while only at high-dose regimen of sensitization/challenge in AO rats (however lower compared to DA rats at both DNCB doses regimens). Both spontaneous as well as hapten-specific IFN- γ production were increased dose-dependently by dLN cells of DNCB-treated DA rats, while in AO rats they stayed unchanged compared to controls, and lower compared to DA rats.

When IL-17 gene expression and production were measured (Fig. 4B) no significant changes in mRNA for IL-17 were observed (owing to variability of data) in DA rats, but spontaneous and hapten-

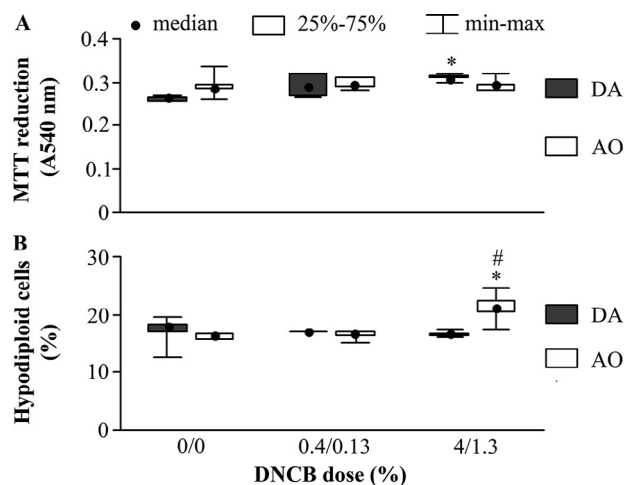


Fig. 3. Draining lymph node cells viability after challenge of CHS reaction. Auricular draining lymph node (dLN) cells were isolated 24 h after challenge of sensitized rats. MTT assay (A) and the percentage of hypodiploid cells (B). Results were pooled from two experiments with 4 animals per group and presented as median and range (min–max). Significantly different at **p* < 0.05 vs 0/0% DNCB; #*p* < 0.05 vs DA rats.

specific production of this cytokine was increased (the latter dose-dependently). In AO rats, numerical increase (*p* = 0.070) of IL-17 mRNA expression was detected after challenge with high DNCB dose, while cytokine production remained unchanged compared to controls, and lower at high doses regime compared to DA rats.

Measurements of IL-10, anti-inflammatory/regulatory cytokine, showed no changes in spontaneous production in AO rats either following high-dose (median 146 pg/ml, range 57–264) or low-dose treatment (median 165 pg/ml, range 100–419) compared to (median 195 pg/ml, range 119–259) controls. Negligible or no response to hapten stimulation was observed (not shown). Unchanged levels of IL-10 produced were noted in DA rats as well (median 274 pg/ml, range 99–363, and median 216 pg/ml, range 139–400, in high and low DNCB dose treatment, respectively compared to median 299 pg/ml, range 145–377 in controls).

In light of the observed differences in IFN- γ and IL-17 production and mRNA expression between DA and AO rats during challenge, it was further analyzed mRNA expression for subunits of IL-12 and IL-23, considered as Th1-promoting and Th17-inducing cytokines, respectively (Table 2). Increased mRNA expression for p40 subunit, which is shared by both IL-12 and IL-23, was detected in DNCB treated DA rats (tendency of increase for both DNCB dose regimen, *p* = 0.055) compared to controls, while in AO rats p40 mRNA expression remained unchanged compared to controls, but numerically (*p* = 0.078) lower at higher DNCB dose treatment compared to DA rats. Expression of mRNA for p35 and p19, specific subunits of IL-12 and IL-23 heterodimers respectively, was significantly increased only in DA rats treated with high DNCB dose compared to controls. In AO rats, mRNA expression for these proteins was unchanged compared to controls and lower for p19 at high dose compared to DA rats.

3.5. Draining lymph node cell activity 48 hours following challenge

Having in mind that differences in ear thickness were noted 48 hours following challenge, selected parameters of draining lymph node cell activity were examined at this time point in separate experiment and included dLN cellularity, IL-2-driven and hapten-specific cell proliferation, number of CD25⁺ cells and hapten-specific proinflammatory cytokine production (Table 3). Higher (vs 24 h, *p* < 0.01) dLN cellularity was noted in sensitized and

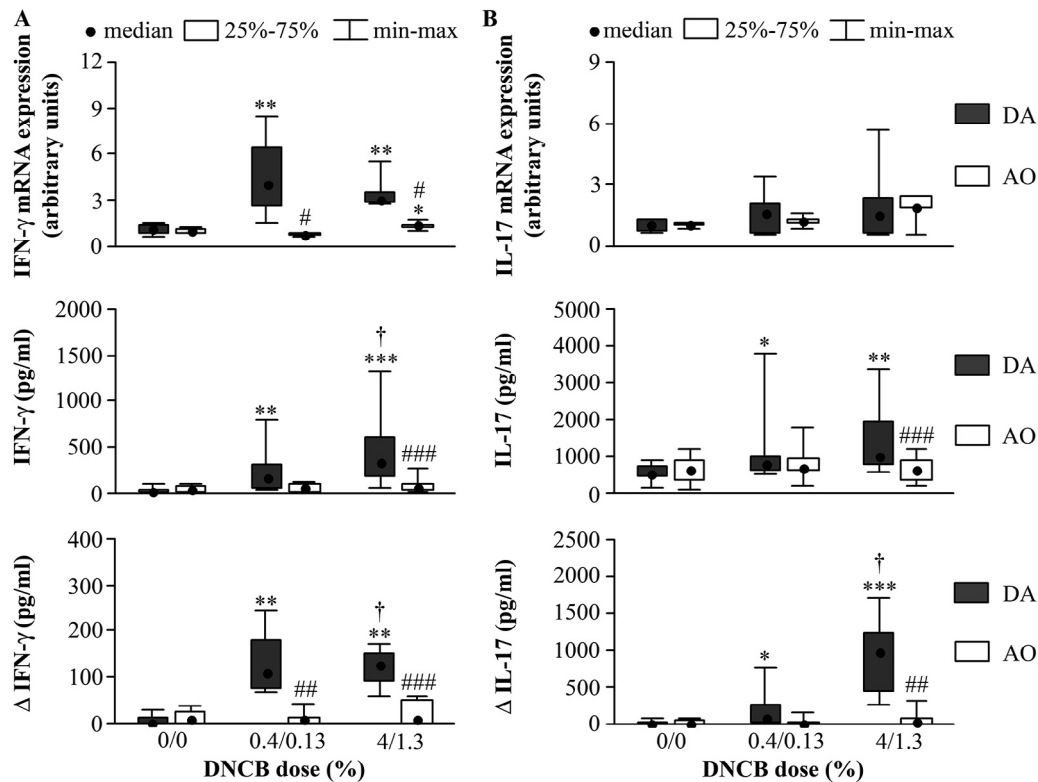


Fig. 4. Cytokine mRNA expression and production by draining lymph node cells after challenge of CHS reaction. Auricular draining lymph node (dLN) cells were isolated 24 h after challenge of sensitized rats. IFN- γ mRNA expression (above), spontaneous (middle) and specific production (below) (A). IL-17 mRNA expression, spontaneous and specific production (B). Cytokine gene expression was analyzed with RT-PCR and presented as relative value. Hapten specific IFN- γ and IL-17 production was presented as differences between DNBS-stimulated and spontaneous production. Results were pooled from at least three experiments with 4–6 animals per group for production and from two experiments for RT-PCR, and presented as median and range (min–max). Significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs 0/0% DNCB; † $p < 0.05$ vs 4/1.3% DNCB and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DA rats.

challenged DA and AO rats and dLN cell numbers were higher compared to controls in both strains, but no strain-related differences were observed at this time point. Although both IL-2-stimulated and hapten-stimulated cell proliferation were lower compared to 24 h ($p < 0.05$) in DA rats, they stayed higher in this strain as compared to AO strain (however higher in both strains compared to controls). Measurements of membrane expression of CD25 molecule showed lower ($p < 0.05$) percentages of CD25⁺ cells compared to values noted 24 h following challenge. Relative number of CD25⁺ cells was higher in DNCB-treated DA and AO rats compared to controls, however lower in AO compared to DA rats. Increased (compared to values measured 24 h following challenge and compared to respective controls) hapten-stimulated production of IFN- γ was noted in both strains, but the levels were higher in DA compared to AO

rats. In contrast, lower (vs 24 h) levels of IL-17 production were noted in both rat strains which were similar to those in controls.

3.6. Skin appearance following sensitization in DA and AO rats

Remodeling of dermal microvascular plexus, small but progressive accumulation of mononuclear cells in dermis and adaptation of skin barrier via hyperkeratosis and hypergranulosis were seen in AO rats sensitized by 0.4% DNCB 3 days following sensitization. Dermal vascular plexus remodeling seemed less prominent 5 days following sensitization. Sensitization with higher DNCB dose resulted in more pronounced remodeling process (day 3) and development of diffuse dermal edema and accumulation of discrete numbers of mononuclear cells in dermis (day 5 following

Table 2
p35, p40 and p19 mRNA expression by dLN cells during challenge phase.

mRNA expression (arbitrary units)	DA			AO		
	DNCB (%)			DNCB (%)		
	0/0	0.4/0.13	4/1.3	0/0	0.4/0.13	4/1.3
p35	0.97 (0.58–1.39)	1.32 (0.86–2.62)	1.83* (0.85–2.93)	0.99 (0.65–1.23)	1.18 (0.57–2.69)	1.49 (0.34–1.88)
p40	1.00 (0.48–1.60)	1.56 (0.73–2.13)	1.79 (1.06–3.70)	0.99 (0.80–1.23)	1.13 (0.80–1.72)	1.09 (0.50–1.45)
p19	1.00 (0.28–1.54)	0.96 (0.37–1.77)	1.40* (1.22–1.99)	0.98 (0.23–1.57)	0.69 (0.34–1.41)	0.76* (0.35–1.94)

Auricular draining lymph node (dLN) cells were isolated 24 h after challenge of sensitized rats.

Results are presented as median and range (min–max).

Significantly different at: * $p < 0.05$ vs 0/0% DNCB; # $p < 0.05$ vs DA rats.

Table 3
Draining lymph node cell activity 48 hours following challenge.

Parameters	DA		AO	
	DNCB (%)		DNCB (%)	
	0/0	4/1.3	0/0	4/1.3
Cell number ($\times 10^6$)	12.3 (2.5–18.1)	24.5* (18.4–28.4)	9.7 (2.8–17.5)	16.2* (12.3–35.3)
Δ Proliferation (c.p.m.)				
Hapten-stimulated ^a	72.5 (42.0–118.3)	387.0** (133.6–645.6)	98.8 (50.9–305.0)	142.9**,# (8.0–210.0)
IL-2-stimulated ^a	19.2 (0.0–62.7)	690.4** (327.6–1278.8)	0.0 (0.0–94.0)	114.6*,### (15.2–278.7)
CD25 ⁺ cells (%)	5.4 (4.9–5.8)	7.9* (7.5–8.3)	3.7# (3.7–3.9)	4.3*,## (3.8–4.7)
Δ IFN- γ production (pg/ml) ^b	0.0 (0.0–0.0)	353.9* (278.4–770.0)	11.2 (0.0–23.9)	180.0*,# (45.0–305.0)
Δ IL-17 production (pg/ml) ^b	59.4 (0.0–264.1)	115.0 (2.0–281.2)	0.0 (0.0–0.0)	0.0 (0.0–31.0)

Auricular draining lymph node (dLN) cells were isolated 48 h after challenge of sensitized rats.

^a Results are expressed as differences between stimulated (with hapten or IL-2) and non-stimulated proliferation.

^b Results are expressed as differences between stimulated (with hapten) and non-stimulated production.

Results are presented as median and range (min–max).

Significantly different at: * $p < 0.05$ and ** $p < 0.01$ vs 0/0% DNCB; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs DA rats.

sensitization). In skin of sensitized DA rats immunoinflammatory events in epidermis (lymphocyte eozycytosis with their satellitosis around keratinocytes, epidermal spongiosis with exudation, parakeratosis and sporadic erosions) as well as abundant focal and diffuse mononuclear dermal infiltration were seen. These changes intensified 5 days following sensitization and were more prominent at higher (4%) DNCB dose (not shown).

3.7. Draining lymph node cellularity, T cell subset composition and proliferative activity following sensitization with DNCB

As presumed that the strength of sensitization determines the intensity of ACD (Friedmann, 2007), basic aspects of activity of lymph nodes that drain skin sensitized with 0.4% or 4% DNCB were determined next (Table 4). Measurements were conducted five days following sensitization, i.e. at time point when rats should be challenged. Increased (compared to non-sensitized rats) dLN cellularity was observed in DA rats, while it was not changed in AO rats and thus was lower in comparison to individuals of DA rats. While both low and high DNCB sensitization doses exerted effect on CD4⁺ cells (significant decrease) and CD8⁺ cells (significant increase) in AO rats, differential type of changes was noted in DA rats (increase in per-

centages of CD4⁺ and a decrease in CD8⁺ cell numbers at high and low hapten doses, respectively) that resulted in higher numbers (vs AO rats) of CD4⁺ cells at higher DNCB dosing regime and in lower numbers of CD8⁺ cells at both sensitization/elicitation DNCB regimen. Owing to the gain in dLN cellularity, increases in total cell numbers of both CD4⁺ and CD8⁺ were observed in DA rats. Because no changes in dLN cellularity were observed in AO rats no changes in either T cell subset were noted, but total cell numbers were lower compared to DA rats. Such pattern of changes resulted in increased (compared to controls) CD4⁺ to CD8⁺ cell ratio in DA, but in lower values in AO rats.

Measurements of proliferative capacity showed dose-dependent increase in *ex vivo* dLN cell proliferation in DA rats, while increase was observed at high DNCB dose solely in AO rats compared to controls (but it was lower compared to DA rats at both DNCB doses) (Fig. 5A). Interleukin-2 stimulated proliferation of dLN cells was increased in both strains (Fig. 5B), dose-dependently in DA rats, but significantly lower at high dose in AO compared to DA rats. Sensitization resulted in increase (compared to controls) in numbers of cells that expressed CD25 in both strains, but lower numbers of CD25⁺ cells were noted in suspensions of dLN cells of both control and sensitized AO compared to DA rats (Fig. 5C).

Table 4
Draining lymph node cellularity and basic T cell subset composition during sensitization phase.

Parameters	DA			AO		
	DNCB dose (%)			DNCB dose (%)		
	0	0.4	4	0	0.4	4
Cell number ($\times 10^6$)	21.7 (10.3–33.2)	43.5** (32.4–97.5)	81.7*** (40.6–113.1)	22.4 (5.4–31.9)	21.9## (7.8–24.4)	32.4### (20.8–48.4)
CD4 ⁺ cells						
%	59.6 (56.6–61.2)	58.2 (57.8–60.6)	62.8* (61.6–64.2)	59.4 (58.6–61.0)	58.4* (57.4–58.6)	54.0*,† (52.7–55.7)
$\times 10^6$	13.0 (6.1–27.2)	25.5*** (19.0–57.2)	51.3***,† (25.5–71.1)	14.7 (16.4–19.0)	12.9## (4.5–14.2)	17.5# (11.5–26.2)
CD8 ⁺ cells						
%	23.9 (23.4–26.5)	23.0* (21.4–23.9)	24.3 (23.1–25.2)	26.7# (25.4–27.7)	31.2*,# (28.2–33.3)	32.1*,# (29.5–36.5)
$\times 10^6$	5.3 (2.5–11.2)	9.9** (7.4–22.2)	19.9***,† (9.9–27.5)	7.3 (7.3–8.5)	6.9## (2.4–7.6)	8.8# (6.9–11.2)
CD4 ⁺ /CD8 ⁺	2.4 (2.2–2.5)	2.5* (2.5–2.6)	2.6* (2.6–2.7)	2.3 (2.2–2.3)	1.9*,# (1.7–2.0)	1.7*,# (1.6–1.9)

Suprascapular and axillary dLN cells were isolated five days after last DNCB treatment.

Results are presented as median and range (min–max).

Significantly different at: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs 0% DNCB; † $p < 0.05$ vs 0.4% DNCB; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DA rats.

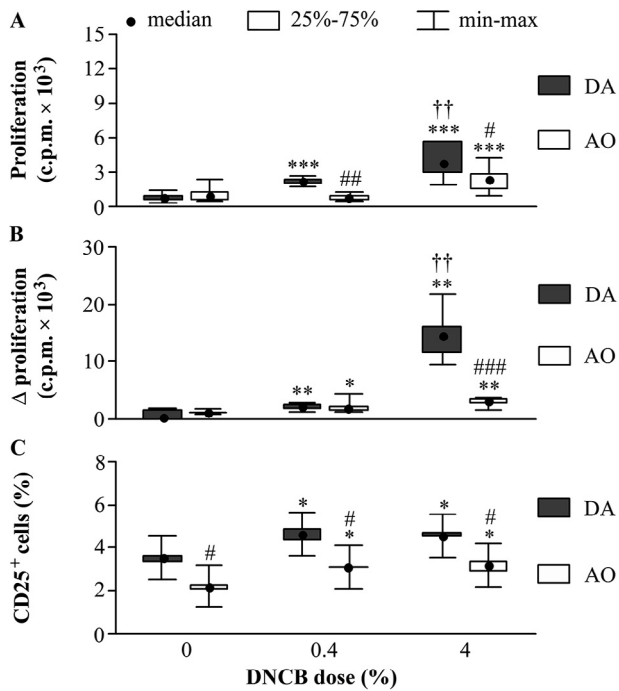


Fig. 5. Draining lymph node cell proliferative activity in sensitization phase of CHS. Suprascapular and axillary dLN cells were isolated five days after last DNCB treatment. *Ex vivo* proliferative activity (A). IL-2 stimulated proliferative activity (presented as differences between IL-2-stimulated and non-stimulated proliferation) (B). Number of CD25⁺ cells (C). Results were pooled from at least two experiments with 4 animals per group and presented as median and range (min–max). Significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs 0% DNCB; † $p < 0.01$ vs 0.4% DNCB and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs DA rats.

3.8. Cytokine mRNA expression and production by draining lymph node cells in sensitization phase

Having in mind that lymph nodes that drain sensitized skin are places of generation of cytokine producing cells, cytokine gene expression as well as specific (hapten-stimulated) cytokine production were measured next (Fig. 6). Dose-dependent increase in mRNA expression for IFN- γ was observed in DA rats, while no changes in the levels of message for this cytokine were observed in dLN cells of sensitized AO rats compared to controls, and were lower compared to DA rats (Fig. 6A). Hapten-specific production of this cytokine was increased in both rat strains after sensitization, but was significantly lower in AO compared to DA rats.

In contrast to IFN- γ , IL-17 mRNA expression by dLN cells was increased after sensitization of both DA and AO rats, but hapten-specific production of this cytokine was noted only in sensitized DA rats (significantly higher compared to AO rats at high and with a tendency, $p = 0.059$, to be higher at lower DNCB dose) (Fig. 6B).

Expression of mRNA for p35 was increased in DA rats sensitized with 4% DNCB while unchanged in AO rats compared to controls and lower after high dose sensitization compared to DA rats. p40 mRNA expression was increased in both DA and AO rats after 0.4% and 4% DNCB sensitization, compared to controls (however numerically lower, $p = 0.06$, at 0.4% in AO compared to DA rats) (Table 5). Expression of mRNA for IL-23 specific subunit, p19, was increased in DA rats sensitized with both DNCB doses, but stayed unchanged in AO rats compared to controls and lower mRNA at 4% DNCB compared to DA rats.

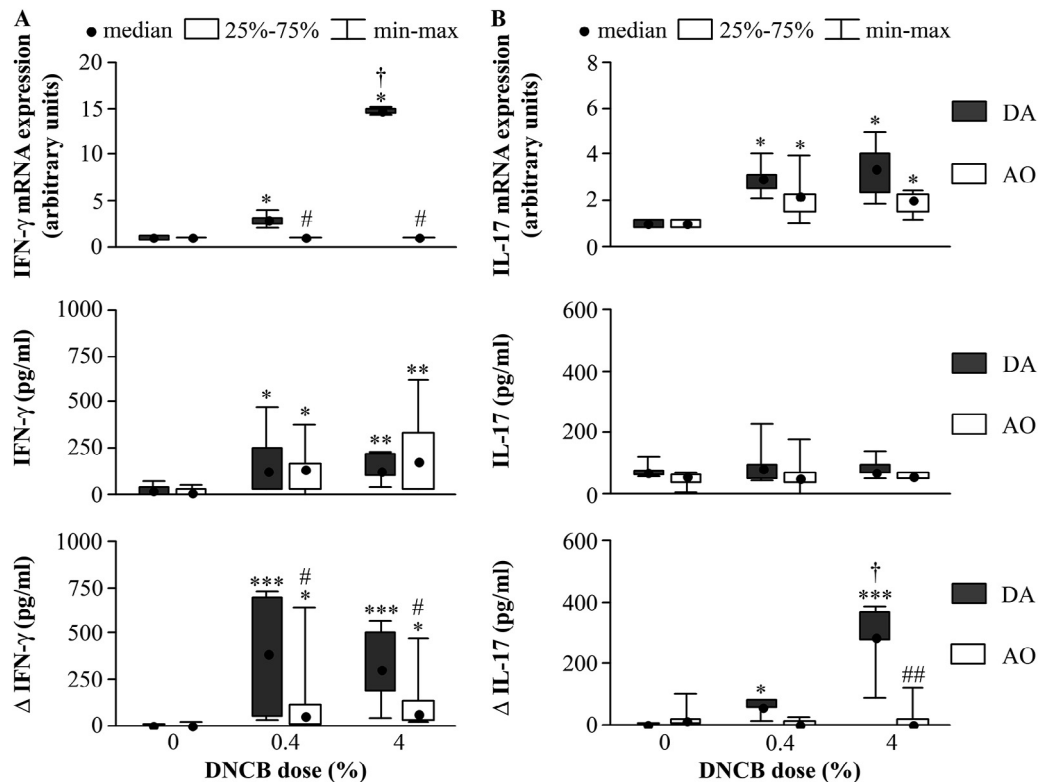


Fig. 6. Cytokine mRNA expression and production by draining lymph node cells in sensitization phase of CHS reaction. Suprascapular and axillary dLN cells were isolated five days after last DNCB treatment. IFN- γ mRNA expression (above), spontaneous (middle) and specific production (below) (A). IL-17 mRNA expression, spontaneous and specific production (B). Cytokine gene expression was analyzed with RT-PCR and presented as relative value. Hapten specific production was presented as differences between DNBS-stimulated and spontaneous cytokine production. Results were pooled from at least three experiments with 4–6 animals per group for production and two experiments for RT-PCR, and presented as median and range (min–max). Significantly different at * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs 0% DNCB; † $p < 0.05$ vs 0.4% DNCB and # $p < 0.05$, ## $p < 0.01$ vs DA rats.

Table 5
p35, p40 and p19 mRNA expression by dLN cells during sensitization phase.

mRNA expression (arbitrary units)	DA			AO		
	DNCB (%)			DNCB (%)		
	0	0.4	4	0	0.4	4
p35	1.02 (0.83–1.10)	0.88 (0.16–1.45)	1.92* (0.71–2.97)	1.11 (0.44–1.46)	0.94 (0.30–1.84)	0.73## (0.24–1.34)
p40	1.00 (0.76–1.30)	2.14* (1.29–3.15)	2.03** (1.55–3.01)	0.95 (0.72–1.54)	1.48* (0.99–2.29)	1.71** (1.19–2.54)
p19	0.97 (0.52–1.55)	1.84* (0.89–2.88)	1.76* (1.06–3.25)	0.91 (0.43–1.72)	1.50 (0.50–2.45)	0.67## (0.17–1.67)

Suprascapular and axillary dLN cells were isolated five days after last DNCB treatment. Results are presented as median and range (min–max).

Significantly different at: * $p < 0.05$ and ** $p < 0.01$ vs 0% DNCB; ## $p < 0.01$ vs DA rats.

4. Discussion

In this study, cardinal aspects of CHS reaction were analyzed in DA and AO rats, two strains known to differ in susceptibility to several experimentally induced inflammatory diseases. Less intense CHS expression in AO rats was associated with lower levels of cell infiltration to challenged skin, lack of or lower intensity of changes in draining lymph node cell proliferative activity and proinflammatory cytokine responses in challenge phase, but with unchanged immunoregulatory cytokine IL-10 production. Lower responsiveness of AO rats to sensitization was observed as well.

Lower intensity of leukocyte infiltration to challenged skin, most probably, was responsible for less intense ear swelling response 24 hours following challenge of AO rats, as effector activities of these cells were responsible for skin inflammation following challenge of sensitized mice (He et al., 2009). As no strain differences were noted in numbers of infiltrating leukocytes 48 hours following challenge and even lower number of mononuclear cells was present in DA rats at this compared to earlier time point, less intense ear swelling response in AO rats might be ascribed to lower levels of activity of these cells.

Increased cellularity of draining lymph nodes 24 hours following challenge of sensitized rats was in line with data from CHS to DNFB in mice that showed accumulation of T cells in draining lymph nodes during challenge phase (Larsen et al., 2007), and the present study showed existence of strain differences in that process.

Given the fact that both CD4⁺ and CD8⁺ cells contribute to cytokine milieu (critical for development of effector cells) (Dearman et al., 1996), but that contribution of specific subset might vary, as shown for CD4⁺ (He et al., 2009; Xu et al., 1996) and CD8⁺ cells (Moussavi et al., 1998), differential ratio of these T cell subsets in DA compared to AO rats might be responsible for differences in proinflammatory cytokine production in sensitized rats.

Augmented proliferative activity noted in challenged skin draining lymph nodes of DA rats might have accounted for increased dLN cellularity in challenged DA rats, as high ($r = 0.81$) and significant ($p < 0.05$) correlation ($Y = 7.88 + 0.01 \times X$) between these two parameters was observed. It seemed closely related to IL-2 in DA rats as responsiveness to this cytokine, increased numbers of cells that express CD25⁺ (inducible α -chain of receptor for IL-2) as well as increased density of expression on per cell basis were shown in these rats. Dose-dependent increase in IL-2 stimulated proliferation of dLN cells from DA rats, detected during both challenge and sensitization phases, is in line with early studies of sensitization with DNCB which showed increase in dLN proliferation in the presence of IL-2 with the increase in DNCB doses used for sensitization of mice (Kimber and Weisenberger, 1989). Lack of changes in numbers of CD25⁺ cells might have accounted for proliferative unresponsiveness in AO rats 24 h following challenge. Importance of IL-2 for strain-related difference in challenge phase was shown later in challenge response (i.e. 48 h following challenge) as well. Higher numbers of cells that expressed this molecule in sensitized compared to control

AO rats during sensitization might have been sufficient for (weak, but significant) proliferative response to IL-2. Generally lower levels of CD25⁺ cells in control AO vs control DA rats indicated that this difference was inherent in these animals. Indeed, basal differences between AO and DA rats in IL-2 responses were reported previously (Vukmanović et al., 1989). Although metabolic viability of dLN cell in challenge phase was not compromised, lack of changes in spontaneous proliferation in AO rats might be explained (at least for high DNCB dose regimen) with the presence of hypodiploid cells in AO rats. Of note, lower sensitivity of AO rats to experimental inflammation in nervous tissue was ascribed to greater susceptibility to apoptosis of inflammatory cells compared to more susceptible DA rats (Lukic et al., 2001).

While cytokine responses to sensitization were subject of several studies (Rennett et al., 2001), cytokine producing activity of dLN during challenge phase has been less known. Increased spontaneous and hapten-specific production of IFN- γ and IL-17 by dLN cells of DA rats 24 hours following challenge show involvement of draining lymph nodes in proinflammatory cytokine response to skin challenge and increased levels of mRNA for IFN- γ indicated dLN cell activation *in vivo*. Absence of production of IFN- γ by dLN cells of AO rats after high DNCB dose (when an increase in cytokine mRNA was observed) suggested posttranscriptional regulation. Increased spontaneous IL-17 production by dLN cells of DA rats 24 hours following challenge in the absence of significant changes of cytokine gene expression might possibly reflect the effect of the cell culture (Haskill et al., 1988) and augmentation of IL-17 production in response to DNBS-stimulation supported an assumption of increased propensity of cells from this strain to produce the cytokine. Absence of changes in IL-17 gene expression and production by dLN cells of AO rats, along with undetectable IFN- γ production, might be the consequence of their lower tendency toward differentiation of Th1 and Th17 cells (judging by unchanged expression of message for both IL-12 and IL-23 heterodimers). Lack of changes of proinflammatory cytokine responses 24 hours following challenge might be explained (at least for high DNCB dose regimen) by the presence of hypodiploid cells in AO rats. In murine model of CHS to TNCB, it was shown that apoptosis of hapten-specific effector T cells located in dLN led to tolerance (Luckey et al., 2011).

Higher levels of specific production of IFN- γ 48 hours following challenge of DA compared to AO rats, supported assumption of this cytokine involvement in strain differences in response to challenge. Decrease of hapten-specific IL-17 production 48 h following challenge (which resulted in lack of differences between controls and challenged animals in both strains) suggested importance of this cytokine early in response. Indeed, the involvement of IL-17 in CHS was measured 24 hours following challenge (He et al., 2006; Nakae et al., 2002).

Disparate hapten-specific production of IFN- γ and IL-17 in AO and DA rats during sensitization phase might have resulted from the differential bias toward differentiation of Th1 and Th17 cells and

might be of major importance for observed differences in the intensity of CHS, as development of hapten-specific effector cytokine producing T cells during this phase was required for full expression of CHS to DNFB in mice (He et al., 2006, 2009; Nakae et al., 2002). The reason for absence of hapten-specific IL-17 production in dLN of sensitized AO rats is unknown at the moment, but regardless of that, hapten-specific cells producing IFN- γ seemed sufficient for development of ear swelling response after challenge. These results suggested caution when considering activity of dLN after challenge for identification of chemicals with potential to elicit CHS response. In corroboration, IL-17 deficiency did not affect the challenge of CHS in mice (Nakae et al., 2002). However, the possibility that absence of hapten-specific IL-17 response in dLN of AO rats could have affected CHS response after challenge should not be completely ruled out, as sensitization and challenge of IL-17R^{-/-} mice with DNFB induced CHS response though reduced compared to one measured in wild type counterparts (He et al., 2009). Differences between AO and DA rats concerning production of proinflammatory cytokines were in line with data that showed lower levels of IFN- γ and IL-17 production in AO compared to DA rats during induction of experimental inflammation in tissue of central nervous system (Markovic et al., 2009).

In conclusion, the present study provides new data concerning mechanisms of strain-dependent differences in CHS reaction to DNCB in rats. Higher proliferative activity and proinflammatory cytokine responses induced in dLN after challenge, as well as more pronounced IFN- γ and IL-17 production by dLN during sensitization phase suggested these activities as underlying mechanisms of higher susceptibility of DA rats to CHS response to DNCB.

Conflict of interest

The authors declare that there are no conflicts of interest in this study. The authors alone are responsible for the content of this manuscript.

Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Grant #173039.

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