Neutrophils are required for both the sensitization and elicitation phase of contact hypersensitivity

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Contact hypersensitivity (CHS), the animal model of human allergic contact dermatitis (ACD), is an inflammatory skin disease induced by contact allergens. Though numerous cellular and molecular players are known, the mechanism of chemical-induced sensitization remains poorly understood. Here, we identify neutrophils as crucial players in the sensitization phase of CHS. Genetic deficiency of neutrophils caused by myeloid-specific deletion of Mcl-1 or antibody-mediated depletion of neutrophils before sensitization abrogated the CHS response. Neutrophil deficiency reduced contact allergen-induced cytokine production, gelatinase release, and reactive oxygen species production in naive mice. Mast cell deficiency inhibited neutrophil accumulation at the site of sensitization. In turn, neutrophils were required for contact allergen–induced release of further neutrophil-attracting chemokines, migration of DCs to the draining lymph nodes, and priming of allergen-specific T cells. Lymph node cells from mice sensitized in the absence of neutrophils failed to transfer sensitization to naive recipients. Furthermore, no CHS response could be induced when neutrophils were depleted before elicitation or when normally sensitized lymph node cells were transferred to neutrophil–deficient recipients, indicating an additional role for neutrophils in the elicitation phase. Collectively, our data identify neutrophils to be critically involved in both the sensitization and elicitation phase of CHS.

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of other innate immune cells to the sensitization phase of CHS is poorly understood.

Neutrophils provide the first line of defense against invading bacterial and fungal pathogens (Mócsai, 2013), but their improper activation may also contribute to tissue damage during various diseases (Mantovani et al., 2011; Németh and Mócsai, 2012). Neutrophils can exert a robust antimicrobial and pro-inflammatory reaction through ROS production, exocytosis of granule proteins (including proteases such as gelatinase), and the release of various cytokines (Mantovani et al., 2011). Interestingly, neutrophils are found in the inflammatory skin lesions of ACD patients (Goebeler et al., 2001). Studies using anti–Gr-1 antibodies before allergen reexposure suggested a role for neutrophils in the elicitation phase of CHS (Engeman et al., 2004), though interpretation of those experiments is complicated by the depletion of various other lineages such as inflammatory monocytes, macrophages, DCs and activated T cells by anti–Gr-1 antibodies (Dunay et al., 2008; Wojtasjak et al., 2010). The role of neutrophils in the sensitization phase of CHS has not yet been investigated.

The aforementioned issues prompted us to test the role of neutrophils in both phases of the CHS response using genetic deletion and antibody-mediated depletion approaches combined with trans-sensitization by adoptive transfer of lymph node cells to naive recipients. Our results provide the first evidence for a critical role for neutrophils in the sensitization phase of CHS.

RESULTS AND DISCUSSION

Genetic deficiency of neutrophils abrogates the CHS response

To investigate the role of neutrophils in CHS, we used mice with a myeloid-specific conditional deletion of the antiapoptotic Mcl-1 protein (LysMCre/CreMcl-1fl/−/fl mice referred to as Mcl-1ΔMyelo mice). Those mice have a selective neutrophil deficiency caused by the requirement of Mcl-1 for the survival of neutrophils, whereas other myeloid-lineage cells (even those that express the LysMCre knock-in allele) are not affected because they do not rely on Mcl-1 for their survival (Dzhagahov et al., 2007). As shown in Fig. 1 A, the Mcl-1ΔMyelo mutation abrogated the ear thickness increase upon reexposure of 2,4,6-trinitrochlorobenzene (TNCB)-sensitized mice to TNCB challenge (P = 2.9 × 10−3), indicating that neutrophil-deficient mice are resistant to CHS.

We also assessed the effect of the Mcl-1ΔMyelo mutation on various leukocyte lineages, including known crucial players in CHS. We observed a nearly complete absence of neutrophils in the peripheral blood (98% reduction; P = 2.41 × 10−14) and spleen (91% reduction; P = 0.015) of Mcl-1ΔMyelo mice, whereas the number of circulating monocytes and T cells (P = 0.11 and 0.84, respectively), and splenic dendritic cells (P = 0.35) were not affected, and the number of splenic macrophages was even slightly, but not significantly (P = 0.81), increased (Fig. S1, A and B). Interestingly, Mcl-1ΔMyelo mice did not show any gross phenotypes and survived normally up to 6 mo of age, suggesting that the low but clearly present number of neutrophils was sufficient to cope with the commensal flora under our animal housing conditions.

Because the LysMCre component of the Mcl-1ΔMyelo mutation is a loss-of-function knock-in mutation of the lysozyme M-encoding gene (Clausen et al., 1999), we also tested whether LysMCre/Cre mice are resistant to CHS. As shown in Fig. 1 B, the LysMCre/Cre mutation did not affect CHS development (P = 0.51), indicating that the defective response in Mcl-1ΔMyelo mice is not caused by the lack of lysozyme M.

As an additional model of neutrophil deficiency, we also tested the effect of genetic deletion of the G-CSF receptor. For those experiments, we used bone marrow chimeras generated by transplanting bone marrow cells from G-CSF receptor-deficient mice (Hermans et al., 2003) on the FVB/N genetic background into WT FVB/N recipients. Such chimeras had a 92% reduction of circulating neutrophil counts compared with control WT chimeras (P = 1.67 × 10−12). As shown in Fig. 1 C, G-CSF receptor deficiency caused a substantial reduction in the CHS response (P = 0.0068).

Collectively, the aforementioned observations provide the first genetic evidence for a functional role of neutrophils in contact hypersensitivity, both on the C57BL/6 and the FVB/N genetic backgrounds. The less dramatic effect of G-CSF receptor deficiency was likely due to the less severe reduction of circulating neutrophil numbers in those animals.

Neutrophil depletion abrogates the CHS response

As another approach to test the functional importance of neutrophils during the sensitization phase of CHS, we depleted neutrophils using an antibody against the Ly6G antigen (Charmoy et al., 2011). As shown in Fig. S1 (C and D), whereas neutrophil depletion dramatically reduced circulating neutrophil numbers (P = 0.00037) and splenic (P = 9.6 × 10−5) neutrophil numbers, it did not affect circulating monocytes or T cells (P = 0.38 and 0.43, respectively), or splenic macrophages or dendritic cells (P = 0.88 and 0.46, respectively). Importantly, as shown in Fig. 1 D, depletion of neutrophils 24 h before sensitization abrogated the overall CHS response (P = 1.1 × 10−39), providing an independent confirmation of the role of neutrophils in contact hypersensitivity.

Kinetic analysis of neutrophil depletion

We next tested the time-course of circulating neutrophil numbers after antibody-mediated neutrophil depletion. As shown in Fig. 2 A, circulating neutrophils were almost completely absent 1 d after the depletion (which corresponds to the time of sensitization in CHS experiments) and remained at very low levels for two additional days. Neutrophil numbers were normalized (P = 0.40) 6 d after depletion, which corresponds to the time of elicitation. Those results suggest that the effect of neutrophil depletion on the CHS response is caused by the absence of neutrophils during the sensitization phase, rather than a prolonged effect causing neutrophil depletion also during the elicitation phase.
Brief Definitive Report

Neutrophils infiltrate the sensitization site

Next, we tested whether neutrophils infiltrate the sensitization site. To this end, we digested TNCB-treated skin samples of naive WT mice and quantified the number of neutrophils by flow cytometry. We observed a profound infiltration of neutrophils to the skin beginning at a few hours after sensitization and plateauing at around 24 h (Fig. 2 B). Other contact allergens such as 2,4-Dinitrochlorobenzene (DNCB) or oxazolone and the irritant croton oil also induced recruitment of neutrophils to the skin of naive WT animals (Fig. 2 C).

Neutrophils are required for the contact allergen-induced inflammatory response

To analyze the function of neutrophils during the sensitization phase of CHS, we tested various signs of contact allergen-induced skin inflammation in naive WT mice. First, we measured the level of IL-1β, a master regulator of the inflammatory reaction and a known central component of the CHS response (Shornick et al., 1996; Weber et al., 2010), at the sensitization site. As shown in Fig. 3 A, neutrophil depletion strongly inhibited the TNCB-induced up-regulation of IL-1β in the skin of naive WT mice (P = 0.0019).

Recent studies on arthritis development indicated that neutrophils trigger a positive-feedback loop by releasing mediators, attracting additional neutrophils to the site of inflammation (Kim et al., 2006; Kovács et al., 2014). This coordinated action, termed neutrophil swarming (Chtanova et al., 2008), is partially mediated by neutrophil-derived CXC chemokines (KC and MIP-2, also known as CXCL1 and CXCL2, respectively) acting as potent neutrophil chemoattractants. As shown in Fig. 3 B, sensitization of naive control mice with TNCB triggered robust up-regulation of MIP-2, which was strongly reduced in neutrophil–depleted mice (P = 0.0013). Similar results were obtained with KC (unpublished data). Another function of neutrophils is the release of proteolytic enzymes such as gelatinase, which leads to extracellular matrix degradation, possibly contributing to the CHS response (Wang et al., 1999). In-gel zymography of tissue extracts revealed up-regulation of gelatinase activity in the affected skin of TNCB-sensitized naive control animals, which was strongly decreased upon prior depletion of neutrophils (Fig. 3 C). Recent studies also demonstrated a crucial role for ROS in the skin in the sensitization and elicitation phases of CHS (Esser et al., 2012). Because neutrophils are able to release large amounts of ROS, we tested in vivo ROS production using a bioluminescence approach. Sensitization with TNCB caused significant ROS production in the skin of naive mice, which was strongly reduced (P = 0.029) by prior depletion of neutrophils (Fig. 3, D and E). In addition, the contact allergens DNCB and oxazolone also potently induced in vivo ROS production, which was also inhibited by neutrophil depletion (P = 0.018 and 0.0015, respectively; Fig. 3 E).

Figure 1. Neutrophils are essential for the CHS response. Mice were sensitized with TNCB or acetone and were challenged with TNCB 5 d after sensitization. The increase in ear thickness 24 h after challenge is depicted. (A and B) CHS response in WT, Mcl-1ΔMyelo, and LysMCre/Cre mice. (C) CHS response in WT mice treated with a neutrophil-depleting anti-Ly6G antibody (PMN depletion) or a control rat IgG 24 h before sensitization. The graphs show mean and SEM from 8–13 (A), 4–6 (B), 5–8 (C), or 5–24 (D) individual mice per group from 3 (A–C) or 5 (D) independent experiments. **, P < 0.01; ***, P < 0.002; n.s., statistically not significant.

Figure 2. Neutrophils in the CHS sensitization phase. (A) Analysis of the number of circulating neutrophils before or at the indicated times after treatment with the neutrophil-depleting anti-Ly6G antibody. (B and C) Infiltration of neutrophils was tested at the indicated times after sensitization with TNCB (B) or 24 h after sensitization with TNCB, DNCB, oxazolone (Oxa), or croton oil (Cr O) on the ears. Neutrophil infiltration was assessed by digestion of the ear skin followed by flow cytometry. The graphs show mean and SEM from 7–26 (A), 8–11 (B), or 9 (C) mice per group from 6 (A) or 4 (B and C) independent experiments.
The role of neutrophils in DC migration and priming of allergen-specific T cells

A critical step in the CHS sensitization phase is the priming of allergen-specific T cells by DCs that migrate from the affected skin to the draining lymph nodes in an IL-1β-dependent manner (Cumberbatch et al., 2002). Because we observed reduced IL-1β production in neutrophil-depleted mice, we next addressed whether neutrophils are required for contact allergen-induced migration of DCs to the draining lymph nodes. As shown in Fig. 4 C, the contact allergen FITC triggered DC migration to the local draining lymph nodes and this response was severely impaired in neutrophil-depleted mice (P = 0.044).

Because DC migration is a crucial step in the sensitization of naive mice, we tested whether priming of allergen-specific T cells was altered in neutrophil-depleted mice. We assessed IFN-γ production by allergen-specific T cells from neutrophil-depleted or control-treated mice in response to allergen reexposure during an in vitro restimulation assay (Fig. 4 D). Lymph node cells from TNCB-sensitized mice were restimulated 5 d after sensitization by modification with TNBS, the water soluble analogue of TNCB that modifies proteins to generate TNP epitopes for T cells. Although lymph node cells from sensitized nondepleted mice responded with strong production of IFN-γ, a strongly decreased IFN-γ release was observed from the lymph node cells of neutrophil-depleted mice (P = 0.0036; Fig. 4 D), indicating a critical role for neutrophils in contact allergen-induced T cell priming.

We performed adoptive CHS (trans-sensitization or passive CHS) experiments to further investigate the role of neutrophils in the priming of allergen-specific T cells. The skin-draining
neutrophils) in the elicitation phase of CHS (Engeman et al., 2004). We also aimed to assess the role of neutrophils in the elicitation phase using the more neutrophil-specific anti-Ly6G antibody and the neutrophil-deficient McI-1\(^{ΔMyelo}\) mouse strain.

As shown in Fig. 5 A, anti–Ly6G-mediated depletion of neutrophils 24 h before elicitation in sensitized mice abrogated the ear swelling response (P = 1.6 × 10\(^{-7}\)). Additional adoptive transfer experiments revealed that no TNCB-induced ear swelling response could be observed when lymph node cells isolated from TNCB-sensitized WT donor mice were adoptively transferred to neutrophil-depleted or McI-1\(^{ΔMyelo}\) recipient mice (P = 0.011 and 0.029, respectively; Fig. 5, B and C).

Those results provide direct evidence for the essential role of neutrophils in the elicitation phase of CHS.

In summary, we demonstrate that neutrophils are crucially involved in both the sensitization and elicitation phases of CHS. In case of sensitization, mast cells trigger the recruitment of neutrophils, which further promote their own recruitment in a swarming manner, and are then required for contact allergen-induced local inflammation, activation, and lymph node cells were isolated 5 d after TNCB sensitization, and were transferred to naive recipients, which were then challenged by TNCB application. As shown in Fig. 4 (E and F), mice receiving lymph node cells from TNCB-sensitized neutrophil-depleted WT donors displayed a strong ear swelling response. No such response could be observed in recipients of lymph node cells from TNCB-sensitized neutrophil-depleted (P = 0.00068; Fig. 4 E) or genetically neutrophil-deficient (P = 0.00025; Fig. 4 F) donors.

Collectively, neutrophil deficiency at the time of sensitization leads to defective allergen-induced migration of DCs to the draining lymph nodes and defective priming of allergen-specific T cells. These results indicate that neutrophils are indispensable for the sensitization phase of CHS.

**Neutrophils play a crucial role in the elicitation phase of CHS**

Prior studies showed that treatment of mice with an anti-Gr-1 antibody before elicitation inhibited the CHS response, indicating an important role for Gr-1–positive cells (possibly neutrophils) in the elicitation phase of CHS (Engeman et al., 2004). We also aimed to assess the role of neutrophils in the elicitation phase using the more neutrophil-specific anti-Ly6G antibody and the neutrophil-deficient McI-1\(^{ΔMyelo}\) mouse strain. As shown in Fig. 5 A, anti–Ly6G-mediated depletion of neutrophils 24 h before elicitation in sensitized mice abrogated the ear swelling response (P = 1.6 × 10\(^{-7}\)). Additional adoptive transfer experiments revealed that no TNCB-induced ear swelling response could be observed when lymph node cells isolated from TNCB-sensitized WT donor mice were adoptively transferred to neutrophil-depleted or McI-1\(^{ΔMyelo}\) recipient mice (P = 0.011 and 0.029, respectively; Fig. 5, B and C).

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migration of DCs and the subsequent priming of allergen-specific T cells. These results indicate that neutrophils play critical roles in various phases and diverse models of allergic skin inflammation, making them attractive targets for the development of future therapeutic strategies.

MATERIALS AND METHODS

Animals. Mice carrying the Mcl1tm1Ywh (Mcl-1floxed) floxed allele of the Mcl-1–encoding gene (Dzhagalov et al., 2007) were obtained from Y. He (Duke University, Durham, NC) and were crossed to mice carrying the LysM Cre tm1Ifo (LysMCre) knock-in strain expressing the Cre recombinase in the myeloid compartment (Clausen et al., 1999). LysM Cre tm1Ifo Mcl-1 floxed mice were used to maintain the strain and to obtain LysM Cre tm1Ifo Mcl-1 floxed homozygous animals (referred to as Mcl-1 ΔMlyso mice). The genotype of the mice was confirmed by allele-specific PCR reactions. Due to the limited availability of Mcl-1 ΔMlyso mice, bone marrow chimeras with an Mcl-1 ΔMlyso hematopoietic system were occasionally generated as previously described (Jakus et al., 2009) and used for CHS experiments. Identical results were obtained with intact Mcl-1 ΔMlyso mice and Mcl-1 ΔMlyso bone marrow chimeras. All mice were on the C57BL/6 genetic background. Control C57BL/6 animals were obtained from our colony or purchased from the Hungarian National Institute of Oncology (Budapest, Hungary). G-CSF receptor-deficient (Csf3rtm1Eur/tm1Eur) mice referred to as GCSF-R−/− mice on the FVB/N genetic background (Hermans et al., 2003) were generously provided by I.P. Touw (Erasmus University, Rotterdam, Netherlands). Mice were kept in individually sterile ventilated cages (Tecniplast) in a conventional facility.

Mcp5-Cre iDTN mice (C57BL/6J background) were generated as previously described (Dudek et al., 2011) and housed at the Experimental Centre at the University of Technology Dresden, Medical Faculty Carl-Gustav Carus, under specific pathogen–free conditions.

All procedures were in accordance with institutional guidelines on animal welfare and were approved by the Sønnefelds University Animal Experimentation Review Board or the Landesdirektion Dresden.

Chemicals and antibodies. TNCB, DNCB, oxazolone, FITC, laminol, and croton oil were obtained from Sigma-Aldrich. Isoflurane was purchased from Baxter, and Liberase II kit was obtained from Roche. Antibodies specific for the following surface markers were used: CD4 (L3T4), CD8 (53–6.7), CD45.2 (104), CD11c (HL3), I-A^v (AF6–120.1), CD11b (M1/70), Gr-1 (RB6–8C5), CD3 (145–2C11), Ly6G (IA8), and CCR7 (4B12) were obtained from BD; F4/80 (BM8) were purchased from eBioscience; F4/80 (Cl:A3–1) was purchased from AbD Serotec; and anti-Ly6A (7/4) was obtained from Abcam. The IL-1β and the MIP-2 ELISA kits were purchased from R&D Systems, and the OptEIA murine IFN-γ ELISA kit was obtained from BD. The neutrophil-depleting anti-Ly6G antibody NIMP-R14 (Lopez et al., 1984; Charmoy et al., 2011) was purified from Hybridoma Supernatant and used for neutrophil depletion as previously described (Sesarman et al., 2008).

Digestion of skin samples. Ear or back skin from mice was collected and cut into small pieces, and then digested with the Liberase II kit (Roche) on an Eppendorf Thermomixer at 1,400 rpm for 1 h at 37°C according to the manufacturer’s protocol. Single-cell suspensions were obtained by passing the digest through a 40-µm cell strainer (BD), after which they were analyzed by flow cytometry.

Depletion of neutrophils and mast cells. Neutrophils were depleted by an i.p. injection of 62.5 µg NIMP-R14 anti-Ly6G antibody in PBS. Control mice received 62.5 µg rat IgG, κ (BioLegend) or PBS.

To induce efficient depletion of mast cells, Mcpt5-Cre iDTN mice received 2 successive i.v. injections of 25 ng/g body weight DT in weekly intervals, and 2 successive intradermal injections of 5 ng/g DT into the ear 6 d and 2 d before allergen application onto the ear. Experiments were performed at least 1 wk after the second systemic DT treatment. DT-treated Cre− littermates served as mast cell–competent controls.

Flow cytometry. The number of various leukocyte types was tested by flow cytometry from blood and spleen samples. Neutrophils were identified as Ly6G-positive or, in the case of Ly6G-mediated depletion, as Ly6A-positive cells in the characteristic forward and side scatter gates. Blood T cells were identified by CD3 staining; blood monocytes as Ly6G-negative/CD11b-positive leukocytes; splenic macrophages as F4/80 and CD11b double-positive cells; and splenic DCs as CD11c-positive and I-A^v-high double-positive cells.

Contact hypersensitivity. For sensitization, the mice were treated with epicutaneous application of 100 µl 3% TNCB in acetone or acetone alone as a vehicle control to the shaved abdominal skin. 3 d after sensitization, the initial ear thickness of the mice was measured, using a pocket thickness gauge (Mitutoyo). After the measurement, all mice (even vehicle-sensitized ones) were challenged by epicutaneous application of 20 µl 1% TNCB on both ears. The ear thickness was measured 24 h after the challenge. The increase in ear thickness as difference between the values before and 24 h after the challenge are displayed. For sensitization of mice on the ear skin, 20 µl of the contact allergen or irritant were applied to the ear. The following concentrations were used: 1% (vol/vol) croton oil in 4:1 acetone/olive oil, 3% (wt/vol) DNCB in acetone, and 3% (wt/vol) oxazolone in EtOH. Acetone, EtOH, or 4:1 acetone/olive oil were used as vehicle controls.

Figure 5. Neutrophils are required for the elicitation phase of CHS. (A) Mice were sensitized by TNCB or acetone, treated with the neutrophil-depleting anti-Ly6G antibody (PMN depletion) 4 d later, and challenged with TNCB after an additional 24 h. The increase of ear thickness during an additional 24 h was measured. (B and C) Nondepleted WT naive mice were sensitized by TNCB or acetone, and their lymph node cells were isolated 5 d later and injected into neutrophil-depleted (B), Mcl-1 ΔMlyso (C), or appropriate control recipients which were then challenged with TNCB and the increase of their ear thickness after 24 h was measured. Graphs show mean and SEM from 3–12 (A) 11–12 (B), or 4–9 (C) individual mice per group from 3 (A and C) or 4 (B) experiments. *, P < 0.05; ***, P < 0.002.
Passive CHS model. For the passive (adoptive transfer) CHS model, mice were sensitized by epicutaneous application of 100 µl 3% TNCB to the shaved abdominal skin and 20 µl 1% TNCB to the dorum of both ears. 5 d after sensitization, the mice were sacrificed, the superficial inguinal and auricular lymph nodes were collected, and a single-cell suspension was prepared. 2 × 10^7 lymph node cells were transferred by i.v. injection to each mouse. After sensitization, the mice were sacrificed, the superficial inguinal and auricular lymph nodes were collected, and a single-cell suspension was prepared. 5 d after allergen application, we sacrificed the mice, collected the auricular lymph nodes and analyzed FITC fluorescence in the DC compartment.

Restimulation of LN cells. Lymph node cells from sensitized mice were collected 5 d after sensitization. A single-cell suspension of the lymph node cells was modified by incubation for 7 min at 37°C in the dark in a 3-mM solution of 2,4,6-Trinitrobenzene sulfonic acid in PBS (TNBS), the water soluble equivalent of TNCB, or PBS as control treatment. After washing thoroughly, 10^6 cells were incubated in 100 µl RP-10 medium (Martin, 2004) in a 96-well plate for 48 h. After the incubation, the contact allergen-induced IFN-γ production was measured by ELISA according to the manufacturer's instructions.

Presentation of data and statistical analysis. Experiments were performed the indicated number of times. Bar graphs show mean and SEM of all mice or samples from the indicated number of independent experiments. Statistical analysis was performed by the StatSoft STATISTICA software on all mice or samples from the indicated number of independent experiments. For all individual data points by determining the significance of the interaction among individual data points, the Bonferroni's post-hoc test was used and the analysis of blood and spleen leukocyte populations which was tested by Student's t test. P values <0.05 were considered statistically significant.

Online supplemental material. Fig S1 shows leukocyte populations of neutrophil-deficient and neutrophil-depleted mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20130062/DC1.

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SUPPLEMENTAL MATERIAL

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Figure S1. Leukocyte populations of neutrophil-deficient and neutrophil-depleted mice. Flow cytometric analysis of different leukocyte populations in the peripheral blood (A and C) or spleen (B and D) of WT and Mcl-1ΔMyelo mice (A and B), or neutrophil-depleted (PMN depletion) or control treated mice 24 h after the depletion (C and D). Graphs show mean and SEM from 12–30 (A), 6 (B), 6–7 (C), or 7 (D) mice per group from 7 (A) or 3 (B–D) independent experiments. *, P < 0.05; ***, P < 0.002, n.s. means statistically not significant.