

Nerve Growth Factor and Neurotrophin-3 Mediate Survival of Pulmonary Plasma Cells during the Allergic Airway Inflammation¹

Melanie Abram,* Michael Wegmann,*[†] Verena Fokuhl,[‡] Sanchaita Sonar,* Elke Olga Luger,^{‡¶} Sebastian Kerzel,[§] Andreas Radbruch,[‡] Harald Renz,^{2*} and Michael Zemlin^{2,3§}

Allergen-specific Abs play a pivotal role in the induction and maintenance of allergic airway inflammation. During secondary immune responses, plasma cell survival and Ab production is mediated by extrinsic factors provided by the local environment (survival niches). It is unknown whether neurotrophins, a characteristic marker of allergic airway inflammation, influence plasma cell survival in the lung. Using a mouse model of allergic asthma, we found that plasma cells from the lung and spleen are distinct subpopulations exhibiting differential expression patterns of neurotrophins and their receptors (Trks). In vitro, the nerve growth factor (NGF) and neurotrophin-3 (NT3) led to a dose-dependent increase in viability of isolated pulmonary plasma cells due to up-regulation of the antiapoptotic Bcl2 pathway. In parallel, the expression of transcription factors that stimulate the production of immunoglobulins (X-box binding protein 1 and NF- κ B subunit RelA) was enhanced in plasma cells treated with NGF and NT3. These findings were supported in vivo. When the NGF pathway was blocked by intranasal application of a selective TrkA inhibitor, sensitized mice showed reduced numbers of pulmonary plasma cells and developed lower levels of allergen-specific and total serum IgE in response to OVA inhalation. This suggests that in the allergic airway inflammation, NGF/TrkA-mediated pulmonary IgE production contributes significantly to serum-IgE levels. We conclude that the neurotrophins NGF and NT3 act as survival factors for pulmonary plasma cells and thus are important regulators of the local Ab production in the allergic airway disease. *The Journal of Immunology*, 2009, 182: 4705–4712.

In allergic asthma, the local pulmonary inflammation includes the development of Ag-specific plasma cells (PC)⁴ and local IgE production (1, 2). Bound to mast cell Fc ϵ RI, the allergen-specific IgE functions as the immunological interface between the allergen and the immune system. Upon cross-linkage of several adjacent Fc ϵ RI-bound IgE molecules, mast cell degranulation is triggered followed by the release of proinflammatory mediators that orchestrate the allergic response (3–5). Thus IgE and IgE-secreting PC play a pivotal role in the initiation and progression of allergic inflammation.

The activation and differentiation of B cells into PC can take place locally in the inflamed tissue, providing high-level production of Ag-specific Abs at the inflammatory site, as described in autoimmune and

allergic diseases (2, 6, 7). Several in vitro studies demonstrated the impact of a microenvironment on PC survival (8–11). Thus, for the survival of airway PC and for a long term Ab production, the local pulmonary environment must provide a complex milieu of cellular and soluble components, which is still poorly understood. In bone marrow, the pool of these survival factors for PC is referred to as survival niches. Cassese et al. (12) identified IL-6 and hyaluronic acid, which are secreted by bone marrow stroma cells, as essential factors for PC survival. The composition of putative analogous pulmonary survival niches for Ab-producing PC in allergic airway inflammation is unknown. Neurotrophins, especially the nerve growth factor (NGF), are among other factors involved in the induction and maintenance of the allergic airway inflammation (13–15). Originally described as a neurotrophic factor, NGF is linked to a plethora of effects in the peripheral neuronal system, as well as inflammatory cells, and is being increasingly described as an important player in asthma pathogenesis (16–18). Primary, local T cells and macrophages have been identified as the sources of NGF production (19–21) and further studies have revealed the correlation of human serum-NGF concentrations with the severity of allergic disease and IgE titers (22). NGF has also been described as a survival factor for lung eosinophils in humans (23, 24).

Moreover, in vitro studies have demonstrated the anti-apoptotic effect of NGF on B cells (25, 26). Therefore, the present study aimed to test the hypothesis that NGF could play a critical role in creating a survival niche for Ab-secreting PC within the locally inflamed airways by induction of anti-apoptotic pathways.

Materials and Methods

Animals

Female BALB/c mice (6- to 8-wk-old; Harlan Laboratories) were maintained under specific pathogen-free conditions. OVA-free diet and water were supplied ad libitum. All animal studies were approved by the local animal ethics committee.

*Department of Clinical Chemistry and Molecular Diagnostics, Philipps-University Marburg, Marburg, Germany; [†]Division of Experimental Pneumology, Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Borstel, Germany; [‡]German Rheumatism Research Center, Berlin, Germany; [§]Department of Pediatrics, Philipps-University Marburg, Marburg, Germany; and [¶]Charité University Hospital Berlin, Allergy-Centrum-Charité, Berlin, Germany

Received for publication August 29, 2008. Accepted for publication February 2, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by the Deutsche Forschungsgemeinschaft Re737/14-1 (to M.A., M.W., V.F., S.S., E.O.L., A.R., and H.R.) and Transregio 22 Pulmonary Allergies; Project A17 (to M.Z.) and Project Z2 (to H.R.).

² H.R. and M.Z. contributed equally to this work.

³ Address correspondence and reprint requests to: Dr. Michael Zemlin, Philipps University Marburg, Department of Pediatrics, Baldingerstr. 1, 35033 Marburg, Germany. E-mail address: zemlin@staff.uni-marburg.de

⁴ Abbreviations used in this paper: PC, plasma cell; ASC, Ab-secreting cell; BAL, bronchoalveolar lavage; ER, endoplasmic reticulum; i.n., intranasal; MNC, mononuclear cell; NGF, nerve growth factor; SAC, *Staphylococcus aureus* cells; siRNA, small interfering RNA; qPCR, quantitative PCR; UPR, unfolded protein response; 7-AAD, 7-aminoactinomycin D; XBP-1, X-box binding protein-1.

Animal treatment protocols

Mice were i.p. sensitized to OVA and challenged with aerosolized OVA as previously described (27). OVA aerosol treatment was given every third day for two weeks, beginning at day 43. For the inhibition of the TrkA receptor, mice were challenged with OVA aerosol on two consecutive days, and received 6 nmol of a highly selective TrkA inhibitor (Calbiochem) in PBS/DMSO intranasally (i.n.) (Fig. 4A). Control mice obtained OVA aerosol and PBS/DMSO i.n.

NGF transgenic mice are on the C57BL/6 background. To investigate the numbers of PC, wild type controls and NGF transgenic mice were chronically exposed to OVA aerosol twice a week on two consecutive days for 12 wk. After 12 wk, mice were killed and analyzed.

Isolation and culture of plasma cells and B cells

For B cell and PC isolation the lungs were perfused with PBS + 1% FCS, without involvement of the local lymph nodes, to eliminate contamination with draining blood cells. Lung lymphocytes were prepared from single cell suspension through density centrifugation using Pancoll (Cytogen). Bone marrow cells were isolated by flushing femurs and tibias with PBS + 1% FCS. CD138⁺/B220^{low/-}/CD19^{low/-} PC were isolated from lung, bone marrow, and spleen with the PC isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions with a purity of >95%. Enriched B220⁺/CD138⁺ PC were sorted by FACS using monoclonal anti-CD138-PE and anti-CD45R-APC Abs (BD Bioscience). For additional experiments, PC were incubated in RPMI 1640 medium (Invitrogen) supplemented with different concentrations (0.5, 1, 5, and 25 ng/ml) of recombinant NGF or NT3 (both from ImmunoTools) at 37°C for 16 or 72 h. Cell viability and detection of apoptotic cells were determined through 7-aminoactinomycin D (7-AAD) and annexin V/propidium iodide (both obtained from BD Bioscience) staining according to the manufacturer's instructions.

For analysis of mRNA expression, isolated PC were incubated with NGF (10 ng/ml) for 16 h

B cells were isolated using the B cell-isolation kit from Miltenyi Biotec according to manufacturer's instructions following culture and activation with 10 ng/ml *Staphylococcus aureus* cells (SAC) (Pansorbin, Calbiochem), 10 ng/ml rIL-4 (Invitrogen) and 2.5 µg/ml anti-CD40 (BD Bioscience) at 37°C for 5 days. At day 6, cells were stimulated with NGF (10 ng/ml) or NT3 (5 ng/ml) for 16 h.

For transcription analysis, total RNA was purified from cultured PC and B cells using the RNeasy Micro Kit (Qiagen).

Western blot

Isolated plasma cells were resuspended in M-PER buffer (Pierce)/0.1% Triton X-100 and centrifuged for 10 min at 14,000 rpm and 4°C. Supernatants (protein fraction) were used for protein assay against a standard of BSA. Defined amounts (25 µg) of proteins were electrophoresed through a 10% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose paper and blocked with blocking solution containing 10% milk powder. Polyclonal rabbit anti-mouse TrkA and TrkB Abs (Santa Cruz Biotechnology) were diluted 1/1000 and 1/200 in blocking solution and incubated over night at 4°C. A secondary HRP-conjugated anti-rabbit Ab (Sigma-Aldrich) was used in PBS Tween 20. The reaction was detected using the Western blotting Luminol Reagent (sc-248, Santa Cruz Biotechnology) and developed using a chemiluminescence Hyperfilm (Amersham Biosciences).

Determination of mRNA expression

cDNA synthesis of total RNA from isolated lung- or spleen-derived PC was performed using SuperScript II reverse transcriptase and random hexamers (Invitrogen). Standard PCR with the cDNA was performed using the Hot-Start Master-Mix (Qiagen) for 40 cycles. The primer sequences (sense/antisense) used were: TrkA, 5'-CCA CAA CTG GTG TGT CC-3'/5'-ATT GTT CCT GTG CCC TGT TC-3'; TrkB (gp145), 5'-AAC CTG CTG GTG AAA ATT GG-3'/5'-TGC TTG CCG TAG GTG AAG AT-3'; TrkC, 5'-GAA GGA GAC AAT GCC GTG AT-3'/5'-TGG CAT GTA CAT TGG TCC AG-3'; NGF, 5'-CCA AGC TCA CCT CAG TGT C-3'/5'-TAC GGT TCT GCC TGT ACG-3'; NT3, 5'-CTG AGT GAC AGC ACC CCT TT-3'/5'-CAG CAC TGT GAC CTG GTG TC-3'; brain-derived neurotrophic factor, 5'-AGG ACG CGG ACT TGT ACA CT-3'/5'-TCA GTT GGC CTT TGG ATA CC-3'.

Real-time PCR was performed with the Rotogene RG-3000 using the QuantiTect-SYBR Green PCR kit (Qiagen) according to the manufacturer's instructions. The relative quantity of each transcript was normalized to the expression of the housekeeping gene *L32* and the relative expression

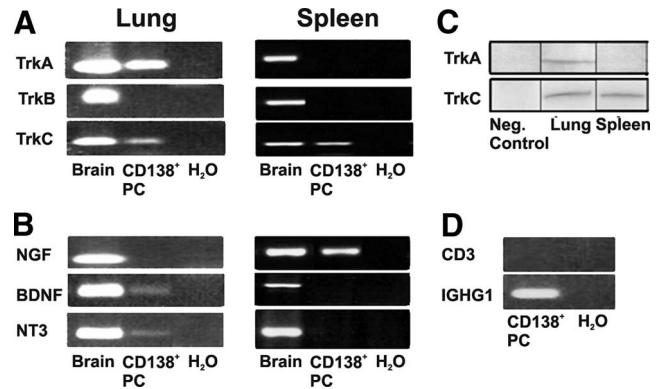


FIGURE 1. Different expression pattern of the Trk-receptors in PC from lung and spleen. PC from the airways and spleen of OVA-sensitized and challenged mice ($n = 20$), were isolated using MACS and FACS sorting. mRNA expression for the Trk-receptors (A) and their ligands (B) and of Trk-receptor protein by Western Blot (C). D, To confirm purity of B lineage cells, isolated cells were tested for T cell-specific CD3 and B lineage specific IGHG1 expression.

level has been calculated by the $\Delta\Delta Ct$ method (28). For analysis the following mouse specific primers were used: *L32* 5'-AACCCAGAGGC ATTGACAAC 3'/5' ATTGTGGACCAGGAACCTTGC 3; *mIL-6*, 5'-TGC CTTCTTGGGACTGATGC-3'/5'-GCCTCCGACTTGTGAAGTGG-3'; *mRelA*(p65), 5'-GACCTGGAGCAAGCCATTAG-3'/5'-ATCGTATGT GAGAGGACAGG-3'; *mEdem1*, 5'-AAGCCTGCAATGAAGGAGAA-3'/5'-CTGCAGTCCAGGGAAGAAAG-3'; *mHspA5*, 5'-TGCAGCAG GACATCAAGTC-3'/5'-TTTCTTCTGGGGCAAATGTC-3'; *mBcl2*, 5'-GGACTTGAAGTGCCATTGGT-3'/5'-TAGCCCCCTCTGTGACAGCTT-3'; *mXBP-1*, 5'-TATCCTTTTGGGCATTCTGG-3'/5'-AAAGGGAGG CTGGTAAGGAA-3'.

Inhibition of the NGF/TrkA signaling in vitro

Isolated lung PC were cultured in medium supplemented with 25 ng/ml NGF and treated with low concentration (6 nmol) of a highly selective TrkA receptor ($IC_{50} = 6$ nM) (Calbiochem) (29) and with 100 ng/ml polyclonal rabbit anti-mouse NGF Ab (Sigma-Aldrich) diluted in medium, for 72 h.

For silencing of the TrkA receptor expression in activated lung mononuclear cells (MNCs) via TrkA-specific small interfering RNA (siRNA; Qiagen), lungs of OVA-sensitized and OVA-challenged mice were perfused and single cell suspensions were produced. MNCs were enriched by centrifugation over an isotonic Pancoll gradient followed by culturing in RPMI 1640 medium and stimulation with 10 ng/ml SAC, 10 ng/ml rIL-4, and 2.5 µg/ml anti-CD40 Abs for 7 days. On day 8, activated MNCs were transfected with 5 pmol/10⁶ cells of TrkA-specific siRNA together with Hi-PerFect transfection reagent (Qiagen) for 3 h following the manufacturer's instructions for primary cells. The specificity of the siRNA was tested by treating control cells with a nontargeting siRNA (Qiagen) or with the transfection reagent alone. After 8 and 24 h, RNA were isolated and *Bcl2* expression was quantified via quantitative PCR (qPCR) as described above. After 72 h levels of IgG1 were determined in the supernatant using anti-mouse IgG1 (A85-3, BD Bioscience), biotin anti-mouse Ab (R 12-3, BD Bioscience), and ELISA. To investigate the involvement of an apoptotic pathway, the Apo-ONE homogeneous caspase-3/7 assay (Promega) was performed 24 h after siRNA transfection according to the manufacturer's instructions. Transfection efficiency (~60%) was determined with a fluorescence-labeled siRNA (Qiagen).

Measurements of Ig concentrations

OVA-specific and total Ab titers were determined in serum as previously described (30). The Ab concentrations of the samples were calculated by comparison with the appropriate standards: mouse IgG1 (S1-68.1, BD Biosciences), mouse IgE (C38-2, BD Biosciences), mouse OVA-IgG1 (OVA-14, Sigma-Aldrich), and mouse OVA-IgE (2C6, Serotec).

Differentiation of cells in the bronchoalveolar lavage (BAL) fluids

Animals were sacrificed 24 h after last treatment with the TrkA inhibitor, the trachea was cannulated, and the BAL cells were obtained by lavage

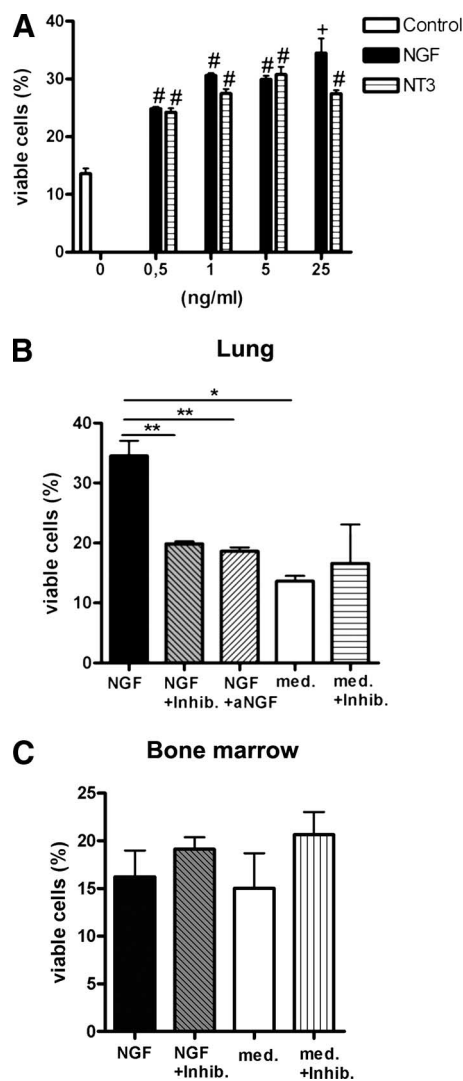


FIGURE 2. NGF and NT3 mediate PC survival. Lung PC were cultured with increasing concentrations of NGF or NT3 for 72 h. Cell viability was determined using 7-AAD staining and FACS. Stimulation with NGF or NT3 led to dose-dependent increased cell viability (A) that could be antagonized by i.n. TrkA inhibitor (B). C, As control, TrkA-negative bone marrow PC treated with NGF alone or together with the TrkA inhibitor were refractory against the inhibition and showed no changes in cell viability. *, $p < 0.05$; +/**, $p < 0.01$.

with 1 ml of PBS. The total cell numbers were determined and cytopins were prepared and fixed for each sample by centrifugation of 200 μ l of BAL fluid at $100 \times g$ for 5 min. The cytopins were stained with Diff Quick (Baxter Merz & Dade) and classified using standard morphological criteria.

Lung histology

For immunofluorescence staining lungs were inflated with 1 ml of 50% Tissue-Tek (Sakura) in PBS. Sections were blocked with PBS/3% BSA and anti-mouse Fc γ receptor (BD Biosciences). For κ/λ staining, FITC-conjugated anti-mouse κ (187.1, SouthernBiotech) and anti-mouse Ig λ_1 , λ_2 , and λ_3 L chain (R26-46, BD Biosciences) were used. Formaldehyde-fixed and paraffin-embedded lungs sections were stained for apoptotic cells with TUNEL assay according to manufacturer's instructions (Roche). For controls, we used lung sections from OVA aerosol-challenged mice, pretreated with recombinant DNaseI for 10 min at 25°C (positive control) and lung sections treated identically but without the enzyme (negative control). Nuclear Fast Red was used for the counter staining (DakoCytomation). Formaldehyde-fixed and paraffin-embedded lung sections were stained with H&E or periodic acid-Schiff. The slides were analyzed using a BX51 microscope and CellF software (both from Olympus).

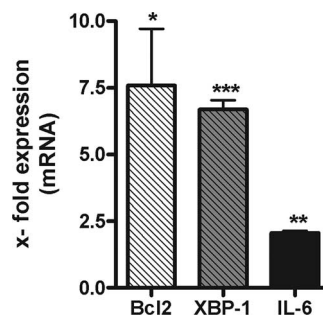


FIGURE 3. NGF enhances expression of Bcl2, XBP-1, and IL-6. The mRNA expression levels were quantified with qPCR and $\Delta\Delta C_t$ method. Culturing of lung PC together with NGF (10 ng/ml) for 16 h led to significantly increased expression Bcl2, XBP-1, and IL-6. Expression of untreated PC (control) was equivalent to one. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Statistical analysis

Results are presented as mean values $\pm$ SEMs. Data were analyzed for significance with the help of unpaired Student's *t* test using the GraphPad Prism software (v. 4.0).

Results

Differential expression of neurotrophin receptors on plasma cells from inflamed airway tissue and spleen

Following sensitization of BALB/c mice to OVA and OVA aerosol challenge, CD138⁺, B220^{low/-}, and CD19^{low/-} PC were isolated from airway tissue and spleen using MACS technology and FACS sorting. The mRNA expression of the neurotrophin-receptors (Trk) differed between the airways and spleen. PC from spleen and inflamed airway tissue expressed the TrkC receptor, whereas only the latter coexpressed TrkA receptor, enabling the pulmonary PC to respond to NGF-signaling (Fig. 1A). In neither population mRNA of the TrkB-receptor was detectable. In agreement with these data, expression of the TrkC-receptor protein (145 kDa) could be detected in PC from both organs, but the TrkA receptor (140 kDa) protein was only detectable in pulmonary PC (Fig. 1C). PC from the lung also expressed the mRNA of the TrkC-receptor ligand NT3 and the TrkB-receptor ligand BDNF but not NGF. Spleen-derived PC were tested positive only for NGF mRNA expression (Fig. 1B). Control experiments showed that PC of both organs expressed mRNA of IGHG1, but not T cell specific CD3, indicating purity of the cells analyzed (Fig. 1D).

NGF promotes survival of lung PC

Isolated pulmonary PC cultured with increasing concentrations of NGF for 72 h showed, in a dose-dependent fashion, a significantly increased number of viable cells compared with the untreated control group, as shown by 7-AAD staining ($p < 0.01$; $p < 0.001$). Increased number of viable cells was also detectable after NT3 stimulation, reaching optimal values at a dose of 10 ng/ml (Fig. 2A). The increased viability in the presence of NGF was reversible by addition of the TrkA inhibitor (31) ($IC_{50} = 6$ nM) for 24 h ($p > 0.01$; Fig. 2B), thus proving that the increased viability in the presence of NGF was mediated via signaling through TrkA. Addition of the TrkA inhibitor did not affect the viability of the cells that were cultured in the absence of NGF (Fig. 2B). As a control to rule out an unspecific toxic effect of the inhibitor, concomitant treatment of isolated lung PC with NGF (25 ng/ml) and a neutralizing anti-mouse NGF Ab led also to a significant reduction of viable cells after 24 h ($p > 0.01$; Fig. 2B). To ensure that loss of viability was

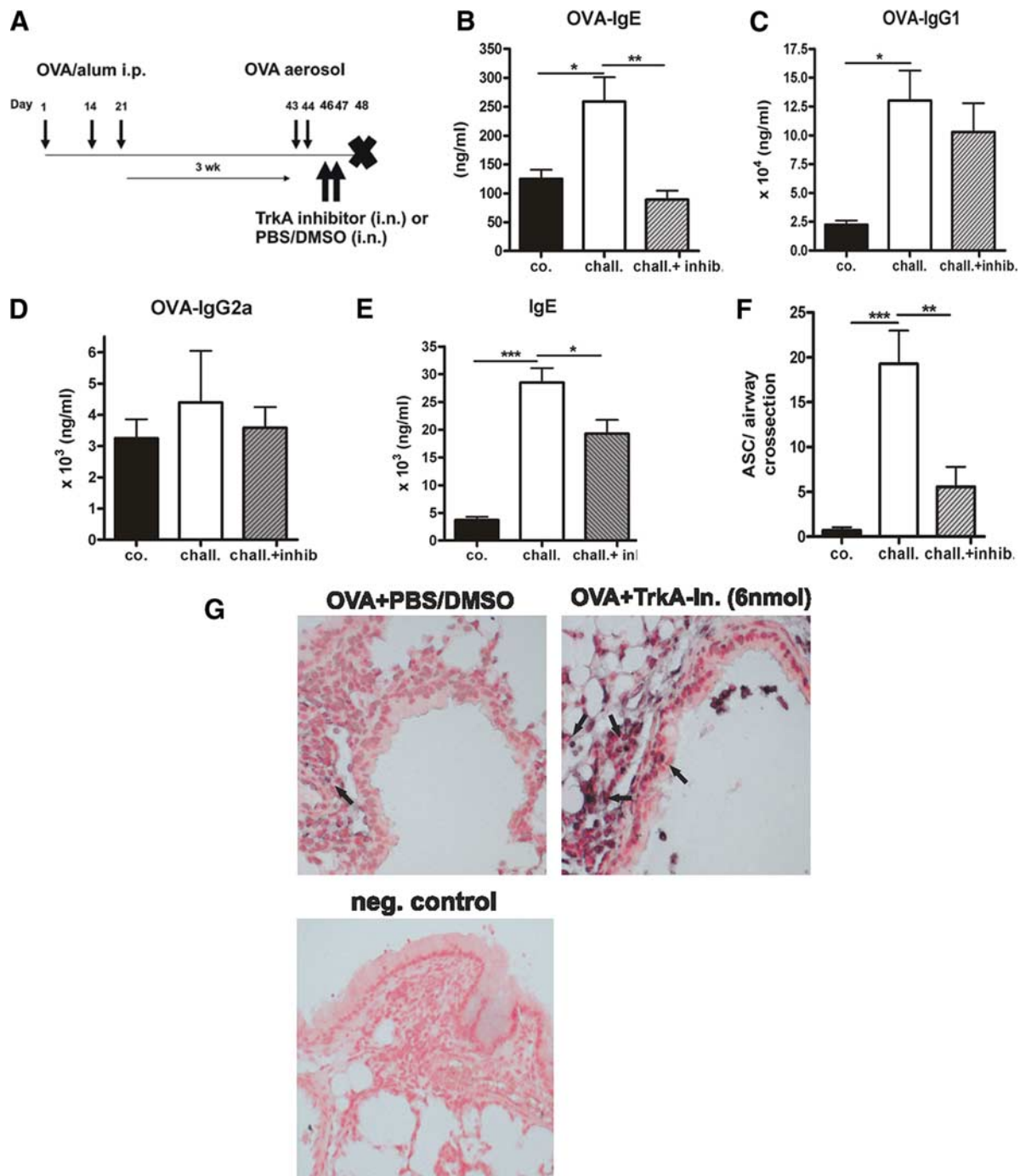


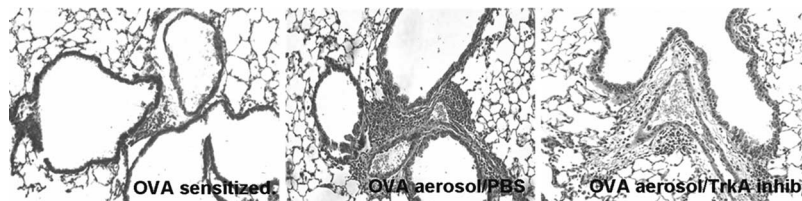
FIGURE 4. Inhibition of TrkA reduces ASC numbers and airway inflammation. Mice were sensitized with a standardized protocol of allergic airway inflammation (A). OVA-sensitized (co.) and challenged mice were treated i.n. with the TrkA inhibitor (chall.+inhib.) or PBS/DMSO (chall.). Total serum OVA-specific IgE was reduced (B and E), whereas IgG1 (C) and IgG2a (D) were unchanged. Pulmonary ASC numbers were significantly reduced in immunofluorescence stainings of cryosections (F). Representative TUNEL-stained lung sections revealed a high number of apoptotic cells (dashes) in mice treated with 6 nmol of TrkA inhibitor (OVA + TrkA-In.) (G). Less apoptosis was detectable in OVA-sensitized control mice treated with the solvent lacking TrkA inhibitor (OVA + PBS/DMSO) and the negative control staining $n = 6$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

due to apoptosis, isolated lung PC were treated with NGF (25 ng/ml) together with TrkA inhibitor (6 nmol) or cultured in medium without treatment for 72 h and stained with V-Annexin and propidium iodide. Staining and analysis by flow cytometry revealed a strong induction of apoptosis in both groups, whereas freshly isolated lung PC showed no V-Annexin- and propidium iodide-specific staining (see supplemental Fig. 1⁵). To verify the specificity of

lung PC to TrkA inhibitor treatment bone marrow-derived PC were used as references. Bone marrow PC did not express TrkA and TrkC receptor specific mRNA (data not shown) and viability was unchanged after treatment with NGF (25 ng/ml) alone or together with the TrkA inhibitor (6 nmol) (Fig. 2C). To test whether increased cell survival by NGF is mediated via expression of genes implicated with survival, isolated lung PC were incubated with NGF (10 ng/ml) for 16 h. After 16 h, qPCR revealed an enhanced expression of Bcl2 ($p < 0.05$), XBP-1 ($p < 0.001$), and IL-6 ($p < 0.01$) (Fig. 3).

⁵ The online version of this article contains supplemental material.

FIGURE 5. Blocking of the TrkA receptor results in reduced inflammatory infiltrate. Representative H&E staining of paraffin-embedded lung showed a reduction of the inflammatory cells after treatment with a TrkA inhibitor in comparison the PBS/DMSO-treated control group. OVA sensitization alone led to the formation of a small inflammatory infiltrate within the airways. Representative pictures.



Reduced inflammation and IgE production after blocking of TrkA in vivo

Because NGF plays a role in the pulmonary allergic inflammation of OVA-sensitized mice (32, 33), we studied the effect of NGF on the homeostasis of local PC in the inflamed airways. BALB/c mice were sensitized with OVA/alum i.p. and further challenged two times with OVA aerosol. Administration i.n. of the TrkA inhibitor on two consecutive days, starting 24 h after the last challenge (Fig. 4A), resulted in significantly reduced serum levels of OVA-specific ($p < 0.001$; Fig. 4B) and total IgE ($p < 0.05$; Fig. 4E). In contrast, no changes were detectable for anti-OVA IgG1 and anti-OVA IgG2a levels ($p = 0.4952$ and $p = 0.663$, respectively; Fig. 4, C and D) at this time point. The histology revealed that the total numbers of Ab-secreting cells (ASCs) in the lung was significantly reduced after i.n. administration of the TrkA inhibitor ($p < 0.05$; Fig. 4F. See supplemental Fig. 2 for representative cryo-sections). In addition, in harmony with previous experiments, NGF-transgenic mice (34) revealed increased numbers of CD138⁺-PC compared with the respective wild type control locally within the inflammation site after 12 wk of OVA aerosol exposition (supplemental Fig. 3). Consistent with the above findings, H&E staining of paraffin-embedded lung sections revealed reduced inflammatory cell numbers in the airways, especially a reduction of eosinophils, in sensitized wild-type mice that were treated with TrkA inhibitor (Fig. 5, supplemental Fig. 4). Examination of apoptosis-specific TUNEL-stained paraffin-embedded lung sections revealed that the TrkA inhibitor-treated animals showed a high number of apoptotic cells within the inflammatory infiltrate than the PBS/DMSO-treated control group (Fig. 4G). To address the role of the pulmonary TrkA receptor expression for the pulmonary inflammatory cells and its influence on local Ab production, mononuclear cells from lungs and spleen of OVA-sensitized and OVA aerosol-exposed mice were isolated and activated with SAC and rIL-4, followed by inhibition of TrkA receptor mRNA expression using gene-specific siRNA. Only in lung-derived MNCs did treatment with TrkA-specific siRNA lead to a reduced Bcl2 expression and increased caspase-3 and -7 activities 8 and 24 h after transfection ($p < 0.001$; Fig. 6). Treatment of splenic MNCs yielded neither Bcl2 reduction nor an enhanced caspase activity (Fig. 6, A and B). IgG1 production was significantly reduced in TrkA-specific siRNA-treated lung MNCs 72 h after transfection ($p < 0.05$; Fig. 6C). IgE Abs were measured only at detection limit (data not shown).

NGF and NT3 up-regulate expression of X-box-binding protein 1 (XBP-1) and NF- κ B

To further explore the role of NGF and NT3 in PC cell survival, we addressed the question whether increased cell viability after NGF stimulation coincides with increased expression of factors involved in anti-apoptotic pathways through reduction of intracellular stress during protein synthesis. Such factors are involved in the unfolded protein response (UPR), a set of cellular control mechanisms that mediate survival by prevention of apoptosis caused by accumulation of unfolded proteins and overwhelming oxidative

stress during long-term Ab production. Therefore we studied the effect of NGF and NT3 on the expression of XPB-1, RelA, Hspa5, and Edem1, factors known to be involved in the UPR, in vitro generated pulmonary ASC. As a control, IgG Ab production was measured in the supernatant of the cell culture (data not shown).

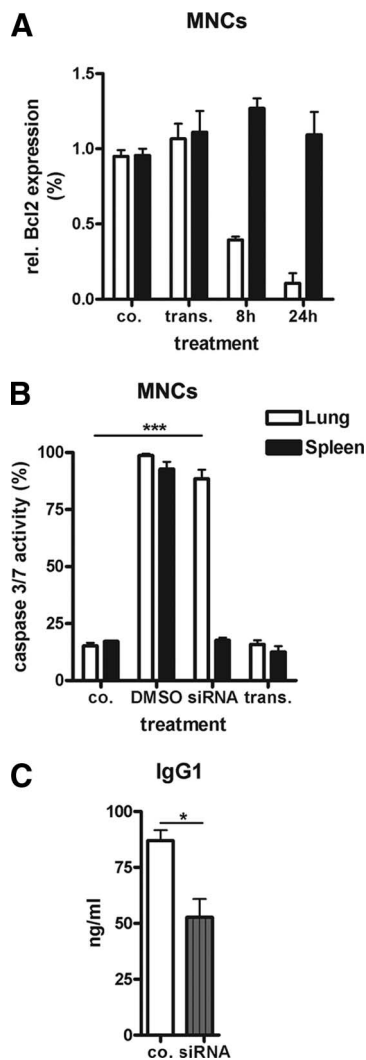


FIGURE 6. Reduction of Bcl2 expression and activation of caspase activity in lung MNCs after treatment with TrkA-specific siRNA. Lung MNCs of OVA-sensitized and OVA-challenged BALB/c mice were cultured and restimulated with SAC, IL-4, and anti-CD40 Abs for 7 days. On day 8, TrkA-specific siRNA was transfected for 3 h. For negative control a nontargeting siRNA (co.) and transfection reagent alone (trans.) were used. A, Bcl2 mRNA expression was quantified after 8 and 24 h using qPCR. B, The caspase-3/7 activity was measured using caspase-3/7 assay (Promega) after 24 h. As positive control DMSO-treated MNCs and as negative control untreated cells (co.) were measured. C, After 72 h, newly secreted IgG1 in the supernatant was quantified with ELISA. Nontransfected restimulated cells were used as reference. *, $p < 0.05$.

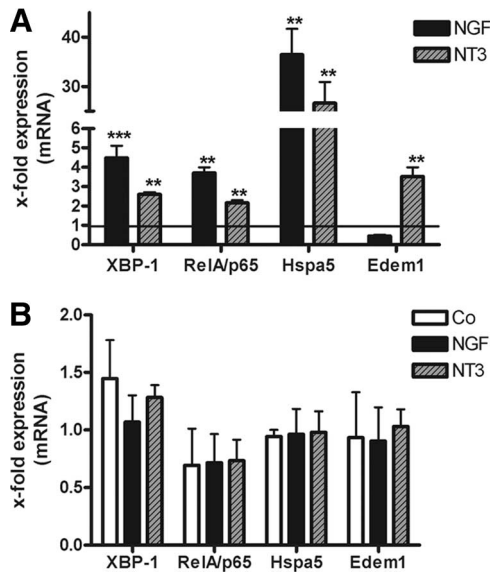


FIGURE 7. NGF and NT3 altered gene expression of ASC. Generated ASC were cultured with NGF (10 ng/ml) or NT3 (5 ng/ml) for 16 h. **A**, Expression levels of XBP-1, RelA, and Hspa5 were significantly increased after stimulation. Edem1 expression was 4-fold increased by NT3, but not altered by NGF. **B**, Expression was compared with untreated cells (black line). Treatment of isolated bone marrow PC with NGF and NT3 showed no changes in gene expression compared with pulmonary PC. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Both NGF and NT3 treatment for 16 h lead to an enhanced expression of the transcription factors XBP-1 ($p < 0.01$ and $p > 0.01$, respectively) and RelA ($p < 0.001$ and $p > 0.01$, respectively) (Fig. 7A). Two factors involved in the quality control machinery of the endoplasmic reticulum (ER), Hspa5, and Edem1, were differentially regulated by NGF and NT3. The treatment with NT3 led to an enhanced expression of Hspa5 ($p < 0.01$) and Edem1 ($p < 0.01$), whereas NGF treatment enhanced Hspa5 ($p < 0.01$) expression but not Edem1-expression (Fig. 7A). As a comparison, TrkA-positive lung PC and bone marrow-derived PC were isolated and stimulated under the same conditions (Fig. 7B). The expression patterns of the analyzed genes did not differ between TrkA-positive PC from lung and bone marrow after NGF treatment.

Discussion

In this study, we could identify for the first time the role of NGF and NT3 as putative survival factors for local PC in inflamed airways of mice with experimental asthma. Furthermore, we found that pulmonary PC differ from splenic PC in their expression pattern of neurotrophin-receptors and their respective ligands. Only the lung PC express the TrkA receptor that binds NGF, whereas the TrkC-receptor that binds NT3 was detectable in both PC populations. In lung PC, concomitant expression of the TrkC receptor and its ligand NT3 could indicate an autocrine mechanism of activation. In contrast, NGF/TrkA-mediated survival of pulmonary PC appears to be a paracrine mechanism. A previous study demonstrated the importance of specific environmental factors on the homeostasis of bone marrow PC (10, 12). Both NGF and NT3 are known to be potent factors mediating growth, proliferation and survival of various cell types including B cells (35–38). Furthermore, NGF is involved in the maintenance of chronic inflammation processes and consecutive remodelling (39–41). NGF might also be an essential part of a survival niche for PC in the allergic airway inflammation, because in mice that conditionally over-express

NGF in the airway epithelium, more CD138⁺ PC are present in the perialveolar area. NT3 is also known to be an inflammatory mediator, but its role in asthma is not well understood. We demonstrated that both NGF and NT3 mediate the survival of isolated lung PC but not in bone marrow-derived PC. The significantly increased viability of NGF-treated cells was mediated by TrkA signaling because this effect could be antagonized by inhibition of the TrkA receptor. This blocking effect also ruled out the possibility that the observed effects of NGF were mediated by other receptors such as the low affinity pan-neurotrophin receptor p75^{NTR}, a well-known death-signaling receptor that acts through the JNK kinase pathway or through NF- κ B/RelA (42–44).

To examine the influence of NGF/TrkA signaling on local Ab production in the lung in vivo, the TrkA receptor was blocked locally in the inflamed airways by i.n. application of the inhibitor. We found that inhibition of the TrkA receptor resulted in a sharp reduction of ASC and inflammatory cells, such as lymphocytes and eosinophils, in the lung. These findings are in harmony with a marked decrease of allergen-specific serum IgE titers in all animals after TrkA inhibitor treatment to control level within 24 h. Application i.n. of TrkA inhibitor did not alter ASC numbers in the spleen (data not shown). Thus it is likely that specific IgE production by pulmonary PC contributes significantly to the systemic IgE levels. Interestingly, the serum levels of IgG1 and IgG2a diminish much more slowly after application of TrkA inhibitor. The rapid drop of IgE levels might be caused by a shorter half-life time of serum IgE (12 h, Ref. 45) compared with IgG (10 days, Ref. 45). In addition, pulmonary IgE-producing PC might be more sensitive to TrkA inhibitor treatment than IgG-producing PC. Significant interindividual differences in IgE levels between individual mice were excluded by measurement of IgE levels before TrkA inhibitor treatment (data not shown).

However, this experimental design cannot rule out the possibility that in addition to the local effects in the lung, i.n. application of the TrkA inhibitor could also have indirect effects on PC populations in other organs, through prevention of migration of newly generated plasma blasts or through other mechanisms. The role of NGF on PC formation and migration processes should be further investigated.

All together, these results indicate that NGF plays an essential role in regulating the local Ab production. Local inhibition of the TrkA receptor and consecutive blocking of NGF signaling may either directly lead to the apoptosis of pulmonary PC, or it may act indirectly via other TrkA-expressing inflammatory cells such as T cells (19) and eosinophils (32) and thus prevent the regulation of other extrinsic factors. In support of this, a previous study shows that reduction of NGF levels leads to a significant reduction of IL-4 levels and prevents the development of airway hyperresponsiveness in OVA-sensitized mice (33). Additionally, IgE and IgG1 production in vitro was diminished by treatment with an anti-NGF Ab (33). In support of these findings, we show that treatment of isolated lung MNCs of OVA-sensitized mice with a specific siRNA against the TrkA receptor mRNA leads to a strong reduction of Bcl2 expression, associated with an increase in caspase-3/7 activity as well as reduced Ab production. In contrast, treatment of splenic MNCs with this siRNA did not influence these parameters. These results again confirm the differences in pulmonary and splenic PC and underscore the role of NGF/TrkA signaling in maintaining the inflammation process and local Ab production, particularly in pulmonary PC.

To identify the mechanism through which the NGF/TrkA signal leads to increased cell viability in pulmonary PC, we studied the expression of proteins involved in the regulation of survival/apoptosis, transcription induction, and protein synthesis. Long term

production and secretion of proteins can induce chronic cellular stress due to an accumulation of unfolded and misfolded proteins in the ER lumen and due to oxidative stress. Such chronic cellular stress is associated with induction of apoptosis. To survive, PC must avoid or reduce such cellular stress during high-rate Ab production. To prevent apoptosis and to maintain homeostasis of ER conditions, a prosurvival pathway, the UPR, can be induced which leads to high expression of ER-resident chaperones such as Hspa5, chaperone cofactors, and components of the ER-associated degradation pathway during ER stress. An essential factor of the UPR pathway is the transcription factor XBP-1, which controls the expression of the above mentioned factors (46). Stimulation of in vitro generated ASC with NGF lead to an enhanced expression of RelA/p65 and a subsequent increase in Bcl2 expression, indicating that NGF-mediated increase of PC viability is at least in part due to the activation of the anti-apoptotic Bcl2-pathway (13, 47–49). In addition, we found that the expression of the transcription factor XBP-1, a major regulator of cellular protein synthesis and folding, was increased in NGF-treated PC. XBP-1 is important to maintain protein synthesis, particularly in PC. Moreover, the expression of the chaperone Hspa5 was strongly increased in NGF as well as NT3-treated PC. This ER-resident chaperone is described to bind to unfolded proteins to prevent their aggregation, which is of great relevance for secreting cells (50).

Interestingly, the expression of the ER degradation enhancer mannosidase α -1 protein, which catalyzes the removal of potentially harmful misfolded luminal glycoproteins from the ER (51), was up-regulated by the TrkC-agonist NT3 but not influenced by the TrkA ligand NGF.

Taken together, NGF- and NT3-signaling via their respective receptors induced increased PC viability in vitro, and enhanced expression of the transcription factors XBP-1 and NF- κ B, essential genes in mediating cellular survival or controlling protein synthesis. Thereby, NGF and NT3 show different gene regulating activities in the case of protein degradation. These findings provide new insights into the cellular mechanisms that lead to regulation of survival and Ab production in PC. The appearance of enhanced expression of IL-6 after NGF stimulation in lung PC implicates that IL-6 might be also involved in cellular homeostasis. IL-6 is important in late stages of PC differentiation and regulation of cell cycle progression (46, 52, 53). In addition, IL-6 is a highly potent survival factor for bone marrow PC in vitro (10, 12), but it seems negligible for Ab production in vivo. Thus, further investigations should address the role of IL-6 in PC survival and its impact on airway inflammation.

In summary, we could identify the neurotrophins NGF and NT3 as putative survival factors for PC in the allergic airway inflammation. Furthermore, we could demonstrate the influence of TrkA receptor signaling on airway inflammation and local Ab production. Our results support the hypothesis that NGF/TrkA signaling may influence the survival of PC via regulation of the Ire1/XBP-1 pathway, essential for prevention of apoptosis under conditions of cellular stress, and thus NGF/TrkA signaling could be involved in long-term Ab production in chronic inflammation. Identification of signals to maintain PC survival as well as local Ab production in inflamed airways could be relevant for a new therapeutic approach of allergic airway inflammation.

Acknowledgments

We are grateful to Stefanie Achenbach and Thomas Ruppertsberg for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

- Beier, K. C., A. Hutloff, M. Lohning, T. Kallinich, R. A. Kroczeck, and E. Hamelmann. 2004. Inducible costimulator-positive T cells are required for allergen-induced local B-cell infiltration and antigen-specific IgE production in lung tissue. *J. Allergy Clin. Immunol.* 114: 775–782.
- Chvatchko, Y., M. H. Kosco-Vilbois, S. Herren, J. Lefort, and J. Y. Bonnefoy. 1996. Germinal center formation and local immunoglobulin E (IgE) production in the lung after an airway antigenic challenge. *J. Exp. Med.* 184: 2353–2360.
- Gould, H. J., and B. J. Sutton. 2008. IgE in allergy and asthma today. *Nat. Rev. Immunol.* 8: 205–217.
- Poole, J. A., P. Matangkasombut, and L. J. Rosenwasser. 2005. Targeting the IgE molecule in allergic and asthmatic diseases: review of the IgE molecule and clinical efficacy. *J. Allergy Clin. Immunol.* 115: S376–S385.
- Busse, W. W., and R. F. Lemanske, Jr. 2001. Asthma. *N. Engl. J. Med.* 344: 350–362.
- Hoyer, B. F., K. Moser, A. E. Hauser, A. Peddinghaus, C. Voigt, D. Eilat, A. Radbruch, F. Hiepe, and R. A. Manz. 2004. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. *J. Exp. Med.* 199: 1577–1584.
- Cassese, G., S. Lindenau, B. de Boer, S. Arce, A. Hauser, G. Riemekasten, C. Berek, F. Hiepe, V. Krenn, A. Radbruch, and R. A. Manz. 2001. Inflamed kidneys of NZB/W mice are a major site for the homeostasis of plasma cells. *Eur. J. Immunol.* 31: 2726–2732.
- Minges Wols, H. A., J. A. Ippolito, Z. Yu, J. L. Palmer, F. A. White, P. T. Le, and P. L. Witte. 2007. The effects of microenvironment and internal programming on plasma cell survival. *Int. Immunol.* 19: 837–846.
- Tokoyoda, K., T. Egawa, T. Sugiyama, B. I. Choi, and T. Nagasawa. 2004. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* 20: 707–718.
- Minges Wols, H. A., G. H. Underhill, G. S. Kansas, and P. L. Witte. 2002. The role of bone marrow-derived stromal cells in the maintenance of plasma cell longevity. *J. Immunol.* 169: 4213–4221.
- Sze, D. M., K. M. Toellner, C. Garcia de Vinuesa, D. R. Taylor, and I. C. MacLennan. 2000. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. *J. Exp. Med.* 192: 813–821.
- Cassese, G., S. Arce, A. E. Hauser, K. Lehnert, B. Moewes, M. Mostarac, G. Muehlinghaus, M. Szyska, A. Radbruch, and R. A. Manz. 2003. Plasma cell survival is mediated by synergistic effects of cytokines and adhesion-dependent signals. *J. Immunol.* 171: 1684–1690.
- Freund-Michel, V., and N. Frossard. 2008. The nerve growth factor and its receptors in airway inflammatory diseases. *Pharmacol. Ther.* 117: 52–76.
- Nockher, W. A., and H. Renz. 2006. Neurotrophins in allergic diseases: from neuronal growth factors to intercellular signaling molecules. *J. Allergy Clin. Immunol.* 117: 583–589.
- Nockher, W. A., and H. Renz. 2002. The role of neurotrophins in the pathogenesis of asthma and related diseases: allergy and asthma as prototypic neuro-immune diseases? *Clin. Exp. Allergy* 32: 1266–1268.
- Renz, H., S. Kerzel, and W. A. Nockher. 2004. The role of neurotrophins in bronchial asthma: contribution of the pan-neurotrophin receptor p75. *Prog. Brain Res.* 146: 325–333.
- Path, G., A. Braun, N. Meents, S. Kerzel, D. Quarcoo, U. Raap, G. W. Hoyle, W. A. Nockher, and H. Renz. 2002. Augmentation of allergic early-phase reaction by nerve growth factor. *Am. J. Respir. Crit. Care Med.* 166: 818–826.
- Otten, U., J. L. Scully, P. B. Ehrhard, and R. A. Gadiant. 1994. Neurotrophins: signals between the nervous and immune systems. *Prog. Brain Res.* 103: 293–305.
- Ricci, A., L. Felici, S. Mariotta, F. Mannino, G. Schmid, C. Terzano, G. Cardillo, F. Amenta, and E. Bronzetti. 2004. Neurotrophin and neurotrophin receptor protein expression in the human lung. *Am. J. Respir. Cell Mol. Biol.* 30: 12–19.
- Ricci, A., S. Greco, S. Mariotta, L. Felici, F. Amenta, and E. Bronzetti. 2000. Neurotrophin and neurotrophin receptor expression in alveolar macrophages: an immunocytochemical study. *Growth Factors* 18: 193–202.
- Ehrhard, P. B., P. Erb, U. Graumann, B. Schmutz, and U. Otten. 1994. Expression of functional trk tyrosine kinase receptors after T cell activation. *J. Immunol.* 152: 2705–2709.
- Bonini, S., A. Lambiasi, S. Bonini, F. Angelucci, L. Magrini, L. Manni, and L. Aloe. 1996. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc. Natl. Acad. Sci. USA* 93: 10955–10960.
- Nassenstein, C., A. Braun, V. J. Erpenbeck, M. Lommatzsch, S. Schmidt, N. Krug, W. Luttmann, H. Renz, and J. C. Virechow, Jr. 2003. The neurotrophins nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4 are survival and activation factors for eosinophils in patients with allergic bronchial asthma. *J. Exp. Med.* 198: 455–467.
- Kobayashi, H., G. J. Gleich, J. H. Butterfield, and H. Kita. 2002. Human eosinophils produce neurotrophins and secrete nerve growth factor on immunologic stimuli. *Blood* 99: 2214–2220.
- Rosini, P., G. De Chiara, P. Bonini, M. Lucibello, M. E. Marcocci, E. Garaci, F. Cozzolino, and M. Torcia. 2004. Nerve growth factor-dependent survival of CESS B cell line is mediated by increased expression and decreased degradation of MAPK phosphatase 1. *J. Biol. Chem.* 279: 14016–14023.
- Torcia, M., G. De Chiara, L. Nencioni, S. Ammendola, D. Labardi, M. Lucibello, P. Rosini, L. N. Marlier, P. Bonini, S. P. Dello, et al. 2001. Nerve growth factor inhibits apoptosis in memory B lymphocytes via inactivation of p38 MAPK, prevention of Bcl-2 phosphorylation, and cytochrome c release. *J. Biol. Chem.* 276: 39027–39036.

27. Wegmann, M., H. Fehrenbach, A. Fehrenbach, T. Held, C. Schramm, H. Garn, and H. Renz. 2005. Involvement of distal airways in a chronic model of experimental asthma. *Clin. Exp. Allergy* 35: 1263–1271.
28. Emmanuele, L., J. Ortmann, T. Doerflinger, T. Traupe, and M. Barton. 2003. Lovastatin stimulates human vascular smooth muscle cell expression of bone morphogenetic protein-2, a potent inhibitor of low-density lipoprotein-stimulated cell growth. *Biochem. Biophys. Res. Commun.* 302: 67–72.
29. Lippa, B., J. Morris, M. Corbett, T. A. Kwan, M. C. Noe, S. L. Snow, T. G. Gant, M. Mangiaracina, H. A. Coffey, B. Foster, E. A. Knauth, and M. D. Wessel. 2006. Discovery of novel isothiazole inhibitors of the TrkA kinase: structure-activity relationship, computer modeling, optimization, and identification of highly potent antagonists. *Bioorg. Med. Chem. Lett.* 16: 3444–3448.
30. Renz, H., H. R. Smith, J. E. Henson, B. S. Ray, C. G. Irvin, and E. W. Gelfand. 1992. Aerosolized antigen exposure without adjuvant causes increased IgE production and increased airway responsiveness in the mouse. *J. Allergy Clin. Immunol.* 89: 1127–1138.
31. Santos, S. D., P. J. Verveer, and P. I. Bastiaens. 2007. Growth factor-induced MAPK network topology shapes Erk response determining PC-12 cell fate. *Nat. Cell Biol.* 9: 324–330.
32. Hahn, C., A. P. Islamian, H. Renz, and W. A. Nockher. 2006. Airway epithelial cells produce neurotrophins and promote the survival of eosinophils during allergic airway inflammation. *J. Allergy Clin. Immunol.* 117: 787–794.
33. Braun, A., E. Appel, R. Baruch, U. Herz, V. Botchkarev, R. Paus, C. Brodie, and H. Renz. 1998. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. *Eur. J. Immunol.* 28: 3240–3251.
34. Hoyle, G. W., R. M. Graham, J. B. Finkelstein, K. P. Nguyen, D. Gozal, and M. Friedman. 1998. Hyperinnervation of the airways in transgenic mice overexpressing nerve growth factor. *Am. J. Respir. Cell Mol. Biol.* 18: 149–157.
35. Nassenstein, C., A. Braun, W. A. Nockher, and H. Renz. 2005. Neurotrophin effects on eosinophils in allergic inflammation. *Curr. Allergy Asthma Rep.* 5: 204–211.
36. Schuhmann, B., A. Dietrich, S. Sel, C. Hahn, M. Klingenspor, M. Lommatzsch, T. Gudermann, A. Braun, H. Renz, and W. A. Nockher. 2005. A role for brain-derived neurotrophic factor in B cell development. *J. Neuroimmunol.* 163: 15–23.
37. Bayas, A., N. Kruse, N. F. Moriabadi, F. Weber, V. Hummel, G. Wohleben, R. Gold, K. V. Toyka, and P. Rieckmann. 2003. Modulation of cytokine mRNA expression by brain-derived neurotrophic factor and nerve growth factor in human immune cells. *Neurosci. Lett.* 335: 155–158.
38. Kimata, H., A. Yoshida, C. Ishioka, T. Kusunoki, S. Hosoi, and H. Mikawa. 1991. Nerve growth factor specifically induces human IgG4 production. *Eur. J. Immunol.* 21: 137–141.
39. Freund-Michel, V., C. Bertrand, and N. Frossard. 2006. TrkA signalling pathways in human airway smooth muscle cell proliferation. *Cell. Signal.* 18: 621–627.
40. Kerzel, S., G. Path, W. A. Nockher, D. Quarcoo, U. Raap, D. A. Groneberg, Q. T. Dinh, A. Fischer, A. Braun, and H. Renz. 2003. Pan-neurotrophin receptor p75 contributes to neuronal hyperreactivity and airway inflammation in a murine model of experimental asthma. *Am. J. Respir. Cell Mol. Biol.* 28: 170–178.
41. Micera, A., E. Vigneti, D. Pickholtz, R. Reich, O. Pappo, S. Bonini, F. X. Maquart, L. Aloe, and F. Levi-Schaffer. 2001. Nerve growth factor displays stimulatory effects on human skin and lung fibroblasts, demonstrating a direct role for this factor in tissue repair. *Proc. Natl. Acad. Sci. USA* 98: 6162–6167.
42. Papa, S., F. Zazzeroni, C. G. Pham, C. Bubici, and G. Franzoso. 2004. Linking JNK signaling to NF-kappaB: a key to survival. *J. Cell Sci.* 117: 5197–5208.
43. Beattie, M. S., A. W. Harrington, R. Lee, J. Y. Kim, S. L. Boyce, F. M. Longo, J. C. Bresnahan, B. L. Hempstead, and S. O. Yoon. 2002. ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. *Neuron* 36: 375–386.
44. Chapman, B. S., and I. D. Kuntz. 1995. Modeled structure of the 75-kDa neurotrophin receptor. *Protein Sci.* 4: 1696–1707.
45. Vieira, P., and K. Rajewsky. 1988. The half-lives of serum immunoglobulins in adult mice. *Eur. J. Immunol.* 18: 313–316.
46. Brewer, J. W., and L. M. Hendershot. 2005. Building an antibody factory: a job for the unfolded protein response. *Nat. Immunol.* 6: 23–29.
47. Madrid, L. V., C. Y. Wang, D. C. Guttridge, A. J. Schottelius, A. S. Baldwin, Jr., and M. W. Mayo. 2000. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kB. *Mol. Cell Biol.* 20: 1626–1638.
48. Pahl, H. L. 1999. Activators and target genes of Rel/NF-kB transcription factors. *Oncogene* 18: 6853–6866.
49. Grumont, R. J., I. J. Rourke, and S. Gerondakis. 1999. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev.* 13: 400–411.
50. Hendershot, L., J. Wei, J. Gaut, J. Melnick, S. Aviel, and Y. Argon. 1996. Inhibition of immunoglobulin folding and secretion by dominant negative BiP ATPase mutants. *Proc. Natl. Acad. Sci. USA* 93: 5269–5274.
51. Olivari, S., and M. Molinari. 2007. Glycoprotein folding and the role of EDEM1, EDEM2 and EDEM3 in degradation of folding-defective glycoproteins. *FEBS Lett.* 581: 3658–3664.
52. Nakagawa, T., H. Zhu, N. Morishima, E. Li, J. Xu, B. A. Yankner, and J. Yuan. 2000. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid- β . *Nature* 403: 98–103.
53. Brewer, J. W., and J. A. Diehl. 2000. PERK mediates cell-cycle exit during the mammalian unfolded protein response. *Proc. Natl. Acad. Sci. USA* 97: 12625–12630.