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Differential regulation of Nr4a subfamily nuclear receptors following mast cell activation

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ABSTRACT

The biological function of the Nr4a subfamily of nuclear receptors is only partially understood. Here we show for the first time that mast cell (MC) activation processes involve the regulation of Nr4a factors. Exposure of murine bone marrow-derived MCs (BMMCs) to live bacteria causes a robust and selective upregulation of all Nr4a members (Nr4a1–Nr4a3). In response to purified LPS, strong upregulation of Nr4a3, but not of Nr4a1 or Nr4a2 was seen. Nr4a3 expression was also induced after the activation of BMMCs by IgE receptor cross-linking. Moreover, Nr4a expression was induced in activated human MCs. As shown by Western blot analysis, Nr4a phosphorylation was induced by IgE receptor cross-linking and calcium ionophore stimulation of BMMCs and LAD2 cells, respectively. By using various inhibitors of signaling pathways, Nr4a3 induction in BMMCs was shown to be strongly dependent on G δ 6976-sensitive kinases and partially dependent on the nuclear factor of activated T-cells (NFAT) pathway, while nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) inhibition failed to inhibit Nr4a3 expression in BMMCs. Together, these data reveal selective induction of Nr4a family members in activated MCs and implicate Nr4a family nuclear receptors in the regulation of MC function.

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1. Introduction

The family of nuclear receptors (NRs) include a large number of ligand-dependent transcription factors such as the various steroid receptors (Aranda and Pascual, 2001; Giguere, 1999; Glass and Saijo, 2010; Mangelsdorf et al., 1995). Upon ligand binding, these types of NRs are translocated into the nucleus where they induce multiple cell- and ligand-specific effects on gene expression patterns. In addition to the ligand-dependent NRs, a number of orphan NRs with atypical ligand-binding domains have been identified (Glass and Saijo, 2010). The latter group of NRs include a large number of transcription factors, all having in common that their physiological ligands have not been identified.

The Nr4a subfamily of orphan NRs include three members – Nr4a1 (Nur77), Nr4a2 (Nurr1) and Nr4a3 (NOR-1). They are immediate early genes that can be induced by a wide array of stimuli,

including stress, prostaglandins, growth factors, inflammatory signals, antipsychotic drugs and fatty acids (Beaudry et al., 2000; Glass and Saijo, 2010; Maheux et al., 2005; Maxwell and Muscat, 2006; Pols et al., 2007). Nr4a subfamily members are known to be widely expressed among various cell types and tissues, e.g., macrophages, central nerve system, vascular smooth muscle cells and T lymphocytes (Beaudry et al., 2000; de Vries et al., 2000; Glass and Saijo, 2010; Maheux et al., 2005; Maxwell and Muscat, 2006; Pols et al., 2007; Zetterstrom et al., 1996). The Nr4a family members have been implicated in numerous contexts, including regulation of proliferation, glucose metabolism, apoptosis, macrophage inflammatory gene expression and atherosclerosis (Bonta et al., 2006; Pei et al., 2006a,b). However, gene knockout studies have revealed relatively mild phenotypes of mice lacking individual Nr4a family members, possibly due to functional redundancy among the Nr4a subgroup members (Maxwell and Muscat, 2006). For example, mice lacking Nr4a1 or Nr4a3 only display relatively mild phenotypic alterations whereas mice double deficient in Nr4a1 and Nr4a3 spontaneously develop lethal acute myeloid leukemia (Mullican et al., 2007).

Mast cells (MCs) are currently emerging as important effector cells in a variety of pathological conditions (Kalesnikoff and Galli, 2008). For example, MCs not only have important pathogenic roles in several types of disease, including allergic airway inflammation, atherosclerosis, experimental autoimmune encephalitis, arthritis and cancer, but also have essential beneficial functions

Abbreviations: MC, Mast cell; BMMC, Bone marrow-derived mast cell; NR, Nuclear factor; SCF, Stem cell factor; NFAT, Nuclear factor of activated T-cells; NF κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; PGN, Peptidoglycan; MCP-1, Monocyte chemoattractant protein-1; PBMC, Peripheral blood-derived mast cell.

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in the context of innate immunity towards bacteria and parasites (reviewed in Kalesnikoff and Galli, 2008; Marshall, 2004). MCs can be activated by a variety of stimuli, including activation through antigen-mediated cross-linking of IgE molecules bound to the high affinity IgE receptors (FcεRI) present on the MC surface, which leads to degranulation and release of preformed mediators stored in the secretory granules (Blank and Rivera, 2004; Lundequist and Pejler, 2011; Turner and Kinet, 1999). However, MCs can also be activated by a number of additional stimuli, such as various bacterial and viral components, complement factors and toxic peptides. Moreover, MC activation can lead, in addition to the release of preformed granule compounds, to the activation of transcriptional responses and release of compounds synthesized *de novo*, the latter including a variety of inflammatory cytokines and lipid mediators (Boyce, 2007; Galli et al., 2005).

To study the global impact of bacterial insult on MC gene expression, we previously performed a gene array expression analysis of MCs after co-culture with live group C streptococci (Rönnerberg et al., 2010). Co-culture of MCs with bacteria resulted in induction of a large number of inflammation-related genes (Rönnerberg et al., 2010). In addition, the Affymetrix gene chip analysis indicated that Nr4a family members were highly expressed in MCs co-cultured with the live streptococci. Nr4a subfamily NRs have not previously been implicated in MC activation processes and we therefore studied the expression and regulation of the Nr4a family of transcription factors in MCs. We show that, out of all NRs, Nr4a family members are strongly and differentially induced by various MC-activating stimuli in both murine and human MCs. Hence, this study implicates the Nr4a subfamily in MC function.

2. Materials and methods

2.1. Reagents

Gö6976, Gö6983, NFAT inhibitor (11R-VIVIT) and NFκB inhibitor (6-amino-4-(4-phenoxyphenylethylamino)quinazoline) were from Calbiochem (Darmstadt, Germany). Phosphatase inhibitor cocktail (P5726) containing sodium orthovanadate, sodium molybdate, sodium tartrate and imidazole was from Sigma–Aldrich (Saint Louis, MO). Odyssey Blocking Buffer (P2711) was from Li–Cor Biosciences (Cambridge, UK). Rabbit anti-phospho-NR4A1 (PAB0547) was from Abnova (Taipei, Taiwan) while goat anti-mouse β-actin (I-19, sc-1616) was from Santa Cruz (Santa Cruz, CA) and the secondary antibodies conjugated to IRDye 800 or IRDye 680 were from Li–Cor Biosciences. The monocyte chemoattractant protein-1 (MCP-1) ELISA kit (88-7391) was from eBiosciences (San Diego, CA). StemPro-34 (10639011), L-Glutamine (25030024), Penicillin–Streptomycin (10378016) were from Invitrogen (Stockholm, Sweden), whereas DMEM and heat-inactivated fetal bovine serum were from SVA (Uppsala, Sweden). Cytokines (IL-3, IL-6, stem cell factor (SCF)) were from Peprotech (Rocky Hill, NJ). Ficoll–Paque Plus (17-1440-02) came from GE Healthcare (Uppsala, Sweden). Annexin V-FITC Apoptosis kit (APOAF) was from Sigma–Aldrich (Saint Louis, MO).

2.2. Bone marrow-derived MCs

Bone-marrow cells from C57BL/6 mice were obtained from femoral and tibial bone and cultured in DMEM, supplemented with 10% heat-inactivated fetal bovine serum, 60 μg/ml Penicillin, 50 μg/ml Streptomycinsulfate, 2 mM L-glutamine and 30% WEHI-3B-conditioned media (which contains IL-3). The cells were kept at a concentration of $0.5\text{--}1 \times 10^6$ cells/ml with weekly changes of medium. Animal experiments were approved by the local ethical committee.

2.3. LAD2 and peripheral blood derived MCs

The LAD2 line isolated from the bone marrow of a patient with MC leukemia was a gift from A. Kirshenbaum and Dean D. Metcalfe (National Institutes of Health). LAD2 cells were cultured in StemPro-34 supplemented with 2 mM L-glutamine, 100 IU/ml Penicillin–Streptomycin, and 100 ng/ml SCF. Cell culture medium was hemidepleted every week with fresh medium supplemented with 100 ng/ml SCF. Human peripheral blood derived MCs (PBMcs) were derived from peripheral blood mononuclear cells isolated by density gradient centrifugation on Ficoll–Paque Plus. The cells were maintained in StemPro-34 supplemented with SCF (100 ng/ml), IL-6 (50 ng/ml) and passed once a week. The PBMcs were used when >95% of the cells stained with toluidine blue (typically 6 wk).

2.4. In vitro co-culture of BMMcs and bacteria

BMMcs grown with 30% WEHI-3B-conditioned media were washed twice in PBS and resuspended in antibiotic-free medium (otherwise as above) at a density of 1×10^6 cells/ml and plated in 24-well tissue plates. Group C streptococci (*Streptococcus equi* (S. equi); strain 62) were grown overnight in Todd–Hewitt broth (Oxoid, Basingstoke, Hampshire, UK) supplemented with 0.5% yeast extract (THB-yeast), washed twice in PBS and added to cells at a multiplicity of infection (MOI) of 1:25.

For IgE-induced activation, cells were sensitized with IgE anti-TNP at 1 μg/ml overnight, washed, resuspended in new medium and stimulated with TNP-OVA (0.4 μg/ml). Alternatively, 2 μM A23187, 1 μg/ml LPS or 10–50 μg/ml peptidoglycan (PGN) was added. For inhibition experiments, 1 μM Gö6976, 1 μM Gö6983, 100 nM NFκB inhibitor or 5 μM NFAT inhibitor were incubated with the cells 30 min before the addition of the stimuli. Cells were collected at various time-points by centrifugation; media and cell fractions were frozen and stored at -20°C . As an indicator of MC activation, the concentration of MCP-1 in the medium fraction was determined using ELISA.

2.5. Western blot analysis

Cell pellets were solubilized in reducing SDS-PAGE sample buffer containing a phosphatase inhibitor cocktail and samples were subjected to SDS-PAGE on 12% gels. Thereafter, proteins were blotted onto nitrocellulose membranes, followed by blocking in Odyssey Blocking Buffer diluted 1:1 in TBS for 1 h. Next, the membranes were incubated with primary antibodies diluted in Odyssey Blocking Buffer:TBS (1:1), containing rabbit anti-phospho-NR4A1 (diluted 1:500) or goat anti-mouse β-actin (diluted 1:500) overnight at 4°C . After washing with TBS/0.1% Tween-20, the membranes were incubated with appropriate secondary antibodies conjugated to IRDye 800 or IRDye 680 (diluted 1:1000 in Odyssey Blocking Buffer:TBS) for 1 h at room-temperature. The membranes were subsequently washed twice in TBS/0.1% Tween-20 and once in TBS before detecting the signal using an Odyssey Infrared Imaging System (Li–Cor Biosciences, 9201-01).

2.6. RNA preparation and quantitative real-time PCR

Total RNA preparation and quantitative real-time PCR (qPCR) was performed as previously described (Duelli et al., 2009). For primers used, see Table 2.

2.7. Apoptosis

Growth factor deprivation-induced apoptosis was accomplished by washing BMMcs once in PBS and subsequently resuspending the cells at 1×10^6 cells/ml in medium lacking WEHI-

3B-conditioned medium. Samples were taken after 0, 24 and 48 h, stained with FITC-labeled Annexin V and Propidium iodide according to the manufacturer instructions followed by flow cytometric analysis. Annexin V and Propidium iodide-positive cells were used to determine the degree of apoptosis at each time-point.

2.8. Immunofluorescence

Cytospins of BMMCs or LAD2 cells were prepared (5 min, 500 rpm) and allowed to dry (1 h, RT). Slides were fixed in 4% paraformaldehyde in PBS for 20 min and permeabilized by incubation in 0.5% Triton X-100 for 10 min. Next, the cells were incubated with goat serum (diluted 50×) before incubation with NR4A3-specific antibody (Imgenex, 5 µg/ml, overnight, 4 °C) or isotype control (IgG, Lifespan Biosciences (LS-C109221)), followed by incubation with Alexa546-labeled goat anti-rabbit IgG (Invitrogen) diluted 1000×. Finally, the nuclei were visualized by DAPI before the slides were mounted using fluorescent mounting medium (Dako, S3023). As positive controls, mouse brain and human placenta tissues were used. The images were collected using confocal microscope Carl-Zeiss LSM700 and the ZEN 2009 software (Carl-Zeiss).

2.9. Statistical analysis

Statistical analysis using two tailed, paired Student's *t*-test was performed using GraphPad Prism 4. Differences were considered significant if the *p*-values were 0.05 or less.

3. Results

3.1. Nr4a factors are selectively upregulated in response to live bacteria and bacterial cell-wall products

In order to screen for NR expression in bone marrow-derived MCs (BMMCs), previously collected gene array data (Rönnerberg et al., 2010) were analyzed for levels of mRNAs coding for various known NRs. Moreover, the levels of NR expression in response to co-culture with live group C streptococci (*S. equi*) were analyzed. As shown in Table 1, out of all NRs expressed, only the Nr4a family members showed significant and more than 2-fold induction in response to co-culture with bacteria. In fact, among the 10 genes showing the highest extent of upregulation out of all genes, all three members of the Nr4a subfamily (Nr4a1–Nr4a3) were found (Table 1 and Rönnerberg et al., 2010). When comparing the levels of expression for the various NRs, the gene array analysis indicated that BMMCs co-cultured with bacteria expressed the Nr4a family members at levels far exceeding those for any of the other NRs (Table 1). Hence, NRs of the Nr4a subfamily are selectively upregulated in BMMCs in response to co-culture with live streptococci, suggesting an important role for Nr4a factors in the regulation of MC-mediated events. At baseline conditions, Nr3c1 (glucocorticoid receptor) and Nr1h2 (Liver X receptor β) showed the highest levels of expression out of the various NRs, in agreement with the known expression of these NRs in MCs (Daeron et al., 1982; Nunomura et al., 2010).

In order to verify the expression of Nr4a members in MCs, we designed and validated qPCR primers for all three members (Table 2), and quantified the extent of Nr4a1–3 upregulation in response to co-culture with live bacteria. As shown in Fig. 1A, co-culture of BMMCs with live *S. equi* caused a dramatic (up to ~1000-fold) upregulation of all three members. To further study the mechanism of Nr4a upregulation, their expression in response to purified bacterial cell surface components was studied. As displayed in Fig. 1B, purified LPS caused robust upregulation of Nr4a3 expression, but had no significant effect on Nr4a1 or Nr4a2 expres-

Table 1

Effect of co-culture with live *S. equi* on the expression of nuclear receptors in BMMCs. BMMCs were cultured either alone (Control) or in co-culture with live *S. equi*, followed by RNA purification and Affymetrix gene array analysis. Based on original data from Rönnerberg et al. (2010).

Gene symbol	Log 2 fold change	Adj. P. Val	Log 2 transformed signal values	
			Control	Co-culture
Nr4a3	6.78	0.000010	6.53	13.31
Nr4a2	5.27	0.000013	6.54	11.81
Nr4a1	4.97	0.000010	8.47	13.44
Esr2	0.57	0.017	5.50	6.07
Nr3c2	0.57	0.039	4.96	5.53
Nr5a2	0.50	0.032	5.01	5.51
Esr1g	0.47	NS ^a	5.51	5.98
Nr5a1	0.44	NS	6.24	6.69
Ppard	0.40	NS	8.07	8.47
Nr2f2	0.40	NS	5.58	5.98
Nr2f1	0.36	NS	5.35	5.70
Nr2e1	0.30	NS	5.28	5.57
Nr2e3	0.28	NS	5.88	6.16
Pparg	0.27	NS	5.10	5.37
Nr0b2	0.23	NS	4.89	5.12
Rarg	0.22	NS	7.19	7.41
Esr1b	0.22	NS	5.66	5.88
Nr1h3	0.21	NS	5.94	6.15
Nr0b1	0.19	NS	4.17	4.36
Nr1i2	0.19	NS	5.79	5.97
Nr6a1	0.17	NS	6.52	6.69
Rarb	0.16	NS	4.96	5.12
Thra	0.16	NS	5.26	5.41
Ppara	0.14	NS	5.27	5.41
Nr1h4	0.12	NS	4.45	4.57
Rorc	0.12	NS	5.93	6.05
Nr1i3	0.11	NS	5.48	5.59
Nr1h5	0.10	NS	4.14	4.25
Hnf4g	0.08	NS	3.76	3.85
Rxrg	0.07	NS	4.86	4.93
Sra1	0.07	NS	7.75	7.81
Rorb	0.06	NS	5.30	5.36
Thrb	0.05	NS	5.07	5.12
Nr1d2	0.04	NS	7.87	7.91
Esr1	0.04	NS	6.53	6.57
Rxra	0.03	NS	6.06	6.10
Rora	0.02	NS	6.16	6.17
Nr1d1	−0.04	NS	6.60	6.56
Rxrb	−0.11	NS	8.08	7.97
Vdr	−0.23	NS	6.46	6.23
Nr1h2	−0.27	NS	9.86	9.59
Nr2f6	−0.32	NS	7.94	7.62
Esr1a	−0.43	NS	8.11	7.68
Pgr	−0.44	NS	7.48	7.04
Rara	−0.44	NS	8.70	8.25
Nr3c1	−0.49	NS	9.73	9.24
Nr2c2	−0.75	0.016	8.95	8.21
Nr2c1	−1.27	0.00082	7.57	6.31

^a NS, not significant.

sion. The induction of Nr4a3 expression by LPS was dose dependent, with increased induction of Nr4a3 at LPS concentrations up to 10 µg/ml (data not shown). In contrast, PGN did not cause any measurable upregulated expression of either of the Nr4a members (Fig. 1C). In fact, PGN caused a somewhat downregulated expression of Nr4a2.

3.2. Nr4a factors are upregulated in response to IgE receptor cross-linking and to calcium ionophore stimulation

In order to clarify whether the upregulated expression of Nr4a family members is specifically induced by bacterial insults, or a general consequence of MC activation processes, we studied the effect of other MC stimulants on Nr4a expression. First, the effect of activation through IgE receptor cross-linking was studied. As shown in Fig. 2A, IgE receptor cross-linking caused a massive upregulation of

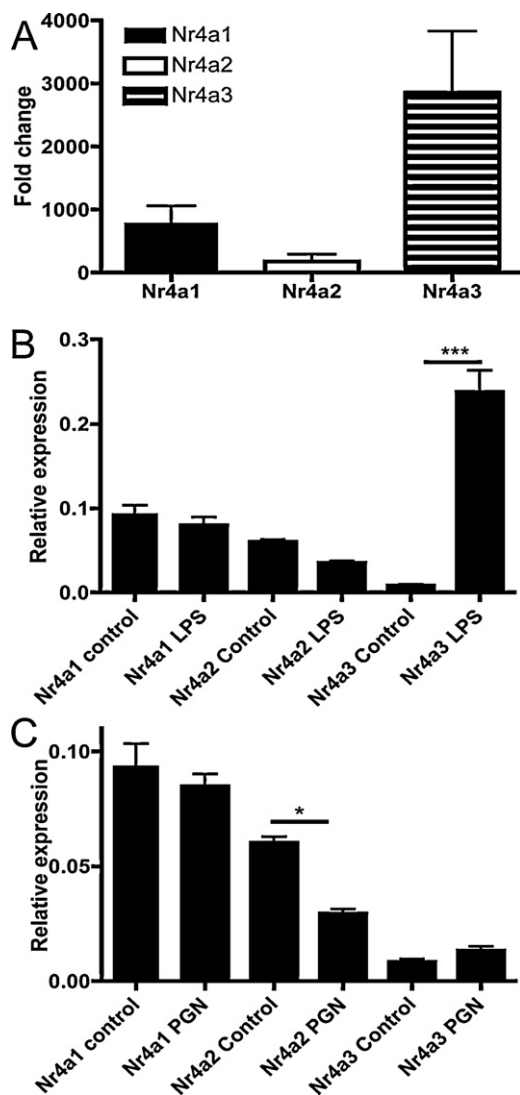


Fig. 1. Nr4a family members are upregulated in BMMCs activated with live bacteria and with bacterial cell wall products. BMMCs (1×10^6 cells) were cultured for 4 h in the presence of (A) live group C streptococci (*S. equi*), (B) LPS ($1 \mu\text{g/ml}$) or (C) PGN ($10 \mu\text{g/ml}$). Total RNA was isolated and analyzed by qPCR for levels of mRNA coding for Nr4a1, Nr4a2 and Nr4a3 as indicated. Expression levels are related to the expression level of the house-keeping gene used (Hprt). Results in A are expressed as mean $\pm$ SEM ($n = 5$) whereas B, C are expressed as mean $\pm$ SEM ($n = 3$).

Nr4a3 expression and a tendency of Nr4a1 upregulation, but no significant upregulation of Nr4a2. The extent of Nr4a3 induction was dose dependent up to $0.4 \mu\text{g/ml}$ TNP-OVA, whereas higher amounts of added antigen resulted in decreased Nr4a3 induction (data not shown).

A hallmark of IgE receptor-mediated MC activation is induction of Ca^{2+} influx into the cytosol, and we therefore also assessed whether calcium ionophore could induce similar effects on Nr4a expression. Indeed, similar to IgE-mediated BMMC activation, calcium ionophore A23187 induced not only a dramatic increase in the expression of Nr4a3, but also a robust upregulation of Nr4a1, whereas no significant effect on Nr4a2 expression was seen.

3.3. Nr4a factor expression is not induced during MC maturation

Next, we investigated whether the expression of Nr4a factors is influenced by the MC maturation process. To this end, Nr4a expression was analyzed at different stages of BMMC maturation. However, neither of the NR4a members was induced during

Table 2
Primers used for qPCR. All primers were designed using the software Primer Express Version 1.0.

Target	Sequence	Amplicon size (bp)
Mouse		
Hprt, fw ^a	5'-GAT TAG CGA TGA TGA ACC AGG TTA-3'	133
Hprt, rev ^b	5'-GAC ATC TCG AGC AAG TCT TTC AGT C-3'	
Nr4a1, fw	5'-TGC ACA GCT TGG GTG TTG ATG TTC-3'	138
Nr4a1, rev	5'-TGT GCT CCT TCA GAC AGC TAG CAA-3'	
Nr4a2, fw	5'-ACA CAC ACA CCT TAA TGG GAC CCT-3'	114
Nr4a2, rev	5'-CAT GCC ACC CAC GCA ACA TTT AGT-3'	
Nr4a3, fw	5'-TCA CCA TCA CCA TCA TCA CCA CCA-3'	119
Nr4a3, rev	5'-AAG GCG GAG ACT GCT TGA AGT ACA-3'	
Human		
GAPDH, fw	5'-TCG ACA GTC AGC CGC ATC TTC TTT-3'	94
GAPDH, rev	5'-ACC AAA TCC GTT GAC TCC GAC CTT-3'	
NR4A1, fw	5'-AAG CCA CAT TGT TGC CAA GAC CTG-3'	146
NR4A1, rev	5'-TGG TGT CCC ATA TTG GGC TTG GAT-3'	
NR4A2, fw	5'-AGC ATG GAC CTC ACC AAC ACT GAA-3'	186
NR4A2, rev	5'-GCT GTG TTG CTG GTA GTT GTG CAT-3'	
NR4A3, fw	5'-TAC ACC AAG CTG ACC ATG GAC CTT-3'	134
NR4A3, rev	5'-ATT TGG TAC ACG CAG GAA GGC TTG-3'	

^a fw, forward primer.

^b rev, reverse primer.

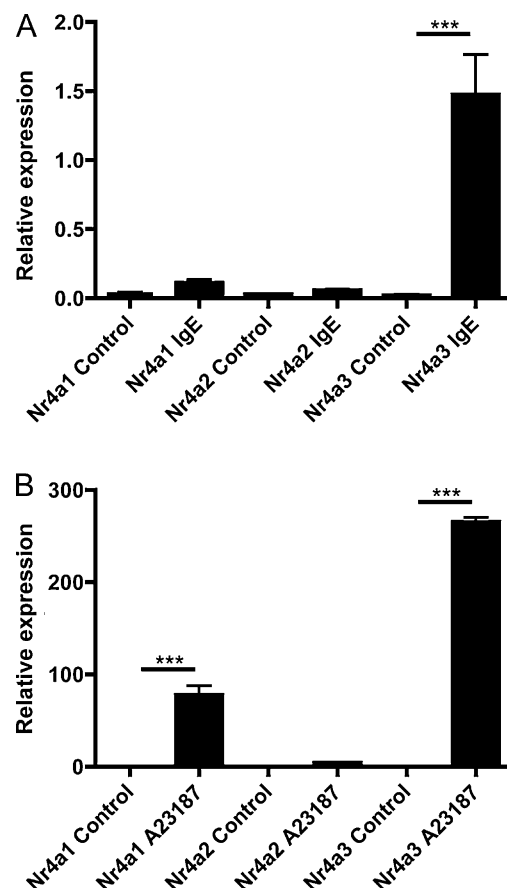


Fig. 2. Nr4a family members are upregulated in BMMCs activated by IgE receptor cross-linking or with calcium ionophore. (A) BMMCs (1×10^6 cells) were incubated with anti-TNP IgE followed by IgE receptor cross-linking by addition of TNP-OVA as described in Section 2. As a control, cells were incubated with anti-TNP IgE without subsequent addition of TNP-OVA. (B) BMMCs (1×10^6 cells) were activated by incubation for 4 h with calcium ionophore A23187 ($2 \mu\text{M}$ final concentration). (A, B) Total RNA was isolated and analyzed by qPCR for levels of mRNA coding for Nr4a1, Nr4a2 and Nr4a3 as indicated. Results (mean $\pm$ SEM, $n = 3$) from three separate BMMC cultures, generated from individual animals are expressed relative to expression of the house-keeping gene (Hprt).

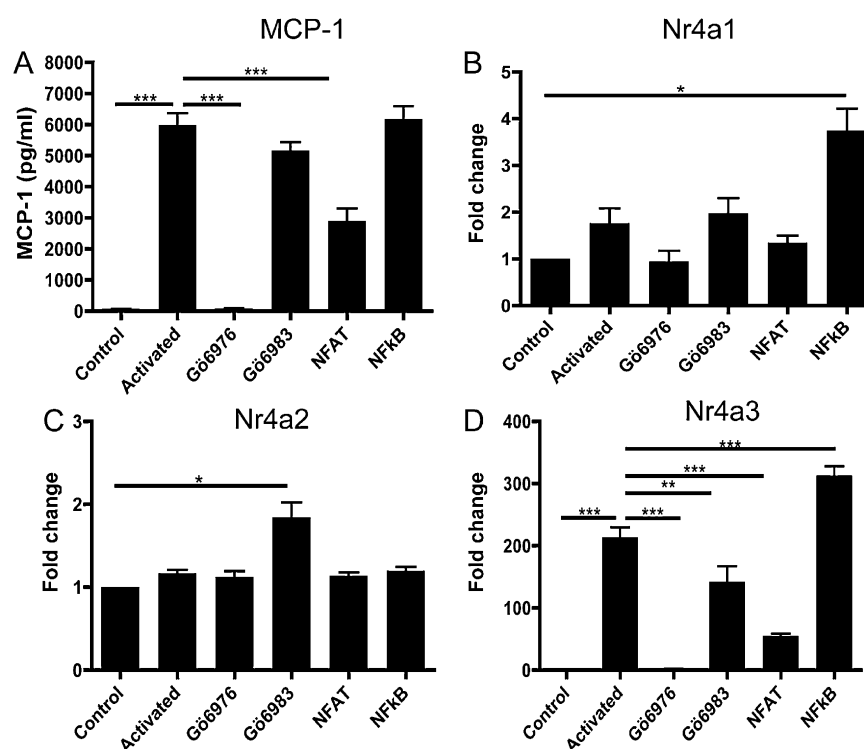


Fig. 3. Nr4a expression in BMMCs is dependent on PKC and on the NFAT pathway. BMMCs (1×10^6 cells) were activated by IgE receptor cross-linking (see Section 2), either in the absence or presence of the following inhibitors of signaling pathways: Gö6976 (1 μ M); Gö6983 (1 μ M); NFκB inhibitor (100 nM) or NFAT inhibitor (5 μ M). (A) As a positive control for MC activation, the secretion of MCP-1 in response to IgE receptor cross-linking was assessed. (B–D) After 4 h, total RNA was isolated and analyzed by qPCR for levels of mRNA coding for (B) Nr4a1, (C) Nr4a2 and (D) Nr4a3 as indicated. Results are derived from three separate BMMC cultures, generated from individual animals (mean $\pm$ SEM, $n = 3$) and expressed as fold change relative to non-stimulated controls.

the process of MC maturation (data not shown). In contrast, the expression of typical MC markers, the serglycin and mouse MC protease 6 (mMCP-6) genes, were low at early stage cultures but showed a gradual increase as the cells matured to BMMCs (data not shown).

3.4. Nr4a3 induction is dependent on Gö6976-sensitive kinases and on the NFAT pathway

In order to investigate which signaling pathways that are involved in the activation of Nr4a expression in response to IgE-mediated MC activation, specific inhibitors were used. As a control for efficient MC activation, the effect of IgE receptor cross-linking on monocyte chemoattractant protein-1 (MCP-1) secretion was also assessed. As shown in Fig. 3A, IgE receptor cross-linking caused a profound release of MCP-1, and the release of MCP-1 was strongly inhibited by Gö6976, an inhibitor of PKC α /β and PKD1 (also denoted PKC μ) (Murphy et al., 2007; Young et al., 2005). MCP-1 secretion was also significantly inhibited by an NFAT inhibitor, while no inhibition was seen when adding Gö6983, a compound that inhibits various types of PKC (PKC α /β/γ/δ/ζ) but is without effect on PKD1 (Young et al., 2005). Further, no effect of NFκB inhibition was observed (Fig. 3A). Again, the expression of Nr4a3 was dramatically enhanced by IgE-mediated BMMC activation, whereas no significant (Nr4a2) or low (Nr4a1) effects on other Nr4a members were seen (Fig. 3B–D). Notably, the upregulated Nr4a3 expression in response to IgE receptor cross-linking was completely abrogated by Gö6976, while Gö6983 had a lower effect. Inhibition of NFκB signaling did not affect Nr4a3 expression, whereas inhibition of the NFAT pathway caused significant inhibition of Nr4a3 expression. Hence, these data implicate Gö6976-sensitive kinases and the NFAT pathway in the induction of Nr4a3 expression in BMMCs.

3.5. MC activation causes Nr4a phosphorylation

Previous studies have indicated that, upon activation, Nr4a1 is phosphorylated in the DNA-binding domain (Martinez-Gonzalez and Badimon, 2005; Pekarsky et al., 2001). Potential phosphorylation sites have also been identified in Nr4a2 and Nr4a3, but phosphorylation of Nr4a2 or Nr4a3 has not been documented. Notably, the potential phosphorylation sites in Nr4a2 and Nr4a3 are identical to the identified site in Nr4a1 (Wingate et al., 2006). Therefore, antibodies towards phosphorylated Nr4a1 (P-Nr4a1) may also detect phosphorylated Nr4a2 and Nr4a3. To investigate if IgE-mediated activation of BMMCs caused Nr4a phosphorylation, an antibody recognizing phosphorylated Nr4a1 (P-Nr4a1) was used. Indeed, Western blot analysis indicated that IgE-mediated BMMC activation caused rapid Nr4a phosphorylation in murine BMMCs (Fig. 4A; left panel). The Nr4a phosphorylation in response to IgE receptor cross-linking was completely inhibited by Gö6976, and partially by Gö6983 and by the NFAT inhibitor, whereas NFκB inhibition resulted in potentiated Nr4a phosphorylation (Fig. 4B).

3.6. Nr4a2 is upregulated in apoptotic BMMCs

Previous reports have suggested that Nr4a factors may have roles in apoptosis, as shown in activation-induced cell death of T and B lymphocytes (Liu et al., 1994; Woronicz et al., 1994) and in caspase-independent macrophage cell death (Kim et al., 2003). To investigate the potential involvement of Nr4a factors in MC apoptosis, apoptosis was induced in BMMCs by growth factor deprivation, followed by assessment of the extent of apoptosis and levels of Nr4a expression. As judged by Annexin V staining, ~35% of the BMMCs were apoptotic after 24 h of culture without growth factors and ~70% of the cells were apoptotic at 48 h of culture (Fig. 5A). Analysis of Nr4a factor expression showed that the induction of

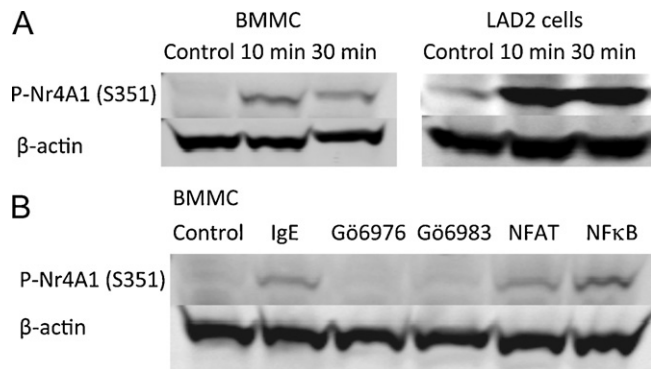


Fig. 4. Nr4a1 is phosphorylated in activated murine and human MCs. (A; left panel) BMMCs (1×10^6 cells) were activated by IgE receptor cross-linking. At the time points indicated, cell extracts were prepared and analyzed for the presence of phosphorylated Nr4a1 (P-Nr4a1) by Western blot. (B) Effects of inhibitors on phosphorylation of Nr4a1 in response to IgE receptor cross-linking. Inhibitors used were: 1 μ M Gö6976; 1 μ M Gö6983; 100 nM NF κ B inhibitor or 5 μ M NFAT inhibitor. (A; right panel) Human MCs (LAD2; 0.5×10^6 cells) were activated by calcium ionophore stimulation (2 μ M). At the time points indicated, cell extracts were prepared and analyzed for the presence of phosphorylated NR4A1 by Western blot.

apoptosis caused a significant upregulation of Nr4a2 expression at 24 h after growth factor deprivation, whereas the expression of Nr4a1 and Nr4a3, in contrast, were somewhat decreased (Fig. 5B). At 48 h, the expression of Nr4a2 was reduced to below baseline levels, and the expression of Nr4a1 and Nr4a3 were further decreased as compared with their levels at 24 h (Fig. 5B).

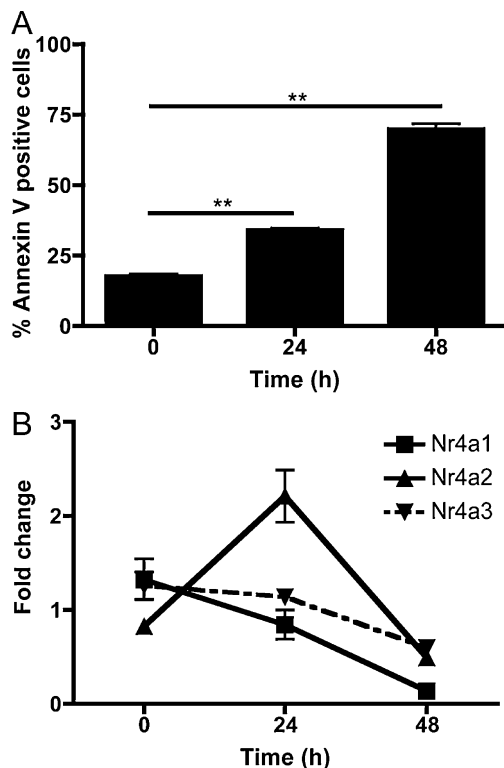


Fig. 5. Nr4a2 expression is upregulated during growth factor deprivation-induced BMMC apoptosis. BMMCs developed in the presence of growth factor-containing WEHI-3B-conditioned medium were deprived of growth factors and were further cultured. (A) To assess the extent of apoptosis, Annexin V positivity was measured by flow cytometry. (B) At the time points indicated, total RNA was recovered and analyzed for levels of mRNA coding for Nr4a1–Nr4a3 as indicated. Results are derived from three separate BMMC cultures, generated from individual animals (mean $\pm$ SEM, $n=3$) and expressed as fold change relative to cytokine sufficient controls.

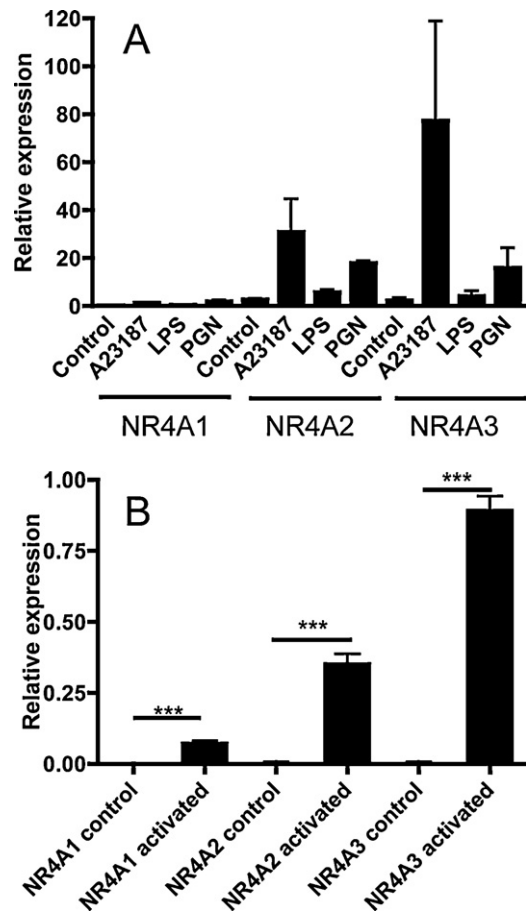


Fig. 6. Upregulated expression of NR4A factors in activated human MCs. (A) Primary human PBMCs ($n=2$) were cultured in the absence or presence of calcium ionophore A23187 (2 μ M final concentration), LPS (1 μ g/ml) or PGN (10 μ g/ml) for 4 h. Total RNA was recovered and analyzed for levels of mRNA coding for NR4A1–NR4A3 as indicated. (B) Human mast cell-like LAD2 cells ($n=3$) were cultured in the absence or presence of calcium ionophore A23187 (2 μ M final concentration) for 4 h. Total RNA was recovered and analyzed for levels of mRNA coding for NR4A1–NR4A3 as indicated. The results (mean $\pm$ SEM) show the expression of the respective NR4A genes relative to the expression levels of the house-keeping gene (GAPDH).

3.7. NR4A factors are upregulated in activated human MCs

Next, the regulation of NR4A factor expression in human MCs was assessed. To this end, their expression was studied in human peripheral blood derived MCs (PBMCs). Additionally, the expression of NR4A factors in a human MC-like cell line, LAD2, was investigated. As shown in Fig. 6A, calcium ionophore stimulation of PBMCs caused a robust upregulation of NR4A2 and, in particular, NR4A3, while NR4A1 expression was only moderately affected. In addition, NR4A2 and NR4A3 expression was induced by LPS and by PGN (Fig. 6A). In line with these findings, calcium ionophore stimulation of LAD2 cells resulted in a marked upregulation of all three NR4A factors. Similar to the situation in murine BMMCs, the effect was largest on NR4A3 expression, while lower effects on NR4A1 and NR4A2 expression were seen. As shown in Fig. 4A (right panel), activation of LAD2 cells with calcium ionophore resulted in rapid phosphorylation of NR4A1.

3.8. MC activation causes increased Nr4a3 protein synthesis

To verify the strongly upregulated expression of Nr4a3 in activated MCs, cells were stained with an anti-NR4A3 antibody followed by analysis with confocal microscopy. In the absence of MC stimulant, BMMCs and LAD2 cells stained only faintly (Fig. 7; left

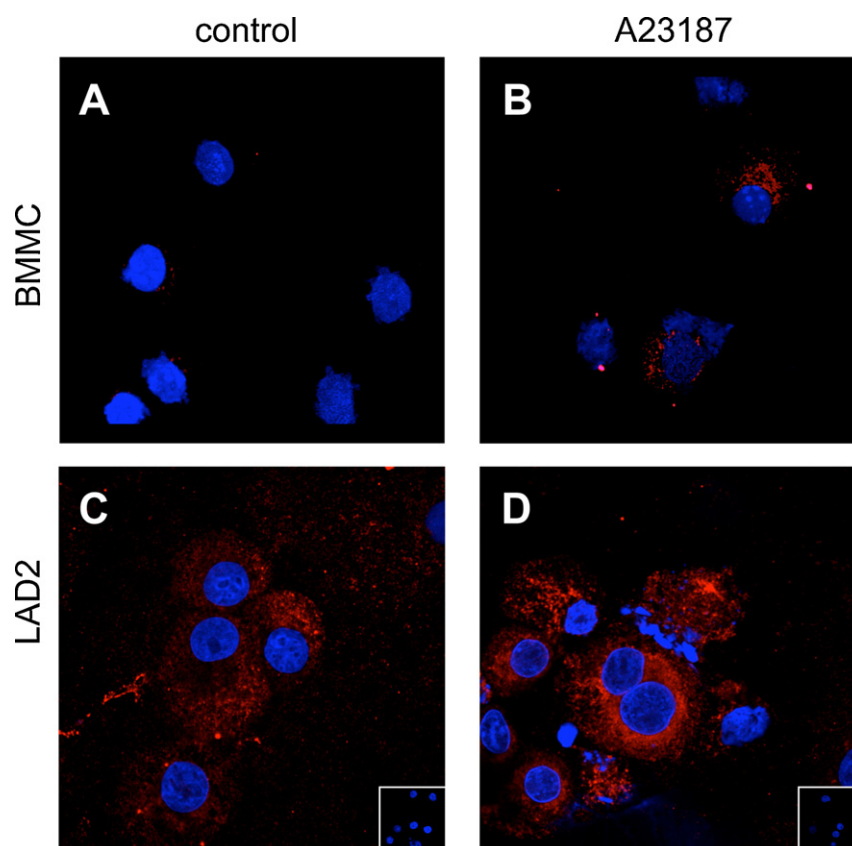


Fig. 7. MC activation causes increased levels of Nr4a3 protein. BMMCs or LAD2 cells were either left untreated or incubated for 24 h with calcium ionophore A23187 as indicated. Cells were stained with an antibody towards NR4A3 and nuclei were visualized with DAPI. The analysis was done by confocal fluorescence microscopy. Insets show the lack of staining when using isotype control antibody.

panels), and there was no staining in cells stained with isotype control antibody (Fig. 7; insets). However, when cells were activated with calcium ionophore, pronounced staining of both BMMCs and LAD2 cells was evident (Fig. 7), confirming that the increased mRNA levels are also reflected by increased Nr4a3 protein synthesis.

4. Discussion

The large family of NRs has an enormous impact on the regulation of gene expression in all cell types, including MCs. For example, MCs have been shown to respond to glucocorticoids (Daeron et al., 1982), thus implicating the glucocorticoid receptor (Nr3c1) in MC function. Further, MCs are known to express liver X receptor- β (Nr1h2) (Nunomura et al., 2010) and peroxisome proliferator-activated receptor γ (Pparg) (Paruchuri et al., 2008). Here we show that MC activation causes a massive and selective upregulation of NRs of the Nr4a family, thus implicating yet another class of NRs in MC function. Notably, when MCs were co-cultured with live group C streptococci, all three members of the Nr4a family were profoundly induced. Interestingly, when purified bacterial cell wall LPS was used, only upregulation of Nr4a3 was seen, whereas purified PGN did not induce the expression of any of the Nr4a members. Hence, Nr4a3 expression appears to be highly sensitive to LPS whereas streptococci-induced upregulation of Nr4a1 and Nr4a2 is caused by, as yet unidentified, bacterial products.

To extend these observations we also investigated whether Nr4a expression was induced in MCs activated by other means, with a major focus on IgE-mediated MC activation. Strikingly, we show that IgE-mediated MC activation caused a massive upregulation of Nr4a3, with the extent of upregulation being much more profound than that caused by LPS. In contrast, effects on other Nr4a factors

were minimal. As IgE-mediated MC activation is a hallmark event during allergic responses, these findings implicate Nr4a-mediated pathways in disease in which IgE-mediated MC activation occurs, e.g. allergic asthma.

IgE-mediated MC activation involves a downstream increase in intracellular Ca^{2+} levels (Blank and Rivera, 2004; Turner and Kinet, 1999). To directly investigate whether Nr4a activation is induced by Ca^{2+} -mediated events, we also stimulated the MCs with calcium ionophore. Indeed, calcium ionophore activation caused a massive upregulation of Nr4a3, and also upregulated Nr4a1 expression. Hence, in agreement with Nr4a regulation in other cell types (Liu et al., 2003; Martinez-Gonzalez et al., 2003), calcium signaling is a key event in the regulation of Nr4a3 expression in MCs.

To further study the signaling pathways involved in Nr4a induction, we used inhibitors specific for various signaling molecules previously implicated in Nr4a regulation. Previous investigations have indicated that Nr4a induction in other cell types can occur via the calcineurin–NFAT pathway (Youn et al., 2000) or through NF κ B activation (Bonta et al., 2006; Pei et al., 2005). When assessing the relative importance of these pathways, we obtained evidence suggesting that the calcineurin–NFAT pathway was active in inducing Nr4a3 expression in IgE-activated MCs, whereas the NF κ B pathway appeared to be of minor importance. Previous investigations have suggested that Nr4a gene induction in endothelial cells is dependent on PKC (Liu et al., 2003). In line with this, IgE-mediated Nr4a3 induction in MCs was partially prevented by the broad-spectrum PKC inhibitor Gö6983. Interestingly, although Nr4a3 induction was partially inhibited by Gö6983, a compound that inhibits PKC $\alpha/\beta/\gamma/\delta/\zeta$ but is without effect on PKD1 (Young et al., 2005), Gö6976, a compound that inhibits PKC α/β and PKD1 (Murphy et al., 2007; Young et al., 2005), completely abrogated

Nr4a3 induction. Combined, these observations are compatible with a role for PKD1 in the signaling cascade leading to upregulation of Nr4a3 expression in MCs. In agreement with this notion, PKD1 was shown to be activated in response to various MC stimulants including IgE receptor cross-linking (Murphy et al., 2007) and, moreover, PKD1 activation in MCs was shown to be sensitive to Gö6976 (Murphy et al., 2007).

An important question was to address the species-specificity of Nr4a upregulation in activated MCs, i.e. to investigate if similar upregulation also occurs in activated human MCs. For this purpose, we investigated Nr4a regulation in human PBMCs and in the human MC-like cell line LAD2. Indeed, Nr4a factors were profoundly induced upon activation of both these human MC types, suggesting that Nr4a upregulation is a general consequence of MC activation over species. Similar to the murine MCs, NR4A3 expression showed the most profound induction also in human MCs. However, whereas murine Nr4a2 expression was relative insensitive to calcium ionophore stimulation, human NR4A2 showed a robust induction.

Previous investigations have implicated Nr4a factors, in particular Nr4a2, in apoptosis (Kim et al., 2003; Liu et al., 1994; Woronicz et al., 1994). To assess the importance of Nr4a members in MC apoptosis, apoptosis was induced by growth factor deprivation, followed by the assessment of the extent of Annexin V positivity and levels of Nr4a expression. Indeed, induction of MC apoptosis caused a significant upregulation of Nr4a2, whereas no induction of Nr4a1 and -3 was seen. Clearly, these data are compatible with a role for Nr4a2 in MC apoptosis. Intriguingly, we may also propose that the upregulation of Nr4a2 seen when MCs are co-cultured with live bacteria may be a result of pro-apoptotic events, effects that are not reproduced when instead challenging MCs with purified cell wall products.

Together, the findings presented here implicate for the first time NRs of the Nr4a family in MC activation. Considering the large impact of MC activation on numerous diseases, we propose that induction of Nr4a factors may be an important event in the transcriptional activities of MCs in response to agents that cause MC activation.

Conflict of interest

The authors have no conflicts of interest.

Acknowledgements

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