

Short communication

**MOLECULAR CLONING AND CHARACTERIZATION
OF A NOVEL ANTI-TLR9 INTRABODY**ELISA REIMER^{1,§}, STEFAN SOMPLATZKI^{1,§}, DIANA ZEGENHAGEN¹,
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Abstract: Toll-like receptor 9 (TLR9) is a component of the innate immune system, which recognizes the DNA of both pathogens and hosts. Thus, it can drive autoimmune diseases. Intracellular antibodies expressed inside the ER block transitory protein functions by inhibiting the translocation of the protein from the ER to its subcellular destination. Here, we describe the construction and characterization of an anti-TLR9 ER intrabody (α T9ib). The respective single-chain Fv comprises the variable domains of the heavy and light chain of a monoclonal antibody (mAb; 5G5) towards human and murine TLR9. Co-expression of α T9ib and mouse TLR9 in HEK293 cells resulted in co-localization of both molecules with the ER marker calnexin. Co-immunoprecipitation of mouse TLR9 with α T9ib indicated that α T9ib interacts with its cognate antigen. The expression of α T9ib inhibited NF- κ B-driven reporter gene activation upon CpG DNA challenge but not the activation

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Abbreviations used: mAb – monoclonal antibody; Intrabody – intracellular antibody

of TLR3 or TLR4. Consequently, TLR9-driven TNF α production was inhibited in RAW264.7 macrophages upon transfection with the α T9ib expression plasmid. The α T9ib-encoding open reading frame was integrated into an adenoviral cosmid vector to produce the recombinant adenovirus (AdV)- α T9ib. Transduction with AdV α T9ib specifically inhibited TLR9-driven cellular TNF α release. These data strongly indicate that α T9ib is a very promising experimental tool to block TLR9 signaling.

Key words: Recombinant antibodies, Recombinant adenovirus, Protein knockdown, Intracellular toll-like receptors, TLR9, ER intrabodies, Macrophage activation

INTRODUCTION

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) that trigger the host defense against invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs) [1, 2]. TLR ligands are produced by viruses, pathogenic bacteria, pathogenic fungi and parasitic eukaryotes.

TLRs 1, 2, 4, 5, 6 and 10 are expressed on the cell surface, while TLRs 3, 7, 8, 9, 11, 12 and 13 are expressed endosomally [3]. TLR9, which recognizes DNA, resides in the endoplasmic reticulum (ER) constitutively. Endocytosed DNA resides in early endosomes and is subsequently transported to a tubular compartment. Concurrent with the movement of DNA in cells, TLR9 redistributes from the ER to endosomes [4].

Endosomal TLRs have been implicated in autoimmune disease pathologies, such as those of rheumatoid arthritis and systemic lupus erythematosus [5, 6]. Dysregulation of TLR7/TLR9 signaling has been found to promote autoimmune disease [6].

The role of TLR9 in rheumatoid arthritis is not yet fully understood. Systemic injection of CpG ODN does sensitize mice, resulting in an exaggerated arthritis if the mice are subsequently challenged with an intra-articular injection of a low dose of CpG ODN [7]. However, the pathogenesis of arthritis in TLR9^{-/-} mice was no different to that in wild-type mice, which indicates that this receptor does not have an obvious role in this model [8].

Inhibitors of TLR signaling targeting cell surface and endosomal receptors have been developed. For example, for cell surface receptors, there are neutralizing antibodies [9, 10], chaperonin 10 [11] and small-molecule antagonists [12]. Endosomal receptors can be blocked by antimalarial chloroquine and short DNA segments (immunoregulatory sequences, IRS), among others [12, 13].

IRS are suppressive oligonucleotides [14, 15] that are often thought to lack exclusive specificity. Effects on other TLR-family members have been shown and some inhibitory DNA segments bind signal transducer and activator of transcription 1 (STAT1) and STAT4 [16]. Furthermore, CpG-containing inhibitory sequences also interact with TLRs 3, 7 and 8 [17].

To bypass any lack of specificity, we used an intrabody with intrinsic specificity for its target [18, 19]. Intrabodies can be targeted to the nucleus, mitochondria or endoplasmic reticulum (ER), where they bind their target proteins and thus persistently inhibit their function. For instance, intrabodies targeted to the ER arrest transitory target proteins preventing their further subcellular trafficking [18, 19]. Intrabodies are a promising alternative to RNA-based knockdown strategies in cases of failure of the latter [20-22]. We previously developed an anti-TLR2 intrabody which inhibited TLR2 ligand-driven cell activation *in vitro* and *in vivo* [23]. Endosomal TLRs (including TLR9) that cannot be reached by normal antibodies due to their endosomal location are ideal targets for ER intrabodies.

Here, we describe the generation and characterization of an anti-TLR9 scFv ER intrabody (α T9ib). α T9ib bound specifically to TLR9 and co-localized together with TLR9 inside the ER. As a consequence, TLR9-specific activation of RAW264.7 macrophages was inhibited. Therefore, this new intrabody appears to be a powerful tool for the inhibition of TLR9 function.

MATERIALS AND METHODS

Cell lines

Murine RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, USA). They were cultured in DMEM (Gibco, Darmstadt, Germany) supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Darmstadt). Human embryonic kidney (HEK293) cells were purchased from the German strain collection (DSMZ, Braunschweig, Germany). Hybridoma cell line 5G5, which secretes mouse IgG2a lambda light-chain TLR9-specific antibody and was developed by Prof. S. Bauer (Marburg, Germany) [24] was cultured in RPMI 1640 (PAN-Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (PAN-Biotech, Aidenbach, Germany), 1% penicillin and streptomycin (Lonza, Basel, Switzerland).

α T9ib construction

mRNA from the hybridoma 5G5, secreting a mouse anti-TLR9 IgG2a/lambda antibody, was isolated using an RNeasy Kit (Qiagen). Oligo-dT(15) primer and a cDNA Synthesis Kit (Roche) were used for the generation of double-stranded cDNA (ds-cDNA). The blunted ds-cDNA was circularized with a T4-DNA Ligase (Roche) at 16°C. For the amplification of both unknown variable antibody gene domains, oligonucleotides specific for the constant region of gamma 2a (2a-back, 5'-CATGCAAATGCCAGCACCTAACCTCTGGGTG-3'; 2a-for, 5'-GAGGACAGGGCTTGATTGTGGGCCCTCTGGGCT-3') and lambda (lambda-for, 5'-TGTTTCCACCTTCCYCTGA-3'; lambda-rev, 5'-GCCTTCCAGKCCACTGTCAC-3') were used in an inverse PCR. Specific amplicons (heavy 2a, 600 bp; lambda 900 bp) were ligated into the TOPO

pCR2.1 vector (Invitrogen) and the unknown VH and VL domains were sequenced.

For the generation of a single-chain Fv fragment, VH and VL domains were amplified with overlapping primers adding an oligonucleotide stretch coding for a glycine-serine linker, (G₄S)₃, and spliced together by overlapping extension PCR. VH was amplified with VHBACKTLR9: 5'-CAGATCCAG TTGGTGCAGTCTGGACCT-3' and VHFORTLR9: 5'-GGCTGCAGAGACA GTGACCAGAGTCCC-3', and VL was amplified with Primer VLBACKTLR9: 5'-CAGGCTGTTGTGACTCAGGAATCTGCA-3' and Primer VLFORTLR9: 5'-TAGGACAGTCAGTTTGGTTCCTCCACC-3'. The synthetic linker VH-(G₄S)₃-VL 5'-ACTCTGGTCACTGTCTCTGCAGCCGGTGGAGGCGGTTTCAGGCGAGG TGGCTCTGGCGGTGGCGGATCGCAGGCTGTTGTGACTCAGGAATCT-3' was amplified with LINKBACKTLR9: 5'-ACTCTGGTCACTGTCTCTGCA GCCGGT-3' and LINKFORTLR9: 5'-AGATTCTGAGTCACAACAGC CTGCCA-3'. The assembly PCR was performed in two steps as described previously [19].

The resulting construct contained the complete scFv coding sequence (cds) derived from 5G5 hybridoma. The scFv sequence was cloned into pCMV/myc/ER vector (Invitrogen) using *SalI* and *NotI* restriction sites. After cloning, a myc-tag and an ER retention motif were fused 3'-terminally to the preceding anti-TLR9 scFv sequence to form the α T9ib construct. Moreover, an α T9ib in which the myc-tag was exchanged with an HA-tag was generated with site-directed mutagenesis of pCMV/myc/ER- α T9ib (Stratagene Quick Change II Kit). The pCMV/HA/ER- α T9ib expression plasmid construct was used for co-localization analyses.

Anti-TLR2 ER intrabody (α T2ib) was generated in the scFv format from an antagonistic monoclonal antibody towards human and murine TLR2 (T2.5) and inhibits the function of TLR2 [23]. α VR-ib recognizes vascular endothelial growth factor receptor-2 (VEGFR-2/KDR), mediates complete inhibition of cell surface expression of the receptor, and inhibits *in vitro* angiogenesis [25]. This antibody was derived from scFv A7 selected from a V-gene phage display library [26].

Construction of an adenoviral vector encoding α T9ib, production of the recombinant virus and infection *in vitro*

An adenoviral vector to carry a bicistronic expression cassette driving expression of the ER intrabody α T9ib and the reporter gene eGFP was constructed via two subcloning steps. In the first step, the coding sequence of the anti-TLR9 ER intrabody, including the ER signal peptide, myc-tag and ER retention signal, was amplified via PCR from the pCMV/myc/ER vector carrying the cds of α T9ib using the primers TLR9-ERRS-BACK-phosph: 5'-ATGGGATGGAGCTGTATCATCCTC-3' and TLR9-ERRS-FOR-phosph: 5'-CTACAGCTCGTCTTCTCGCTTGC-3'. The amplicon was ligated into the *SmaI* linearized vector pGEM/IRES/EGFP [27] containing the IRES sequence of

the polio virus followed by the reporter gene eGFP. After transformation in *E. coli* DH5 α , positive clones (pGEM/ α T9ib/IRES/EGFP) containing the bicistronic expression cassette of the α T9ib gene and the reporter gene eGFP served as templates in the second step to derive AdV α T9ib particles and infect RAW264.7 cells [23]. RAW264.7 cells were infected with recombinant adenovirus at a multiplicity of infection (moi) of 50 for AdVGFP, AdV α T2ib and AdV α T9ib. The adenoviral vector contains the genome of a replication-deficient adenovirus type 5 subgenus C in which the E1 and E3 region is deleted [27].

Subcellular co-localization and co-immunoprecipitation of α T9ib and murine TLR9

For the co-localization experiments, HEK293 cells were grown on sterile coverslips and transiently transfected with mTLR9-myc expression plasmid (murine TLR9-myc pcDNA3.1, obtained from M. Brinkmann, HZI, Braunschweig, Germany), HA-tagged anti-TLR9 intrabody expression plasmid, and cherry-CD63 expression plasmid (obtained from M. Brinkmann, HZI, Braunschweig, Germany). Immunostaining was performed as described [23] using a rabbit anti-HA antibody (Santa Cruz, clone Y11) and Cy5-labeled goat anti-rabbit antibody (Dianova) for detection of HA-tagged α T9ib and an FITC-labeled goat anti-myc antibody for detection of mTLR9-myc. Calnexin was detected by mouse anti-Calnexin antibody (Abcam, clone AF18) and Cy3-labeled goat anti-mouse antibody (Dianova). Analyses were performed with a laser scanning confocal microscope (LSM 510 META, Carl Zeiss).

Co-immunoprecipitation was performed as described [23] with the modification that the cells were lysed with 20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5% glycerol, 0.25% sodium deoxycholate, 0.05% SDS, 0.05% Triton-X 100 and 0.5 mM EDTA after 72 h of transient expression.

NF- κ B-dependent luciferase assay in HEK293 cells overexpressing specific TLRs and α T9ib

3×10^4 human embryonic kidney (HEK293) fibroblastoid cells were seeded in each well of a 96-well cell culture plate and transfected with plasmids directing constitutive expression of mTLR9 or other TLRs, α T9ib or control intrabody (anti-VEGFR 2 intrabody scFv A7, α VR-ib [25]) and Renilla luciferase, as well as NF- κ B-dependent expression of firefly luciferase. Challenge with TLR agonists and the assay itself were performed as described [23].

Visualization of intracellular accumulation of TNF α by flow cytometry

RAW264.7 macrophages were co-transfected with expression plasmids for eGFP and α T9ib or control plasmid pCMV/myc/ER. After 4 days, the cells were challenged with TLR agonists and intracellular flow cytometry was performed according to the method described in [23]. Intracellular TNF α in GFP-positive cells was detected with allophycocyanin-labeled rat anti-mouse TNF α antibody (BD Biosciences, clone MP6-XT22). Flow cytometry analyses were performed using a FACS-Calibur equipped with CellQuest software (Becton Dickinson).

Immunoblot analysis

Immunoblot analysis was performed as described [23]. Cell lysis was performed using lysis buffer with 20 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM EDTA, 2% SDS and 0.5% benzonase.

ELISA

TNF α concentration was determined using a mouse TNF α ELISA Kit (BD Biosciences, BD OptEIA) according to the manufacturer's instructions.

RESULTS

Construction of the anti-TLR9 scFv intrabody-coding sequence

Amplification of a DNA fragment encoding the variable domain of the heavy chain (VH) of the hybridoma 5G5 resulted in a 386-bp PCR product. The corresponding variable domain of the light chain (VL) encompassed 329 bp. The linker-encoding fragment VH-(G₄S)₃-VL encompassed 95 bp. PCR assembly of VH, VL and the synthetic linker-encoding fragments resulted in a 754-bp construct (Fig. 1A). Ligation of the anti-TLR9 scFv DNA into the plasmid pCMV/myc/ER resulted in an anti-TLR9 intrabody (α T9ib) construct comprising a myc-tag and the ER retention sequence fused to the 3' terminus of the anti-TLR9 scFv sequence (Fig. 1B).

Co-localization of α T9ib and TLR9 inside the ER compartment and intracellular binding of α T9ib to murine TLR9

Co-localization of α T9ib and mTLR9 was visualized by immunofluorescence microscopy (Fig. 2A). Both resided within a lattice structure identical with the ER compartment. Co-staining of α T9ib, mTLR9 and ER resident marker calnexin indicated localization of α T9ib and mTLR9 inside the ER (upper panel). Co-transfection with cherry-CD63 (endosomal marker) expression plasmid showed no localization of α T9ib and mTLR9 inside the endosomal compartment (lower panel). Specific intracellular binding of mTLR9 and α T9ib was further verified by co-immunoprecipitation performed with anti-HA antibody and with anti-myc antibody (Fig. 2B). Lack of immunoprecipitation of mTLR9 with the control intrabodies α T2ib and α VR-ib in lysates of HEK293 cells expressing respective protein pairs demonstrated α T9ib specificity for its cognate antigen.

Functionality of α T9ib upon transient overexpression in HEK293 cells

In order to analyze α T9ib function, NF- κ B-driven reporter gene activity was determined in HEK293 cells overexpressing both mTLR9 and α T9ib upon TLR9 specific challenge. Co-transfection of α T9ib and mTLR9 expression plasmid DNA effectively inhibited TLR9 activity (Fig. 3A). By contrast, cellular activation through either TLR4 or TLR3 was not influenced by co-expression of α T9ib. Moreover, another ER intrabody (α VR-ib) recognizing the vascular endothelial growth factor receptor-2 (VEGFR-2/KDR) did not block TLR9

activation. Immunofluorescence staining of HEK293 cells co-transfected with α T9ib and mTLR9 expression plasmids demonstrates co-expression of both proteins in almost every transfected cell (Fig. 3B).

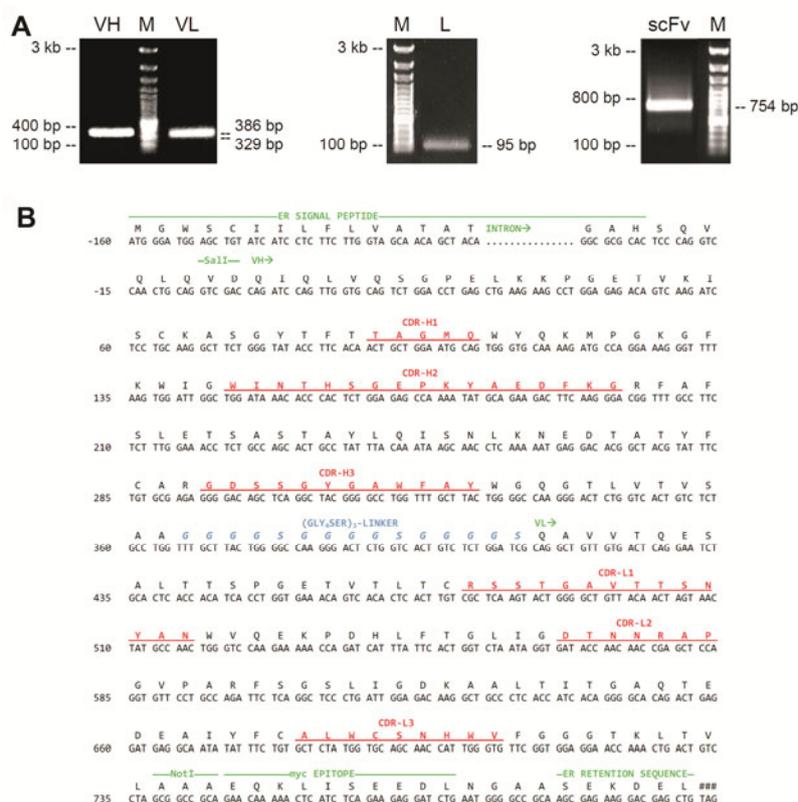


Fig. 1. Assembly and primary sequence of human/murine TLR9 cross-reactive intrabody. A – PCR amplified variable domain encoding sequences of mAb 5G5, the linker DNA, and the product of the assembly PCR. The amplification products were separated using agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Molecular weights 386 and 329 bp respectively correspond to the expected size of the variable domains of the immunoglobulin G (IgG) heavy (VH) and light chain (VL), 95 bp to the amplified linker DNA fragment (L) and 754 bp to the expected apparent size of the product of scFv assembly PCR. B – Primary sequence of human/murine TLR9-cross-reactive intrabody. The coding (lower line) and amino acid (upper line; ####: stop codon) sequences of α T9ib are shown, including the ER signal peptide, the myc-epitope, and the ER retention sequence, which are all shown in green. The complementarity-determining regions (CDR1-CDR3) of the variable domains of the heavy and light chain are printed in bold red. The synthetic linker (shown in bold blue italic letters), localized between the VH and VL domains, was introduced by assembly PCR. *SalI* and *NotI* restriction sites were used to clone the anti-TLR9 scFv fragment into pCMV/myc/ER.

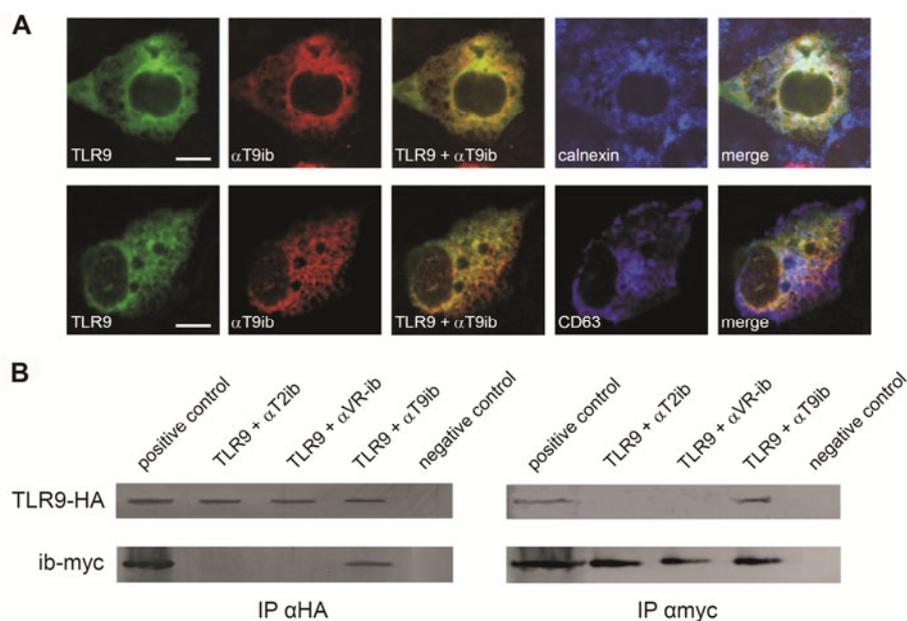


Fig. 2. Subcellular co-localization and co-immunoprecipitation of α T9ib and murine TLR9 upon transfection. A – Immunofluorescence analysis by laser scanning confocal microscopy of fixed and permeabilized HEK293 cells transiently transfected with the mTLR9-myc expression plasmid, HA-tagged α T9ib and cherry-CD63 (endosomal marker). Expression of mTLR9-myc was visualized using FITC-labeled goat anti-myc antibody. Expression of HA-tagged α T9ib was detected using rabbit anti-HA antibody and Cy5-labeled goat anti-rabbit antibody. The expression of calnexin was visualized using anti-calnexin antibody and Cy3-conjugated goat anti-mouse antibody. Scale bar: 10 μ m. B – For immunoprecipitation, 1×10^6 HEK293 cells were co-transfected with HA-tagged mTLR9 expression plasmid together with myc-tagged α T2ib expression plasmid, myc-tagged α VR-ib expression plasmid, or myc-tagged α T9ib expression plasmid. After 72 h, the cells were lysed and incubated overnight with rabbit anti-HA-agarose-conjugated antibody (IP α HA, left) or anti-myc antibody (IP α myc, right) for 1 h, followed by incubation with Protein G PLUS Agarose overnight. The precipitates were analyzed using 10% SDS-PAGE and probed either with mouse anti-myc and goat anti-mouse PO-labeled antibodies to visualize ib-myc (lower panel) or with rabbit anti-HA and goat anti-rabbit PO-labeled antibodies to detect TLR9-HA (upper panel). Non-precipitated cell lysates that were single-transfected with HA-tagged mTLR9 expression plasmid or myc-tagged α T9ib expression plasmid were used as positive controls. Lysate with HA-tagged mTLR9 was used as a negative control for detection with anti-myc antibody and vice versa.

Inhibition of TLR9-specific signal transduction in RAW264.7 macrophages through α T9ib expression

To demonstrate that α T9ib inhibits TLR9-specific signaling in macrophages, the α T9ib expression plasmid DNA was co-transfected with an eGFP expression plasmid into RAW264.7 macrophages that were challenged with either LPS,

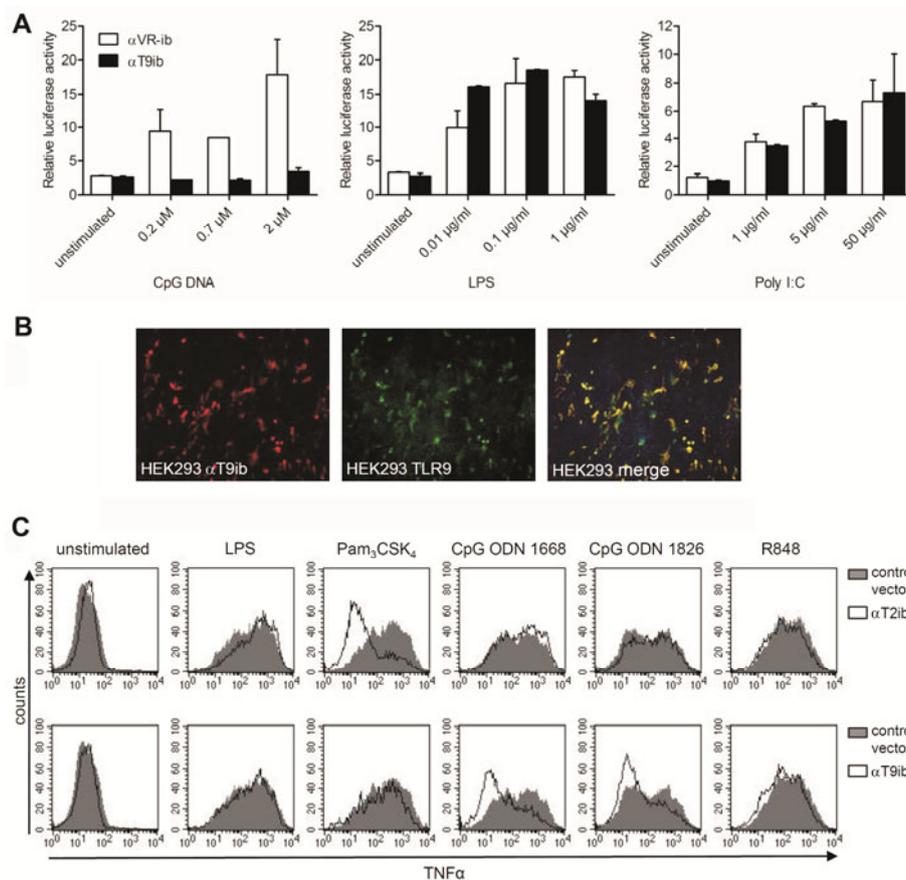


Fig. 3. Inhibition of TLR9 activity upon transient overexpression of α T9ib and murine TLR9 in HEK293 cells and inhibition of intracellular accumulation of TNF α in RAW264.7 macrophages transfected with α T9ib. A – Assay of NF- κ B-driven reporter gene activation in HEK293 cells transiently co-expressing mTLR9, mTLR4 or hTLR3 and α T9ib or α VR-ib. The cells were respectively stimulated with CpG DNA, LPS or Poly I:C. The results are presented as the mean \pm SD for one representative experiment out of 3 independent iterations. B – HEK293 cells were co-transfected with α T9ib and HA-tagged mTLR9 and stained for immunofluorescence with mouse anti-myc antibody and Cy3-labeled goat anti-mouse antibody or rabbit anti-HA antibody and FITC-labeled goat anti-rabbit antibody. C – RAW264.7 macrophages were co-transfected with eGFP expression plasmid and pCMV/myc/ER vector or eGFP expression plasmid and α T9ib expression plasmid or α T2ib expression plasmid as a control. Four days after transfection, the cells were stimulated with LPS (100 ng/ml), Pam₃CSK₄ (100 ng/ml), CpG-oligonucleotide 1826 (1 μ M), CpG-oligonucleotide 1668 (1 μ M) or R848 (10 μ g/ml). The cells were fixed and permeabilized, and the intracellular TNF α was analyzed in GFP-positive cells using flow cytometry with allophycocyanin-labeled rat anti-mouse TNF α .

Pam₃CSK₄, CpG ODN 1826, CpG ODN 1668, or R848. TNF α production was determined by intracellular flow cytometry in GFP-positive intrabody-expressing cells (Fig. 3C). α T9ib inhibited intracellular accumulation of TNF α upon ODN 1826 or 1668 challenge, whereas TLR2-, TLR4- and TLR7-specific activation induced by Pam₃CSK₄, LPS or R848 was not affected.

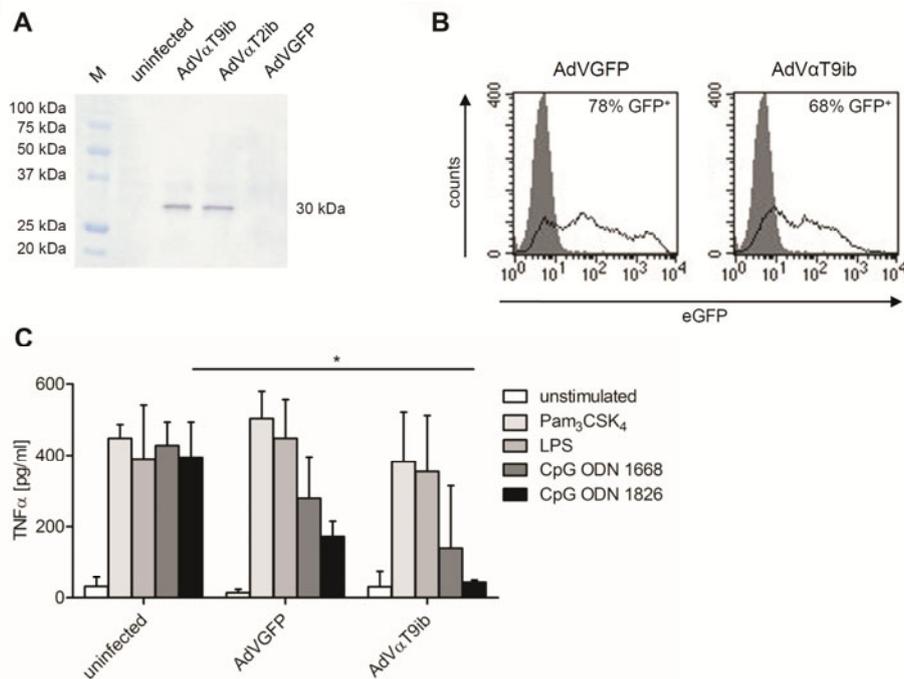


Fig. 4. Adenovirally transduced α T9ib inhibits the TNF α secretion induced in RAW264.7 macrophages by CpG ODN 1668 and CpG ODN 1826 but not that induced by lipopeptide and LPS. 1×10^6 RAW264.7 macrophages in a 6-well microtiter plate were infected for 10 days with AdV α T9ib or AdVGFP as a control. A – Myc-tag specific immunoblot analysis of RAW264.7 cell lysates. Proteins bands of α T9ib and α T2ib control were visualized by anti-myc antibody. B – Transduction efficiency of RAW264.7 cells infected with AdV α T9ib or AdVGFP analyzed by flow cytometry. The portion of infected cells was determined by analysis of green fluorescence (grey area: uninfected cells; white area: cells infected as indicated). C – RAW264.7 macrophages were challenged with CpG ODN 1826 (5 μ g/ml), CpG ODN 1668 (5 μ g/ml), LPS (100 ng/ml), or Pam₃CSK₄ (100 ng/ml) for 4 h at 37°C. Cytokine release to the cell supernatant was determined using ELISA. Data are shown as means \pm SD for 3 independent experiments. *P < 0.05, two-tailed Student's t test.

To increase the transduction efficiency of α T9ib in macrophages, we constructed an adenoviral vector and generated AdV α T9ib particles. Myc-tag-specific immunoblot analysis of RAW264.7 cell lysates upon infection with AdV α T9ib or AdV α T2ib revealed an apparent size of adenovirally transduced α T9ib of approximately 30 kDa (Fig. 4A). Simultaneous expression of α T9ib and bicistronic eGFP was demonstrated by immunofluorescence microscopy (data

not shown). Furthermore, eGFP expression was analyzed in RAW264.7 cells by flow cytometry. Macrophages were transduced equally effectively with the adenoviral construct AdV α T9ib and the control AdVGFP (Fig. 4B). Infection rates ranged from 70-80%. After 10 days, a maximum of eGFP-expressing cells was observable and the amount of living cells ranged from 80-90%.

The recombinant AdV α T9ib particles were applied to demonstrate that α T9ib inhibits TNF α secretion from RAW264.7 macrophages upon challenge with CpG DNA (Fig. 4C). Non-infected cells and AdVGFP- and AdV α T9ib-infected cells responded almost equally to TLR2- or TLR4-specific challenge. The cells released TNF α in substantial amounts compared to the controls. In AdVGFP-infected cells, the release of TNF α was reduced to some extent upon challenge with CpG DNA. By contrast, cells that were infected with AdV α T9ib were mostly unable to respond to a TLR9-specific challenge.

DISCUSSION

Toll-like receptors play a central role in the development and sustainment of chronic inflammatory diseases [28]. Specific research focuses on finding TLR antagonists as novel therapeutics, for example against systemic lupus erythematosus, multiple sclerosis, inflammatory bowel disease and rheumatoid arthritis, in which the immune system is inappropriately overactive [12].

To inhibit intracellular TLRs, which cannot be targeted by classical mAbs, anti-malarial drugs and short DNA sequences are used, but they often lack sufficient specificity [12, 16, 17]. By contrast, ER-retained intrabodies do act specifically [18] and are capable of persistently blocking receptor functions if the intrabody gene is stably expressed upon retroviral or lentiviral virus infection [29, 30]. Here, we developed an ER intrabody (α T9ib) for the specific knockdown of the intracellularly localized TLR9. To the best of our knowledge, this is the first description of an ER-targeting intrabody that inhibits the function of an intracellularly localized receptor.

α T9ib was constructed from the variable domains of mAb 5G5 recognizing both human and murine TLR9 [24]. Functional analysis of α T9ib demonstrated efficient and highly specific inhibition of TLR9 signaling in HEK293 cells overexpressing mTLR9 and in RAW264.7 macrophages (Figs 3 and 4C) due to the retention of TLR9 inside the ER by α T9ib. Complex formation of both molecules inside the ER was clearly demonstrated (Fig. 2A).

For efficient transduction of RAW264.7 macrophages and for future experiments with mice, we subcloned the α T9ib expression cassette into an adenoviral vector that drives bicistronic expression of eGFP and α T9ib. Cellular release of TNF α from AdV α T9ib-infected RAW264.7 macrophages upon TLR9-specific challenge with CpG ODN 1668 and 1826 was predominantly blocked to a level similar to the background level of unstimulated cells. Interestingly, AdVGFP-infected cells also showed a decreased release of TNF α . Referring to this, it has been demonstrated that adenoviruses may induce TLR9-dependent

TNF α secretion in macrophages [31]. This might lead to a lower potential of AdVGFP-infected cells to respond to a TLR9-specific challenge, although a higher release of TNF α in unstimulated cells would be expected in this case. Other possibilities would include the influence of eGFP overexpression on TLR9 signaling.

Our data show for the first time the highly specific and efficient neutralization of nascent TLR9 in the ER by an ER intrabody. Therefore, α T9ib represents a very promising experimental tool to study TLR9-driven cell activation. For example, α T9ib might be used to study the effect of TLR9 signaling on the function of specific immune cells. We have started to test the capability of α T9ib to interrupt the inflammatory reactions leading to chronic inflammatory diseases in appropriate experimental mouse models.

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REFERENCES

1. Akira, S., Uematsu, S. and Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124** (2006) 783-801.
2. Kawai, T. and Akira, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* **11** (2010) 373-384.
3. Lee, B.L., Moon, J.E., Shu, J.H., Yuan L., Newman, Z.R., Schekman, R. and Barton G.M. UNC93B1 mediates differential trafficking of endosomal TLRs. *eLife*.00291.
4. Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K.A., Monks, B.G., Knetter, C.F., Lien, E., Nilsen, N.J., Espevik, T. and Golenbock, D.T. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* **5** (2004) 190-198.
5. Trivedi, S. and Greidinger, E.L. Endosomal Toll-like receptors in autoimmunity: mechanisms for clinical diversity. *Therapy* **6** (2009) 433-442.
6. Celhar, T., Magalhaes, R., and Fairhurst, A.-M. TLR7 and TLR9 in SLE: when sensing self goes wrong. *Immunol. Res.* **53** (2012) 58-77.
7. Zeuner, R.A., Verthelyi, D., Gursel, M., Ishii, K.J. and Klinman, D.M. Influence of stimulatory and suppressive DNA motifs on host susceptibility to inflammatory arthritis. *Arthritis Rheum.* **48** (2003) 1701-1707.
8. O'Neill, L.A.J. Primer: Toll-like receptor signaling pathways-what do rheumatologists need to know? *Nat. Clin. Pract. Rheumatol.* **4** (2008) 319-327.
9. Daubeuf, B., Mathison, J., Spiller, S., Hugues, S., Herren, S., Ferlin, W., Kosco-Vilbois, M., Wagner, H., Kirschning, C.J., Ulevitch, R. and Elson, G. TLR4/MD-2 monoclonal antibody therapy affords protection in experimental models of septic shock. *J. Immunol.* **179** (2007) 6107-6114.
10. Meng, G., Rutz, M., Schiemann, M., Metzger, J., Grabiec, A., Schwandner, R., Lippa, P.B., Ebel, F., Busch, D.H., Bauer, S., Wagner, H. and Kirschning, C.J.

- Antagonistic antibody prevents toll-like receptor 2-driven lethal shock-like syndromes. **J. Clin. Invest.** 113 (2004) 1473-1481.
11. Vanags, D., Williams, B., Johnson, B., Hall, S., Nash, P., Taylor, A., Weiss, J. and Feeney, D. Therapeutic efficacy and safety of chaperonin 10 in patients with rheumatoid arthritis: a double-blind randomized trial. **Lancet** 368 (2006) 855-863.
 12. Hennessy, E.J., Parker, A.E. and O'Neill, L.A.J. Targeting Toll-like receptors: emerging therapeutics? **Nat. Rev. Drug. Discov.** 9 (2010) 293-307.
 13. Kuznik, A., Bencina, M., Svajger, U., Jeras, M. and Rozman, B. Mechanism of endosomal TLR inhibition by antimalarial drugs and imidazoquinolines. **J. Immunol.** 186 (2011) 4794-4804.
 14. Klinman, D.M., Zeuner, R., Yamada, H., Gursel, M., Currie, D. and Gursul, I. Regulation of CpG-induced immune activation by suppressive oligodeoxynucleotides. **Ann. NY Acad. Sci.** 1002 (2003) 112-123.
 15. Barrat, F.J., Meeker, T., Chan, J.H., Guiducci, C. and Coffman, R.L. Treatment of lupus-prone mice with a dual inhibitor of TLR7 and TLR9 leads to reduction of autoantibody production and amelioration of disease symptoms. **Eur. J. Immunol.** 37 (2007) 3582-3586.
 16. Marshak-Rothstein, A. Toll-like receptors in systemic autoimmune disease. **Nat. Rev. Immunol.** 6 (2006) 823-835.
 17. Graham, K.L., Lee, L.Y., Higgins, J.P., Steinmann, L., Utz, P.J. and Ho, P.P. Treatment with a toll-like receptor inhibitory CpG oligonucleotide delays and attenuates lupus nephritis in NZB/W mice. **Autoimmunity** 43 (2010) 140-155.
 18. Böldicke, T. Blocking translocation of cell surface molecules from the ER to the cell surface by intracellular antibodies targeted to the ER. **J. Cell Mol. Med.** 11 (2007) 54-70.
 19. Böldicke, T., Somplatzki, S., Sergeev, G. and Mueller, P.P. Functional inhibition of transitory proteins by intrabody-mediated retention in the endoplasmatic reticulum. **Methods** 56 (2012) 338-350.
 20. Bilanges, B. and Stokoe, D. Direct comparison of the specificity of gene silencing using antisense oligonucleotides and RNAi. **Biochem. J.** 388 (2005) 573-583.
 21. Qiu, S., Adema, C.M. and Lane, T. A computational study of off-target effects of RNA interference. **Nucleic Acids Res.** 33 (2005) 1834-1847.
 22. Cao, T. and Heng, B.C. Intracellular antibodies (intrabodies) versus RNA interference for therapeutic applications. **Ann. Clin. Lab. Sci.** 35 (2005) 227-229.
 23. Kirschning, C.J., Dreher, S., Maaß, B., Fichte, S., Schade, J., Köster, M., Noack, A., Lindenmaier, W., Wagner, H. and Böldicke, T. Generation of anti-TLR2 intrabody mediating inhibition of macrophage surface TLR2 expression and TLR2-driven cell activation. **BMC Biotechnol.** 10 (2010) 31.
 24. Ahmad-Nejad, P., Häcker, H., Rutz, M., Bauer, S., Vabulas, R.M. and Wagner, H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like

- receptors at distinct cellular compartments. **Eur. J. Immunol.** 32 (2002) 1958-1968.
25. Böldicke, T., Weber, H., Mueller, P.P., Barleon, B. and Bernal, M. Novel highly efficient intrabody mediates complete inhibition of cell surface expression of the human vascular endothelial growth factor receptor-2 (VEGFR-2/KDR). **J. Immunol. Meth.** 300 (2005) 146-159.
 26. Böldicke, T., Tesar, M., Griesel, C., Rohde, M., Gröne, H.-J., Waltenberger J., Kollet, O., Lapidot, T., Yayan, A. and Weich, H. Single-chain antibodies recognizing the human vascular endothelial growth factor receptor-2 (VEGFR-2, flk-1) on the surface of primary endothelial cells and preselected CD34+ cells from cord blood. **Stem Cells** 19 (2001) 24-36.
 27. Mayer, H., Bertram, H., Lindenmaier, W., Korff, T., Weber, H. and Weich, H. Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation. **J. Cell Biochem.** 95 (2005) 827-839.
 28. Ospelt, C. and Gay, S. TLRs and chronic inflammation. **Int. J. Biochem. Cell Biol.** 42 (2010) 495-505.
 29. Thomas, C.E., Ehrhardt, A. and Kay, M.A. Progress and problems with the use of viral vectors for gene therapy. **Nat. Rev. Genet.** 4 (2003) 346-358.
 30. Swan, C.H., Bühler, B., Steinberger, P., Tschann, M.P., Barbas III, C.F. and Torbett, B.E. T-cell protection and enrichment through lentiviral CCR5 intrabody gene delivery. **Gene Ther.** 13 (2006) 1480-1492.
 31. Cerullo, V., Seiler, M.P., Mane, V., Brunetti-Pierri, N., Clarke, C., Bertin, T.K., Rodgers, J.R. and Lee, B. Toll-like receptor 9 triggers an innate immune response to helper-dependent adenoviral vectors. **Mol. Ther.** 15 (2007) 378-385.