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Inhibitor of Apoptosis Proteins as Novel Targets in Inflammatory Processes

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Objective—Inhibitor of apoptosis proteins (IAPs), such as X-linked or cellular IAP 1/2 (XIAP, cIAP1/2), are important regulators of apoptosis. IAP antagonists are currently under clinical investigation as anticancer agents. Interestingly, IAPs participate in the inflammation-associated TNF receptor signaling complex and regulate NFκB signaling. This raises the question about the role of IAPs in inflammation. Here, we investigated the anti-inflammatory potential of IAP inhibitors and the role of IAPs in inflammatory processes of endothelial cells.

Methods and Results—In mice, the small molecule IAP antagonist A-4.10099.1 (ABT) suppressed antigen-induced arthritis, leukocyte infiltration in concanavalin A-evoked liver injury, and leukocyte transmigration in the TNFα-activated cremaster muscle. In vitro, we observed an attenuation of leukocyte–endothelial cell interaction by downregulation of the intercellular adhesion molecule-1. ABT did not impair NFκB signaling but decreased the TNFα-induced activation of the TGF-β–activated kinase 1, p38, and c-Jun N-terminal kinase. These effects are based on the proteasomal degradation of cIAP1/2 accompanied by an altered ratio of the levels of membrane-localized TNF receptor-associated factors 2 and 5.

Conclusion—Our results reveal IAP antagonism as a profound anti-inflammatory principle in vivo and highlight IAPs as important regulators of inflammatory processes in endothelial cells. **(*Arterioscler Thromb Vasc Biol*. 2011;31:2240-2250.)**

Key Words: Endothelium ■ Pharmacology ■ NF-kappaB ■ inflammation ■ inhibitor of apoptosis proteins (IAPs)

X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein-1 (cIAP1) and -2 (cIAP2) are the best characterized mammalian members of the inhibitor of apoptosis protein (IAP) family, whose common feature is the presence of baculoviral IAP repeat (BIR) domains. The IAPs, in particular XIAP, have been implicated in the regulation of cell death by interaction with caspases via their BIR domains.[1,2] Because IAPs are frequently overexpressed in human malignancies,[3,4] they became highly attractive targets for the development of promising anticancer therapeutics. One strategy to develop IAP-targeting compounds was based on the structure of the endogenous IAP antagonist Smac. The peptidic Smac mimetic ABT [compound 11 reported in Oost et al[5]], which we used as an experimental IAP inhibitor in this work, is a small molecule inhibitor modeled to bind to the BIR3 domain of XIAP with a nanomolar affinity and has successfully been used as an apoptosis-inducing compound in anticancer strategies.[5]

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In addition to its caspase-inhibiting function, XIAP is known to interact with NFκB and mitogen-activated protein kinase (MAPK) signaling.[6,7] The cIAPs have been reported to participate in the TNF receptor (TNFR)-associated signaling complex where they are involved in the ubiquitin-mediated signaling and in the associated activation of NFκB.[8–11] Besides TNFR signaling, IAPs were found to be involved in TRAIL-R, CD40, BAFF-R, and nucleotide-binding and oligomerization domain-containing protein 1/2 signaling.[12] Moreover, recent publications indicate a regulatory role of IAPs in immune functions by controlling cell survival[13] or activation of immune cells. IAPs have been shown to regulate the cytokine production of macrophages via participating in the nucleotide-binding and oligomerization domain signaling,[14] and Bauler et al[15] has identified XIAP as a regulator of innate immunity to Listeria infections. These findings point toward a complex role of IAPs in cellular processes beyond the regulation of apoptosis.

The TNFR signaling is of utmost importance for the induction of a wide variety of inflammatory processes and their activation plays a crucial role in the pathogenesis and progression of many severe disorders, such as atherosclerosis, arthritis, or sepsis.[16] One hallmark of inflammation is the

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extravasation of circulating leukocytes from the blood into the inflamed tissue. The endothelium tightly regulates and, on activation by proinflammatory stimuli, such as TNFα, strongly promotes this process and is thus a crucial player in inflammation.

We inferred from the existing knowledge that IAPs could be crucial mediators of inflammation and that IAP antagonists might thus exert anti-inflammatory actions. Consequently, we analyzed the general capability of IAP inhibition to influence inflammation in vivo and investigated the underlying molecular mechanisms of action in vitro in endothelial cells (ECs).

Methods

An expanded version of this section can be found in the supplemental material available at http://atvb.ahajournals.org.

Smac Mimetics

A-4.10099.1 (ABT) [compound 11 reported in Oost et al[5]] was kindly provided by Abbott Bioresearch Corporation, Worcester, MA. The monomeric compound Smac066 [compound 40d reported in Seneci et al[17]] and the dimeric Smac085 (unpublished) were kindly provided by the group of Prof. Pierfausto Seneci (Department of Organic and Industrial Chemistry, University of Milano, Milan, Italy).

Animals

All experiments were performed with male C57BL/6 mice (Charles River, Sulzfeld, Germany) according to the German legislation for the protection of animals and approved by the local governmental authorities.

Murine Antigen-Induced Arthritis

The antigen-induced arthritis experiment was performed as previously described by Veihelmann et al.[18] Briefly, mice were immunized against methylated bovine serum albumin (Sigma-Aldrich, Taufkirchen, Germany) and arthritis was induced by injection of methylated bovine serum albumin into the left knee joint.

Concanavalin A-Induced Murine Hepatitis

The model has been described previously in detail.[19] Briefly, concanavalin A (ConA; Sigma-Aldrich) was administered to mice intravenously at 15 mg/kg. One μg ABT was administered intravenously 15 minutes before ConA. Mice were euthanized 8 hours after ConA application. Plasma enzyme activity of alanine aminotransferase was assessed using an automated procedure with COBAS MIRA (Roche, Basel, Switzerland). Liver tissue paraffin sections were stained with a naphthol-AS-D-chloroacetate-esterase kit (Sigma-Aldrich).

Analysis of Leukocyte Adhesion and Transmigration by Intravital Microscopy of the Mouse Cremaster Muscle

The surgical preparation of the cremaster muscle and the intravital microscopy were performed as described by Baez.[20]

Cell Culture and Media

Primary human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords. HUVECs and HeLa cells were cultured as previously described.[21,22] Human neutrophil granulocytes were separated from heparinized peripheral blood of healthy volunteers by using CD15 MicroBeads (Miltenyi, Bergisch Gladbach, Germany).

Granulocyte Adhesion Assay

Neutrophil granulocytes were added to confluent HUVECs that were pretreated or not with CD54 blocking antibody/isotype control (Biolegend, San Diego, CA) and allowed to adhere for 30 minutes. After lysis, the amount of adhered granulocytes was analyzed by measurement of myeloperoxidase activity.

Flowcytometric Analysis

Oxidative Burst

Neutrophils were primed with dihydrorhodamine (1 μmol/L) for 10 minutes.

CD11b Surface Expression

Granulocytes were incubated with a FITC-labeled anti-CD11b antibody (AbD Serotec, Düsseldorf, Germany).

ICAM-1 Expression

HUVECs were trypsinized, formalin-fixed, and incubated with a FITC-labeled anti-CD54 antibody (Biozol, Eching, Germany). Cells were analyzed with a FACSCanto II flow cytometer (Becton Dickinson, Heidelberg, Germany).

ICAM-1 and Interleukin-8 mRNA Expression

HeLa cells were treated as indicated and ICAM-1/interleukin-8 mRNA expression was measured by real-time RT-PCR as previously described.[23]

Quantification of Cell Death

Apoptosis rates were measured by determination of subdiploid DNA content as previously described.[24] Briefly, permeabilized cells were stained with propidium iodide and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson). *General cell death rates* were quantified by staining nonpermeabilized cells with propidium iodide and subsequently analyzing the propidium iodide positive cells by flow cytometry.

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Munich, Germany) via electroblotting. The following antibodies were used: Phospho-TGF-β–activated kinase 1 (TAK1), phospho-extracellular signal-regulated kinase, TNF receptor-associated factor 2 (TRAF2), phospho-p38, phospho-cJun N-terminal kinase (JNK), phospho-IκBα, ubiquitin, and receptor-interacting protein1 (RIP1; Cell Signaling/NEB, Frankfurt am Main, Germany). MAPK phosphatase-1, IκBα, p52 (NFκB), TRAF5 (Santa Cruz, Heidelberg, Germany), cIAP1 (R&D Systems, Wiesbaden, Germany), cIAP2 (Epitomics/Biomol, Hamburg, Germany), and XIAP (Becton Dickinson). Jurkat cell lysate was from Cell Signaling/NEB.

Analysis of NF-κB p65 Intracellular Localization

Formalin-fixed HUVECs were incubated with an anti-NFκB p65 (Santa Cruz) primary and AlexaFluor 488-linked secondary antibody (Molecular Probes, Eugene, OR). The translocation of NFκB p65 was analyzed using a Zeiss LSM 510 Meta confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

Electrophoretic Mobility Shift Assay

Nuclear protein extracts were prepared from HUVECs and electrophoretic mobility shift assay was performed as previously described.[25]

Dual Luciferase Reporter Assay

Firefly luciferase reporter vector pGL4.32[*luc*2P/NF-κB-RE/Hygro] and Renilla luciferase reporter vector pGL4.74[*hRluc*/TK] were from Promega (Heidelberg, Germany). HUVECs were transfected using the Amaxa HUVEC Nucleofactor Kit (Lonza, Cologne, Germany). Luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega).

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Immunoprecipitation

HUVEC lysates were incubated with cIAP1 (R&D Systems) or RIP1 (Cell Signaling) antibody. Proteins were precipitated by adding Protein A agarose beads (Sigma-Aldrich). Proteins were extracted from the beads with Laemmli sample buffer and subjected to Western blot analysis.

TAK1 Kinase Assay

Active TAK1 kinase was precipitated from cell lysates with TAK1 antibody (Cell Signaling). [γ-^{32}P]ATP and the TAK1 substrate MAPK kinase 3 (Biaffin, Kassel, Germany) were added to start the kinase reaction. Samples were analyzed by SDS gel electrophoresis and phosphorylated MAPK kinase 3 was detected by autoradiography.

Gene Silencing

HUVECs were transfected with XIAP siRNA (ON-TARGETplus SMARTpool siRNA), cIAP1 siRNA (ON-TARGETplus duplex siRNA), cIAP2 siRNA (ON-TARGETplus duplex siRNA) (Thermo Scientific/Dharmacon, Bonn, Germany), or with the corresponding nontargeting siRNA by electroporation using the Amaxa HUVEC Nucleofector kit (Amaxa/Lonza).

Cytosol/Membrane Fractionation

Confluent HUVECs were treated as indicated, lysed, and separated into a soluble (cytosolic) and a particulate (membranous) fraction, as described previously by Li et al.[26]

Statistical Analysis

Bar graph data represent means±SEM. Statistical analysis was performed with the GraphPad Prism software version 3.03 (GraphPad Software, San Diego, CA). Unpaired *t* test was used to compare 2 groups. To compare 3 or more groups, 1-way ANOVA followed by Bonferroni posthoc test was used. $P \leq 0.05$ was considered as statistically significant.

Results

The IAP Inhibitor ABT Exhibits Anti-Inflammatory Actions in Murine Models of Inflammation and Blocks Leukocyte Extravasation

The overall anti-inflammatory potential of ABT was tested in 2 different animal models of inflammation: (1) In the antigen-induced arthritis model, mice that were treated intraperitoneally with 5 μg ABT per day did not evolve any joint swelling in the antigen-treated knee (Figure 1A). (2) In the ConA-evoked hepatitis model, ABT (1 μg, IV) diminished (≈40%) the rise in the serum levels of alanine aminotransferase, a marker for liver cell injury (Figure 1B). Moreover, the ConA-provoked neutrophil recruitment to the liver, which crucially contributes to the liver injury, is reduced significantly when mice were treated with ABT as shown in histological images and by determining the neutrophil count (Figure 1C).

In postcapillary venules of the TNFα-treated mouse cremaster muscle, ABT affected leukocyte extravasation: The group of mice that was pretreated with 5 μg ABT (IA) for 30 minutes showed a slight (nonsignificant) reduction of leukocyte adhesion (≈20%) and a strong decrease of leukocyte transmigration (≈60%) in comparison to the control group (Figure 1C). Leukocyte rolling, vessel diameter, blood flow velocity, wall shear rate, and systemic count of leukocytes were not influenced by ABT (data not shown).

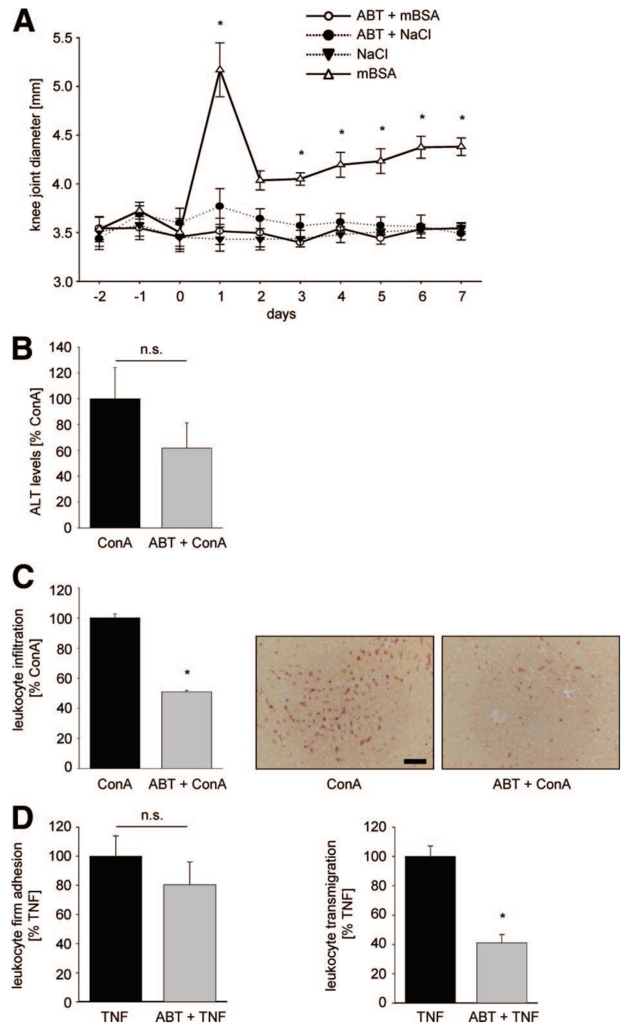

Figure 1. Antagonist A-4.10099.1 (ABT) protects against antigen-induced arthritis, diminishes ConA-induced liver injury, and attenuates leukocyte extravasation in mice. **A**, Mice were immunized with methylated BSA (mBSA). One group was additionally treated IP with 5 μg ABT every day beginning 2 days before the induction of the arthritis with mBSA in the left knee joint. The right knee joints were injected with NaCl solution (internal controls). The knee joint diameter was determined daily. Control mice: $N=5$. ABT-treated mice: $N=6$. *$P \leq 0.05$ vs ABT+mBSA. **B**, **C**, Mice were treated IV with 1 μg ABT 15 minutes before IV administration of 15 mg/kg ConA. Liver tissues were removed 8 hours after ConA application. ConA-treated mice: $N=12$. ConA+ABT-treated mice: $N=12$. **B**, Plasma levels of alanine transaminase (ALT) were assessed. **C**, Liver sections were stained for naphthol-AS-D-chloroacetate esterase to analyze neutrophil infiltration. Left: the bar graph shows the quantification of the neutrophil count in the liver sections. *$P \leq 0.05$ vs ConA. Right: representative images. Black bar=100 μm. **D**, Leukocyte recruitment to the mouse cremaster muscle was induced by intrascrotal injection of TNFα (500 ng) 4 hours before intravital microscopy. Five micrograms of ABT was injected IA 30 minutes before application of TNFα. During a 15-minute observation period, leukocyte adhesion and transmigration were assessed. TNFα-treated mice: $N=5$. ABT+TNFα-treated mice: $N=5$. *$P \leq 0.05$ vs TNFα.

IAP Inhibition Influences Leukocyte Adhesion to Endothelial Cells and Reduces TNFα-Induced ICAM-1 Expression

Aiming at mechanistic analyses, we confirmed the attenuation of leukocyte–EC interaction in vitro. The adherence of

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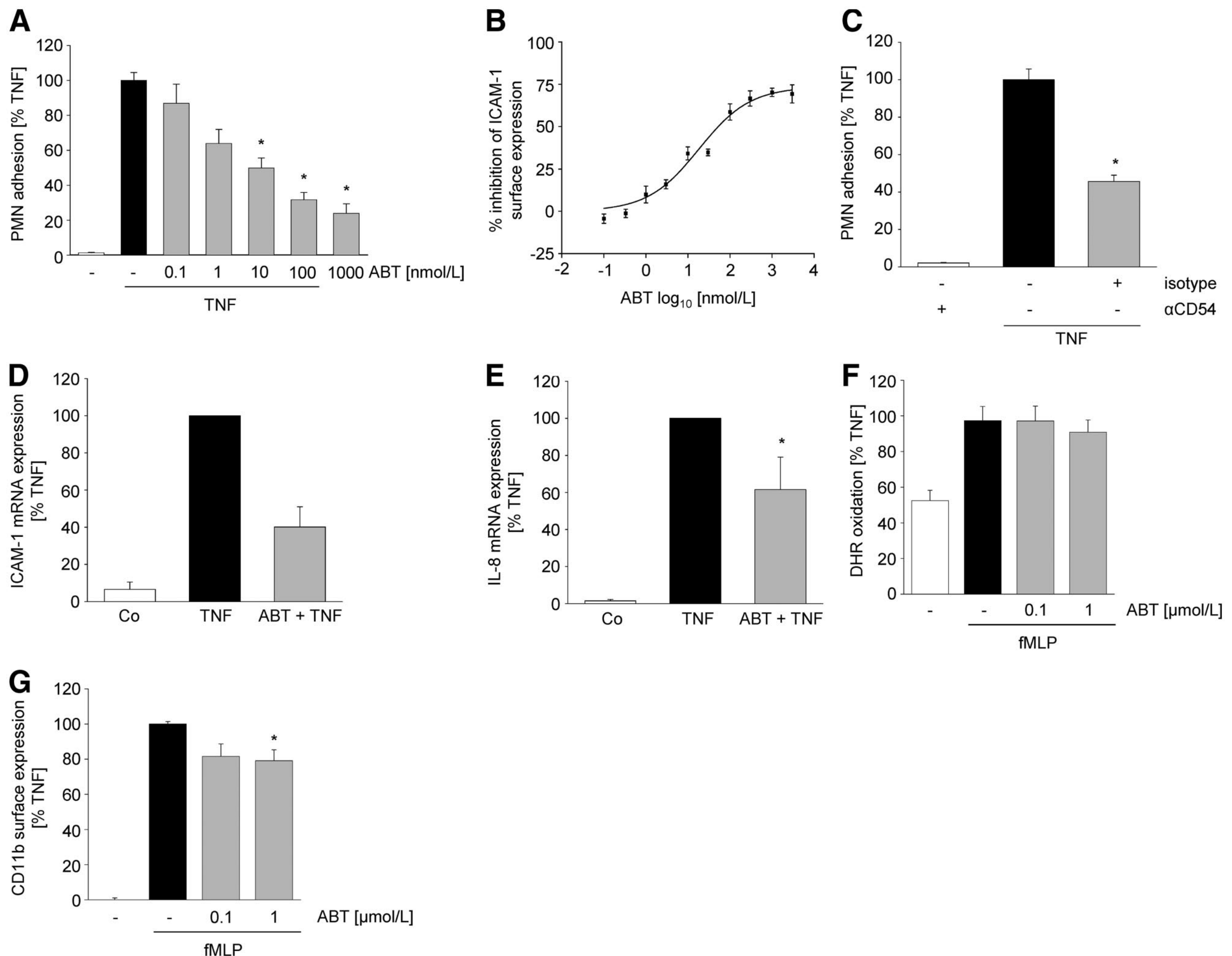

Figure 2. Influence of antagonist A-4.10099.1 (ABT) on TNFα-induced leukocyte–endothelial cell interaction as well as on TNFα-activated pathways in endothelial cells, HeLa cells, and leukocytes. Human umbilical vein endothelial cells (HUVECs) were preincubated for 30 minutes with ABT (**A**) or with intercellular adhesion molecule-1 (ICAM-1) blocking antibody (αCD54, 20 μg) for 1 hour (**C**) and treated with TNFα (10 ng/mL) for 24 hours. Isolated human neutrophil granulocytes were added (10^5 cells per well) and allowed to adhere for 45 minutes. Myeloperoxidase activity kinetics were measured to determine the amount of adhered granulocytes. **A**, *N*=7. **P*≤0.05 vs TNFα alone. **B**, HUVECs were pretreated with ABT for 30 minutes and treated with TNFα (10 ng/mL) for 24 hours. The levels of ICAM-1 surface expression were determined by flow cytometry. *N*=3. **C**, *N*=3. **P*≤0.05 vs TNFα alone. **D**, **E**, HeLa cells were treated with ABT (1 μmol/L) for 30 minutes. TNFα (20 ng/mL) was applied for 3 hours. ICAM-1 (**D**; *N*=3) and IL-8 (**E**; *N*=5) mRNA expression were analyzed by real-time RT-PCR. **P*≤0.05 vs TNFα alone. **F**, **G**, Isolated human neutrophil granulocytes were treated for 30 minutes with ABT before incubation with 100 nmol/L fMLP for 15 minutes. **F**, Granulocytes were loaded with 10 μmol/L dihydrorhodamine and analyzed by flow cytometry. *N*=3. **G**, CD11b surface expression was measured by flow cytometry. *N*=3. **P*≤0.05 vs fMLP alone.

freshly isolated human neutrophils to the EC monolayer was concentration-dependently reduced with an IC_{50} value of ≈10 nmol/L (Figure 2A). Pretreatment of ECs with ABT resulted in a profound concentration-dependent downregulation of endothelial ICAM-1 surface protein expression with an IC_{50} value of 18 nmol/L (Figure 2B). An ICAM-1-blocking antibody strongly reduced the adhesion of neutrophils to ECs, thus corroborating the essential role of ICAM-1 for neutrophil adhesion (Figure 2C). Moreover, ABT influences mRNA levels of both ICAM-1 (Figure 2D) and interleukin-8 (Figure 2E), as shown in HeLa cells. Regarding actions of ABT on neutrophils, ABT had no influence on the formyl-methionyl-leucyl-phenylalanine-induced production of reactive oxygen species (Figure 2F) and slightly reduced the expression of CD11b (Figure 2G). These findings indicate that ABT, besides strongly acting on ECs, might also affect neutrophil activation.

The Effect of ABT on ICAM-1 Expression Can Be Mimicked by Structurally Different IAP Inhibitors and Is Not Due to Increased Apoptosis or Caspase Activation

Two IAP antagonists structurally different from ABT, the monomeric Smac mimetic Smac066 and the homodimeric Smac085, effectively reduced the TNFα-evoked ICAM-1 expression (Figure 3A), suggesting that the action of ABT is not a specific feature of this individual compound due to side or off-target effects. As assessed by measuring the rate of apoptosis (subdiploid DNA content), ABT treatment for 24 and 48 hours did not cause any induction of apoptosis (Figure

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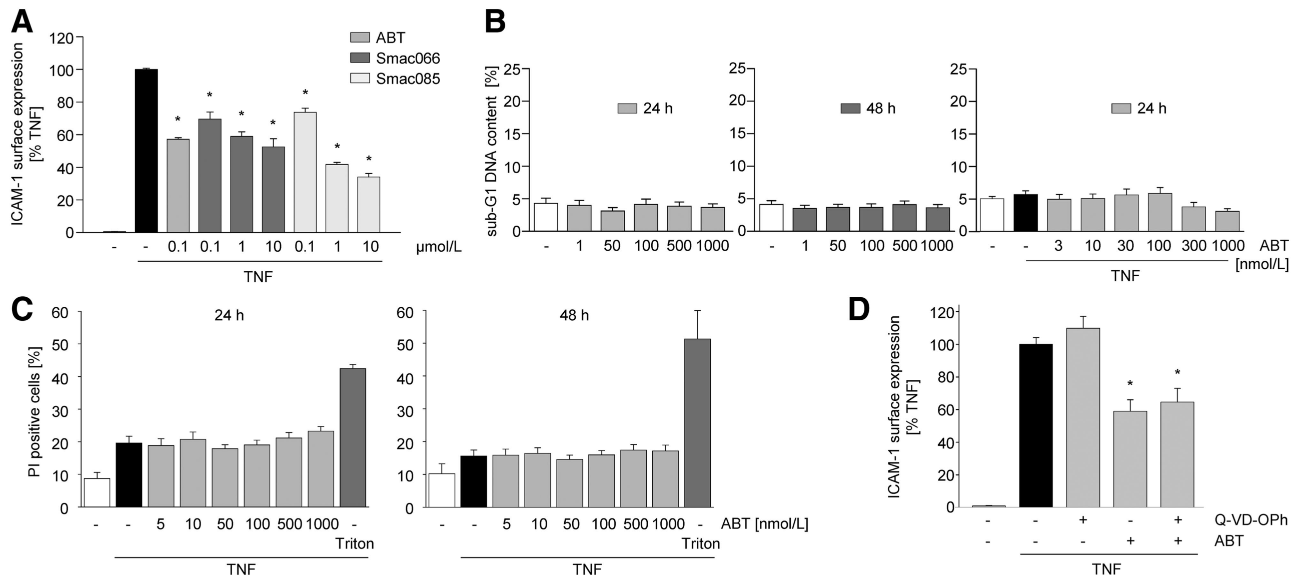

Figure 3. The effect of antagonist A-4.10099.1 (ABT) on intercellular adhesion molecule-1 (ICAM-1) expression can be mimicked by structurally different inhibitor of apoptosis protein (IAP) inhibitors and is not due to increased apoptosis or caspase activation. **A**, Human umbilical vein endothelial cells (HUVECs) were treated with Smac066 (monovalent) or Smac085 (bivalent) for 30 minutes before incubation with TNFα (10 ng/mL) for 24 hours. The levels of ICAM-1 surface protein expression were determined by flow cytometry. $N=3$. $^*P\leq0.05$ vs TNFα alone. **B**, HUVECs were treated with ABT for 24 or 48 hours or they were pretreated with ABT for 30 minutes before incubation with TNFα (10 ng/mL) for 24 hours. Subdiploid DNA content was determined by flow cytometry. $N=3$. **C**, HUVECs were pretreated for 30 minutes with ABT and subsequently treated with TNFα (10 ng/mL) for 24 or 48 hours. Cells were then incubated with propidium iodide (PI; 10 μg/mL) for 30 minutes. PI positive cells were analyzed by flow cytometry. Cells permeabilized with Triton X-100 (0.1%) were used as a positive control. $N=3$. **D**, HUVECs were pretreated with the pan-caspase inhibitor Q-VD-OPh (10 μmol/L, 30 minutes) before treatment with ABT (100 nmol/L) for 30 minutes and TNFα (10 ng/mL) for 24 hours. The levels of ICAM-1 surface protein expression were determined by flow cytometry. $N=3$. $^*P\leq0.05$ vs TNFα alone.

3B, left and middle panel). Even the additional presence of TNFα for 24 hours did not evoke apoptotic cell death (Figure 3B, right panel). Moreover, by quantification of propidium iodide-positive cells after 24 and 48 hours of treatment with ABT and TNF (Figure 3C), we revealed that the IAP antagonist does not trigger cell death in HUVECs. Thus, it can be excluded that the effects of ABT on ECs are simply due apoptosis induction. Furthermore, it can also be excluded that caspases participate in the action of ABT on the TNFα-induced ICAM-1 expression: Figure 3D shows that the application of a pan-caspase inhibitor (Q-VD-OPh) had no impact on this effect.

ABT Does Not Interfere With the Endothelial NFκB Signaling

NFκB is the major transcription factor that regulates ICAM-1 expression; however, as shown in Figure 4A, ABT did neither influence the phosphorylation of the NFκB inhibitor IκBα, nor its degradation evoked by TNFα. Also, both the TNFα-induced translocation of the NFκB subunit p65 to the nucleus (Figure 4B) and the DNA-binding capacity of NFκB were left unaffected by ABT (Figure 4C). Besides this canonical way of NFκB activation, ABT treatment did also not lead to an induction of the noncanonical NFκB signaling, because neither ABT alone nor in combination with TNFα did evoke the processing of p100 to p52 (Figure 4D). Finally, the TNFα-evoked NFκB-dependent gene expression was not affected by ABT in a dual-luciferase reporter gene assay (Figure 4E, left). Curcumin, which is known to inhibit NFκB signaling by blocking IκBα phosphorylation,[27] was used as a positive control (Figure 4E, right).

IAP Inhibition Affects Endothelial MAPK Signaling

Figure 5A shows that ABT diminished the TNFα-induced phosphorylation of the MAPKs p38 and JNK, but not of extracellular signal-regulated kinase, and did not affect the protein levels of the MAPK phosphatase-1. Pharmacological inhibition of p38 and JNK resulted in a significant decrease of TNFα-induced ICAM-1 expression (Figure 5B). The added levels of ICAM-1 reduction caused by the 2 inhibitors SP600125 (JNK) and SB203580 (p38) nicely correspond to the level caused by ABT. In addition, ABT affects the p38- and JNK-activating MAP3K TAK1: The TNFα-induced phosphorylation of TAK1, indicating its activation, was reduced when cells were pretreated with the IAP antagonist (Figure 5C). Moreover, by performing a TAK1 kinase assay that uses MAPK kinase 3 as a TAK1 substrate, we could confirm the inhibitory action of ABT treatment on the TNFα-triggered TAK1 activity (Figure 5D). Taken together, ABT inhibited the TNFα-induced activation of p38, JNK, and TAK1.

Targeting of cIAP1 and cIAP2, But Not of XIAP, Accounts for the ICAM-1-Reducing Effect of ABT

We tested whether gene silencing of XIAP, cIAP1, or cIAP2 results in similar effects on ICAM-1 expression as treating ECs with the IAP antagonist. Surprisingly, a reduction of endothelial XIAP levels on transfection of siRNA resulted in

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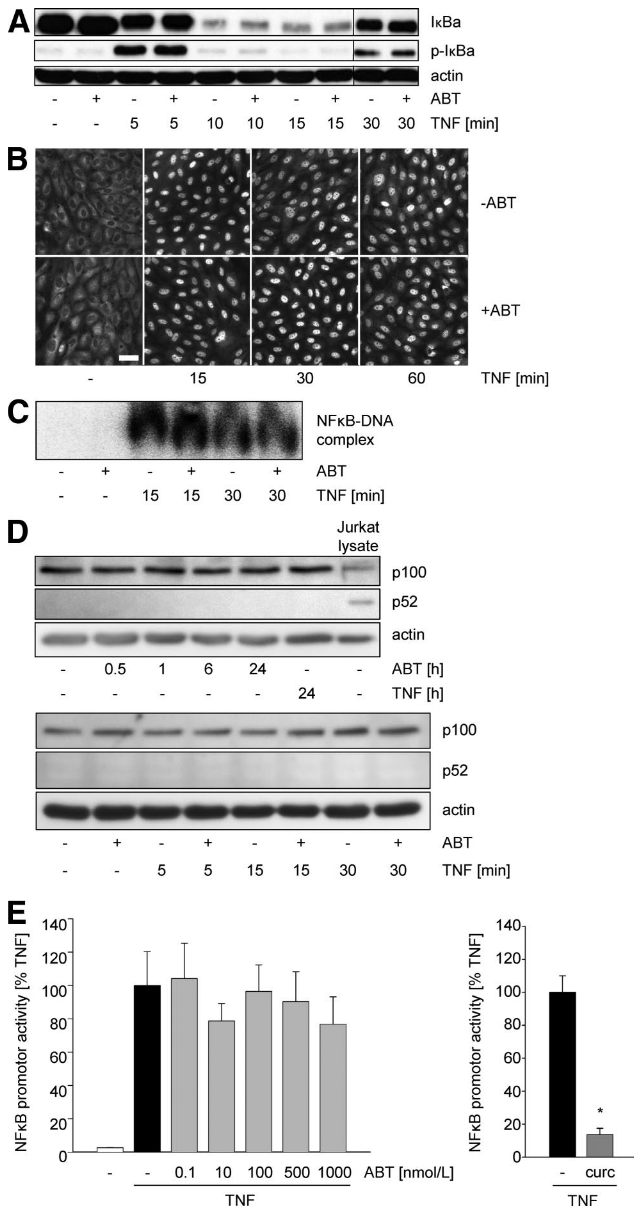

Figure 4. Antagonist A-4.10099.1 (ABT) does not interfere with the endothelial NFκB signaling. **A**, **D**, HUVECs were pretreated with ABT (100 nmol/L) for 30 minutes and subsequently treated with TNFα (10 ng/mL). Levels of IκBα, phospho-IκBα, and actin (**A**) or levels of p100, p52, and actin (**D**) were determined by Western blotting. Jurkat lysates were used as positive control (**D**). One representative out of 3 independently performed experiments is shown, each. **B**, NFκB p65 subunit was visualized via immunocytochemistry. White bar=40 μm. One representative out of 3 independently performed experiments is shown, each. **C**, NFκB DNA-binding activity was measured by electrophoretic mobility shift assay. One representative out of 3 independently performed experiments is shown, each. **E**, TNFα was applied for 6 hours. The well-known NFκB inhibitor curcumin (curc, 20 μmol/L, 30 minutes pretreatment) served as positive control. NFκB promotor activity was analyzed by dual luciferase reporter gene assay. N=3. *P≤0.05 vs TNF.

a huge increase of ICAM-1 expression and ABT was still capable of reducing ICAM-1 levels (Figure 5E, left). Interestingly, these cells exhibited strongly increased protein levels of cIAP1 and cIAP2 (Figure 5E, right). Nevertheless, silencing of cIAP1, cIAP2, and of both significantly reduced TNFα-induced ICAM-1 expression (Figure 5F). Of note, the degree of reduction is similar to that achievable with ABT. These results suggest that the presence of XIAP is not required for the effects of the IAP antagonist and that cIAP1 and cIAP2 are involved in the regulation of TNFα-induced ICAM-1 expression.

ABT Interferes With the TNF Receptor Proximal Signaling

The fact that silencing of cIAP1 and cIAP2, but not of XIAP, reduced ICAM-1 expression gave rise to the question in which way the Smac mimetic influences the activation of p38 and JNK. We analyzed the action of ABT on the protein levels of XIAP, cIAP1, and cIAP2 and found a rapid and sustained reduction of cIAP1, whereas XIAP levels were not affected (Figure 6A). Protein levels of cIAP2 and their reduction by ABT could only be detected when its expression was induced by TNFα for a longer period of time (24 hours) as shown in Figure 6C. Furthermore, we could demonstrate that the IAP antagonist leads to a fast and strong ubiquitination of cIAP1 (Figure 6B). The pharmacological proteasome inhibitor MG132 abolished the ABT-evoked disappearance of cIAP1 and cIAP2 (Figure 6C) and, most importantly, abrogated the effects of ABT on the TNFα-induced expression of ICAM-1 (Figure 6D), indicating that proteasomal degradation is important for these events.

cIAP1 and cIAP2 have been described as members of the TNFR signaling complex.[28] We hypothesized that the degradation of cIAP1/2 mediated by ABT influences this complex. TRAF2 is known to mediate the activation of NFκB and MAP3K signaling[29] and was reported to interact with cIAP1/2.[30] ABT triggers an immediate decrease of TRAF2 protein levels in endothelial membrane fractions (Figure 6E). In contrast, TRAF5, which can compensate the loss of TRAF2 in terms of activation of the NFκB signaling,[31,32] was not affected by ABT (Figure 6E).

Moreover, as shown in Figure 6F, the IAP antagonist led to a reduced ubiquitination of the TNF receptor-associated kinase RIP1, which is known to be essential for the TNFα-mediated activation of TAK1.[9]

In summary, our data indicate that ABT induces the ubiquitination and proteasomal degradation of cIAP1/2, which is accompanied by an altered ratio of the levels of TRAF2 and TRAF5 at the EC membrane and by an altered ubiquitination status of the TAK1 activator RIP1.

Discussion

Activation of inflammatory processes is crucial for the host defense against infections and for repairing tissue damage, but it shows destructive properties in chronic pathological conditions like atherosclerosis, rheumatoid arthritis, or ischemia. The administration of conventional anti-inflammatory therapeutics, such as glucocorticoids or nonsteroidal anti-inflammatory drugs, is not always effective and often causes severe side effects. Consequently, inflammation is still a very important subject of research in terms of unraveling novel signaling interconnections in order to develop new therapeutical strategies. IAPs have been increasingly recognized to affect NFκB signaling[6] and to be part of the TNF receptor-

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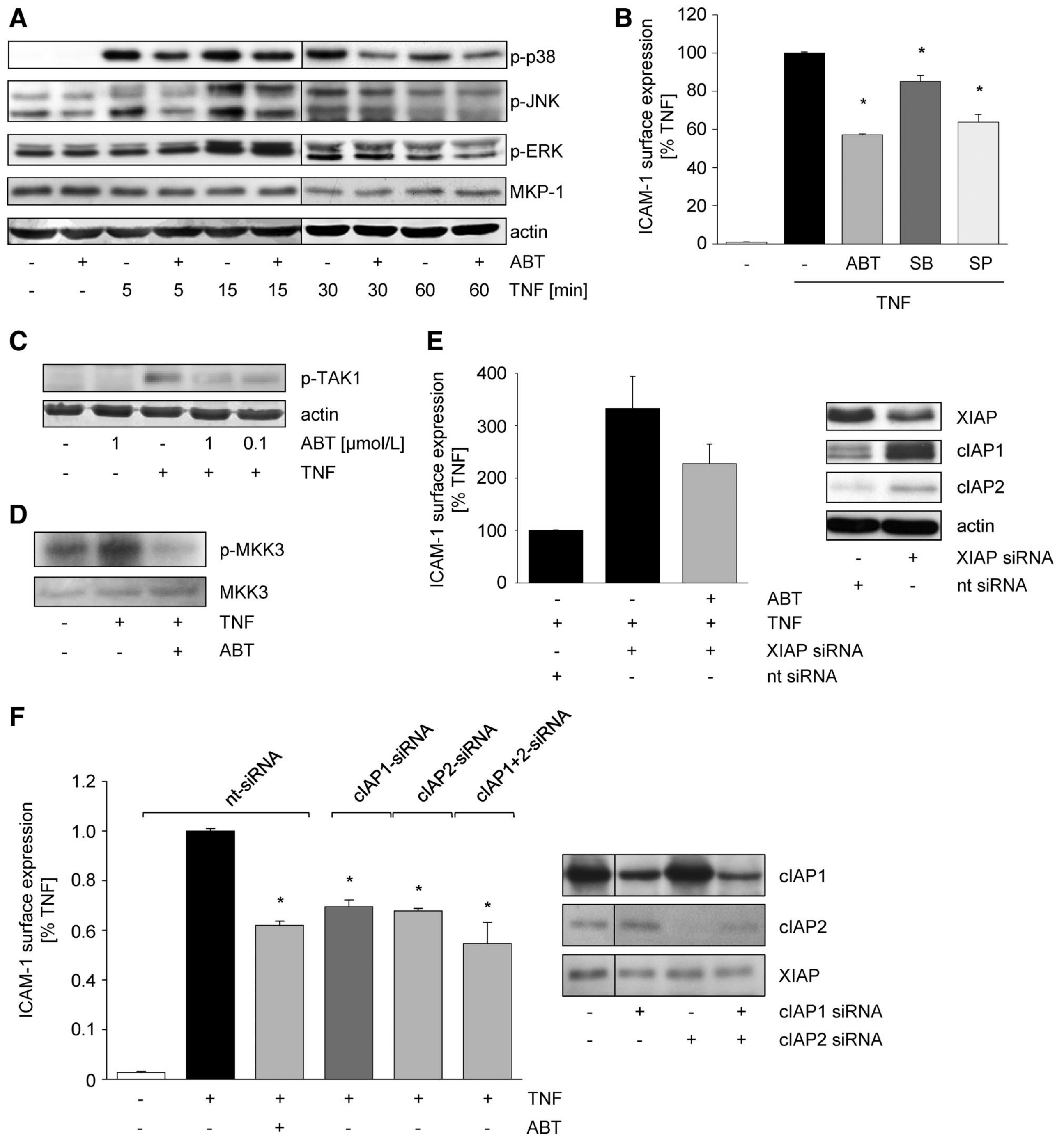

Figure 5. IAP inhibition affects endothelial mitogen-activated protein kinases (MAPK) signaling and silencing of cellular inhibitor of apoptosis protein 1/2 (cIAP1/2), but not of X-linked inhibitor of apoptosis protein (XIAP), inhibits intercellular adhesion molecule-1 (ICAM-1) expression. **A**, Human umbilical vein endothelial cells (HUVECs) were treated with 100 nmol/L ABT for 30 minutes before incubation with 10 ng/mL TNFα. Levels of phospho-p38 mitogen-activated protein kinase (MAPK), phospho-c-Jun N-terminal kinase (JNK), phospho-extracellular signal-regulated kinase (ERK), MAPK phosphatase-1 (MKP-1), and actin were analyzed by Western blotting. One representative out of 3 independently performed experiments is shown, each. **B**, HUVECs were treated with ABT (100 nmol/L), the p38 MAPK inhibitor SB203580 (20 μmol/L), and the JNK inhibitor SP600125 (10 μmol/L) for 30 minutes followed by TNFα (10 ng/mL) for 24 hours. Levels of ICAM-1 surface expression were determined by flow cytometry. $N=3$. $^*P\leq0.05$ vs TNFα alone. **C**, **D**, HUVECs were pretreated with ABT for 30 minutes followed by 5 minutes TNFα (10 ng/mL) treatment. Levels of phospho-TGF-β–activated kinase 1 (TAK1) and actin were determined by Western blotting (**C**). TAK1 was precipitated from cell lysates and, after performing a TAK1 kinase activity assay, levels of the phosphorylated TAK1 substrate MAPK kinase 3 were detected by autoradiography on gel electrophoresis (**D**). One representative out of 3 independently performed experiments is shown, each. **E**, **F**, HUVECs were transfected with nontargeting (nt) or specific siRNA against cIAP1, cIAP2, and XIAP. Cells were then treated with ABT (100 nmol/L, 30 minutes) and TNFα (10 ng/mL, 24 hours). Levels of ICAM-1 surface expression were determined by flow cytometry. N=3. Levels of X-linked inhibitor of apoptosis protein (XIAP), cIAP1, cIAP2, and actin were analyzed by Western blotting. One representative out of 3 independently performed experiments is shown, each. $^*P\leq0.05$ vs TNFα alone.

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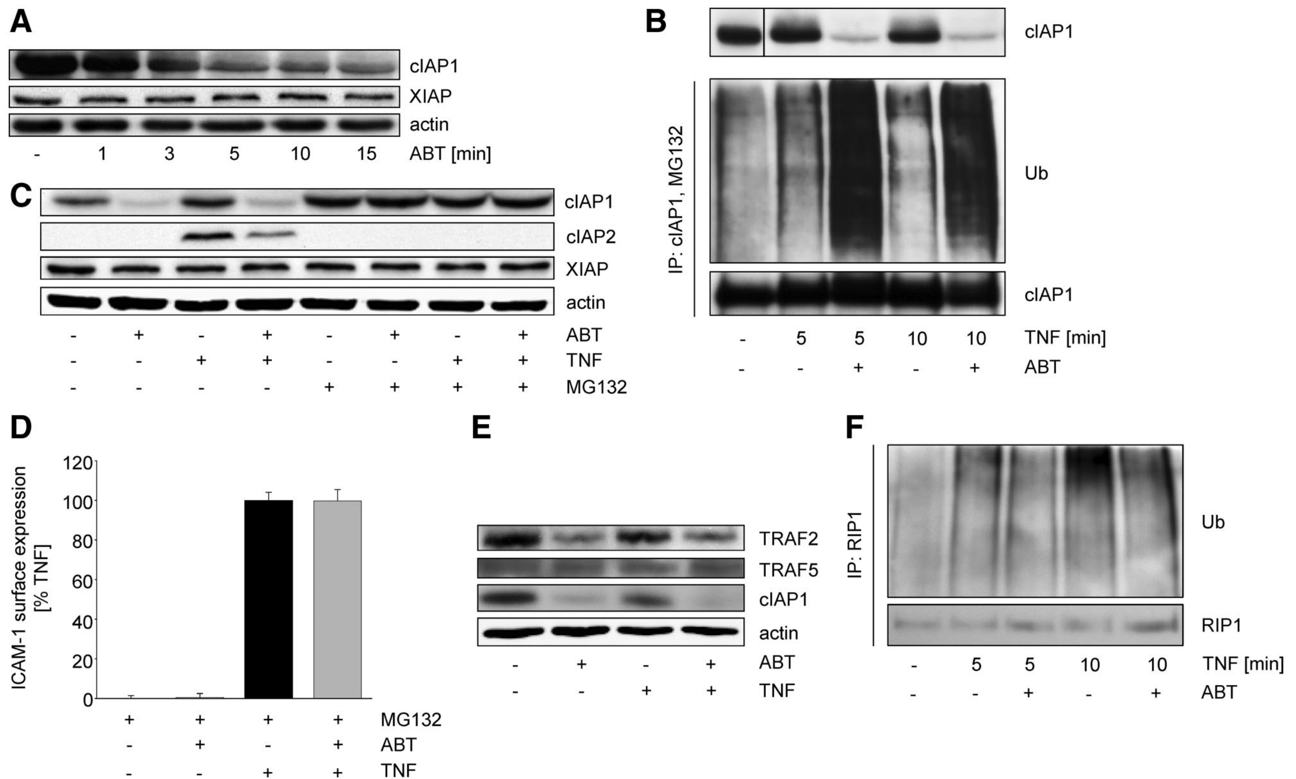

Figure 6. Antagonist A-4.10099.1 (ABT) interacts with the TNF receptor signaling. **A**, Human umbilical vein endothelial cells (HUVECs) were treated with ABT (100 nmol/L). Levels of cellular inhibitor of apoptosis protein 1 (cIAP1), X-linked inhibitor of apoptosis protein (XIAP), and actin were analyzed by Western blotting. One representative out of 3 independently performed experiments is shown. **B**, After 30 minutes pretreatment of HUVECs with ABT (100 nmol/L) and subsequent treatment with TNFα (10 ng/mL), levels of cIAP1 were analyzed by Western blot (upper panel). Additionally, cIAP1 was precipitated from cell lysates of HUVECs that were pretreated with the proteasome inhibitor MG132 (10 μmol/L, 30 minutes) before treatment with ABT and TNF. Levels of ubiquitinated (Ub) cIAP1 and of precipitated cIAP1 were determined by Western blotting (middle and lower panel). One representative out of 3 independently performed experiments is shown, each. **C**, **D**, HUVECs were pretreated with the proteasome inhibitor MG132 (10 μmol/L, 30 minutes) before treatment with ABT (1 μmol/L) for 30 minutes and TNFα (10 ng/mL) for 24 hours. The levels of cIAP1, cIAP2, XIAP, and actin were determined by Western blotting. One representative out of 3 independently performed experiments is shown, each (**C**). Levels of ICAM-1 surface expression were determined by flow cytometry. $N=3$. **D**, **E**, HUVECs were treated with ABT (1 μmol/L, 30 minutes) followed by TNFα (10 ng/mL) for 5 minutes. Cells were then fractionated into cytosol and membrane fractions. The levels of TNF receptor-associated factors 2 and 5, cIAP1, and actin were determined by Western blotting. One representative out of 3 independently performed experiments is shown, each. **F**, Cells were treated with ABT for 30 minutes (100 nmol/L) and subsequently incubated with TNFα (10 ng/mL). Receptor-interacting protein1 (RIP1) was precipitated from cell lysates and levels of ubiquitinated and precipitated RIP1 were analyzed by Western blotting. One representative out of 3 independently performed experiments is shown.

associated complex.[8,30] Besides their role in the regulation of cell survival, there is upcoming evidence that the IAPs are also involved in the regulation of immune functions.[12] In the present work, we demonstrate that IAP inhibitors could open a new and promising anti-inflammatory approach.

In vivo, the IAP antagonist exerted a profound anti-inflammatory effect in an antigen-induced arthritis model and, moreover, inhibited liver injury and leukocyte infiltration in a ConA-evoked hepatitis model in mice. Also leukocyte-EC interactions were significantly attenuated in vivo. The dosing of ABT used in the in vivo models was deduced from the concentrations that caused both the maximum inhibition of ICAM-1 expression and a significant inhibition of leukocyte adhesion in vitro (1 μmol/L). In the arthritis model, ABT was applied IP at 250 μg/kg/d. Based on the theoretical assumption of an immediate and complete absorption, this dosage could generate a maximum plasma level of 10 μmol/L. Regarding the administration of 50 μg/kg ABT directly into the circulation in the hepatitis and cremaster model, a maximum ABT plasma level of 2 μmol/L could be achieved. Noteworthy, subcutaneously applied doses of up to 40 mg/kg/d ABT are tolerable in mice.[5]

Until now, IAP antagonists have only been applied in vivo to proof them as promising candidates for anticancer therapy: ABT was successfully used in a murine breast cancer xenograft tumor model[5] and, noteworthy, some Smac mimetics have already entered phase I clinical trials.[33] Our work, however, is the first study that investigates the ant-inflammatory action of an IAP antagonist in the context of leukocyte–EC interaction, both in vivo and in vitro. On the basis of the profound in vivo actions of ABT, we used this compound as a tool to uncover the role of IAPs in inflammatory processes of ECs.

The up-regulation of EC adhesion molecule expression is a hallmark of inflammatory processes. Endothelial cell adhesion molecules are crucial for leukocyte recruitment to sites of inflammation and they tightly regulate the different stages of rolling, adhesion, and transmigration (diapedesis) of leu-

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kocytes.[34] We infer from our data that the IAP antagonist impairs leukocyte adhesion by inhibiting the expression of ICAM-1 on ECs, because blocking of ICAM-1 on the EC surface by a neutralizing antibody clearly lowered leukocyte adhesion.

Interestingly, the activation of the canonical NFκB pathway, which plays a pivotal role in the upregulation of ICAM-1, is not targeted by ABT. ABT did also not alter the noncanonical NFκB signaling. This is in contrast to studies showing that IAP antagonists can activate this signaling pathway in other cell types.[35,36] Instead, ABT inhibited the activation of the MAPKs p38 and JNK, which have been reported to regulate cell adhesion molecules[37] by activation of different transcription factors.[38] The fact that inhibitors of JNK and p38 significantly reduce TNFα-triggered ICAM-1 expression in ECs suggests that ABT blocks cell adhesion molecule expression predominantly via an inhibition of these 2 MAP kinases but not via affecting NFκB signaling.

The IAP-antagonist ABT, which was modeled to bind to the BIR3 domain of XIAP, reduced the phosphorylation of the MAP3K TAK1 (upstream of JNK and p38). However, silencing of XIAP did not result in an anti-inflammatory effect, but it even amplified TNFα-induced ICAM-1 expression and caused an increase in the levels of cIAP1 and cIAP2. This compensatory upregulation of cIAP1 and cIAP2 has also been reported in XIAP knockout mice.[39] In contrast, the reduction of cIAP1 and/or cIAP2 levels by RNAi led to an inhibition of the TNF-induced ICAM-1 that is comparable to the degree of inhibition caused by ABT. Correspondingly, the IAP antagonist itself evoked a decrease of cIAP1 and cIAP2, but not of XIAP levels in ECs. Monovalent Smac mimetics, such as ABT or Smac066, were designed to mimic the Smac AVPI-binding motif to target the BIR3 domain of XIAP. Nevertheless, it has been shown that this kind of mimetics also exhibit high affinities to the BIR3 domains of cIAP1 and cIAP2.[41] Interaction of Smac mimetics with cIAPs influences their E3-ligase and auto-ubiquitination activity and results in proteasomal degradation of cIAP1/2, whereas XIAP levels are not affected.[35,36,39–41] We could demonstrate that ABT induces ubiquitination of cIAP1, which goes along with the loss of this protein. The exact mode of action of Smac mimetics in this context has not been elucidated yet. However, Darding et al[42] suggested that Smac mimetic-induced auto-ubiquitination of cIAPs requires TRAF2 as scaffolding protein to mediate dimerization of the cIAP RING domain, which supports their E3-ligase activity. Our findings suggest that the anti-inflammatory effect of ABT does not arise from antagonizing XIAP but is connected to the proteasomal degradation of cIAP1 and cIAP2, which is nicely reflected in the results of the cIAP1/2 silencing experiments. Interestingly, cIAP1 and cIAP2 knockout mice are asymptomatic, which might be due to the observed mutual upregulation due to compensatory mechanisms.[13,43] Unfortunately, a cIAP1/2 double knockout mouse, in which the effects of ABT might be mimicked, is not available. However, the importance of influencing cIAP1/2 levels for the anti-inflammatory action of the IAP antagonist is affirmed by our finding that upregulation of cIAP1 and cIAP2 levels in response to XIAP silencing strongly increases endothelial activation.

Moreover, we found that the reduction of cIAP1 and cIAP2 protein levels influences TNFR signaling and, as a consequence, the activation of the MAP3K TAK1. The TNFα-induced activation of TNFR involves the assembly of the so-called TNFR-associated complex resulting in an activation of NFκB and MAPK signaling. The recruitment of TRADD and TRAF2, cIAP1/2 and RIP,[29] as well as the degradative and nondegradative ubiquitination processes exerted by the E3 ligase function of TRAF2 and cIAP1/2 provide platforms for the recruitment and activation of the IκB kinase and the TAB/TAK complex.[28] cIAP1/2 can directly interact with the adaptor protein TRAF2 and with RIP1,[9,30] which function both in NFκB and MAPK signaling.[35,44–46] It has been reported that the auto-ubiquitination–dependent degradation of cIAP1 and cIAP2 induced by IAP antagonists completely abrogates NFκB signaling.[10,11] In contrast, it has also been described that cIAP1/2 degradation evoked by a Smac mimetic is able to stimulate NFκB signaling.[36,47] In our setting, the treatment of ECs with an IAP antagonist resulted in a proteasome-dependent degradation of cIAP1 and cIAP2 but not in an alteration of NFκB activation. Instead, we detected a loss of TRAF2 in the EC membrane fraction mediated by ABT suggesting that the degradation of cIAP1/2 influences the TNFα-induced MAPK activation by affecting TRAF2 in the TNFR-associated signaling complex. However, there was no TNFα-evoked increase of TRAF2 in the membrane fractions, as it could have been expected, but nevertheless our finding is in accordance with the study of Yeh et al reporting that a loss of TRAF2 prevents TNFα-caused activation of JNK.[32] Moreover, we found a reduction of RIP1 ubiquitination in ABT-treated ECs. Bertrand et al[9] showed that cIAP1/2 can directly ubiquitinate RIP1 and that TRAF2 needs to be present for proper ubiquitination of RIP1. In the absence of the cIAPs, the association of RIP1 with TAK1 was impaired, which affected TAK1 signaling. Because TAK1 not only accounts for an activation of MAPK, but also of IκB kinase and thus NFκB,[48] the question arises of how MAPK signaling is impaired by ABT, whereas NFκB signaling remains unaffected. In contrast to TRAF2, the level of TRAF5 in membrane fraction did not change. In knock-out mice, TRAF5 has been found to compensate for the absence of TRAF2 concerning the activation of NFκB, but not in regard to the activation of the MAPK signaling.[31,32] This strongly supports our hypothesis that the degradation of cIAP1 and cIAP2 in the TNFR-associated signaling complex goes along with an impaired initiation of MAPK signaling. Thus, the inflammatory activation of ECs is abrogated, whereas NFκB signaling is not impaired. This may explain why HUVECs did not die on treatment with ABT and TNF as it has been described for cancer cells[36]: The unaffected, ongoing NFκB activation still can mediate a prosurvival signaling.

Inflammation and the regulation of cell death are often closely connected, not only in the development and progression of cancer, but also in pathologies like atherosclerosis.[49] For example, Moran and Agrawal showed an upregulation of IAPs in atherosclerotic plaques of patients with carotid stenosis.[50] Therefore, it could be speculated that targeting IAPs might possess the potential to dually influence inflammatory diseases like atherosclerosis, ie, by suppressing in-

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flammatory endothelial signaling processes as well as inducing apoptosis of proliferating vascular smooth muscle cells and infiltrating immune cells. Most interestingly, Tseng et al[51] describes elimination of cIAPs in macrophages as a highly specific tool to inhibit proinflammatory genes without affecting the anti-inflammatory and tumor suppressive IFN response and suggests IAP antagonists as potentially promising anti-inflammatory drugs.

In summary, our study provides for the first time evidence that IAP-inhibiting compounds possess profound anti-inflammatory properties in vivo. Moreover, we could show that IAPs play an important regulatory role in the inflammatory activation of ECs and, most importantly, that this might open a novel therapeutic strategy for the treatment of inflammation-associated diseases.

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Disclosures

None.

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