DISSERTATION

The Effect of the long non-coding RNA IFNG-AS1 on T Cells in Acute Coronary Syndrome with Intact Fibrous Cap

Die Wirkung der langen nicht-kodierenden Ribonukleinsäure IFNG-AS1 auf T-Zellen beim akuten Koronarsyndrom mit intakter fibröser Kappe

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ACEI	Angiotensin-converting enzyme inhibitors
ACS	Acute coronary syndrome
AhR	aryl hydrocarbon receptor
anti-CCP-Ab	Anti-cyclic citrullinated peptide antibodies
APC	Antigen-presenting cell
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
BACE1-AS	Beta-secretase-1-antisense transcript
BAT	Brown adipose tissue
BP	Blood pressure
CAD	Coronary artery disease
CANTOS	Canakinumab Antiinflammatory Thrombosis Outcome Study
CCR5	C-C chemokine receptor 5
CD	Cluster of differentiation
CM	Central memory
COLCOT	Colchicine Cardiovascular Outcomes Trial
CRP	C-reactive protein
CTCF	CCCTC-binding factor
CXCL1	Chemokine (C-X-C motif) ligand 1
DAPT	Dual antiplatelet therapy
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	dextran sulfate sodium

EC	Endothelial cells
ECG	Electrocardiogram
EGM	Endothelial cell growth medium
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
EndMT	Endothelial to mesenchymal transition
ESR	Erythrocyte sedimentation rate
ESS	Endothelial shear stress
FACS	flow cytometry
FSC	forward Scatter
GC	Guanine-cytosine
H3K4	histone H3 at lysine 4
HAEC	Human aortic endothelial cells
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HGP	Human genome project
HIV	Human immunodeficiency virus
HOCl	hypochlorous acid
HT	Hashimoto's Thyroiditis
IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
IFC-ACS	Acute coronary syndrome with intact fibrous cap
IMPROVE-IT	Improved Reduction of Outcomes: Vytorin Efficacy International Trial
ISR	In-stent restenosis
ITP	Immune thrombocytopenia

LDL-C	low-density-lipoprotein cholesterol
LODOCO	Low-Dose Colchicine
lncRNA	Long non-coding Ribonucleic Acid
LRP1-AS	Lipoprotein receptor-related protein 1-antisense transcript
L-Trp	L-tryptophan
LV	left ventricular
MACS	Magnetic Activated Cell Sorting
MG	Myasthenia gravis
MI	Myocardial infarction
miRNA	Micro Ribonucleic Acid
MM	Multiple myeloma
MMP	metalloproteinase
MPO	myeloperoxidase
mRNA	Messenger Ribonucleic Acid
MS	Multiple sclerosis
mTOR	mammalian target of rapamycin
ncRNA	Non-coding Ribonucleic Acid
NETs	Neutrophil extracellular traps
NFAT	nuclear factor of activated T cells
NK	natural killer cells
NSCLC	Non-small cell lung cancer
NSTEMI	Non–ST-segment elevation myocardial infarction
OCT	Optical coherence tomography
PAD	Peripheral arterial disease
PBMC	Peripheral blood mononuclear cells

PCI	Percutaneous coronary intervention
piRNA	Piwi protein interacting Ribonucleic Acid
PKC	protein kinase C
PKD1	Polycystic kidney disease 1
PS	phosphatidylserine
PVD	peripheral vascular disease
RA	Rheumatoid arthritis
RB	Retinoblastoma
RBC	Red blood cell
RCF	Rotational centrifugal force
RF	Rheumatoid factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal Ribonucleic Acid
siRNA	Small interfering Ribonucleic Acid
SLE	Systemic lupus erythematosus
SSC	Side scatter
STEMI	ST-segment elevation myocardial infarction
T2D	Type 2 diabetes
TC	Total cholesterol level
TLR2	Toll-like receptor 2
TNFalpha	tumor necrosis factor-alpha
tRNA	Transfer Ribonucleic Acid
TACS	Traceless Affinity Cell Selection
TEMRA	Terminally differentiated effector memory

TF	Transcription factors
TIMP	Tissue inhibitor of metalloproteinases
TNF-alpha	Tumor necrosis factor-alpha
UC	Ulcerative colitis
UK	United Kingdom
UV	ultraviolet
VCAM-1	Vascular adhesion molecule 1
VEST	vesnarinone trial
VSMC	Vascular smooth muscle cells

Abstract (English)

Introduction

Long non-coding RNAs (lncRNAs) are involved in varieties of disease pathologies and regulate the progression of diseases, including Alzheimer's disease (AD), diabetes mellitus type 2 (T2D) and cancer. Furthermore, lncRNAs seem to participate in the progression of atherosclerosis to its final manifestation acute coronary syndrome. However, their exact regulatory effects on the underlying pathologies of acute coronary syndrome are still incompletely understood. Recent studies have indicated that T cells accumulate at the lesion of acute coronary syndrome with intact fibrous cap (IFC-ACS) as compared to acute coronary syndrome with ruptured fibrous cap (RFC-ACS). In this regard, we investigated the hypothesis that the lncRNA IFNG-AS1 could also affect the function of T cells and their subsets in IFC-ACS differently from RFC-ACS.

Methods

Twenty patients presenting with IFC-ACS were matched by age, biological sex and diabetes mellitus type 2 status to twenty patients with RFC-ACS and to twenty controls with stable coronary artery disease. The lncRNA expression patterns were analyzed in peripheral blood mononuclear cells by RT-PCR of a pre-selected library. PBMCs and T cells were isolated from whole blood samples of healthy donors and were transfected using electroporation of small interfering RNA targeted at IFNG-AS1. The activation of T cells and the count of T cell subtypes were analyzed by flow cytometry analysis, as well as the induction of endothelial cell death in a co-culture with the transfected T cells. Cytokine release was assessed in supernatants. The expression of other inflammatory genes and their regulation upon IFNG-AS1 silencing was analyzed by RT-PCR.

Results

The general expression of IFNG-AS1 in PBMCs was lower in IFC-ACS than in RFC-ACS patients. The expression of IFNG-AS1 showed a negative correlation with $CD3^+$ T cell count while positively correlated with granulocyte and neutrophil absolute cell count at the lesion site of IFC-ACS. Silencing of IFNG-AS1 impaired the expression of the inflammatory genes IFNG and CD69, as well as the IFNγ cytokine release. The downregulation

of IFNG-AS1 also impaired the activation of $CD4^+$ T cells and reduced the count of Th1 cells. Silencing of IFNG-AS1 attenuated the capacity of inducing apoptosis by Th1 cells in a co-culture with endothelial cells.

Conclusions

The downregulation of IFNG-AS1 in T cells may represent a novel mechanism to reduce the T cell activation in IFC-ACS patients and thereby resolve inflammation, which may provide a promising therapeutic strategy for this population.

Abstract(German)

Einleitung

Long non-coding RNAs (lncRNAs) sind an verschiedenen Krankheitspathologien beteiligt und regulieren das Fortschreiten von Krankheiten, einschließlich der Alzheimer-Krankheit (AD), Diabetes mellitus Typ 2 (T2D) und malignen Erkrankungen. Darüber hinaus scheinen lncRNAs am Fortschreiten der Atherosklerose bis hin zur Endmanifestation des akuten Koronarsyndroms beteiligt zu sein. Ihre genauen regulatorischen Auswirkungen auf die zugrunde liegenden Pathologien des akuten Koronarsyndroms sind jedoch noch unvollständig geklärt. Frühere Studien haben gezeigt, dass sich T-Zellen an der Läsion des akuten Koronarsyndroms mit intakter fibröser Kappe (IFC-ACS) ansammeln, im Vergleich zum akuten Koronarsyndrom mit rupturierter fibröser Kappe (RFC-ACS). In der vorliegenden Arbeit soll daher die hypothesis geprüft werden, ob die lncRNA IFNG-AS1 auch die Funktion von T-Zellen und ihren Untergruppen bei IFC-ACS im Vergleich zu RFC-ACS beeinflussen könnte.

Methoden

Zwanzig Patienten mit IFC-ACS wurden nach Alter, biologischem Geschlecht und Diabetes mellitus Typ 2 zu Patienten mit 20 RFC-ACS und mit 20 Kontrollpersonen mit stabiler Koronararterienerkrankung zugeordnet. Die lncRNA-Expressionsmuster wurden in mononukleären Zellen des peripheren Blutes durch RT-PCR einer vorgewählten Bibliothek analysiert. PBMCs und T-Zellen wurden aus Vollblutproben gesunder Spender isoliert und mittels Elektroporation small interfering RNA, die auf IFNG-AS1 abzielte, transfiziert. Die Aktivierung von T-Zellen und die Anzahl der T-Zell-Subtypen wurden mittels Durchflusszytometrie-Analyse analysiert, ebenso wie die Induktion des Endothelzelltods in einer Co-Kultur mit den transfizierten T-Zellen. Die Zytokinfreisetzung wurde in den Überständen beurteilt. Die Expression anderer Entzündungsgene und deren Regulation bei der Stummschaltung von IFNG-AS1 wurde mittels RT-PCR analysiert.

Ergebnisse

Die allgemeine Expression von IFNG-AS1 in PBMCs war bei IFC-ACS-Patienten geringer als bei RFC-ACS-Patienten. Die Expression von IFNG-AS1 zeigte eine negative Korrelation mit der $CD3^+$-T-Zellzahl, während sie positiv mit der absoluten Zellzahl der Granulozyten und Neutrophilen an der Läsions-Stelle von IFC-ACS korrelierte. Das Silencing von IFNG-AS1 beeinträchtigte die Expression der Entzündungsgene IFNG und CD69 sowie die IFNγ-Zytokinfreisetzung. Die Herunterregulierung von IFNG-AS1 beeinträchtigte auch die Aktivierung von $CD4^+$ T-Zellen und verringerte die Anzahl der Th1-Zellen. Das Silencing von IFNG-AS1 verringerte die Fähigkeit, Apoptose durch Th1-Zellen in einer Co-Kultur mit Endothelzellen zu induzieren.

Schlussfolgerung

Die Herunterregulierung von IFNG-AS1 in T-Zellen könnte einen neuen Mechanismus darstellen, um die T-Zell-Aktivierung bei IFC-ACS-Patienten zu reduzieren und dadurch Entzündungen zu beseitigen, was eine vielversprechende Therapiestrategie für diese Population darstellen könnte.

1 Introduction

1.1 Acute coronary syndrome (ACS)

Acute coronary syndrome is the final manifestation of myocardial ischemia due to partial or complete coronary artery occlusion(1). Thirty percent of all ACS are STEMI and the rest are NSTEMI and unstable angina(2-4). ST-segment elevation myocardial infarction (STEMI) is characterized by a complete coronary artery occlusion, resulting in transmural myocardial infarction and ST-segment elevation in the Electrocardiogram (ECG) (5). In contrast, non–ST-segment elevation myocardial infarction (NSTEMI) presents no significant or even no ST-segment elevation on the ECG(6) and is characterized by a non-transmural myocardial infarction. Unstable angina occurs at rest and the duration is usually over 20 minutes(7).

ACS is still the primary cause of death in the world, accounting for an estimated 31% of all kinds of deaths(8). Although tremendous efforts have been made for the prevention, diagnosis and treatment of ACS, there are still a lot of diagnostic and therapeutic gaps.. For example, the mortality rate of ACS decreased from 294 to 225 per 10000 people in America in the recent twenty years(9). In Europe, the mortality rate of ACS has been decreasing by 5% annually in the past 5 years(10). But ACS is still the primary contributor to global high morbidity and mortality, leading to huge clinical, economic and social burdens worldwide. For instance, there are more than 25 million ACS patients hospitalized worldwide every year(11).

1.1.1 Difficulties in diagnosis, treatment and secondary prevention of of ACS

The Diagnosis of ACS is based on the combination of symptoms, the presentation of ECG and changes in serum biochemical markers, like cardiac troponin.

Chest pain is the main symptom of ACS patients, while 33% of ACS patients are without typical symptoms. This makes timely and accurate diagnosis difficult, leading to worse outcomes(12).

A 12-lead electrocardiogram (ECG) is used to diagnose ACS, but ECG has limited sensitivity (50%-60%) in acute MI diagnosis. For instance, STEMI presents significant ST-segment elevation but NSTEMI (40%-50%) does not show a significant change in ECG(13).

Cardiac troponin (cTnI and cTnT) is demonstrated as a highly sensitive cardiac injury biomarker, indicating irreversible damage to cardiomyocytes in NSTEMI, STEMI and type 2 myocardial infarction (T2MI) but not in unstable angina patients. T2MI is a non-atherosclerotic MI, characterized by irreversible damage to cardiomyocytes secondary to the insufficient supply of oxygen to the myocardium in conditions like sepsis, anemia etc(14). However, troponin is not specific for atherosclerosis-driven myocardial infarction and therefore lncRNAs may contribute to increasing the specificity of the early diagnosis. Attempts toward the combination of surveillance of changes in expression patterns of lncRNAs and traditional biomarkers have already been reported. For example, the combination of lncRNA-ABHD11-AS1 increases 24.3% diagnostic accuracy in screening early gastric cancer compared to the only application of serum carcinoembryonic antigen (CEA)(15)

Therefore, the diagnostic efficacy of the combination of lncRNAs and traditional methods for ACS remains to be investigated.

Percutaneous coronary intervention (PCI) is the standard treatment for ACS patients(16). Immediate recanalization of the target vessel and balloon dilatation/stent implantation restores the blood supply, reduces the infarction area and improves the prognosis of STEMI(17). PCI for STEMI should be performed as quickly as possible within 2 hours of the onset of STEMI. If conditions do not allow immediate treatment, PCI within 12 hours also benefits the prognosis (18). While the timeframe of PCI treatment for NSTEMI patients depends on the risk classification evaluated with the GRACE score. Extremely high-risk NSTEMI patients should get PCI treatment as fast as possible within 2 hours. High-risk patients could also benefit from PCI treatment within 24 hours if the delayed time is beyond 2 hours (19).

Besides PCI, another important therapy is dual antiplatelet therapy (DAPT). DAPT is used to prevent further myocardial infarction and stent thrombosis after PCI(20). Although PCI and DAPT have improved clinical prognosis significantly, the risk of recurrent cardiovascular adverse events in STEMI patients still remains high (15%) due to in-stent restenosis (ISR) and residual risk (21-24). The rate of ISR within one year of PCI remains at 10% with the continuous optimization of the stent materials and application of DAPT. We also face the risk of major bleeding events after using DAPT(25).

Other treatments, like statins, anti-inflammatory treatments, antihypertensive drugs, glucose-lowering agents, antiplatelet drugs, smoking cessation, diet control and appropriate exercise form the cornerstone for the secondary prevention of ACS.

Secondary prevention mainly targets patients who have already experienced ACS due to a significantly high risk of recurrence (26). In the past, secondary prevention mainly focused on strategies for lowering cholesterol. In 1997, the Scandinavian Simvastatin Survival Study (4S) proved statin reduces recurrent cardiovascular adverse events in secondary prevention by lowering the TC and low-density-lipoprotein cholesterol (27). From then on, large amounts of clinical trials have clearly indicated that ACS patients benefit from the usage of statin in secondary prevention(28). Further decreasing LDL-C to resolve residual cholesterol risk becomes the overarching theme.

A combination of two or even more medical treatments is common in secondary prevention, often leading to low adherence(29). For example, some studies indicate that 20-30% of ACS patients discontinue preventive medicine after 1 year of PCI due to the side effects of drugs and forgetfulness(30-32). Novel precise and targeted therapy is required to treat ACS patients and improve their recovery as well as reduce their recurrent risk of cardiovascular events.

We also observe a great proportion of post-MI patients with well-controlled LDL cholesterol levels, which still face a great risk of recurrent cardiovascular adverse events. Some studies attribute these events to residual risk factors, like inflammation, smoking and BP(33).

As reported recently, compared to other secondary treatment strategies, anti-inflammatory treatments have made great progress in several trials (22). For example, Improved Reduction of Outcomes: Vytorin Efficacy International Trial (IMPROVE-IT) study demonstrates the combination of ezetimibe and simvastatin lower the LDL-C level and inflammatory indicator, hs-CRP, leading to a significant reduction of the recurrent cardiovascular events rate(34). IMPROVE-IT provides the potential importance of controlling the residual inflammatory risk in secondary prevention. In addition, the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS) reveals antiinflammatory therapy with canakinumab significantly decreases the rate of recurrent cardiovascular events without affecting cholesterol levels (35). Besides, the Colchicine Cardiovascular Outcomes Trial (COLCOT) and Low-Dose Colchicine (LODOCO) also provide evidence that antiinflammatory therapy with colchicine benefits ACS patients and

patients with stable coronary disease in secondary prevention(36, 37). There is no doubt that controlling inflammatory residual risk is still a long road to go.

In this context, lncRNA may be a promising individualized therapy targeting inflammatory residual risk. For example, silencing lncRNA E330013P06 may reduce the expression of inflammatory cytokines, like IL6, in the diabetes mouse model(38). But the exact role of lncRNA in inflammatory residual risk is incompletely understood.

1.1.2 Changes in the ACS population

ACS is becoming more common in younger individuals and in the elderly(39). As reported recently, there are approximately 23% of ACS patients younger than 55 years old(40). In the past, ACS patients were characterized by major risk factors, such as hypertension, diabetes mellitus type 2 and hypercholesterolemia. Nowadays, a great proportion of young ACS patients (36%) is presenting only one or even no traditional risk factors(41). Therefore, novel risk factors and risk prediction models for young ACS patients are required.

Besides, the increasing aging population with ACS significantly brings challenges to global clinical service and social finances due to multiple comorbidities, increased dismal prognosis and expensive cost(42). Advanced age is a known risk factor for ACS, while the aging population commonly presents higher possibilities of other unknown risk factors(43). Thus, a better understanding of unknown risk factors for old ACS patients is required.

In conclusion, we are nowadays facing a demographic shift in the ACS population with mostly poorly understood novel risk factors, which requires better examination and understanding of novel risk factors.

1.1.3 The prevalence and mechanism of IFC-ACS

Thrombotic occlusion of the coronary artery is the primary pathological mechanism of ACS(44). As demonstrated in autopsy studies, vulnerable plaques result in thrombotic occlusion(45). About 55-60% are plaque prone to rupture and subsequent thrombosis, 30-35% are plaque erosion and 2-7% by calcified nodules (CN)(46). The ruptured plaques are characterized by a large necrotic core containing lipids, a thin fibrous cap, infiltration of inflammatory cells in the plaque, like macrophages, and intra-plaque hemorrhage(47). Compared to ruptured plaques, eroded plaques are characterized by intact fibrous caps and local detachment of endothelial cells with thrombus formation(44). CN is

characterized by a calcified mass that protrudes into the lumen of the vessels(48). According to the pathologic features, ACS patients with a ruptured plaque and ACS patients who present plaque with intact fibrous cap are classified into RFC-ACS and IFC-ACS.

With modern intracoronary imagining, like optical coherence tomography (OCT), the proportion of in-vivo diagnosed plaque erosion is rising (49). For example, as revealed by recent studies, about 40% of ACS patients are presenting with IFC-ACS, showing a higher prevalence than in the past 20 years, where the diagnosis was limited only to post-mortem histological observations during the autopsy of chosen individuals(50). Notably, in female ACS patients, plaque erosion even causes up to 50% of ACS deaths (51).

Nevertheless, the underlying mechanisms of IFC-ACS are still incompletely understood. Recent studies have demonstrated IFC-ACS may be due to high endothelial shear stress (ESS)(52, 53), inflammatory cytokines released in coronary vessels(54), Toll-like receptor 2 (TLR2) activation(55), neutrophil extracellular traps (NETs) and endothelial to mesenchymal transition (EndMT)(56).

For example, in 78% of IFC-ACS patients, thrombosis occurred at high ESS regions(52) and in these regions, endothelial cells (EC) showed a higher ratio of apoptosis(53). Inflammatory cytokines in coronary vessels, like TNFα, increase the expression of membrane type 1-matrix metalloproteinase (MT1-MMP) and activate MMP2, leading to the degrading of the extracellular matrix to induce the desquamation of ECs(54). Elevated TLR2 participates in plaque erosion by recruiting pro-inflammatory neutrophils and by causing apoptosis and detachment of EC(55). NETs trap the platelets to induce thrombosis and cause the desquamation of ECs(55). EndMT induces the overexpression of MMPs, resulting in the degradation of the extracellular matrix and the desquamation of EC to aggravate plaque erosion(56).

The mechanism of IFC-ACS has only recently become better understood, with multiple inflammatory mechanisms involved, including neutrophils(57) and T cells(58). For example, it is revealed in recent studies that IFC-ACS is partially caused by the accumulation and activation of T cells to cause the desquamation of EC(58). Th1 and Th17 cells have also been demonstrated to induce a pro-inflammatory response by releasing cytokines like IFNγ, IL17 and IL2(59). For instance, Th1 cells release IFNγ to activate macrophages to increase the inflammatory cytokine release of TNFα and IL1(60).

Regulating T-cell function might therefore be a potentially promising strategy for IFC-ACS by regulating T-cell function. For example, lncRNAs like IFNG-AS1 have been shown to regulate the function of T cells to promote the progression of diseases like rheumatoid arthritis (RA)(61) and multiple sclerosis (MS), etc.(62).

1.2 Long non-coding RNA (lncRNA)

LncRNAs were first reported to regulate the expression of their adjacent protein-coding gene to promote the development of different diseases(63).

1.2.1 History of lncRNAs

In 1947 André Boivin found that deoxyribonucleic acid (DNA) is transcripted into ribonucleic acid (RNA), which is responsible for the production of proteins(64). Afterwards, in 1950, Raymond Jeener further developed the understanding that RNA is synthesized in the nucleus and promotes the production of proteins in the cytoplasm(65). Furthermore, metabolically active RNA was first reported by Al Hershey's research group(66), and then Elliot 'Ken' Volkin, and Lazarus Astrachan found it was complementary to at least one strand of DNA(67). In 1961, Jacob and Monod defined this RNA as messenger RNA (mRNA)(68), which triggered great scientific interest and laid the foundation for a large amount of studies of RNA. With the development of new laboratory techniques, scientists recognized that there are different types of RNAs and even some which do not encode proteins. These RNAs are called non-coding RNA (ncRNA), for example, in 1955, George Palade found the first ncRNA was harbored in ribosomes and was therefore named ribosomal RNA (rRNA)(69). In 1957, the second group of ncRNA-the transfer RNA (tRNA) was identified by Mahlon Hoagland and Paul Zamecnik(70). LncRNAs are a group of ncRNAs with a length of over 200 nucleotides, which distinguishes them from shorter regulatory RNAs, like microRNA (miRNA) and PIWI protein-interacting RNA (piRNA)(71). Although plenty of ncRNAs have been identified, only limited functional long non-coding RNA (lncRNAs) are found. For instance, H19 was the first lncRNA found in eukaryotes, which was believed to be different from traditional mRNA, and in recent years, it has been proven to combine with miR-17 leading to the proliferation of non-small cell lung cancer (NSCLC), to promote the development of NSCLC(72, 73). Fluorescence in situ hybridization experiments located the lncRNA Xist to the X chromosome deactivation-related regulatory region(74), but its function was not clear. Recently, there are some

pieces of evidence supporting the silencing effect of Xist on the X chromosome by recruiting proteins like PTBP1, MATR3, TDP-43, and CELF1(75, 76). The human genome project (HGP) opens a new era for the studies of lncRNA. In this project, comprehensive genomic sequencing reported new lncRNAs existing in the human genome(77, 78). The function of lncRNAs is not well-established, identification of their potential function is still challenging for scientists all over the world. Scientists found that many lncRNAs often co-express with their adjacent protein-coding gene in previous studies, so they suggested that the function of lncRNAs was to regulate their adjacent protein-coding gene and verified their function in different disease models(63).

1.2.2 Regulatory function of lncRNAs in other diseases

LncRNAs have been shown as important contributors to different disease pathologies, for example, the lncRNA MALAT1 shows multiple functions in the pathology of different cancer types including retinoblastoma (RB), multiple myeloma (MM), epithelial ovarian cancer, gastric cancer, and hepatocellular carcinoma (79) like proliferation, apoptosis, metastasis, angiogenesis, cancer immunity and chemotherapeutics resistance. MALAT1 silencing causes the upregulation of miR-124 and thereby suppresses the proliferation of RB(80). The downregulation of MALAT1 has also been shown to induce apoptosis in MM by decreasing the expression of the anti-apoptotic protein Bcl-2 (81). MALAT1 downregulation also impairs the migration of epithelial ovarian cancer by suppressing the expression of epithelial-to-mesenchymal transition (EMT) through the PI3K/AKT pathway(82). Furthermore, MALAT1 is a promotor of angiogenesis in GC through binding to the VE-cadherin/β-catenin complex and the involvement of the ERK/MMP and FAK/paxillin pathways(83). In thyroid cancer, MALAT1 regulates tumor-associated macrophages (TAM) to produce and release FGF2 protein which is a very effective growth factor increasing cancer aggressivity. Upregulation of MALAT1 also induces resistance to chemotherapy drugs via the HIF-2α-MALAT1-miR-216b axis in HCC(84).
LncRNAs also affect other diseases apart from cancer, like AD, human immunodeficiency virus (HIV), T2D and systemic lupus erythematosus (SLE). Beta-secretase-1-antisense transcript (BACE1-AS) increases the stability of BACE1 mRNA to promote the production of a key element of AD named oligomers of Aβ 1–42(85). LncRNA lipoprotein receptor-related protein 1-antisense transcript (LRP1-AS) is elevated in AD patients and regulates the activation of chromatin-associated protein HMGB2(86). Some other lncRNAs, like 51A(87) and AD-linc1(88) are also found upregulated in AD.

In HIV patients, the lncRNA NEAT1 and NRON are very important lncRNAs for the replication of HIV(89). Knockdown of NEAT1 impairs the structure of the nuclear paraspeckle leading to the reduction of the replication of HIV. While downregulation of NRON results in the increased activity of the nuclear factor of activated T cells (NFAT) and thereby increases replication of HIV-1(90).

In T2D patients, lncRNAs are also associated with varied pathological processes, like homeostasis of β cells and adipose development. The downregulation of the lncRNA-βlinc1 disrupts the function of the islet and impairs glucose homeostasis severely(91). Brown adipose tissue (BAT) plays a protective role in T2D. A deficiency of lncRNA-Adi inhibits the development of adipose tissue-derived stromal cells (ADSCs) into adipose tissue(92).

Furthermore, the lncRNA NEAT1 triggers the development of SLE through the induction of the mitogen-activated protein kinase (MAPK) signal pathway(93). Although a lot of lncRNAs have demonstrated their important roles in human diseases, understanding the distinct roles of lncRNAs in these diseases is still in infancy.

1.2.3 LncRNAs participate in ACS

Notably, recent studies also identify lncRNAs as important regulators of atherosclerosis and acute coronary syndrome (ACS).

LncRNA ANRIL is located at chromosome 9p21, the strongest susceptibility loci of ACS identified by Genome-wide association studies (GWAS)(94, 95). LncRNA ANRIL increases the abnormal expression of surface adhesion molecules of endothelial cells, the proliferation of vascular smooth muscle cells (VSMC) and mononuclear cells as well as the abnormal glycolipid metabolism in atherosclerosis.

In ACS patients, the lncRNA ANRIL activates endothelial cells to release adhesion molecules (ICAM-1 and VCAM-1) with proinflammatory activity by targeting the let-7b/TGF-βR1 pathway(96). ANRIL also has the ability to combine with the component of polycomb repressive complex-2 (PRC2), SUZ12 to silence $p15^{INK4}$ and thereby to increase the abnormal proliferation of VSMCs in the coronary artery wall(97). Alu element (Alu sequence participating in the combination of the targeted gene promoter) in ANRIL facilitates the proliferation and adhesion of pro-inflammatory mononuclear cells to promote the progression of atherosclerosis (98, 99). Upregulation of ANRIL is associated

with the aggravation of atherosclerosis by decreasing the expression of glycolipid metabolism-related genes, like ADIPOR1 (100).

There are also some other less studied lncRNAs reported in the pathology of ACS like p21, H19, MALAT1 and SENCR.

Some lncRNA show direct effects on cell biology and proliferation in ACS like the lincRNA-p21 exacerbates the progression of atherosclerosis by suppressing the proliferation of endothelial cells and thereby increasing plaque vulnerability (101). MALAT1 downregulation decreases the apoptosis of cardiomyocytes during myocardial infarction (MI) in a murine model (102). The downregulation of SENCR increases the abnormal proliferation of VSMCs to aggregate atherosclerosis(103).

Furthermore, lncRNAs seem to have an effect on gene expression in ACS like the lncRNA H19 could recruit the CCCTC-binding factor (CTCF) and thereby downregulate the expression of the polycystic kidney disease 1 (PKD1) gene, a risk gene for ACS patients with type 1 diabetes (104).

LncRNAs have been continuously identified and their importance in the pathological mechanisms at all stages of atherosclerosis and ACS are emerging in recent years. Nevertheless, the comprehensive exploration of their functions is still one of the main targets in modern science, as lncRNAs may represent novel therapeutic targets for a variety of different diseases.

1.2.4 The regulatory role of IFNG-AS1 in T cell-driven autoimmune and inflammatory diseases

There are pieces of evidence for lncRNAs participating in plaque destabilization leading to plaque rupture or RFC-ACS, for example, Nron causes instability of plaque in a murine model. But there is very little evidence about their role in IFC-ACS and especially in T cell immune response in IFC-ACS patients(105). Therefore, further understanding of the regulatory roles of lncRNAs in T cell-driven inflammatory patterns in atherosclerosis and thereby developing personal therapeutic strategies for IFC-ACS patients remains one of the main goals of modern atherosclerosis research.

IFNG-AS1 may be a potential target for driving atherosclerotic cardiovascular disease. For example, recent studies indicate that IFNG-AS1 has a positive relationship with the Gensini Score, an indicator of the severity of CAD(106). Another study demonstrates

IFNG-AS1 has a positive correlation with pro-inflammatory cytokines in CAD patients, like hs-CRP, TNFα and IL6(107).

Besides, as described in previous studies, ACS plays important roles in immune response and different T cell-driven diseases like autoimmune and inflammatory diseases.

For instance, IFNG-AS1 is the first lncRNA affecting the immune response to the Theiler virus and Salmonella infection in the murine model through epigenetic modification manners. Overexpression of IFNG-AS1 in a mouse model increased the susceptibility to Theiler virus infection while decreasing the infection rates of Salmonella(108). Subsequent studies have shown that IFNG-AS1 plays important roles in different T cell-driven diseases like autoimmune and inflammatory diseases.

IFNG-AS1 has shown its importance in different autoimmune diseases, like Rheumatoid Arthritis (RA), multiple sclerosis (MS), Sjögren syndrome (SS), Hashimoto's Thyroiditis (HT) and immune thrombocytopenia (ITP).

For example, RA patients show a higher expression of IFNG-AS1. The expression of IFNG-AS1 has a positive correlation with laboratory inflammatory parameters like erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and rheumatoid factor (RF), indicating a potential pro-inflammatory effect in RA patients(61).

IFNG-AS1 is demonstrated to promote the development of MS. For instance, there are some pieces of evidence showing the expression of IFNG-AS1 is higher in the relapsing phase compared to healthy control(62).

In SS patients, the expression of IFNG-AS1 helps to exacerbate the development of SS. The expression of IFNG-AS1 shows a positive association with the ratio of Th1 cells and markers of disease severity, like SSA, ESR and IgG(109).

IFNG-AS1 is also revealed to aggravate HT progression. The expression of IFNG-AS1 has a positive correlation with indicators for HT progression, like anti-thyroglobulin antibody (TgAb) and thyroperoxidase antibody (TPOAb)(110).

In addition, IFNG-AS1 is reported to participate in the progression of ITP by promoting the expression of IFNG(111).

IFNG-AS1 plays an important role in different inflammatory diseases as well, like inflammatory bowel disease (IBD) and chronic inflammatory disease, ulcerative colitis (UC).

For example, IFNG-AS1 has been described as playing a pro-inflammatory role in IBD patients because the expression of IFNG-AS1 shows a positive relationship with the expression of inflammatory cytokine, IL2(112).

In UC patients, the expression of IFNG-AS1 is higher than in healthy controls. Overexpression of IFNG-AS1 negatively regulates Mirt2 to induce more apoptosis of colonic epithelial cells(113).

However, the exact role of IFNG-AS1 regulating T cell-driven inflammatory patterns, especially in IFC-ACS still requires further investigation to increase the pathological understanding of this rare IFC-ACS-causing pathology and thereby improve its diagnosis, treatment and prevention.

2 Hypothesis

The main objective of the current study is to characterize the expression patterns and function of IFNG-AS1 in the T-cell-driven immune response after acute coronary syndrome with intact fibrous cap vs. ruptured fibrous cap.

3 Materials and methods

3.1 Materials

3.1.1 Chemicals and reagents

Name	Supplier
Phosphate buffered saline（PBS）	SIGMA
Ethylenediaminetetraacetic acid (EDTA)	SIGMA
Ficoll-Paque	Catalogue Number: GE17-1440-02, SIGMA
RPMI 1640	Gibco
CTS™ Optimizer™ T-cell-Expansion medium	Catalogue number: A37040-01, Thermo Fischer
Trypan blue	Catalog number: 15250061, Gibco
Fetal bovine serum (FBS)	Thermo Fischer
L-glutamine	Thermo Fischer
Penicillin-Streptomycin	Catalog number: C-22111, PromoCell
recombinant IL12	catalog number: 130-096-705, Miltenyi Biotec
recombinant IL 2	catalog number: 200-02-50, Pepro Tech
siRNA	Assay ID: s501414, Ambion
Stealth RNAi negative Control Duplexes	Cat. Number: 12935300, Life Technologies
phorbol 12-myristate 13-acetate (PMA)	SIGMA
ionomycin	SIGMA
Attune™ Wash Solution	Catalog number: A24974, Thermo Fisher
Attune™ Focusing Fluid	Catalog number: A24904, Thermo Fisher

Attune™ Shutdown Solution	Catalog number: A24975, Thermo Fisher
Attune™ Performance Tracking Beads	Catalog number: 4449754, Thermo Fisher
Sytox Orange	Life Technologies
Deoxyribonuclease I from Bovine Pancreas	Life Technologies
T Cell TransAct	Reference number: 130-111-160, Miltenyi Biotec
QIAzol Lysis Reagent	QIAGEN
RNase free water	BBRAUN
80% Ethanol	BDH chemical
100% Ethanol	BDH chemical
RNaseZap	SIGMA
Ambion™ DNase I (RNase-free)	Ambion
RNAse Inhibitor	Promega
SYBR Green	Life Technologies
Primers	Ambion
Annexin V Binding Buffer	Biolegend

3.1.2 Equipment

Name	Supplier
Export Advance™ aspiration catheter	Medtronic
Centrifuge	Eppendorf
-80 Freezing container	BINDER

Thermostat water bath cauldron	Medinglab
Ice maker	ggmagastro
Microscope	Motic
Neubauer chamber	Life Technologies
Handheld tally counter	Life Technologies
Magnetic separator (MACS)	Miltenyi
Vertical flow clean bench	Thermo Fischer
Cell culture incubator	BIONDER
Invitrogen™ Neon™ Transfection System (Neon™ electroporation device, pipette, and pipette station)	Life Technologies
Vacuum pump	Vaccubrand
Attune Flow Cytometer	Thermo Fischer
PCR Workstation	VMR
NANO Vue Plus	Imagination at work
Thermocycler	Eppendorf
Real-Time PCR System	Thermo Fischer

3.1.3 Consumables

Name	Supplier
50ml falcon	Corning Science
Pipettes	Eppendorf
Test tube holder	Eppendorf

Vacuum blood collection tube (EDTA)	Plymouth
Pipette tips	Greiner
LS columns	Miltenyi
15ml falcon	Corning Science
Cell culture dishes	Cellstar
T75 flask	T75 flask

3.1.4 Cell lines

Name	Supplier
HAEC	Cell systems

3.1.5 Commercial kits

Name	Supplier
Human Pan T Cell Isolation Kit	Catalogue number: 130-096-535, Miltenyi Biotec
Neon™ Transfection System 100 µL Kit (Buffer T, Buffer E2, 100 µL Neon™ Tips, and Electroporation Tubes)	Life Technologies
Th1/Th2 Cytokine Kit (Human IL2, IL4 , IL6, IL10, TNF, and IFNγ Capture Beads, Human Th1/Th2 PE Detection Reagent, Human Th1/Th2 Cytokine Standards, Cytometer Setup Beads,	Catalog number: 551809, Becton Dickinson

PE Positive Control Detector, FITC Positive Control Detector, Wash Buffer, Assay Diluent, Serum Enhancement Buffer)	
miRNeasy Micro Kit (Spin columns, Collection tubes, RWT buffer, RPE buffer)	Catalogue Number 1071023, QIAGEN
High-Capacity cDNA Reverse Transcription Kit (10X RT Buffer, RT Random Primers, dNTP Mix (100mM), MultiScribe Reverse Transcriptase)	Life Technologies

3.1.6 Antibodies

Name	Supplier
CD4 BV605	Biolegend
CD45 BV711	Biolegend
CD3 AF488	Biolegend
CCR6 AF488	Biolegend
CCR7 PE	Thermo Fischer
CD3-P-CPeF710	Thermo Fischer
CXCR3-PE-Cy7	Thermo Fischer
CD25 eFluor450	Thermo Fischer
CD45RA eF506	Thermo Fischer
CXCR5 SB 600	Thermo Fischer
CD8 BV711	Biolegend

CCR4 APC	Thermo Fischer
CD4 AF700	Thermo Fischer
CD127–APC-eF780	Thermo Fischer
CD45RA AF488	Biolegend
CCR7 PE	Thermo Fischer
PD-1 PerCP-eF710	Thermo Fischer
CD27 PE Cy7	Biolegend
CD57 Pac Blue	Biolegend
CD45 BV605	Biolegend
CD8a BV605	Biolegend
CD28 BV711	Biolegend
CD4 AF647	Thermo Fischer
CD3 APC-eF780	Thermo Fischer
CD8 PerCp Cy 5.5	Biolegend
Annexin V PB	Biolegend
CD144 PE-Cy7	Biolegend

3.1.7 Medium

CTS complete medium	Concentration
FBS	10%
Penicillin-Streptomycin	1%
L-glutamine	10mM
CTS OpTmizer Expansion Supplement	26ml

CTS OpTmizer Expansion Basal Medium	1000ml

RPMI complete medium	Concentration
FBS	10%
Penicillin-Streptomycin	1%
RPMI 1640	500ml

EGM complete medium	Concentration
FBS	10%
Penicillin-Streptomycin	1%
EBM™ Basal Medium	500ml
EGM™ SingleQuotsTM Supplement Pack	1 x Green Cap Vial with hEGF 0.50 mL
	1 x Natural Cap Vial with Hydrocortisone 0.50 mL
	1 x Red Cap Vial with GA-1000 0.50 mL
	1 Bottle BBE 2.00 mL
	1 x Blue Cap Vial with Ascorbic Acid 0.50 mL
	1 Bottle FBS 10.00 mL

EBM medium	Concentration
FBS	10%
Penicillin-Streptomycin	1%
EBM™ Basal Medium	500ml

3.1.8 Buffers and solutions

Name	Composition
Dilution PBS with EDTA	
	50ml PBS
	200µl 2mM EDTA
Sterile staining buffer	
	49ml PBS
	1ml FBS
	200µl 2mM EDTA
Trypsin/EDTA	0.05% trypsin-EDTA
Stop solution	
	Annexin V Binding Buffer
	10% FBS
FACS fix solution	0.5% PFA in PBS
FACS staining buffer	0.05% NaN3
	50ml PBS
	2% FCS

3.2 Methods

3.2.1 Study plan

Five hundred and fifty-five ACS patients presenting with STEMI(114) and NSTEMI(115) were included in the OPTICO-ACS study (NCT03129503). Details regarding excluded patients have been previously published(58). All of them underwent optical coherence

tomography (OCT) examinations of the ACS-causing culprit lesion in order to distinguish plaque erosion from plaque rupture as the potential pathology as previously described(58). In order to perform a robust sub-analysis, twenty IFC-ACS patients were matched to the same amount of RFC-ACS patients according to the following criteria: age, biological sex, and diabetes mellitus type 2. Patients diagnosed as chronic coronary syndrome (CCS) were included in the control group. To verify the hypothesis, $CD3^+$ $CD4^+$, and $CD8^+$ T cells from healthy donors were used in the ex-vivo experiments.

3.2.2 Samples collection and storage

The lesional blood was collected from the local environment of the culprit lesion during the thrombectomy of the ACS patients cohort using an export catheter. The second sample was collected from the systemic circulation (femoral or radial artery). Afterwards, PBMCs were isolated from the lesional samples by using Ficoll-Paque density gradient centrifugation and the pellets were stored at -80°C for the following experiments.

3.2.3 PBMC isolation

For PBMC isolation, the Ficoll-Paque density gradient centrifugation is commonly used to separate PBMCs from granulocytes and red blood cells due to their lower density.
For this purpose, we use Ficoll-Paque density gradient centrifugation to separate PBMCs. 30ml of ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood samples, obtained from healthy donors, were collected into vacutainers. The whole blood samples were diluted with sterile PBS at a ratio of 1:1. The diluted blood was layered carefully on top of the Ficoll-Paque solution. To collect PBMCs, diluted blood samples were centrifuged at five hundred rotational centrifugal force (500x RCF) for 30 minutes. PBMCs in the interface layer of plasma and Ficoll were washed twice with PBS, resuspended in the ice-cold PBS and kept on ice for subsequent experiments.

3.2.4 Cell counting

For optimal experimental conditions, we estimated the desired cell count by using the Neubauer hemocytometer chamber and trypan blue as a live-dead stain. Alive cells have intact cell membranes so that the trypan blue can not be taken up while dead and apoptotic cells lose their integrity and are marked by trypan blue.

The isolated PBMC suspension was mixed with 0.4% trypan blue at a 1:10 ratio. Afterwards, the absolute numbers of trypan negative cells were estimated on a Neubauer hemocytometer for each of the four quadrants with an average calculated at the end.

3.2.5 T-Cell isolation

There are three methods for isolation of T cells: Traceless Affinity Cell Selection (TACS), REAlease Technology and Magnetic Activated Cell Sorting (MACS) were generally adopted for T-Cell isolation(116).

REAlease Technology uses magnetic beads with REAfinity recombinant antibodies to reversibly bind CD-specific makers of T cells, which could provide labeled T cells and this procedure would take around 50 minutes.

TACS uses the reversible binding ability of Fab fragments with CD-specific makers of T cells to separate T cells, which has the advantage of offering unlabeled and unactivated T cells. But it's taking longer than the other methods.

Based on the MACS system, with the human Pan T cell Isolation Kit, untouched $CD3^+$ T cells were purified after passing the magnetic field by negative selection with labeled non-$CD3^+$ T cells retained in the magnetic columns. The whole procedure takes a shorter time, which is a clear advantage in comparison to the other one.

To investigate the differentiation and activation of $CD3^+$ T cells and to provide high viability in the shortest time, untouched $CD3^+$ T cells were purified by the human Pan T cell Isolation Kit in the MACS system according to the recommendations of the supplier. Purified $CD3^+$ T cells were seeded at a density of 1x10^6/well in12-well-plates with prewarmed CTS-medium. Then the cells were incubated at a 95% humidity atmosphere of 37.0 °C and 5% CO2.

3.2.6 T Cell expansion and Th1 differentiation

3.2.6.1 T Cell polyclonal expansion

In-vivo $CD3^+$ T Cell expansion and differentiation normally requires two signals(117): the first is the antigen presentation by antigen-presenting cells (APC) through binding of the T cell receptor (TCR) and major histocompatibility complex (MHC) of the APCs and the second signal is provided by costimulatory molecules to induce effector functions in the T cells. In-vitro $CD3^+$ T Cell expansion and differentiation were induced by anti-CD3 and

anti-CD28 antibodies to mimic the two in-vivo signals with CD3/CD28 Streptamer or CD3/CD28 beads.

CD3 /CD28 Streptamer exactly determines the initiation and termination of the stimulation by controlling the reversible binding of Fab fragments against CD3 and CD28. The long-term existence of stimulators negatively affects the activity and the expansion efficacy of T cells. CD3/CD28 Streptamer needs an extra reagent, D-Biotin to terminate the stimulation.

On the premise of optimal activation effect, T Cell TransAct is more simple and efficient. It allows the removal of the beads through washing and centrifugation steps without the requirement of other reagents and has better contact with the cell-particle in suspension. Therefore, to induce polyclonal expansion and effector functions, we used T Cell TransAct according to the instructions at a density of 1x10^6 cells/well in 12-well plates with prewarmed CTS-medium for three days(118).

3.2.6.2 Th1 differentiation

To induce the Th1 cells from $CD3^+$ $CD4^+$ T Cells, the above stimulated $CD3^+$ T Cells were simultaneously treated with recombinant IL12 (final concentration: 10mg/mL, Miltenyi Biotec) and recombinant IL2 (final concentration: 20UI/ml, Pepro Tech) and afterwards incubated at a 95% humidity atmosphere of 37.0 °C and 5% CO2 for 3 days(119).

3.2.7 Preparation of endothelial cells

Cell culture with human aortic endothelial cells (HAEC) passage six (p6) was established three to four days prior to the in-vitro experiments with Th1 cells in the endothelial cell growth complete medium (EGM) (EBM Basal Medium, EGM SingleQuots Supplement Pack, FBS 10%, Penicillin-Streptomycin 1%). After de-freezing the HAECs, the cells were added to a T75 cell culture flask and left for one hour to adhere. Afterwards, the old medium was exchanged due to dimethyl sulfoxide (DMSO) content of the freezing medium. HAECs were grown for two to three days until 80-90% growing confluence was reached(120). At this time point, HAECs were believed to be most suitable for further experiments as over-confluency leads to insufficient nutrients in the EGM medium and death of the cells. On the other hand, if cells were passaged too early (less than 60%), the growth of cells would have been insufficient for the purpose of our experiments.

3.2.8 Passage of HAEC

For the collection of the HAECs, trypsinization is known as the most common method not involving mechanical stress and thereby protecting the integrity of the cells. Scraping methods apply more breakage to the cells than chemical methods(121).
HAECs were detached from the flask by 0.05% trypsin-EDTA, which was neutralized by an equal volume of 10% Fetal bovine serum (FBS). Then they were re-seeded at a density of 1x10^5/well in 12-well plates with EGM medium.

3.2.9 IFNG-AS1 silencing

There are three main methods for transfection in general: chemical-based transfection, physical-based transfection and biological-based transfection.
Chemical-based transfection includes calcium phosphate co-precipitation transfection and liposome-mediated transfection. Traditional liposome-mediated transfection is complicated and has higher cytotoxicity than other methods(122). High serum content in the medium leads to low transfection efficacy by affecting the combination of liposome complex. When serum is depleted, liposomes become toxic to cells. In addition, Optimal conditions differ among different cell types based on their susceptibilities to liposomes(123). Calcium phosphate co-precipitation transfection has been shown to have poor repeatability and lower transfection efficacy(124).
Biological-based transfection usually adopts virus-mediated techniques. The limitations of virus-mediated transfection are time-consuming(125), off-target effects(126) and immunogenicity(127).
Physical-based transfection commonly contains electroporation transfection and microinjection transfection. Microinjection transfection is characterized by injecting siRNA fragments directly into the subcellular location (128). The drawbacks of this method are complicated, expensive and also could induce gene mutation (129). The principle of electroporation transfection is efficiently transferring foreign genes into target biosamples by stable electric fields(130). Electroporation induces small pores on the membrane of cells using short electrical impulses and allowing siRNA to pass through for a short time and thereby not destroying the integrity of the membrane permanently. Compared to other techniques, the Neon electroporation transfection system (Thermo Fischer) has wide applicability, high transfection efficacy and survival of the cells(131).

Primary T cells exhibit higher resistance to transfection compared to other primary cells due to their inherent resistance to conventional chemical reagents(132). In order to transfect efficiently and preserve high cell viability, the Neon Electroporation Transfection System was used. According to the protocols of the supplier, 2x10^6 T cells were transfected by pre-designed siRNA for IFNG-AS1 (final concentration: 20µM, Assay ID: s501414, Ambion). The control group was also set up at the same parameters with Stealth RNAi negative Control Duplexes (final concentration: 20µM, Cat. Number: 12935300, Life Technologies). Afterwards, in order to recover cells, they were incubated at a density of 2x10^6/well in 12-well plates with prewarmed CTS-medium containing IL2 (final concentration 20UI/ml, catalog number: 200-02-50, Pepro Tech) for 24 hours. The viability of cells was assessed by flow cytometry analysis after transfection (see below). The efficacy of siRNA silencing was measured by RT-PCR after 48 hours of transfection (see later).

3.2.10 Co-Culture of HAEC and T-Cells

In order to further investigate the effector functions of the transfected Th1 cells, Th1 cells were stimulated with phorbol 12-myristate 13-acetate (PMA, end concentration 20nM, SIGMA) and ionomycin (end concentration 100nM) (133). PMA is a well-known non-specific potent activator of the protein kinase C (PKC) family(134). Ionomycin is a Ca+ ionophore to induce Ca+ influx, which may increase intracellular Ca+ concentration to further activate PKC(135). Activated PKC catalyzes the phosphorylation of intracellular proteins and activates Th1 cells to release effector cytokines(136).
Furthermore, pre-activated and previously transfected Th1 cells were next transferred to HAECs to initiate the in-vitro co-culture experiments. The rate of dead and apoptotic HAECs was assessed by flow cytometry analysis after 24 hours.

3.2.11.2 Flow Cytometry Analysis of activation and differentiation of T cells

The differentiation of $CD3^+$ T cells into different subtypes is characterized by specific expression of surface markers and therefore the cells were labeled with fluorescence-conjugated antibodies: CCR6 AF488, CCR7 PE, CD3-P-CPeF710, CXCR3-PE-Cy7, CD25 eFluor450, CD45RA eF506, CXCR5 SB 600, CD8 BV711, CCR4 APC, CD4 AF700, CD127–APC-eF780. The activation of $CD3^+$ T cells was labeled following fluorescence-conjugated antibodies: CD45RA AF488, CCR7 PE, PD-1 PerCP-eF710, CD27 PE Cy7, CD57 Pac Blue, CD45 BV605, CD8a BV605, CD28 BV711, CD4 AF647, CD3 APC-eF780.

Four differentiated T cell subgroups were analyzed: Naive cells are characterized by CD45RA and CCR7 positive(137). Effector memory (EM) cells are both negative. Central memory (CM) cells are marked with CCR7 positive and CD45RA negative(137). While terminally differentiated effector memory (TEMRA) cells are marked with CCR7 negative and CD45RA positive(138).
Activated T cells can be distinguished by PD-1 and CD57. PD-1 is expressed on the surface of activated T cells(139). While CD57 negative is a marker for senescence(140).

3.2.11 Flow Cytometry Analysis

Flow cytometry Analysis is an automatic rapid, quantitive, sensitive tool for single cells and biological particles in solution with multiple parameters(141). Flow cytometry Analysis can be used for the characterization of different cell populations by detection of their specific surface CD (Cluster of differentiation) markers, for detection of apoptotic/dead cells and even for cytokine measurements in a solution.
In the current study, the flow cytometry analysis was performed on the Attune Flow Cytometer (Thermo Fischer) and analyzed by the Kaluza Software (Beckman Coulter).

3.2.11.1 Flow Cytometry Analysis for HAEC apoptosis

For the detection of apoptosis, there are several methods: TUNEL staining assay, enzyme-linked immunosorbent assay (ELISA) of cell death detection(142), flow cytometry analysis, Caspase 3-7 staining and examination by light microscopy. TUNEL staining and ELISA assay can detect an important biological feature of apoptotic cells-nucleosomal DNA fragmentation (143). Light microscopy differentiates apoptotic cells and dead cells by morphological features. In the western blot method, apoptotic-related proteins like Cas-9 can be detected(144). However, these four methods can not exactly provide the quantitative detection of apoptotic cells. Caspase 3-7 staining sensitively detects the activity of apoptotic-related proteins like Cas-3 in the early stage of apoptosis. The disadvantage is the poor detection of apoptotic cells at the late stage of apoptosis(145).
In order to quantitatively analyze the ratio of dead and apoptotic HAECs affected by Th1 cells, the flow cytometry analysis was adopted. The apoptosis assay was carried out by an Annexin V/Sytox Orange staining of the cell suspension. Annexin V binds to phosphatidylserine (PS), which is normally located on the inside of the cell membrane. In the initial stages of apoptosis and in dead cells PS is relocated to the outer side of the membrane where it can be marked by Annexin V(146). Therefore, in order to distinguish apoptotic

from dead cells a second staining with Sytox Orange is required, as Sytox orange or other DNA-stains can only penetrate the membrane of dead cells and get into the nucleus. Double-positive cells are then considered dead, while Sytox-negative and Annexin-positive cells are considered apoptotic(147). However, there are still limitations, such as a lack of capability to distinguish dead cells due to apoptosis or necrosis.
In order to determine the rate of apoptosis/death in a suspension of different cells, a cocktail of antibodies against specific CD molecules was used to distinguish between T cells and HAECs: CD4 BV 605, CD45 BV711, CD3 AF488, CD8 PerCp Cy 5.5, CD144 PE-Cy7. The stained cells were measured on the Attune Flow Cytometer (Thermo Fischer) and analyzed by the Kaluza Software (Beckman Coulter).

3.2.11.3 Flow Cytometry Analysis for assessment of viability of T cells

In order to assess the viability of T cells after electroporation. T cells were labeled with fluorescence-conjugated antibodies targeting specific CD molecules: CD4 BV 605, CD45 BV711, CD3 AF488 and CD8 PerCp Cy 5.5, as well as with the already mentioned dies for apoptosis/death: Annexin V and Sytox Orange. Afterwards, the viability rates were measured using flow cytometry analysis as already explained.

3.2.12 Th1/Th2 Cytokine Measurement

Enzyme-linked immunosorbent assay (ELISA) and cytometric bead array (CBA) are commonly used for detecting cytokines in solution. ELISA tests a specific protein in solution by antibodies-coated plates. CBA uses dispersed labeled particles in solution to capture cytokines and then the labeled particles-cytokines complex is detected by the flow cytometer. Compared to ELISA, CBA allows for the measurement of multiple proteins. A CBA offers a high throughput tool for measuring a whole pallet of proteins/cytokines(148).
To assess cytokine production in transfected and pre-activated T Cells, the supernatants were collected after 48 hours and were analyzed using the CBA method following the instruction of Th1/Th2 Cytokine Kit (Catalog number: 551809, BD).

3.2.13 Quantitative Real-Time PCR

The routine method for analyzing the efficacy of transfection is quantitative Real-Time polymerase chain reaction (RT-PCR). PCR is a specific DNA and cDNA fragments amplification technique, enabling visual study of these fragments(149). The cycle threshold (CT) value required for exceeding the specific level of threshold indirectly indicates the

content of targeted DNA and cDNA. By adding a fluorescent dye to the PCR reaction system, RT-PCR monitors the accumulation of fluorescent signals in real-time (150).

3.2.13.1 RNA isolation

One of the biggest challenges in studying RNAs is their instability and degradation due to RNases which are ubiquitous. Therefore, the miRNeasy Micro Kit (Catalogue Number 1071023, Qiagen, Germany) offers a fast, reproducible extraction of total RNA. Qiazol destroys the cell membrane and the nuclear membrane so that RNAs are released. RNA has the max absorption at 260nm (A260) and protein absorbs UV (Ultraviolet) maximally at 280nm (A280). Commonly purity of RNA is evaluated by the A260/A280 ratio (A260/A280>1.8). If the A260/A280 ratio is less than 1.6, it may suggest protein contamination(151).

Therefore, cells were lysed in the QIAzol lysis reagent for 5 minutes. 140µl Chloroform was added to the tubes and mixed well by shaking for 15 seconds. Afterward, according to the manual protocol of miRNeasy Micro Kit, total RNA was purified by centrifugation in spin columns. And then the purity of RNA was verified by NANO Vue Plus (Imagination at Work).

3.2.13.2 cDNA Reverse Transcription

RT-PCR is used for amplifying DNA and cDNA. Besides, The total RNA is instable and therefore needs to be converted into cDNA fort he further steps of the PCR.

500ng total RNA was converted to cDNA using the high capacity cDNA reverse transcription kit (Catalogue number: 4368814, ThermoFisher Scientific)

Table 1: cDNA Reverse Transcription mixture 1

Mixture 1	**Volume**
RNA + H2O	13.2µl
DNAse I	0.5µl
Total	13.7µl

Mixture 1 was transferred to Thermocycler (Eppendorf) and underwentthe program for cDNA synthesis.

Table 2: cDNA Reverse condition for mixture 1

Steps	**Temperature**	**Time**
1	37°C	15 minutes
2	75°C	10 minutes
3	70°C	10 minute

Table 3: cDNA Reverse Transcription mixture 2

Mixture 2	**Volume**
10x Buffer	2µl
25x dNTPs	0.8µl
RNAse Inhibitor	0.5µl
Transcriptase	1µl
Total	4.3µl

Mixture 2 was added to each sample and transferred to the thermocycler to start the next program.

Table 4: cDNA Reverse condition for mixture 2

Steps	**Temperature**	**Time**
1	25°C	10 minutes
2	37°C	120 minutes
3	85°C	5 minute

3.2.13.3 RT-PCR

SYBR Green indicates real-time amplification by binding to double-stranded DNA and generating fluorescence(152).

Therefore, RT-PCR was performed by Real-Time PCR System (Thermo Fischer) with SyBr Select PCR Master Mix kit following the instruction of the supplier.

Table 5: RT-PCR mixture

Mixture	**Volume**
SYBR Green	5µl
Primers	2µl
H2O	1µl
cDNA	1µl
Total	10µl

RT-PCR mixture was added to the PCR plate and transferred to the Real-Time PCR System to start the RT-PCR program.

Table 6: RT-PCR condition

Steps	**Temperature**	**Time**
1	50°C	2 minutes
2(40 cycles)	95°C 95°C 60°C	10 minutes 5 seconds 1 minute
3	95°C 60°C 95°C	15 seconds 1 minute 15 seconds

Table 7: RT-PCR primers

Gene	**Forward primer**	**Reverse primer**
IFNG-AS1	GCTGATGATGGTGGTGGCAATCT	TTAGCAGTTGGTGGGCTTCT
FOSB1	GCAGCAGCTAAATGCAGGAAC	GCCATCTTCCTTAGCCGGTG
JUN	CCAACTCATGCTAACGCAGC	CTCTCCGTCGCAACTTGTCA
CD69	GATGCCACCAGTCCCCATTT	ACCCAGTCCTCAGAGCATGA
IL2RA	GTGGTGGGGCAGATGGTTTA	TTGTGACGAGGCAGGAAGTC
TNFA	CATCCAACCTTCCCAAACGC	CTGTAGGCCCCAGTGAGTTC

3.2.14 Statistical Analysis

Normal distribution of continuous data are tested with the Kolmogorov-Smirnov test. Continuous and normally distributed data are presented as mean ± standard deviation (SD). Continuous data without normal distribution are presented as median with interquartile

range (IQR). Three matched groups of data were analyzed by Dunn's multiple comparison tests. Wilcoxon matched-pairs signed rank test is performed to analyze two nonparametric independent data. ANOVA with the Bonferroni corrections test is adopted when there are two involved factors. All statistical analyses were performed with GraphPad Prism Software. The correlation between the expression of IFNG-AS1 and the cell count is analyzed by Spearman correlation analysis with SPSS. *p*-value <0.05 is regarded as statistically significant.

4. Results

4.1 The different expression patterns of IFNG-AS1 in ACS patients and sCAD patients

In comparison to RFC-ACS patients, we observed a significantly lower IFNG-AS1 expression in PBMCs from IFC-ACS patients(IFC-ACS vs. RFC-ACS: 2.15 ± 0.83 vs. 2.89 ± 0.93; *p*=0.0047). We additionally observed lower expression levels of IFNG-AS1 in IFC-ACS patients when comparing to matched sCAD patients (IFC-ACS vs. sCAD: 2.15 ± 0.83 vs. 2.57 ± 1.14; *p*=0.0342) (Figure 1). In IFC-ACS patients, Spearman's correlation analysis showed a significant negative relationship between the expression of IFNG-AS1 and the absolute cell count for $CD3^+$ T cells (r=-0.6223, *p*=0.005) (Figure 2A), while this correlation was not significant in RFC-ACS patients (r=-0.3835, *p*=0.091) (Figure 2B). A significant positive correlation between the expression of IFNG-AS1 and the absolute cell count of granulocytes and neutrophils in IFC-ACS patients was shown by Spearman's correlation analysis (r=-0.6202, *p*=0.006) (Figure 2C), while we did not found this correlation in RFC-ACS patients (IFC-ACS: r=0.6202, *p*=0.006; RFC-ACS: r=-0.2692, *p*=0.251) (Figure 2D).

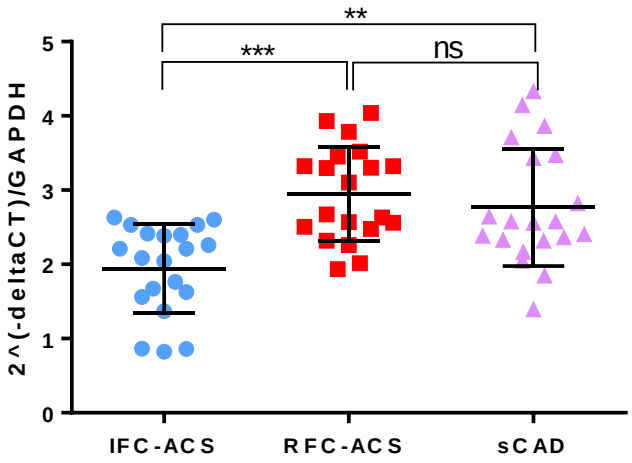


Figure 1: The relative expression of IFNG-AS1 in IFC-ACS patients PBMCs was lower than RFC-ACS patients and sCAD patients. The expression of IFNG-AS1 was normalized to the expression of GAPDH; Dunn's multiple comparison tests; *** p< 0.001, ** p< 0.01, ns p>0.05 (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

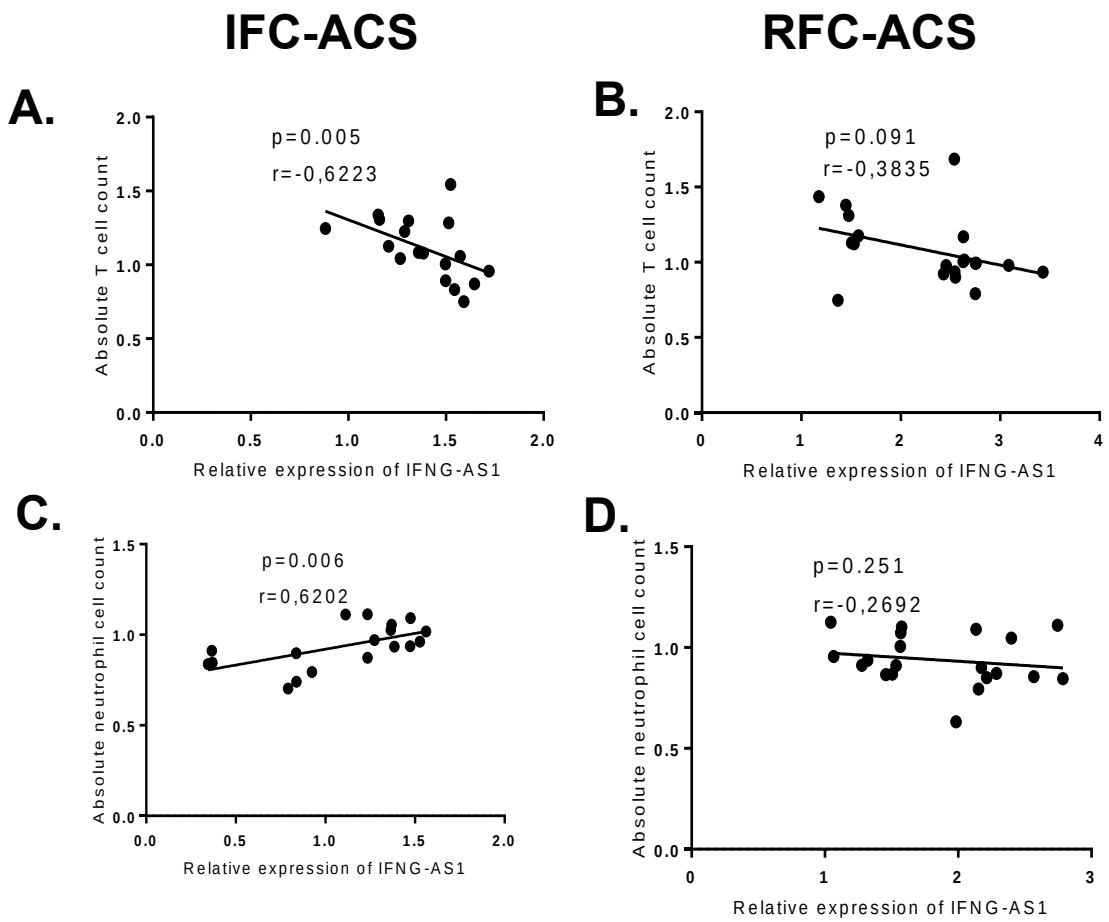


Figure 2: The correlation between the expression of IFNG-AS1 and absolute cell count for $CD3^+$ T cells and the granulocyte and neutrophils in IFC-ACS and RFC-patients. A significant negative correlation between the expression of IFNG-AS1 and the absolute cell count for $CD3^+$ T cells in IFC-ACS patients was indicated by Spearman's correlation analysis (A), while this correlation was not presented In RFC-ACS patients (B). A significant positive relationship between the expression of IFNG-AS1 and the granulocyte and neutrophils absolute cell count in IFC-ACS patients (C), however, there was no significant correlation in RFC-ACS patients (D); Spearman correlation analysis; Statistical significance is deemed when $p < 0.05$. (Figure 2A and 2B modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

4.2 siRNA targeting IFNG-AS1 is efficiently transfected to T cells by electroporation

In order to mimic the expression pattern of IFNG-AS1 in IFC-ACS patients, siRNA targeting IFNG-AS1 was transfected to primary human T cells by electroporation. Upon silencing of IFNG-AS1, RT-PCR showed an over 50% reduction in the expression level of IFNG-AS1 (siIFNG-AS1 vs. siNC: 48.42 ± 27.52% vs. 100%; p=0.0039) (Figure 3). In addition, the viability of T cells was not affected significantly by electroporation (Figure 4).

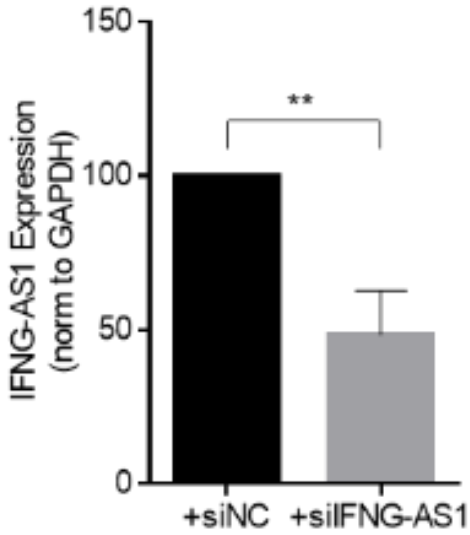


Figure 3: Electroporation with siRNA targeting IFNG-AS1 resulted in decreased expression of IFNG-AS1 in primary human T cells; The expression of IFNG-AS1 was normalized to the expression of GAPDH; Wilcoxon matched-pairs signed rank test; ** $p < 0.01$ (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

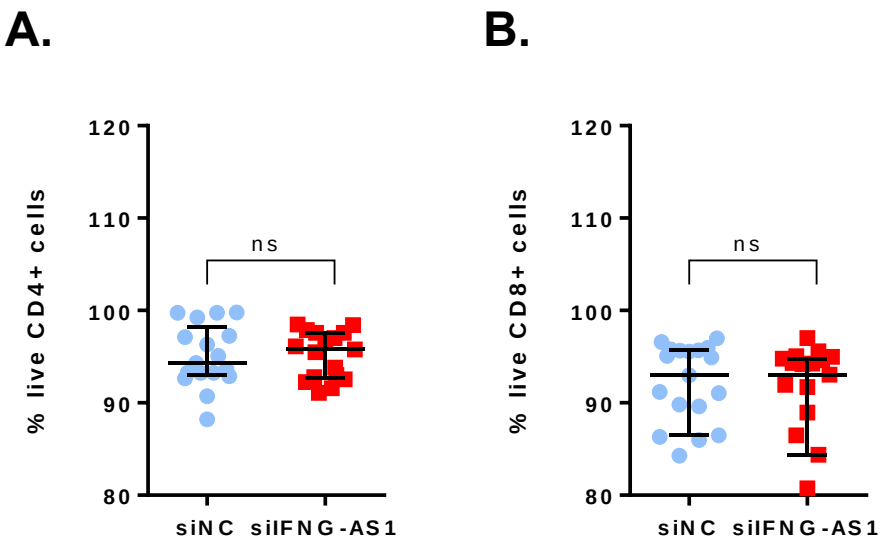


Figure 4: Viability of $CD4^+$T cells and $CD8^+$T cells upon transfection. There is no statistically significant difference in the viability of $CD4^+$ T cells (A) and $CD8^+$ T cells (B) in two groups. Wilcoxon matched-pairs signed rank test; Statistical significance is deemed when $p < 0.05$. (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

4.3 Inflammatory gene expression is suppressed following IFNG-AS1 silencing

IFNG-AS1 was reported to induce inflammatory gene expression in T cells(107). We wondered whether IFNG-AS1 silencing would lead to the reduction of inflammatory gene expression. Subsequently, we found an obvious reduction in expression of inflammatory genes, IFNG and CD69, in siIFNG-AS1 group (IFNG: siIFNG-AS1 vs. siNC: 72.65 ± 33.71% vs. 100%, p=0.0391, Figure 5A; CD69: IFNG: siIFNG-AS1 vs. siNC: 84.18 ± 15.57% vs. 100%, p=0.0078, Figure 5B). However, inflammatory genes including FOSB1, JUN, TNFA, and IL2RA were not significantly affected after IFNG-AS1 silencing (Figure 6A-D).

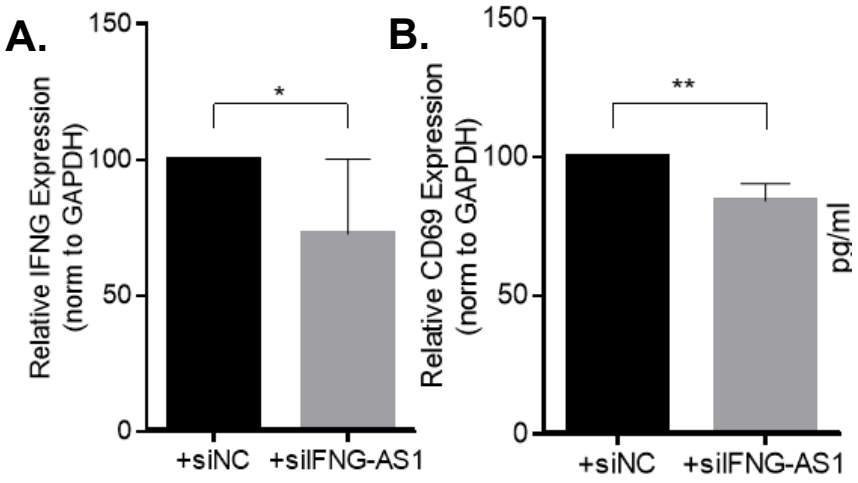


Figure 5: The relative expression of inflammatory genes IFNG and CD69 in the siIFNG-AS1 group and the negative control. The expression of inflammatory genes IFNG (A) and CD69 (B) reduced after IFNG-AS1 silencing; The expression of genes was normalized to the expression of GAPDH; Wilcoxon matched-pairs signed rank test; ** p< 0.01 * p< 0.05. (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

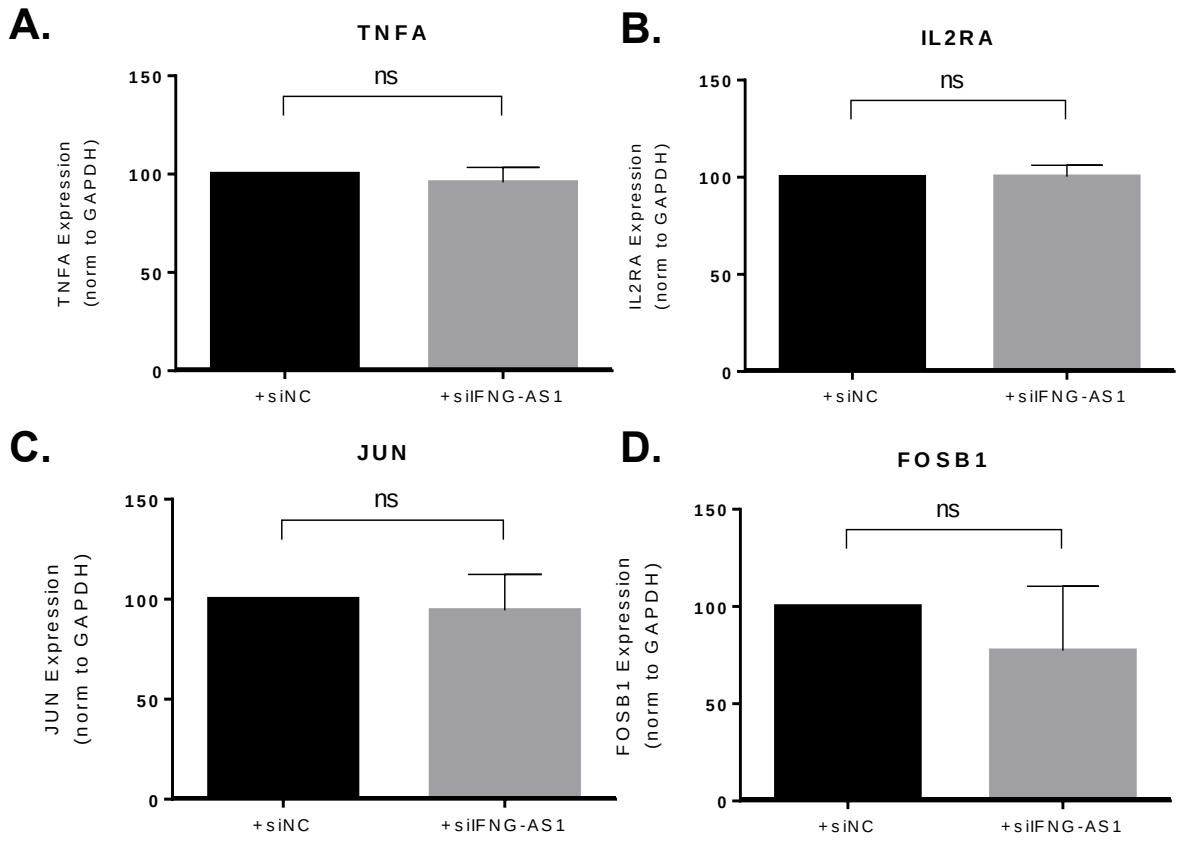


Figure 6: The relative expression of inflammatory genes TNFA, FOSB1, IL2RA, and JUN in the siIFNG-AS1 group and the negative control. Silencing IFNG-AS1 did not induce significant changes in the expression of inflammatory genes, TNFA (A), IL2RA (B), JUN (C) and FOSB1 (D); The expression of genes was normalized to the expression of GAPDH; Wilcoxon matched-pairs signed rank test; ns $p>0.05$ (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

4.4 IFNG-AS1 silencing leads to a reduction in subsequent production of inflammatory cytokines

IFNG-AS1 silencing resulted in decreased expression of IFNG, we questioned whether inflammatory cytokines corresponding to IFNG would be also suppressed. Similarly, the production of IFNγ was also observed to be highly suppressed following IFNG-AS1 silencing. (siIFNG-AS1 vs. siNC: 2023 ± 1037 vs. 2911 ± 937.2, p=0.0005) (Figure 7). While there were no significant changes in the expression of other cytokines, IL10, IL4, IL5 and IL2 (Figure 8).

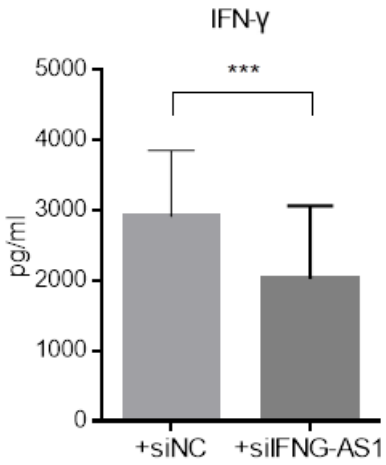


Figure 7: The production of IFN-γ was highly suppressed in the siIFNG-AS1 group compared with the negative control; Wilcoxon matched-pairs signed rank test; *** p< 0.001 (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

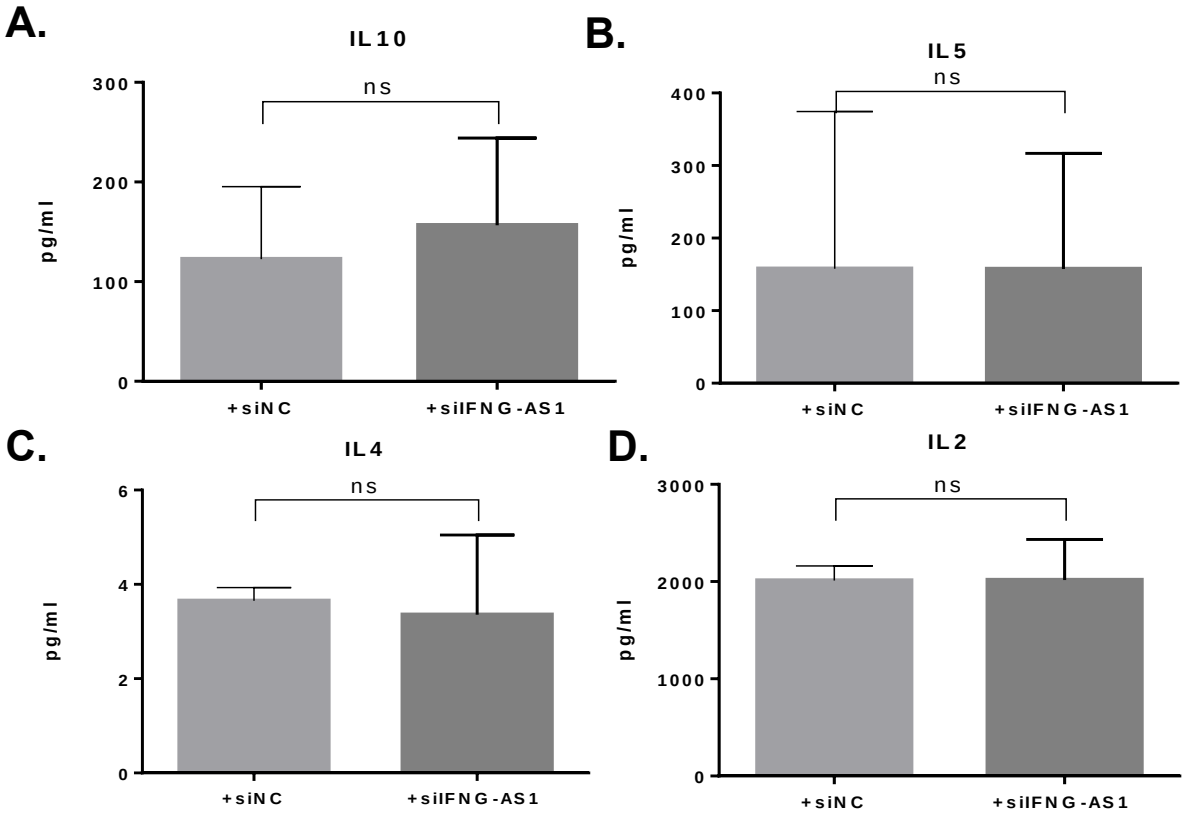
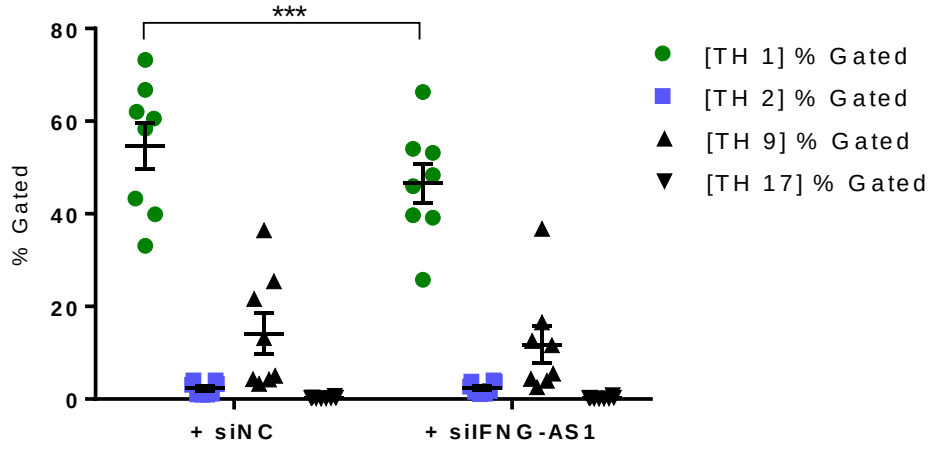


Figure 8: The concentration of cytokines, IL2, IL5, IL4 and IL10 in the IFNG-AS1 siIFNG-AS1 group and the negative control. Upon IFNG-AS1 silencing, there were no significant changes in the production of IL10 (A), IL5 (B), IL4 (C) and IL2 (D); Wilcoxon matched-pairs signed rank test; ns p>0.05. (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

4.5 Silencing IFNG-AS1 reduces the amount of Th1 cells

Th1 cells are the main T lymphocyte subtype cells to produce IFNγ(153). In order to clarify whether the reduction of IFNγ was also induced by the decreased Th1 cell numbers, we measured the number of T-cell subtypes after transfection. We found a significantly decrease in Th1 cell count after IFNG-AS1 silencing (siIFNG-AS1 vs. siNC: 65.89 ± 20.13% vs. 68.43 ± 18.56, *p*=0.0005 (Figure 9A). However, IFNG-AS1 silencing did not influence the Treg cell count (Figure 9B) and memory subtypes (Figure 10A). There was also no difference in memory subtype count of $CD8^+$ T cells following IFNG-AS1 silencing (Figure 10B).

A.



B.

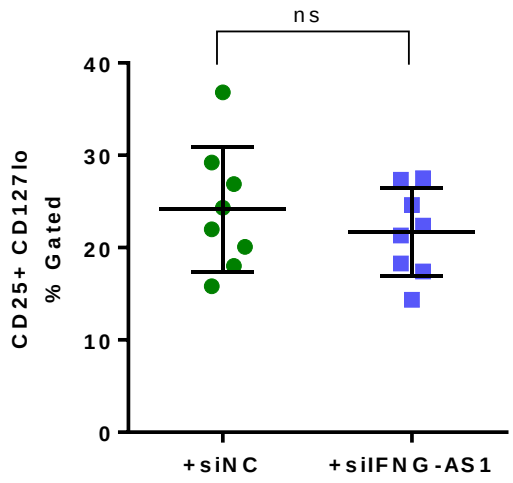
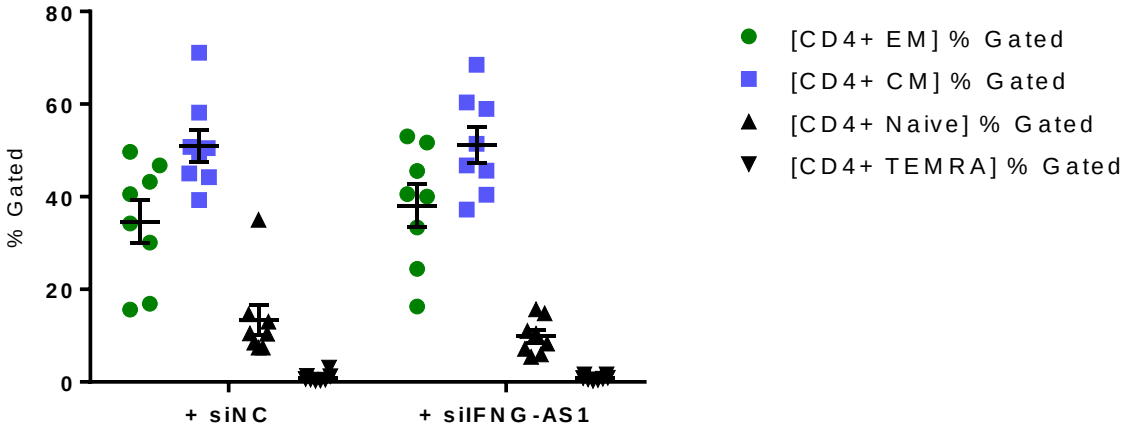


Figure 9: Silencing effect of IFNG-AS1 on the amount of Th1, T regs and Th1 subtypes. Th1 cell count was significantly impaired by IFNG-AS1 silencing (A), while Treg cell count was not affected

(B); Repeated ANOVA with Bonferroni's multiple comparison test; *** p < 0.001 ns p>0.05. (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

A.



B.

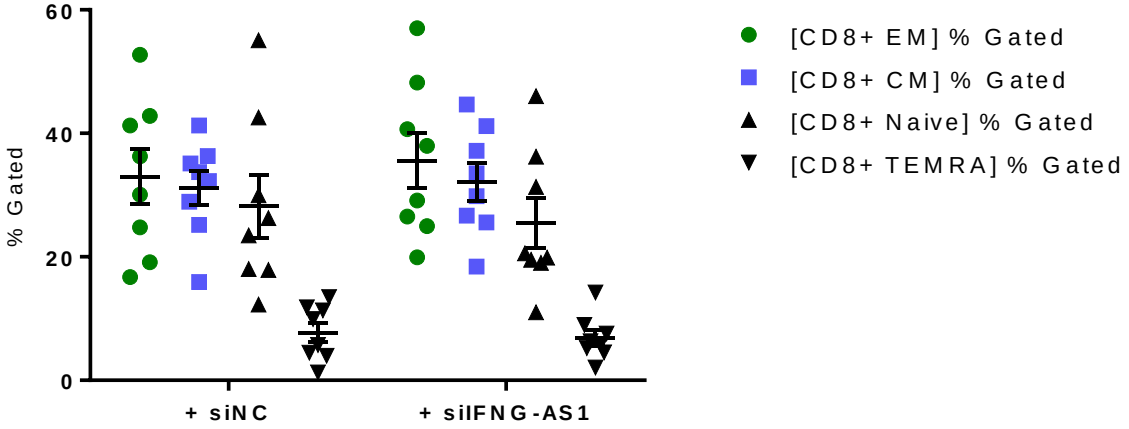


Figure 10: Silencing IFNG-AS1 didn't show a significant effect on the count of memory subtypes of $CD4^+$ T cells (A) and $CD8^+$ T cells (B); Repeated ANOVA with Bonferroni's multiple comparison test. (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

4.6 IFNG-AS1 silencing results in lower T-cells-mediated cytotoxicity

T cells mediated cytotoxicity in ACS patients by producing IFNγ (153). IFNG-AS1 silencing induced decreased expression of IFNγ. So we wanted to figure out if decreased IFNG-AS1 would result in reduced T-cells-mediated cytotoxic effects and inflammation. To investigate this assumption, we co-cultured transfected T cells with endothelial cells and found, upon silencing IFNG-AS1, the dead rate of endothelial cells was reduced

(48.63±54.39% in siIFNG-AS1 as compared to 55.3 ±63.83% in siNC), while the apoptotic rate was enhanced (18.01±46.12% in siIFNG-AS1 as compared to 8.07±40.51% in siNC) (Figure 11), indicating lower cytotoxicity mediated by T cells.

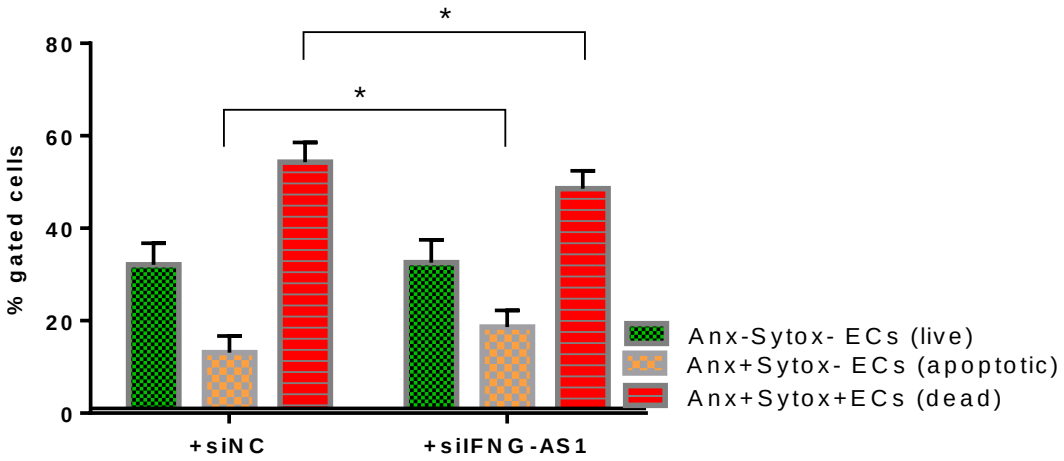


Figure 11: Silencing IFNG-AS1 resulted in increased apoptotic rate and decreased death rate of HAECs co-cultured with transfected T cells; Repeated ANOVA with Bonferroni's multiple comparison test; * $p < 0.05$. (modified according to the original manuscript, Meteva, Y Zhao et al, 2024)

5. Discussion

In the current study, a reduced expression of the lncRNA IFNG-AS1 was observed in patients with IFC-ACS, leading to decreased amount of pro-inflammatory Th1 cells with lower capacity of IFNγ -production and decreased cytotoxicity towards endothelial cells. Hence, we postulate that the downregulation of IFNG-AS1 might be an anti-inflammatory response aiming to compensate excessive activation of T cells in IFC-ACS, as we previously observed an increased T cell activation and cytotoxicity at the culprit lesion site of IFC-ACS patients(58).

5.1 Function of IFNG-AS1 in the context of ACS and other diseases

IFNG-AS1 has been documented as both a pro-inflammatory and an anti-inflammatory contributor across different diseases: RA, inflammatory bowel diseases (IBD), SS, HT and myasthenia gravis (MG) (61, 109, 110, 112, 154). However, little is known about its precise contribution to ACS, particularly to IFC-ACS. Therefore, the current study aims to provide a more comprehensive characterization of the functional properties of IFNG-AS1 in this context.

5.2 Expression of IFNG-AS1 in IFC-ACS and in other diseases

Currently, we observe a downregulation in the expression of IFNG-AS1 in local PBMCs from IFC-ACS patients compared to sCAD and RFC-ACS. Correspondingly, Rafiei et al. noted a similar expression pattern of IFNG-AS1 in CAD patients when compared to the healthy control group(106). In contrast, Xu et al. reported a higher expression of IFNG-AS1 in CAD patients compared to individuals suspected of CAD without stenosis in the coronary artery(107). The divergence among these results might possibly be attributed to the different studied populations (CAD, ACS and healthy subjects) as well as different cell types being analysed. Additionally, we are currently comparing two different ACS pathophysiologies: IFC-ACS and RFC-ACS. Furthermore, the currently described downregulation of IFNG-AS1 was exclusively observed in local PBMCs from occluded coronary arteries, whereby Xu et al. analyzed peripheral blood samples, which may explain the different results.

In other diseases, such as ITP, Ali et al. postulated that the higher expression of IFNG-AS1 is driven by the autoimmune character of the disease marked by Treg cell

dysregulation and therefore lacking of anti-inflammatory mechanisms to resolve inflammation(155). Notably, in our current study, IFNG-AS1 originates from PBMCs, which comprise not only T lymphocytes but also monocytes, B cells, NK cells and dendritic cells(156). Therefore, these results suggest that the cell type, where IFNG-AS1 originates from, may be important for its functional properties.

For instance, Eomes, a crucial transcription factor (TF), plays a significant role in the differentiation and maturation of NK cells. Increased exposure to IL12+IL18 leads to reduced expression of Eomes in immature NK cells, resulting in significantly lower expression of IFNG-AS1 (157). While on the other hand, T cells upregulate IFNG-AS1 in order to release more IFNγ during their polarization to Th1 cells (61). Hence, analyzing the source of IFNG-AS1 in IFC-ACS seems to be important in order to properly understand the results.

5.3 Expression of IFNG-AS1 in different cell types

Therefore, we employed Spearman correlation analysis between the IFNG-AS1 expression patterns and the different cell counts in the blood samples. The IFNG-AS1 expression was currently analyzed in PBMCs, which contain monocytes, T cells, B-cells and NK-cells, but no granulocytes. Interestingly, IFNG-AS1 displayed a positive correlation with the absolute cell count of granulocytes and neutrophils, and a negative correlation with the absolute counts of total $CD3^+$ T cells (including $CD4^+$ and $CD8^+$ T cells), which may be due to the fact that T-cells may change their chemotaxis properties towards neutrophils depending on IFNG-AS1 expression. For example, IFNG-AS1 was reported to enhance the expression of IFNγ in T cells and thereby leading to accumulation of of neutrophils (108, 119, 158). Furthermore, Peng et al. demonstrated that silencing IFNG-AS1 in $CD4^+$ T cells led to lower abundance and lower production of $IFN\gamma^+$ T cells (110), suggesting an important role of IFNG-AS1 in T cell proliferation, differentiation and activation.

5.4 Effect on T cell proliferation, T cell subtype count and survival

In this context, we tested the effect of IFNG-AS1 on the count of T cell subtypes and observed a reduction in the pro-inflammatory Th1 subtype. While no significant effects were observed on the amount of memory subtypes as analyzed by surface markers of activated T cells. Similarly, Wang et al. identified a reduction in the percentage of Th1

cells when silencing IFNG-AS1 in $CD4^+$ T cells purified from healthy individuals(109). We postulate that the decreased Th1 cell count might be attributed to either the vulnerable of Th1 cells to IFNG-AS1 silencing or a reduced differentiation specifically into Th1 cells. Although we measured the viability of total $CD4^+$ T cells, we did not test the viability of individual $CD4^+$T cell subtypes. Therefore, we can not rule out the possibility that the decreased Th1 cell count was caused by a higher death rate of Th1 cells due to their vulnerability to IFNG-AS1 silencing. Decreased differentiation specifically into Th1 cells might be explained by reduced activation effect of IFNG-AS1 on Th1-specific transcription factors (TFs). For instance, Collier et al. demonstrated that IFNG-AS1 increases the production of IFNγ in order to activate Th1-specific TFs(159). Consequently, the deficiency of IFNG-AS1 may impair the differentiation of Th1 cells driven by Th1-specific TFs.

Correspondingly, the IFNG-AS1-silenced T cells produce lower levels of IFNγ, as Th1 cells are described as the primary source of IFNγ in ACS patients(153, 160, 161).

On the other hand, we found that IFNG-AS1 abrogation did not affect the amount of $CD8^+$ T cells and their memory subtypes. The lack of effect on $CD8^+$ T cells might be due to a relatively low expression of IFNG-AS1 in CD8 cells in general (119). Therefore, we believe that IFNG-AS1 plays a neglectable role in $CD8^+$ cell activation and their memory subtype count as stated by other studies as well(110, 119).

5.5 Effect on activation of T cells

Despite the absence of effects on the count of $CD4^+$ T cell memory subtypes, we observed an effect on T cell activation in co-culture with endothelial cells upon IFNG-AS1 silencing. $CD4^+$ T cells have been reported to have cytotoxic effects on ECs, leading to apoptosis and EC death, ultimately resulting in plaque destabilization(162-164). IFNG-AS1 depletion may lead to the incapability of T cells to induce apoptosis and endothelial cell death due to reduced numbers of pro-inflammatory Th1 cells and lower release of IFNγ as shown by the current study.

In this context, our results also illustrated that the downregulation of IFNG-AS1 led to a reduced transcription level of CD69 in T cells. CD69 serves as a recognized marker to reflect T-cell activation and migration to inflamed tissue(165, 166). Similarly, Rankin et al. showed that the conditioned medium of T cells, overexpressing IFNG-AS1, led to a higher expression of CD69 in PBMCs through increased pro-inflammatory cytokines like IL2(167). Furthermore, a decrease in the activation surface marker CD69 may also

indicate impaired transmigration properties of transfected T cells, as shown for inflammatory lesions in colitis, where infiltrated T cells had a higher CD69 expression (168).

Even on the transcription and translational level, we observe an effect upon IFNG-AS1 silencing with a noticeable decrease in the expression of the IFNG gene and subsequent secretion of IFNγ. In line with our findings, patients with HT(110) and MS(62) both manifested a positive association between the expression of IFNG-AS1 and IFNG. Additionally, in vitro experiments demonstrated that the silencing of IFNG-AS1 led to a reduction in IFNG expression in T cells(110, 169). We presume the epigenetic modification of IFNG and the specific structure of IFNG-AS1 might account for this result. Specifically, in $CD8^+$ T cells, IFNG-AS1 has been reported to undergo epigenetic modification by binding with protein WRD5, which facilitates the methylation of histone H3 at lysine 4 (H3K4). The methylation of H3K4 at the IFNG locus results in enhanced expression of IFNG(108). Moreover, the specific structure, such as CCCTC binding factor (CTCF) in IFNG-AS1 is crucial for the expression of IFNG. CTCF in IFNG-AS1 may serve as an insulator, ensuring the physical proximity and binding between IFNG-AS1 and IFNG(170). It has been reported that eliminating the CTCF from IFNG-AS1 leads to a reduction in the expression of IFNG and its corresponding cytokines(170).

However, our results did not demonstrate a significant impact on other cytokines, such as IL10, IL4, IL5 and IL2. In agreement with our results, Rankin et al. did not detect a significant difference in the expression of TNFα and IL10, following overexpression of IFNG-AS1 in Jurkat T cells(167).

Overall, the decline of transcription in CD69- and other inflammatory genes, as well as in the pro-inflammatory cytokine release further supports our hypothesis that the downregulation of IFNG-AS1 may have anti-inflammatory properties.

5.6 Limitations and strengths of the study

In order to overcome the resolution limitations of contemporary intravascular imaging, we employed high-resolution OCT examination to distinguish between two distinct ACS path-ophysiologies: IFC-ACS and RFC-ACS. Additionally, our study 's prospective design builds upon our previous OPTICO-ACS-study(58). The current study further investigated the anti-inflammatory regulatory effect of lncRNA IFNG-AS1 on T cell activation in IFC-

ACS patients. This research may provide additional insights into the pathological mechanisms of IFC-ACS patients and presents a promising intervention target.

However, despite the potential therapeutic benefits, challenges such as compromised pathogen defense exist. The current study also has certain limitations. First of all, our study did not use single-cell techniques and thereby could link IFNG-AS1 expression to T cells only by association. Single-cell techniques would provide an exact information about the cell origin of IFNG-AS1. Secondly, our study was restricted by a relatively small sample size and a caucasian population. Large-scale and cross-sectional clinical trials are required to further validation of our results. Thirdly, we tested the gene expression level but not the protein expression of CD69. Therefore, the currently observed regulation of the CD69 gene may not completely represent the activation states of T cells.

6. Conclusions

Our study suggests a potential anti-inflammatory regulative effect of IFNG-AS1 on T cell activation in IFC-ACS. It points to a promising therapeutic target in IFC-ACS patients aiming at reduction of T-cell-mediated inflammation. Future larger translational studies are required to validate the in-vivo anti-inflammatory effects of IFNG-AS1 on ACS patients and especially on IFNG-AS1 patients.

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Statutory Declaration

I, [Yingjie Zhao], by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [The effect of the long non-coding RNA IFNG-AS1 on T cells in Acute Coronary Syndrome with Intact Fibrous Cap/Die Wirkung der langen nicht-kodierenden Ribonukleinsäure IFNG-AS1 auf T-Zellen beim akuten Koronarsyndrom mit intakter fibröser Kappe], independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

13.05.2024

Date Signature

Declaration of your own contribution to any publications

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Yingjie Zhao contributed to the following manuscript:

Manuscript 1: Meteva D*, Y Zhao*, Seppelt C, Abdelwahed Y, Gerhardt T, Häfner K, Schuhmacher B, Strässler E, Rauch-Kröhnert U, Skurk C, Haghikia A, Kratzer A, Giral H, Joner M, Rai H, Klotsche J, Kränkel N, Landmesser U, Zeiher AM, Dimmeler S, Leistner DM* and Abplanalp WT*. The lncRNA IFNG-AS1 Modulates T Cell Activation in Acute Coronary Syndrome with Intact Fibrous Cap- Insights from the OPTICO-ACS Study. (in submission) *These authors contributed equally to this work.

Detailed contribution:

He collected data independently, including (such as cell count, FACS results, results of PCR and cytokine production etc.)

He analyzed data independently (for example, analyses of PCR results and analyses of FACS results etc.)

He conducted statistical analyses with the assistance of the supervisor.

He wrote the manuscript together with Meteva D., ensuring accurate interpretation of results with the help of the supervisor.

He addressed the feedback of reviewers with guidance from the supervisor.

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Signature, date and stamp of first supervising university professor / lecturer

_____

Signature of doctoral candidate

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publications

[1] Meteva D, Vinci R, Seppelt C, Abdelwahed YS, Pedicino D, Nelles G, Skurk C, Haghikia A, Rauch-Kröhnert U, Gerhardt T, Straessler E, Zhao Y, Golla F, Joner M, Rai H, Kratzer A, Arnal HG, Liuzzo G, Klotsche J, Crea F, Landmesser U, Leistner DM, Kränkel N. Toll-like receptor 2, hyaluronan, and neutrophils play a key role in plaque erosion: the OPTICO-ACS study. Eur Heart J. 2023 Jun 29:ehad379.

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