

**THERAPEUTIC POTENTIAL OF AN IMMUNOSUPPRESSIVE
OLIGODEOXYNUCLEOTIDE ENCAPSULATED WITHIN
LIPOSOMES ON BLEOMYCIN-INDUCED MOUSE MODEL OF
LUNG INFLAMMATION AND FIBROSIS**

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
MASTER OF SCIENCE
IN
MOLECULAR BIOLOGY AND GENETICS

By
Gizem Kılıç
May 2019

**THERAPEUTIC POTENTIAL OF AN IMMUNOSUPPRESSIVE
OLIGODEOXYNUCLEOTIDE ENCAPSULATED WITHIN LIPOSOMES ON
BLEOMYCIN-INDUCED MOUSE MODEL OF LUNG INFLAMMATION AND
FIBROSIS**

By Gizem Kılıç

May 2019

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

İhsan Gürsel (Advisor)

Kamil Can Akçalı

Can Naci Kocabaş

Ali Osmay Güre

Onur Çizmecioğlu

Approved for the Graduate School of Engineering and Science

Ezhan Karaşan

Director of the Graduate School of Engineering and Science

ABSTRACT

THERAPEUTIC POTENTIAL OF AN IMMUNOSUPPRESSIVE OLIGODEOXYNUCLEOTIDE ENCAPSULATED WITHIN LIPOSOMES ON BLEOMYCIN-INDUCED MOUSE MODEL OF LUNG INFLAMMATION AND FIBROSIS

Gizem Kılıç

M.Sc. in Molecular Biology and Genetics

Advisor: İhsan Gürsel

May 2019

Systemic sclerosis (SSc) is an autoimmune/autoinflammatory disease with unknown etiology. It is characterized by vascular dysfunction, inflammation and disseminated fibrosis of skin or internal organs. Although its prevalence is low, development of fibrosis on internal organs and lack of a curative treatment result in high morbidity. Current therapies targeting specific symptoms such as interstitial lung disease, Raynaud's phenomenon and pulmonary arterial hypertension are inefficient, and at best, temporarily relieves the symptoms throughout the course of the treatment. Herein, we investigated the therapeutic potential of an immunosuppressive oligodeoxynucleotide expressing TTAGGG telomeric repeats which is known as the "A151 ODN" on bleomycin-induced mouse model of systemic sclerosis. A151 ODN is the single stranded synthetic form of the telomeric repeat sequence expressed on mammalian chromosome, and it contains four repeats of "TTAGGG" motif. In order to enhance the therapeutic effectivity while protecting its digestion from nuclease activity following administration, we encapsulated A151 ODN within anionic liposomes. Since pattern recognition receptors and their signaling pathways were demonstrated to initiate inflammation in SSc, we first explored the immunosuppressive capacity of A151 ODN by analyzing in vitro cytokine productions and surface marker expression levels. Similar with the previous findings, A151 ODN was highly potent to abolish cytokine production in response to TLR9 induction. Although A151 ODN by itself was not very effective to suppress cytokine secretion following TLR1/2 and TLR4 induction, encapsulation within anionic liposomes further improved the immunosuppressive potential in response to TLR engagement. Furthermore, flow cytometry analyses revealed that A151 ODN decreased antigen presentation capacity and activation of bone-marrow derived macrophages (BMDMs) in response to TLR stimulation which was

demonstrated by the reduction in levels of surface MHCII and co-stimulatory molecules as well as proteins having role on macrophage adherence and migration. A151 ODN also inhibited transcription of two major genes known to play a critical role on the development of fibrosis, TGF β and Col1a1, from fibroblasts. Following these promising results on A151 ODN's immunosuppressive and anti-fibrotic potential, we tested its therapeutic role on bleomycin-induced lung inflammation and fibrosis on mice which reflects different phases of systemic sclerosis. First in vivo experiment that A151 ODN was used prior to bleomycin administration revealed that A151 ODN could prevent development of systemic sclerosis by reducing immune cell recruitment into alveolar space and suppressing the secretion of inflammatory cytokines. After that, we investigated if A151 ODN could abolish established lung inflammation triggered by bleomycin instillation. For that, we treated animals with an A151 ODN either in free form or encapsulated within anionic liposomes after lung inflammation was initiated following bleomycin instillation. Data indicated that A151 ODN reduced macrophage activation marker expressions, monocyte and neutrophil infiltration into alveolar space. Moreover, suppression on immune cells activation in bronchoalveolar lavage fluid (BALF) correlated with the inhibited cytokine production. As a result of reduced inflammation, pro-fibrotic gene expressions were less in A151 ODN-treated mice. Of note, liposomal encapsulation provided reduced gene expressions while failed to further enhance the immunosuppressive potential on surface marker expression or cytokine secretion of A151 ODN. Lastly, we tested whether treatment with liposome-encapsulated A151 ODN is still effective to regress fibrosis once it has been developed; therefore, we treated mice with single injection of liposomal A151 on different time points. Unfortunately, single instillation was insufficient to decrease fibrosis and macrophage activation as well as cytokine production. Taken together, our findings indicated that liposome-encapsulated A151 ODN is very potent to attenuate the lung inflammation whereas single injection was ineffective to regress established lung fibrosis.

Keywords: A151 ODN, systemic sclerosis, liposomes, immunosuppressive, inflammation, fibrosis

ÖZET

LİPOZOMA YÜKLENMİŞ İMMÜNBASKILAYICI BİR OLİGODEOKSİNÜKLEOTİDİN BLEOMİSİN İLE FAREDE OLUŞTURULMUŞ AKCİĞER ENFLAMASYONU VE FİBROZU ÜZERİNDEKİ TEDAVİ EDİCİ POTANSİYELİ

Gizem Kılıç

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: İhsan Gürsel

Mayıs 2019

Sistemik skleroz (SSc), oluşum nedeni bilinmeyen bir otoimmün/otoenflamatuvar hastalığı olup damarsal işlev yetersizlikleri, enflamasyon ve deri ya da iç organlara yayılan fibrozis ile karakterizedir. Görülme sıklığı düşük olmasına rağmen, iç organlarda gelişen fibrozis ve iyileştirici bir terapinin bulunmaması ölüm oranının yüksek olmasına yol açmaktadır. Günümüzde kullanılan tedavi yöntemleri; interstisyel akciğer hastalığı, Raynaud fenomeni ve pulmoner akciğer hipertansiyonu gibi belirli semptomları hedeflemekte ve ancak tedavi sürdürüldüğü sürece bu semptomlar rahatlatılabilmektedir. Bu çalışmada, telomerik TTAGGG dizisini barındıran immünbaskılayıcı bir oligodeoksinnükleotid olan A151 ODN'nin bleomisin uygulamasıyla farede oluşturulmuş sistemik skleroz oluşumundaki tedavi edici etkisini inceledik. A151 ODN, memeli kromozomundaki telomerik bölgeden köken alan ve 4 tekrarlı "TTAGGG" motifinin tek sarmallı sentetik formudur. Bu çalışmada A151 ODN, terapötik etkisini arttırmak ve vücuttaki nükleaz atağından korunmak için aniyonik lipozomlara yüklenerek kullanılmıştır. Örüntü tanıma reseptörlerinin ve bunların sinyal yollarının SSc'de görülen enflamasyonu başlattığının belirlenmesi üzerine öncelikle A151 ODN'nin immünbaskılayıcı özelliklerini *in vitro* hücre kültür ortamında immün hücrelerinin sitokin üretimini ve yüzey belirteç düzeylerini belirledik. Geçmişteki bulgulara benzer olarak A151 ODN, TLR9 etkinleşmesini ve buna bağlı olarak da sitokin salımını güçlü bir şekilde bastırdı. A151 ODN TLR1/2 ve TLR4 indüklenme yanıtlarını durdurmada tek başına fazla etkili olmazken, aniyonik lipozomlara yüklenerek bu TLR'lerin etkinliğini bastırmada daha etkili olduğu saptanmıştır. Dahası, akan hücre ölçer analizleri, A151 ODN'nin kemik iliğinden oluşturulan makrofajlarda (BMDM) TLR uyarımına karşı antijen sunum kapasitesi ile eş uyaran etkinleşmesi, MHCII düzeyinin yanında makrofaj tutunması ve göçünde rol alan protein seviyelerini azalttığını göstermiştir. Bunun yanı sıra A151 ODN fibroblastlarda iki önemli

fibrotik gen olan TGF β ve Colla1 transkripsiyonunu da azaltmıştır. A151 ODN'nin immünbaskılayıcı ve fibrozis karşıtı etkilerini gösteren umut verici sonuçların ardından bu moleküllü bleomisin ile farede oluşturulan akciğer enflamasyon ve fibrozis modelinde denedik. A151 ODN'nin bleomisin uygulamasından 3 gün önce farelere verildiğinde, bronko-alveolar lavaja geçen immün hücre miktarı ile enflamatuvar sitokin salımını azalttığı belirlenmiştir. Bu öncü sonuçlar bize A151 ODN'nin sistemik skleroz oluşumunu engelleyebileceğini göstermiştir. Daha sonra, A151 ODN'nin bleomisin kaynaklı oluşmuş akciğer enflamasyonunu geriletip geriletemeyeceğini araştırdık. Bu çalışmalarda A151 ODN'i farelere serbest ya da aniyonik lipozom içerisine yüklenmiş halde akciğer enflamasyonu oluşturulduktan sonra intraperitoneal yolla enjekte ettik. Elde edilen sonuçlar A151 ODN'nin makrofajlardaki etkinleşmeyi bastırdığını ve alveolar bölgeye nötrofil ve monosit akümülayonunu azalttığını gösterdi. Bronkoalveolar lavaj sıvısına (BALF) geçen immün hücrelerinden salınan sitokin düzeyinde de gerileme olduğu tespit edildi. A151 ODN ile tedavi edilen farelerde enflamasyondaki azalma pro-fibrotik gen ifadelerinde de düşüşe yol açmıştır. A151 ODN'nin lipozom içine yüklenmesi pro-fibrotik gen ifadelerinde düşüşe yol açarken, hücre yüzey belirteçlerine veya sitokin salımı üzerine olan immünbaskılayıcı etkilerine katkı sağlamadığı görülmüştür. Son olarak, lipozoma yüklenmiş A151 ODN'nin tek enjeksiyon ile fibrozis oluştuktan sonra yaralanmayı geriletip geriletemeyeceğini araştırdık. Bu çalışmada farelere bleomisin instilasyonundan sonra A151 ODN'i farklı zamanlarda uyguladık. Ne yazık ki, elde edilen sonuçlardan tek doz tedavinin fibrozisi, makrofaj etkinleşmesini ve sitokin üretimini baskılamakta yetersiz kaldığı anlaşılmıştır. Sonuç olarak bu çalışmadan elde edilen bulgular lipozoma yüklenmiş A151 ODN'nin akciğer enflamasyonunu azaltmakta yeterli olduğunu ancak tek seferlik tedavi uygulamasının geç dönemde fibrozisi önlemekte yetersiz kaldığını göstermektedir.

Anahtar Sözcükler: A151 ODN, sistemik skleroz, lipozomlar, immünbaskılayıcı, enflamasyon, fibrozis



To my precious family,

Acknowledgements

First of all, I would like to express my gratitude to my supervisor Prof. İhsan Gürsel for his guidance, patience and endless support. In addition to be a great scientific mentor, he is also a life mentor for me. It has been a privilege to carry out my master's degree thesis studies in his lab and learn from him.

I appreciate Assoc. Prof. Ali Osmay Güre, Asst. Prof. Onur Çizmecioğlu, Prof. Kamil Can Akçalı and Prof. Can Naci Kocabaş for being jury members in my thesis defense and their invaluable suggestions.

I would like to thank Banu Bayyurt Kocabaş for her knowledge, help and support, especially during the first year of my masters studies. I want to give many thanks to my sweet home mate and old member of THORLAB, Havva Özgen Kılıgöz, for her invaluable support and friendship. It is a great honor for me to be a part of THORLAB family. I want to thank my labmates Göksu Gökberk Kaya, Muzaffer Yıldırım, Tuğçe Canavar Yıldırım, Bilgehan İbibik, Tamer Kahraman, Fehime Eroğlu Kara, Pınar Gür Çetinkaya for their companionship and understanding in the lab during my studies. I am also grateful to İrem Evcili and Naz Bozbeyoğlu for their help, friendship and support. We have shared so many things and collected valuable memories with them that I will cherish forever. I am indebted to my dearest lab mate Özlem Bulut for always motivating and supporting me. She is a great lab partner and an everlasting friend that I can share many things. I cannot imagine how I would survive in the lab without her.

I feel so lucky to make a friend like Said Tiryaki, and I am very thankful to him for friendship and cheering me up. I would like to thank Dr. Seda Sabah Özcan for her companionship and support.

I would like to share my thanks to Dr. Gamze Aykut and Ulaş Saçını for their invaluable helps during animal experiments, Abdullah Ünnü and Okan Erşahan for their support in technical problems and Yavuz Ceylan for being very nice to me and his entertaining conversations.

Last but not least, my greatest thanks are for my family, my father Adnan and my mother Okşan for their everlasting support, guidance and confidence that I always feel. I am indebted for what they have done for me. Also, I am grateful to have a wonderful brother Berkay, who makes me laugh all the time. Without them, I would not be at the point where I am now.

Contents

ABSTRACT.....	II
ÖZET	IV
ACKNOWLEDGEMENTS	VII
CONTENTS.....	VIII
LIST OF FIGURES	XII
LIST OF TABLES	XIVV
ABBREVIATIONS	XV
CHAPTER 1.....	1
1. INTRODUCTION.....	1
1.1 The Immune System	1
1.1. Innate Immunity and Pattern Recognition Receptors (PRRs)	2
1.1.1.1. Toll-like Receptors (TLRs).....	3
1.2 Suppressive Oligodeoxynucleotides	4
1.2.1 A151 ODN	5
1.2.1.1 Physical and Chemical Properties	5
1.2.1.2 Mechanism of Action.....	6
1.2.1.3 A151 as a therapeutic ODN	7
1.3 Liposomes.....	8
1.3.1 General Properties	8
1.3.2 Liposomes Based on Charge	9
1.3.3 Liposomes in Drug Delivery	10
1.4 Systemic Sclerosis	11
1.4.1 Symptoms and Diagnosis	11
1.4.2 Molecular Mechanisms of the Disease	12
1.4.2.1 Role of PRRs in SSc	13
1.4.2.2 Role of Immune Cells in Ssc.....	15
1.4.2.2.1 Neutrophils in Ssc	15
1.4.2.2.2 Macrophages in SSc.....	16

1.4.2.2.3 Lymphocytes in SSc	17
1.4.2.3 Role of Non-Immune Cells in SSc.....	18
1.4.3 Current and Prospective Treatment Options in SSc	19
1.5. Outline of the Study.....	22
CHAPTER 2	24
2. MATERIALS AND METHODS	24
2.1 Materials	24
2.1.1 TLR Ligands and Antigens	24
2.1.2 Lipids.....	24
2.1.3 Cell Culture Media and Standard Buffer Solutions.....	24
2.1.4 Antibodies	25
2.2 Methods	27
2.2.1 Liposome Preparation.....	27
2.2.1.1 A151 Encapsulation within Liposomes.....	28
2.2.1.2 Size Measurement of Liposomes using Dynamic Light Scattering	28
2.2.2 Cell Culture	28
2.2.2.1 Splenocyte Isolation.....	28
2.2.2.2 Bone Marrow-Derived Macrophages (BMDM) Preparation	28
2.2.2.3 Single Cell Isolation from Lung Tissue	29
2.2.2.4 Cell Lines	29
2.2.2.4.1 L-929.....	29
2.2.2.4.2 NIH3T3.....	30
2.2.2.5 Cell Counting and Layering	30
2.2.3 <i>In Vitro</i> Stimulation of Cells with Ligands	31
2.2.4 Enzyme-Linked Immunosorbent Assay (ELISA)	32
2.2.5 Determination of Gene Expression	32
2.2.5.1 Total RNA Isolation.....	32
2.2.5.2 cDNA Synthesis.....	33
2.2.5.3 RT-qPCR.....	33
2.2.6 Flow Cytometry Analysis.....	34
2.2.6.1 Cell Fixation.....	34
2.2.6.2 Surface Marker Staining of Cells	34
2.2.7 Western Blotting.....	35
2.2.7.1 Protein Isolation and Determination of Protein Concentration via BCA Assay	35

2.2.7.2 SDS-PAGE.....	35
2.2.7.3 Wet Transfer.....	35
2.2.7.4 Blocking and Incubation with Antibodies.....	36
2.2.7.5 Gel Imaging.....	36
2.2.8 Animal Experiments.....	36
2.2.8.1 Subcutaneous Bleomycin Administration.....	36
2.2.8.2 Intratracheal Bleomycin Instillation.....	36
2.2.8.3 Free and Liposomal A151 Treatment.....	37
2.2.8.4 Bronchoalveolar Lavage Fluid (BALF) Collection	37
2.2.9 Statistical Analysis	37
CHAPTER 3	38
3. RESULTS	38
3.1 Physicochemical properties of liposomes.....	38
3.1.1 Size and polydispersity index determined by DLS	38
3.2 Determination of free and liposomal A151 ODN's immunosuppressive capacity <i>in vitro</i>	39
3.2.1 Studies with splenocytes involving free or liposome encapsulating A151 ODN.....	39
3.2.2 Studies with macrophages and fibroblasts	44
3.2.2.1 Suppressive effects of A151 ODN on cytokine production and surface activation markers in BMDMs.....	44
3.2.2.2 Inflammasome studies.....	49
3.2.2.3 Conditioned media experiments.....	50
3.3 Determination of A151 ODN's anti-fibrotic effect <i>in vitro</i>	55
3.3.1 Studies with fibroblasts	55
3.4 Establishment of bleomycin induced scleroderma with intratracheal instillation	57
3.4.1 Preventive effect of free A151 ODN on early stage of bleomycin-induced scleroderma	63
3.4.2 Therapeutic implications of free and liposome-encapsulated A151 formulations on bleomycin induced lung inflammation.....	66
3.4.3 Therapeutic effects of liposome-encapsulated A151 formulation on bleomycin induced fibrosis	76
CHAPTER 4	82
DISCUSSION	82

APPENDICES	88
APPENDIX A.....	88
APPENDIX B.....	91
Appendix B1 – ELISA.....	92
Appendix B2 - Immunoblotting.....	93
Appendix B3- Flow Cytometry.....	95
REFERENCES	96
Copyright Permissions.....	114



List of Figures

Figure 1.1 Signaling pathways utilized by different TLRs.	4
Figure 1.2 Mode of action of A151 ODN in different types of immune cells	7
Figure 1.3 Overall disease progression of systemic sclerosis	13
Figure 2.1 Representative hemocytometer grids under the light microscope.	30
Figure 3.1 Size distribution analyses of anionic liposomes by DLS.	38
Figure 3. 2 TNF α , IL-6, IL-12 and IFN γ production from murine splenocytes in response to control ODN (Ctrl ODN), anionic liposomes (AL), A151 or liposome-encapsulated A151 (AL(A151)) stimulation.	39
Figure 3.3 Effects of free and liposome-encapsulated A151 on TNF α and IL-6 production from splenocytes stimulated with TLR1/2, TLR4 and TLR9 ligands.	41
Figure 3.4 Effects of free and liposome-encapsulated A151 on IL-12 and IFN γ production from splenocytes stimulated with TLR1/2, TLR4 and TLR9 ligands.	43
Figure 3.5 Effect of A151 ODN on expressions of co-stimulatory molecules in bone marrow derived macrophages (BMDM) stimulated with TLR1/2, TLR4 and TLR9 ligands.	45
Figure 3. 6 A151 ODN dependent inhibition of BMDM-specific recruitment and activation marker expressions following stimulation with TLR1/2, TLR4 and TLR9 ligands.	47
Figure 3.7 IL-6 production from BMDMs stimulated with bleomycin in the presence or absence of A151 ODN.	48
Figure 3.8 Altered IL-1 β production in response to dose dependent A151 ODN administration in BMDMs activated with p(dA/dT).	50
Figure 3.9 Effect of A151 ODN on nigericin induced NLRP3 inflammasome activation in BMDMs.	51
Figure 3.10 TNF α , IL-6 and IL-12 production from BMDMs stimulated with TLR ligands and NIH3T3 cell line stimulated with conditioned media obtained from BMDMs.	53
Figure 3.11 Col1a1 expression in NIH3T3 cells in response to TLR ligand and TGF β stimulation.	55
Figure 3.12 TGF β gene expression in response to bleomycin in NIH3T3 cells.	56
Figure 3.13 Changes in cytokine levels and cell populations in BALF at day 3, 7 and 21 following bleomycin administration.	60
Figure 3.14 Changes in gene expression levels, macrophage cell population and protein expressions in lung tissue after 3, 7 and 21 days upon bleomycin administration.	63
Figure 3.15 Analysis of BALF cell populations and cytokine levels in mice that were pre-treated with A151 ODN for 3 days, then injected bleomycin.	64
Figure 3.16 Analysis of lung cell populations and collagen gene expressions in mice that were pre-treated with A151 ODN for 3 days, then injected with bleomycin and followed for three days before sacrificing.	65
Figure 3.17 Experimental design of bleomycin induced lung inflammation and change in body weights of mice.	67
Figure 3.18 Changes in BALF cytokine levels and cell population in response to bleomycin instillation and A151 ODN treatment.	69

Figure 3.19 Surface marker expressions of alveolar macrophages obtained from BALF.	72
Figure 3.20 Cell surface expressions of alveolar macrophages obtained from lung following bleomycin instillation.	74
Figure 3.21 Gene and protein expressions of lung samples from bleomycin and A151 delivered mice.	75
Figure 3.22 Liposomal A151 ODN treatment in bleomycin induced scleroderma model and cytokine levels in BALF.	77
Figure 3.23 Alveolar macrophages in BALF samples after a single intraperitoneal liposomal A151 ODN injection into bleomycin-induced scleroderma in mice.	78
Figure 3. 24 MHCII, CD11b and CD80 expressions in BALF of liposome-encapsulated A151 ODN treated mice following bleomycin instillation.	80
Figure 3. 25 Collagen gene expressions in lungs of mice treated with liposomal A151 ODN following bleomycin instillation.	81



List of Tables

Table 2.1. Mouse-specific monoclonal antibody pairs (unlabeled and biotinylated) used throughout the ELISA studies.	25
Table 2.2. Standard recombinant proteins and their respective initial concentrations used throughout the ELISA studies.	25
Table 2.3 Fluorochrome-conjugated mouse-specific antibodies used throughout flow cytometry studies.	26
Table 2.4. Primary and HRP-conjugated secondary antibodies used throughout Western blotting experiments.	27
Table 2.5. Stimulants and their concentrations used during <i>in vitro</i> stimulation assays.	31
Table 2.6. RT-qPCR primers and expected product sizes.	33
Table 2.7. RT-qPCR conditions used in experiments.	34

Abbreviations

Ab	Antibody
AIM2	Absent in melanoma 2
ANA	Anti-nuclear antibodies
APC	Antigen presenting cell
BCA	Bicinchoninic acid
BMDM	Bone marrow derived macrophages
BLM	Bleomycin
bp	Base pairs
BSA	Bovine serum albumin
CCL	Chemokine (C-C) ligand
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CLR	C-type lectin receptors
CM	Conditioned media
ConA	Concanavalin A
CpG	Unmethylated cytosine-phosphate-guanosine motifs
CTL	Cytotoxic T cells
CXCL	C-X-C motif chemokine ligand
DAMP	Damage-associated molecular patterns
DC	Dendritic cell
ddH ₂ O	Double distilled water
dH ₂ O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium

DNA	Deoxyribonucleic acid
DLS	Dynamic light scattering
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FSC	Forward scatter
HEPES	N-2-Hydroxyethylpiperazine-N'-2 Ethanesulfonic Acid
HMGB1	High mobility group box1
IFN	Interferon
IL	Interleukin
i.p.	Intraperitoneal
IRAK	IL-1R associated kinase
i.t.	Intratracheal
LPS	Lipopolysaccharide
mu	Murine
mAb	Monoclonal antibody
MAL	MyD88-adaptor-like protein
M-CSF	Macrophage colony stimulating factor
MCP	Monocyte chemoattractant protein
MHC	Major histocompatibility complex
MLV	Multilamellar vesicles
MyD88	Myeloid-differentiation primary response protein 88
NEAA	Non-essential amino acids

NFκB	Nuclear factor-κB
NLR	NOD-like receptor
NO	Nitric oxide
NOD	Nucleotide oligomerization binding domain
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PD-L1	Programmed death ligand-1
Pen/Strep	Penicillin and streptomycin
PGN	Peptidoglycan
poly (I:C)	Polyinosinic-polycytidylic acid
PNPP	p-nitrophenyl phosphate
PRR	Pattern recognition receptors
PS	Phosphatidylserine
q-PCR	Quantitative PCR
RIG-I	Retionic acid inducible gene-I
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse transcriptase-quantitative PCR
RT	Room temperature
SA-ALP	Streptavidin alkaline phosphatase
SSC	Side scatter

SSc	Systemic sclerosis
ssDNA	single stranded DNA
ssRNA	single stranded RNA
SUV	Small unilamellar vesicles
Th	T helper
TIR	Toll-IL-1 resistance
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF-receptor associated factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cells
TRIF	TIR domain-containing adaptor protein inducing IFN β
ULV	Unilamellar vesicles

CHAPTER 1

1. Introduction

1.1 The Immune System

The immune system protects body from pathogenic microorganisms by detecting and eliminating them while distinguishing self-antigens from non-self. In mammals, immune system is divided into two sub-systems, innate immunity and adaptive immunity. Although the specificity and reaction speed against microbial components differ, elements of innate and adaptive immune system interact with each other [1].

A pathogen must pass the surface barriers such as enzymes and mucus in order to cause an infection in the body. Since keratin on skin and mucus secreting body cavities are not ideal for microbial growth of most of the microorganisms, they need to pass through ectoderm layer to live, grow and proliferate [2]. Once a microorganism breaches first line of defense, it encounters with the cells of immune system.

Innate immune system is an evolutionarily conserved, and it is found even in the simplest animals because innate immune cells are the first to recognize non-self and initiate the inflammatory response. Innate immune system cells of numerous organisms express germ-line encoded receptors that are highly conserved in their genomes. These receptors are specialized to recognize non-self expressed molecular patterns but not protein antigens [3, 4]. Another feature of the innate immune system is the immediate response against intruders. Upon recognition, chemokines produced by the cells in surrounded tissue induce recruitment of immune cells such as neutrophils and macrophages. Infiltrated cells produce pro-inflammatory cytokines, e.g., IL-1, TNF α and IL-6 and chemokines [5]. Monocytes, basophils, eosinophils and mast cells also participate in early recognition and response against pathogens as a part of innate immune activation.

Adaptive immune system provides broader and more efficient recognition of self and non-self antigens and is only found in higher vertebrates. The most important feature of adaptive immune system is to express specific receptors for antigens. Following the first encounter with an antigen presented by antigen presenting cells (APCs), B- and T-cells proliferate and differentiate. Formation of memory cell subsets confers the rapid activation of immune cells, quick and strong clearance in the subsequent detections of that same antigenic epitope [6]. Receptors of T- and

B-lymphocytes are shaped by gene rearrangements in contrast to that of innate immune cells; therefore, they are able to sense numerous specific antigenic determinant sites of a single protein antigen rather than general molecular patterns.

Although it was believed that only B- and T-lymphocytes develop memory, recent studies have shown that some of the innate immune cells may also display signs of long-lasting memory [7]. This phenomenon is called “trained immunity”, and its mechanism was revealed as a result of epigenetic modifications. For instance, Bacillus Calmette-Guerin (BCG) vaccine that normally protects against tuberculosis was found to lead non-specific protective effects against yellow fever viremia by epigenetic reprogramming in monocytes [8]. Therefore, monocytes of vaccinated individuals produced more IL-1 β compared to that of non-vaccinated people, and reduced viremia was correlated with increased IL-1 β secretion from monocytes. This novel finding opened a new characteristic of innate immune system and has a potential for the development of new generation vaccines as well as different treatment strategies [9].

1.1.1 Innate Immunity and Pattern Recognition Receptors (PRRs)

Innate immune cells are defined as the first line of defense after pathogens breach the anatomical barriers of host. Antimicrobial enzymes and peptides produced by immune or non-immune cells present in blood and extracellular fluids, lyse and kill the microorganisms. However, when these are inefficient to kill the pathogen, innate immune cells come into play and detect patterns expressed on the pathogens. Pattern recognition receptors (PRRs) sense microbial patterns called pattern-associated molecular patterns (PAMPs) and endogenous antigens released from damaged cells which are defined as danger-associated molecular patterns (DAMPs) [10]. Bacterial cell wall components like lipopolysaccharide (LPS), peptidoglycan (PGN), unmethylated bacterial CG-rich DNA are some examples of what are classified as PAMPs while remnants of apoptotic cells are called as DAMPs [11, 12].

PRRs can be membrane bound (have role on phagocytosis or signaling), cytosolic or free in blood circulation. There are four types of PRRs defined thus far. These are Toll-like receptors (TLRs), nucleotide oligomerization domain (NOD)-like receptors (NLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and C-type lectin receptors (CLRs) [13]. Activation of these receptors lead to production of pro-inflammatory cytokines, chemokines, type I interferons and antimicrobial proteins which all can initiate inflammation by different pathways. Only TLRs will be discussed in the following section of this thesis.

1.1.1.1. Toll-like Receptors (TLRs)

Toll is first identified in *Drosophila* as a receptor that regulates anti-fungal response [14, 15]. After that, mammalian homolog of Toll receptors was discovered, and different types of Toll-like receptors were characterized [16, 17].

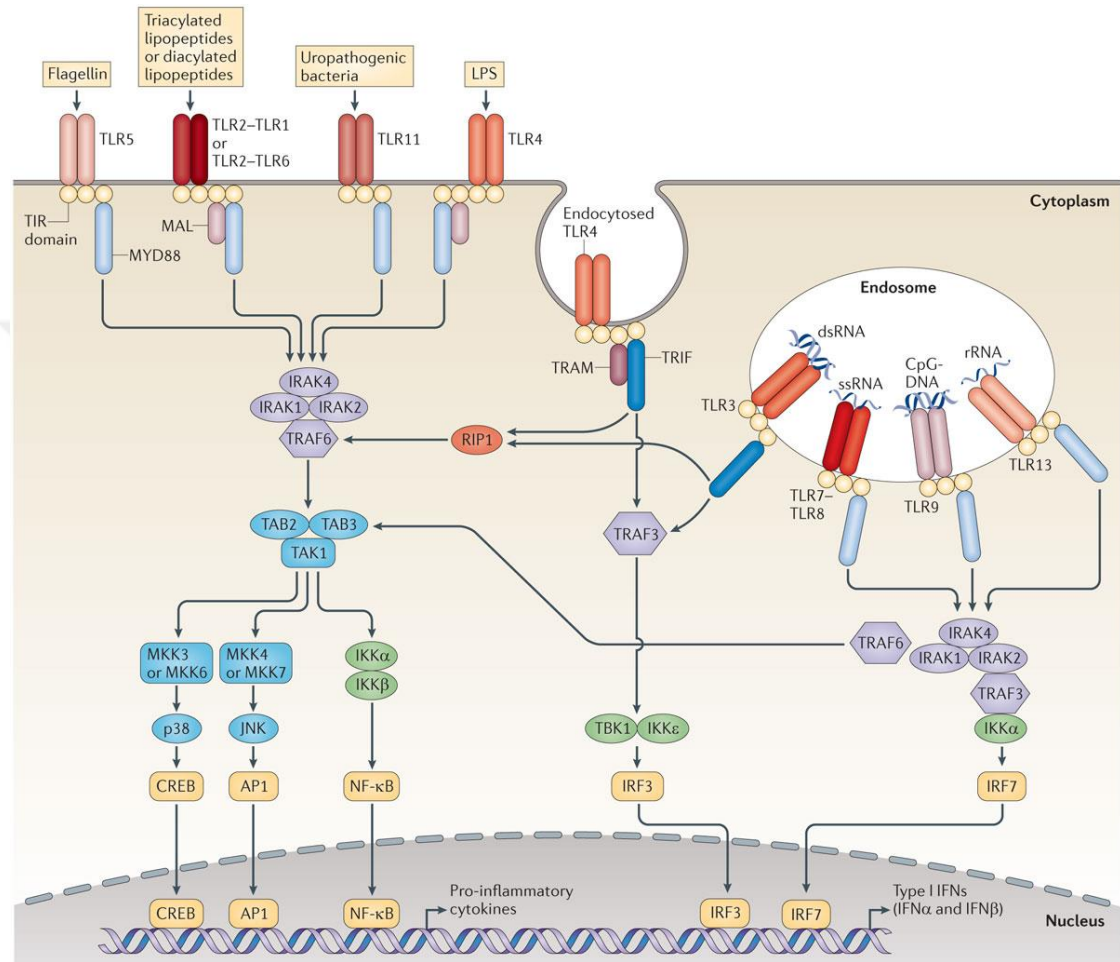
To date, 12 mouse and 10 human TLRs have been identified. While TLR 3, 7/8 and 9 were found on the membrane of intracellular vesicles such as endosomes, the rest were reported to localize on plasma membrane. The first identified member is the TLR4 and is both found on plasma membrane and endosomes [18]. As a result, both extracellular and intracellular pathogens are recognized by TLRs upon engagement of their respective PAMP ligands. These receptors are single-pass transmembrane proteins which have extracellular domain consisting of leucine rich repeats (LRRs) and cytoplasmic domain called Toll/IL-1 receptor (TIR) [19]. Cytoplasmic tails of TLRs are highly similar to that of mammalian IL-1 receptor family whereas structure of LRRs vary. LRRs provide recognition of PAMPs while TIR domain is involved in downstream signaling.

TLRs are expressed in immune cells as well as non-immune cells. Different type of cells can have different TLR expressions. For instance, epithelial cells in the apical surface of intestine continuously interact with bacteria; however, it does not cause excess inflammation since low expression of TLR4 and TLR5 results in weak inflammatory response against the bacteria which are naturally found in intestines [20]. Consequently, expression of TLRs are tightly regulated to prevent unnecessary inflammatory responses.

TLR signaling is a very-well defined pathway (Figure 1.1). Following ligand binding to a TLR, receptor dimerizes and modifies its conformation to recruit adaptor proteins which are either myeloid differentiation primary response protein 88 (MyD88) and MyD88-adaptor-like protein (MAL) or TIR-domain containing adaptor protein inducing IFN β (TRIF) and TRIF related adaptor molecule (TRAM) [21]. Most of the TLRs use MyD88-dependent pathway to elicit the signal except TLR3 which recruits TRIF/TRAM. TLR4 has been reported to use both MyD88 and TRIF pathway [22]. When MyD88 is activated, its death domain recruits IL-1R associated kinase 1 and 4 (IRAK1 and IRAK4). IRAK complex forms a scaffold to recruit and phosphorylate other proteins such as TNF receptor associated factors (TRAFs). TRAF6 is a ubiquitin E3 ligase, and its polyubiquitination forms another scaffold for transforming growth factor- β -activated kinase 1 (TAK1) activation. Two adaptor proteins of TAK1, TAK binding protein 1 and 2 (TAB1/2), bring TAK1 into proximity with IRAK1 to facilitate its phosphorylation, then activated TAK1 results in I κ B degradation by ubiquitination, eventually

releasing nuclear factor- κ B (NF κ B), an important transcription factor involved in production of pro-inflammatory cytokines.

TLR3 contrary to MyD88 it only utilizes TRIF as adaptor. TRIF recruits TRAF3 to form polyubiquitin molecules that recruit kinases, IKK ϵ and TBK1. This complex phosphorylates interferon regulatory factor 3 (IRF3) which is responsible for type I interferon production [19].



Nature Reviews | Immunology

Figure 1.1 Signaling pathways utilized by different TLRs [19].

1.2 Suppressive Oligodeoxynucleotides

Nucleic acids expressed by microorganisms lead to inflammation via induction of TLRs; however, removing bacterial CpG DNA or adding suppressive sequences reverse immune activation [23]. Krieg et al. was the first to show that non-stimulatory or suppressive motifs in viral DNA have suppressive functions in response to TLR9 induced immune activation [24]. After a couple of years, telomeric motifs expressing repeats of TTAGGG have been reported to

downregulate immune response in mammals. Moreover, it was found that immunosuppressive effect of this ODN depends on the repetitive sequence [25]. Following the discovery of this natural immunosuppressive motif expressing 4 repeats of TTAGGG (named as A151 ODN), various types of synthetic suppressive ODNs having distinct sequences and mechanism of action, were synthesized. For example, H154 whose sequence is “CCTCAAGCTTGAGGGG” inhibits pro-inflammatory cytokine production both *in vivo* and *in vitro* against CpG induced immune activation whereas it does not have suppressive ability against LPS or ConA [23]. Another example is ‘G’ ODN which was shown to impede production of pro-inflammatory cytokines via competition for binding to TLR9 mainly in DCs and macrophages, and its immunosuppressive activity was demonstrated in murine models of SLE and endotoxic shock [26-28].

1.2.1 A151 ODN

A151 is the best studied suppressive ODN, and it has a broad immunosuppressive capacity. In the early studies, A151 was shown to co-localize in endosomes with TLR9 and inhibited binding of CpG ODN, hence defined as a TLR9 antagonist [25]. However, further studies indicated that A151 could be more than being TLR9 antagonist, as it will be reviewed in following sections.

1.2.1.1 Physical and Chemical Properties

A151 ODN consists of 4 repeats of “TTAGGG” and has 24-mer structure. Studies demonstrated that repeated motifs are required for immunosuppression, and 4 repeats of TTAGGG confers the maximal inhibition in response to CpG ODN; however, sequence longer than 24 nucleotides did not further enhance its suppressive capacity [25]. A151 ODN contains four poly-G sequences that form high order quadruplex structures with inter-chain Hoogsteen base-pairing via hydrogen bonding, and its suppressive activity is thought to originate from this structure which is similar to structure of some of other suppressive ODNs [29].

Bases in natural telomeric sequence are connected with phosphodiester (PO) bonds. However, freely circulating DNA sequences with phosphodiester bonds are less stable and have shorter half-life *in vivo*, thus ineffective in therapy. Conversely, phosphorothioate linkages have been shown to be more potent and stable against enzymes *in vivo* [30]; therefore, A151 having phosphorothioate backbone is mostly preferred to be utilized in studies exploring its therapeutic applications. A recent study reported increased immunosuppressive ability of A151 loaded into hexapod-like DNA structure which consists of 6 copies of DNA sequence complementary to

A151 ODN [31]. Overall, development of drug carriers or complex molecules can be useful to boost suppressive capacity of A151 ODN.

1.2.1.2 Mechanism of Action

The exact mode of action of A151 ODN on cells are still a mystery. However, there are many studies focused on broad immunosuppressive effects of A151 and which proteins are targeted by it. STATs were demonstrated to be the targets of A151 in the early studies. In 2005, Shirota et. al showed A151 inhibited production of Th1 cytokines IL-12 and IFN γ by binding and inhibiting phosphorylation of STAT4 and STAT1, respectively [32]. In addition, it impairs conversion of CD4⁺ cells into Th1 via inhibiting STAT3 phosphorylation [33]. This study also proved that decrease in Th1 differentiation is independent from TLR9 signaling pathway. Later, it was shown that inhibition of STAT1 phosphorylation by A151 also leads to Treg generation which might be an option to use it in Treg based immunotherapy [34]. Another study argued that A151 prolongs STAT3 phosphorylation by inhibiting SOCS3 activity which eventually causes Th17 cell development [35]. Besides its effects on Th cell differentiation, B cell activation and subsequent antibody production is impeded by A151 ODN [36].

A151 has broad effects on monocytes and macrophages other than inhibiting pro-inflammatory cytokines. AIM2 but not NLRP3 inflammasome pathway is impaired by A151 in response to dsDNA stimulation in mouse BMDMs and DCs [37]. In this work, A151 was shown to bind and inhibit AIM2, prevent the production of ASC specks, IL-1 β and IL-18. A recent study indicated that A151 ODN efficiently inhibits cGAS activation by competing with dsDNA and decrease the production of type I IFN in human monocytes [38].

Overall mechanism of action of A151 ODN on immune cells are summarized in Figure 1.2.

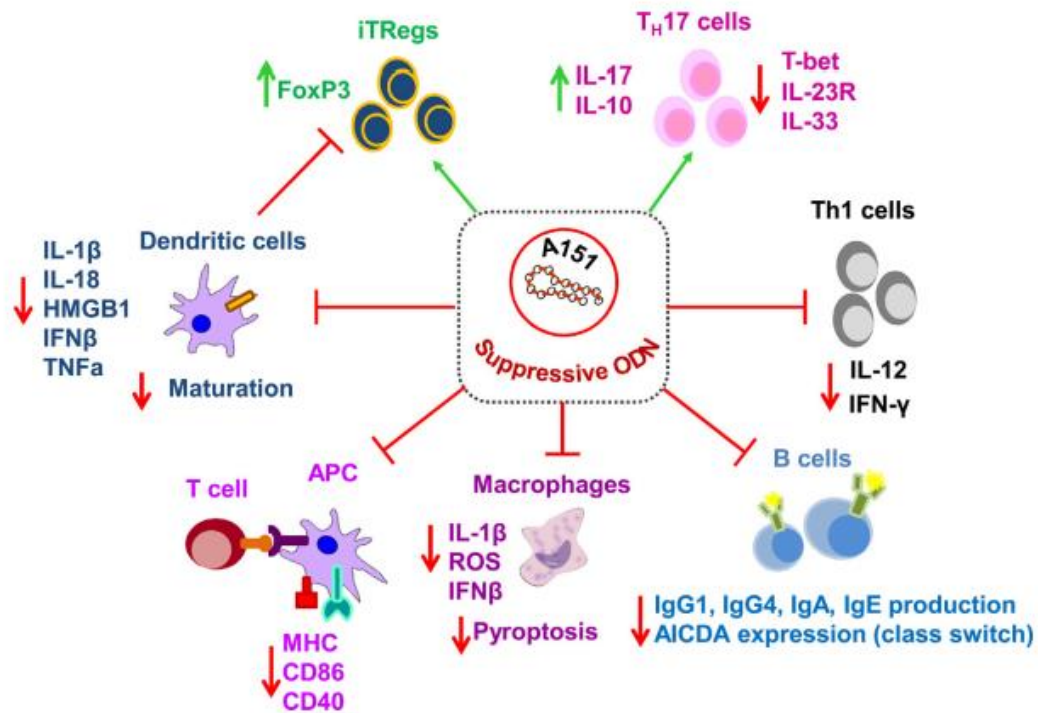


Figure 1.2 Mode of action of A151 ODN in different types of immune cells [29]. Green arrows indicate the elevation while red arrows show the decrease or inhibition. (AICDA: Activation induced cytidine deaminase, ROS: Reactive oxygen species)

1.2.1.3 A151 as a therapeutic ODN

Considering A151 ODN's wide range of immunosuppressive effects *in vitro*, there are various studies investigating the therapeutic potential of A151 on different inflammatory and autoimmune diseases. Protection of mice from LPS-induced endotoxic shock using A151 ODN was one of the earliest showing immunosuppressive effects of telomeric DNA *in vivo* [32]. In 2004, Dong et. al reported that A151 decreased the disease severity in collagen type II-induced murine model of rheumatoid arthritis by reducing serum titers of pathogenic IgG anti-Col II antibody production and IFN γ secretion [39]. In lupus prone NZB/NZW mice, A151 delayed the onset and progression of disease while increased lifespan by reducing anti-dsDNA antibody production from B cells and inhibiting IL-12 and IFN γ secretion [40].

In ApoE^(-/-) mice, A151 ODN decreased atherosclerotic lesions by downregulating expressions of VCAM-1 and MCP-1 which are two key proteins regulating inflammation in atherosclerosis. Furthermore, A151 contributed to inhibit the development of atherosclerosis by decreasing the Th1 dominancy and favoring Th2 cell differentiation and cytokine production such as IL-10 and IL-4 [41, 42].

Lastly, immunosuppressive effects of A151 in organ-specific inflammation was shown with different studies. As an example, pre-treatment of A151 to silica-induced murine model of lung

inflammation improved survival and decreased mortality by increasing macrophage viability and reducing H_2O_2 which is a marker for reactive oxygen species (ROS) generation [43]. Moreover, A151 was shown to reduce ocular inflammation induced by LPS on mice and rabbits; thus, it might be a potential treatment for the therapy of uveitis [44].

1.3 Liposomes

Liposomes were discovered and first observed with electron microscopy at 1964 by Alec Bangham and colleagues [45]. 10 years later, Gregory Gregoriadis has shown that biotherapeutics could be entrapped into liposomes so that they can be used as drug carriers [46-49]. Since then, a lot of progress has been made to utilize liposomes in drug delivery.

1.3.1 General Properties

Liposomes are artificial vesicles in various sizes made up of synthetic or natural phospholipids [50]. They have a similar structure to cell membrane, consisting of lipid bilayer and aqueous core. Depending on the formulation, they can be different in size, lipid composition and charge. They are highly compatible and less toxic, containing hydrophobic and hydrophilic parts; therefore, all of these properties make liposomes ideal drug carriers [51]. Although liposomes have advantages such as active targeting to related organ/tissue, biocompatibility, reducing the toxicity of drug that it encapsulated, increased bioavailability and efficacy of therapeutics, there are also drawbacks which include low solubility, leakage of the drug and oxidation of phospholipids [52].

Liposomes can be differently classified based on preparation method (reverse-phase evaporation or vesicle extrusion), size (small, intermediate or large vesicles) and their lamellar structure (unilamellar vesicles or multilamellar vesicles). Depending on the preparation method, size and lamellarity of liposomes also change.

Liposomes can vary in size; however, size of 40-500 nm are mostly preferred for drug delivery purposes [53]. Size of liposome-based drug must be carefully determined because size affects the fate and success of the drug carrier throughout the treatment. For instance, liposomes smaller than 100 nm was less recognized by macrophages proteins for clearance from the body since they are less likely to interact with plasma proteins [54]. Moreover, they passively accumulate at the tumor sites. Conversely, capacity of smaller liposomes to entrap a drug is low.

Lamellarity is another essential factor that must be taken into consideration. Liposomes can be either unilamellar which has one lipid bilayer or multilamellar which has several bilayers.

Unilamellar vesicles (ULVs) are 50-250 nm size with an aqueous core and used to encapsulate hydrophilic substances [55]. On the other hand, multilamellar vesicles (MLVs) are larger with 1-5 μm size, and they are generally utilized for entrapment of lipid-soluble drugs. Lamellarity also influences release of encapsulated drug. Release of a substrate from MLVs is slower than that of ULVs because it must pass multiple bilayers to go out from liposomes.

Lipid composition is an important determinant for stability and interaction of liposomes *in vivo* [56]. Cholesterol (Chol) induces dense packaging of other phospholipids, thus reducing the permeability and increasing the stability of membrane. Phosphatidylcholine (PC) is another lipid that regulates membrane stability. Even though liposomes are similar to the structure of biological membranes, they can be easily degraded by enzymes in the body or recognized by mononuclear phagocytic system before it has not arrived at the desired location. However, this issue is partly overcome by modifications of lipids and addition of different molecules to lipid composition. In this aspect, PEGylation of liposomes was the first successful attempt to increase the stability and circulation time of liposomes *in vivo*. Polyethylene glycol (PEG) is a polyether diol compound with many advantages such as biocompatibility, high solubility in aqueous or organic solvents and low immunogenicity [57]. The success of PEGylated liposomes underlies escaping from phagocytic cells by forming a protective hydrated layer on the surface of liposomes. One major drawback of PEGylated liposomes has been reported as the reduced uptake rate by target cells [58]. Another strategy to improve drug delivery by liposomes is the development of pH-sensitive liposomes [59]. These liposomes release their cargo in acidic environment of endosomes, and they are commonly used for cancer therapy.

Lipid content and ratio are charge-determining aspects. Likewise the size, lamellarity and charge of liposomes is an influencing factor for stability and half-life. How charge influences the extent of liposome delivery is explained in the next section.

1.3.2 Liposomes Based on Charge

Liposomes can be either neutral, anionic or cationic in terms of net surface charge. Neutral liposomes are relatively stable in blood, non-toxic and commonly used for encapsulation of DNA with special techniques; therefore, they have a potential to be utilized in gene transfer and gene therapy [60, 61]. However, they release their contents in the extracellular space since they form aggregates and poorly interact with cells [56]. Charged liposomes seem to be more advantageous compared to neutral liposomes. For example, zeta potential caused by electrostatic charge on liposomal surface elevates interaction with cells, therefore prevents their aggregation.

Cationic liposomes are made by using positively charged lipids such as DOTAP (1,2-dioleoyl-3-trimethylammonium-propane [chloride salt]) and DC-Chol-HCl (3 β -[N-(N',N'-dimethylaminoethane)-carbonyl] cholesterol hydrochloride) [56]. They are highly internalized by the cells because of the interaction of net negative charge on cell membrane and positive charge on liposomal membrane. Cationic liposomes are preferred to encapsulate TLR ligands, peptides and chemotherapeutics. Moreover, their use as vaccine adjuvants has been reported, they increase the immunogenicity of the encapsulated ligand although they do not have immunogenic effect by themselves [62]. However, they have high toxicity compared to neutral and anionic liposomes, as one study reported that some cationic liposomes cause toxicity in lungs in a dose dependent fashion [63].

Anionic liposomes carry negative charge on their surface. They have been reported to quickly extravasate to tumor area and deliver the cargo compared to cationic and neutral liposomes [64], and they are very useful in monocyte/macrophage targeted delivery of liposomes since scavenger receptors on macrophages recognize phosphatidylserine (PS) and take up the liposomes [65]. Moreover, they have been shown to form a unique and strong conformation with single stranded (ss) ODNs [66]. Therefore, encapsulation of ss-ODNs within anionic liposomes might enhance their stability, cellular uptake and function compared to their encapsulation within other types of liposomes. However, a major drawback of using anionic liposomes is being unstable in blood circulation. Therefore, intravenous administration is not preferred for delivery of anionic liposomes.

Overall, all liposomes have different advantages and drawbacks; thus, type of liposome which is going to be used for a specific condition must be carefully decided. This is another factor hindering transition of liposomes in to clinical applications.

1.3.3 Liposomes in Drug Delivery

Drug entrapment into liposomes is achieved either by passively (during liposome formation) or by DRV (dehydration-rehydration vesicles) method after liposomes have been made [67]. With DRV method, peptides, proteins, DNA and sensitive drugs could be easily encapsulated within liposomes.

Liposomes has already been used to deliver numerous drugs, especially chemotherapeutics such as doxorubicin, cisplatin and annamycin. Doxil, the first FDA-approved nano-drug, is liposomal doxorubicin, used for treatment of Kaposi's sarcoma [68]. Liposome formulation includes cholesterol and PC while liposomal membrane is PEGylated to increase circulation in blood. Delivery of vaccines for hepatitis A and influenza within liposomes are also approved and

currently being used [69, 70]. These formulations contain peptides originating from virus coat since they are shown to be more effective to boost humoral immune response.

“New generation liposomes” such as temperature-sensitive liposomes confer targeted delivery rather than passive accumulation because they release bioactive cargo above the melting temperature (around 41°C) of the phospholipids exist in the liposome formulation while retain the drug at normal body temperature. An example of temperature-sensitive liposomes, ThermoDox® encapsulating doxorubicin, is at phase III trial intended against hepatocellular carcinoma therapy [71].

While most of the liposomal formulations are designed for intravenous delivery, few drugs were encapsulated within liposomes are developed for intradermal and ocular instillation. Oral delivery is not preferred since liposomes are generally labile at low pH and furthermore quickly degraded by the enzymes in GI tract. There are numerous other examples of liposomes approved to use in cancer, viruses and fungi in addition to countless liposomal formulations that are being tested in phase trials [71, 72].

1.4 Systemic Sclerosis

Systemic sclerosis (scleroderma or SSc) is an immune-related rheumatic disease defined by fibrosis on skin or internal organs and vascular abnormalities. Although it has low prevalence, mortality is high [73].

Prevalence of this disease depends on race, sex and age. It was reported that frequency of the disease is higher in USA and Australia than Japan and Europe, and prevalence changes in different regions of Europe [74]. Strikingly, the risk of developing systemic sclerosis increases with age, and women have a higher risk for developing SSc. Of note, correlation between exposing chemical such as silica, asbestos etc. and developing scleroderma has not been determined yet [74].

1.4.1 Symptoms and Diagnosis

Since symptoms are common with other immune-mediated rheumatic diseases such as systemic lupus erythematosus, it might be difficult to diagnose systemic sclerosis in the clinic. Raynaud’s phenomenon, presence of anti-nuclear antibodies (ANA) in blood and vasculopathy are the most frequently encountered symptoms [73]. Classification of SSc types and criteria for diagnosis have not been clear and not well-characterized until American College for Rheumatology/European League Against Rheumatism Collaborative Initiative (ACR/EUCAR)

published a report in 2013 [75]. According to this report, there are three hallmarks of SSc. These are defined as vasculopathy, production of autoantibodies and fibrosis on skin/internal organs. Furthermore, thickening of the skin on fingers extending proximal to the knuckles is sufficient for diagnosis of systemic sclerosis.

Two major subgroups were described as i) limited cutaneous and ii) diffuse cutaneous systemic sclerosis. In limited cutaneous SSc, progression is slower, and fibrosis is mostly seen on skin of hand, face or arm. Raynaud's phenomenon, vasoconstriction of fingers and toes resulting in reduced blood flow and change in skin color [76], is observed before fibrotic development and accepted as an early sign of the disease. Increase in pulmonary artery pressure which is termed as pulmonary hypertension [77] and anti-centromere antibody production are seen in majority of patients having limited cutaneous scleroderma [78]. Of all patients that develops systemic sclerosis, 25% are diagnosed to have diffuse cutaneous SSc [79]. It progresses more rapidly compared to limited cutaneous subtype, is involved in a large area of skin and one or more internal organs. Furthermore, average life expectancy is around 10-15 years due to involvement of internal organs.

1.4.2 Molecular Mechanisms of the Disease

Scleroderma is known to be a complex disease which many types of cells and molecules produced from these cells contribute to its development (Figure 1.3), although its etiology is not known. Overall, endothelial cells and platelets are activated due to cell damage or inflammation. Secreted chemokines/cytokines and proteins from activated cells induce activation of immune cells which also results in fibroblast activation. Activated fibroblasts start to produce extracellular matrix proteins to rebuild the extracellular structure that has been damaged due to inflammatory response [80]. Accumulation of extracellular matrix proteins such as fibronectin, type I and type III collagen are important molecular markers of fibrosis. However, fibrosis persists and could not be resolved due to continuous inflammation which is opposite to what happens in a normal wound healing process.

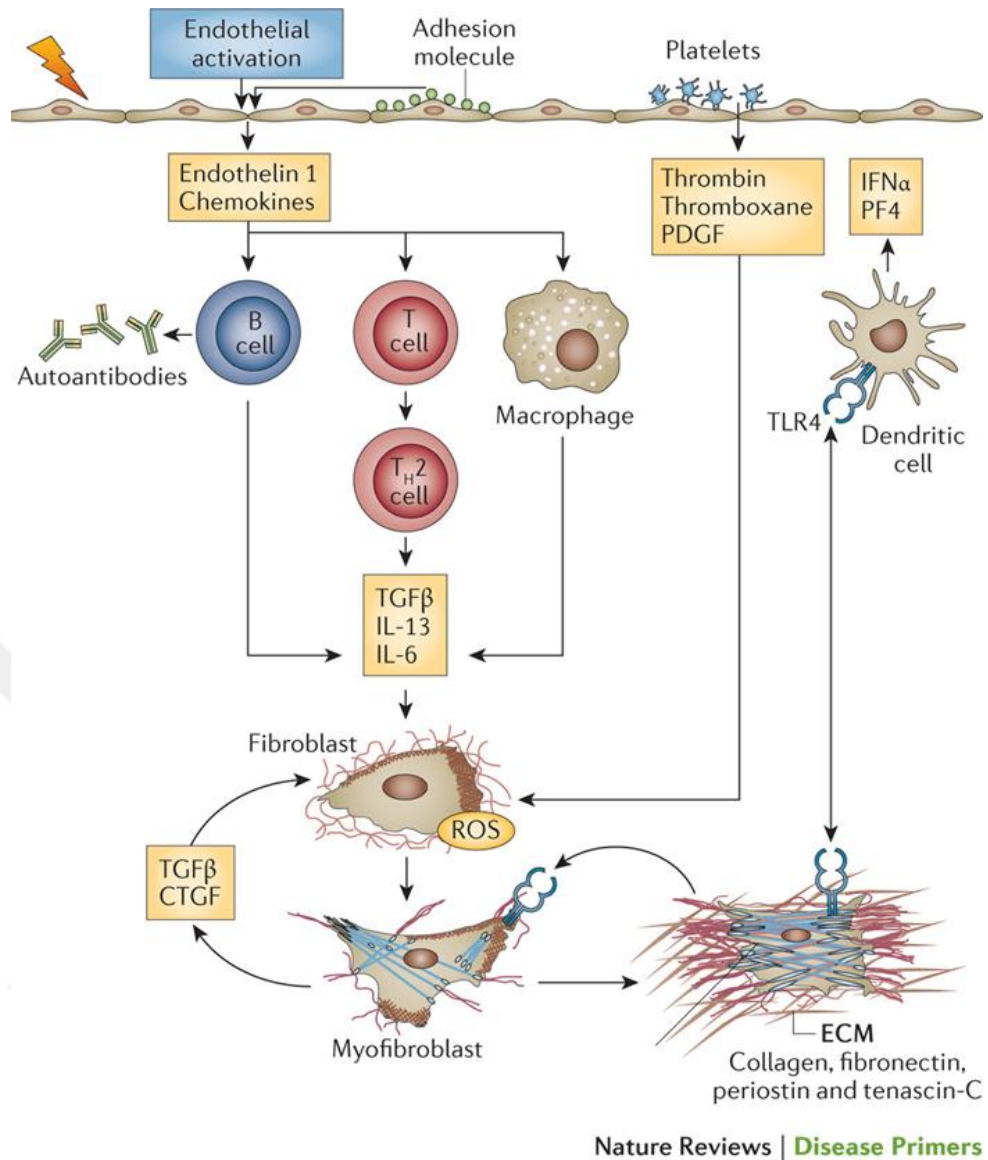


Figure 1.3 Overall disease progression of systemic sclerosis [81].

1.4.2.1 Role of PRRs in SSc

Although what initiates development of systemic sclerosis still remains unknown, it is argued that DAMPs have a crucial role to start the inflammation and subsequent fibrosis likewise in other autoimmune diseases [82]. Molecules that are secreted from damaged cells such as high mobility group box1 (HMGB1), DNA, RNA, histones, ATP and heat-shock proteins (HSPs) induce PRRs including TLRs and lead to pro-inflammatory cytokines and type I interferon production [83]. TLR2, TLR4 and TLR9 has been found to be important in development of inflammation in systemic sclerosis, and some of the ligands bound to these receptors are elevated in serum of patients [84-86].

Serum amyloid A (SAA) is an acute phase reactant and ligand for TLR2, and SAA levels in serum increase in case of an inflammation. O'Reilly et al. demonstrated that SAA binds TLR2, elevating IL-6 production through IRAK4 and NF κ B [87]. Moreover, TLR2 expression was upregulated in SSc dermal fibroblasts compared to healthy dermal fibroblasts, possibly making the cells more sensitive to SAA.

TLR4 is the most studied pattern recognition receptor that is related with fibrosis. One study revealed TLR4 sensitized fibroblasts for fibrotic effects of TGF β [88]. TLR4 activation in these fibroblasts resulted in increase in collagen synthesis and gene expression related to extracellular matrix remodeling, and TLR4 abrogation diminished this effect. Tenascin-C is another danger signal for cells since its expression is restricted to the cells in embryonic development while adult cells do not express it. Piccinini et al. have reported that tenascin-C secreted from the cells after bacterial infection or tissue injury bound to TLR4 and induced production of both cytokines and phosphorylation of extracellular matrix proteins in macrophages, contributing development of fibrosis [89]. HMGB1 protein which is one of the DAMPs mentioned to have role on development of systemic sclerosis, was demonstrated to bind both TLR4 and TLR2 to initiate signaling and subsequent inflammation [90].

TLR9 is another receptor that is known to contribute development of fibrosis in systemic sclerosis. TLR9 was indicated to be upregulated in skin samples of SSc patients, and the major source of upregulation was from myofibroblasts [91]. A study showed nucleosomes, composed of two copies of each histones wrapped by helical DNA, were upregulated in serum of SSc patients and induced TLR9 signaling [92]. Furthermore, TLR9 expression was upregulated in B- and T-cells of SSc patients.

In addition to TLRs, NLRs such as NLRP3 was proved to have crucial role on inflammation and subsequent fibrosis in systemic sclerosis. To summarize NLRP3 inflammasome signaling, receptor subunits constitute a ring-like structure for enabling pro-caspase1 recruitment upon induction of the receptor. Pro-caspase-1 cleavage leads IL-1 β and IL-18 secretion following the cleavage of pro-IL-1 β and pro-IL-18 [93]. NLRP3 activation as well as increased IL-1 β and IL-18 transcription were observed in dermal fibroblasts of SSc patients while caspase-1 inhibition decreased the level of cytokines with the reduction of α -SMA and collagen [94]. Additionally, ASC^(-/-) and NLRP3^(-/-) mice were reported to be resistant to bleomycin-induced fibrosis.

To sum up, many pattern recognition receptors are involved in inflammatory phase of systemic sclerosis, contributing to tissue damage.

1.4.2.2 Role of Immune Cells in SSc

1.4.2.2.1 Neutrophils in SSc

Neutrophils are phagocytic innate immune cells with a diverse range of functions such as production of neutrophil extracellular traps (NETs), antimicrobial proteins and chemokines to recruit other immune cells to infected area, and they are the most abundant innate immune cells in human blood [95]. Because neutrophils are the first immune cells to infiltrate into inflamed areas, they are critical in the management of early inflammatory response. They have a relatively short lifespan in the circulation, and they are cleared by macrophages and immature dendritic cells in bone marrow, liver or spleen [96].

Neutrophils secrete their own DNA wrapped with histones and antimicrobial proteins to trap microbes when they encounter with antigens, and this phenomenon is called neutrophil extracellular traps (NETs) [97]. After the discovery of NETs, association of neutrophils with many diseases including autoinflammatory diseases was described (i.e. systemic lupus erythematosus, rheumatoid arthritis, vasculitis and systemic sclerosis).

Activation and infiltration of neutrophils must be strictly controlled; otherwise, overexuberant activation could severely harm the tissue and worsen the inflammatory process. In a review by Groves and Soehnlein, contribution of neutrophils to acute lung injury (ALI) and acute response distress syndrome (ARDS) were discussed [98]. According to this report, neutrophils modulate lung inflammation by secreting neutrophil-derived serine proteases and enzymes on their granules, matrix metalloproteinases to degrade extracellular matrix components and producing ROS and NOS. An interesting study revealed how neutrophil extracellular traps induce fibroblast activation, proliferation and their differentiation into myofibroblasts [99]. In this study, data revealed that IL-17 expressed in NETs enhanced fibrotic activity of differentiated lung fibroblasts, and NETs were in proximity with α -SMA expressing fibroblasts of patients having systemic sclerosis. Therefore, contribution of NETs on fibrosis has been demonstrated with this study, besides the well-known effects of NETs on inflammatory diseases. Lastly, reasons of autoantibody production in systemic sclerosis and other autoimmune diseases are still unknown. After binding of autoantibodies to deiminated core histones of active neutrophils has been revealed [100], Dwivedi et al. demonstrated neutrophils are the responsible cells that produce citrullinated histones as a component of NETs. [101]. Furthermore, because neutrophil activation and antibody production against citrullinated histones took place simultaneously, it is possible that NETosis induce B-cell activity.

1.4.2.2.2 Macrophages in SSc

Macrophages are highly critical in defense against pathogens since their phagocytic capacity is very high. They also play an essential role on clearance of dead and apoptotic cells. Moreover, they contribute to the formation of humoral immunity by antigen presentation. Every organ/tissue has its own resident macrophage population originated from embryonic yolk sac and fetal liver during embryonic development, and they can proliferate and maintain themselves in the course of inflammation [102]. However, circulating monocytes also help to replenish macrophage population in tissues when there is a severe inflammation [103]. Depending on their location, macrophages are named differently. For instance, macrophages found and specialized in liver are called Kupffer cells while macrophages in central nervous system are microglial cells.

Macrophages either prevent or contribute to progression of diseases such as diabetes, cancer, and atherosclerosis. There are also many studies on how they contribute to pathogenesis of systemic sclerosis and how they can be utilized as a therapeutic target [104-106]. Impaired regulation of macrophage function such as uncontrolled cytokine production, impaired connection between macrophages and non-immune cells might exacerbate inflammation as well as fibrosis [107].

Increase in macrophage-attractant chemokines and macrophage-derived cytokines/molecules in blood, skin and lung of systemic sclerosis patients indicated that macrophages are recruited to inflammatory or fibrotic region and play role on the course of disease [108, 109]. In that regard, CCL2, CCL5 and CCL19 were shown to be positively correlated to SSc progression and severity. In a study conducted by Mathes and co-workers revealed that high CCL19 production on skin was due to CD163⁺ pro-fibrotic macrophages [108]. Furthermore, CCL19 levels were in line with vascular inflammation score in systemic sclerosis patients.

In addition to production of inflammatory molecules such as chemokines, cytokines and ROS, macrophages are also well-known sources of fibrosis-promoting cytokines such as PDGF, TGF β and matrix metalloproteinases. TGF β is the most studied pro-fibrotic cytokine; therefore, its signaling pathway and modulators are well-known. SMAD2, SMAD3 and SMAD7 proteins are downstream elements of TGF β signaling pathway [110]. SMAD2 and SMAD3 positively-regulate signaling pathway while SMAD7 serves as negative-regulator of TGF β . TGF β secreted from macrophages result in transformation of fibroblasts to myofibroblasts. Moreover, inflammatory cytokines, e.g. IL-1 β , TNF α and IFN γ also induce TGF β receptor type I, enhancing signaling activity.

Macrophages are mainly divided into two phenotypic sub-groups as M1 (pro-inflammatory) and M2 (anti-inflammatory) [111]. On one hand, M1-macrophages are known to upregulate glycolysis to satisfy the high energy need and to produce pro-inflammatory cytokines, nitric oxide (NO) and reactive oxygen species (ROS). On the other hand, M2-macrophages engage mainly in oxidative phosphorylation and fatty acid oxidation processes, contributing to tissue remodeling and wound healing activities. Tissue microenvironment has a special importance on regulation of macrophage polarization and metabolism since macrophages are affected by nutrient and oxygen level and modulate their effector function according to these factors. Recent studies demonstrated that macrophages are not terminally differentiated cells and possess phenotypic plasticity. Cellular metabolism plays a crucial role on determining its activation and polarization states [112, 113].

Effects of macrophage polarization on inflammatory and fibrotic phases of systemic sclerosis has been investigated; however, role of M1-macrophages on fibrosis is not fully understood. Studies indicated that M1-macrophages can both initiate and inhibit pulmonary fibrosis [114]. Additionally, M2-macrophages have been shown to contribute fibrotic development by producing TGF β , IL-4 and IL-10, and M2-depletion attenuated bleomycin-induced lung fibrosis in mice [115].

1.4.2.2.3 Lymphocytes in SSc

In addition to myeloid cells such as macrophages and neutrophils, T- and B-lymphocytes are two adaptive immune system cell types which participate in development and progression of systemic sclerosis.

CD8⁺ T cells were detected in blood and recruited to organs such as lungs in SSc patients [116-118]. One study demonstrated that CD8⁺ T-cells increased expressions of granzyme B, granzyme K, CD3 and CD8; moreover, they are highly active and capable of infiltrate into inflamed tissues [119]. A recent work showed that effector/memory T-cells constituted an important portion of CD8⁺ T-cell population isolated from blood, and these cells were responsible for production of pro-fibrotic cytokines, i.e. TGF β , IL-4, IL-10 and IL-13 whose levels are significantly high that of healthy controls [120]. Th2 cytokine production from CD8⁺ T-cells was correlated with impaired lung function and fibrosis [118] while IL-13 secretion was shown to modulate skin fibrosis in SSc patients [117]. Overall, these studies clearly exhibited the role of CD8⁺ T-cells on both inflammation and fibrosis in SSc.

CD4⁺ T-cells are divided into many groups, and this classification has become more diverse in the recent years with the addition of Th9 and Th17 cells besides well-characterized Th1, Th2,

Th17, Treg (regulatory) and Tfh (follicular helper) cells. Th1, Th17 and Th22 cells are more abundant in inflammatory phase of systemic sclerosis while Th2 is the dominant helper T-cell group in fibrotic phase of SSc [121]. Although Th17 cell abundance is more pronounced in defense against extracellular pathogens, bronchoalveolar lavage and serum of SSc patients displayed high levels of IL-17A and IL-22 whose major source is Th17 cells [122]. Furthermore, IL-17A secreted from Th17 cells elevated the production of pro-inflammatory cytokines while inhibited type I collagen expression from fibroblasts, contributing the inflammatory response and abrogating fibrotic development.

Role of Treg cells on systemic sclerosis is still not fully elucidated due to conflicting reports. Although most of the reports argued Tregs abolished in SSc patients [123, 124], some studies claimed cell population increased in circulation [125].

B-lymphocytes are the source of auto-antibodies observed in blood of systemic sclerosis patients, as well as other autoimmune diseases. Studies showed that B cells were recruited to affected organs such as skin and lung [126, 127]. B-cell hyperactivation was also reported with the increased expression of activation marker CD19 and decreased expression of CD22 [128] whereas Breg (regulatory B cell) population and function were impaired in patients [129]. Antibodies against DNA topoisomerase I [130], RNA polymerase III, centromeres, fibrillarin [131] are commonly found in serum of systemic sclerosis patients and regarded as one of the symptoms of the disease.

1.4.2.3 Role of Non-Immune Cells in SSc

Non-immune cells such as endothelial cells, vascular smooth muscle cells, fibroblasts and epithelial cells play essential roles on progression of systemic sclerosis. Epithelial cells are the first line of defense for invading pathogens and harmful molecules. These cells produce molecules such as H₂O₂, ROS, self-nucleic acids and proteins such as HMGB1, heat-shock proteins and fragments of extracellular matrix components upon cell damage to induce immune cell activation and infiltration, contributing inflammation [132]. How these molecules are recognized by immune cells were outlined in Section 1.4.2.1.

Damage on vascular structures and endothelial cells are considered as early signs in SSc development. Upon activation by pathogens, stress or chemicals, endothelial cells produce chemokines such as MIP1 α , MIP1 β and MCP-1 in addition to upregulation of adhesion molecules and selectins [133]. Chemokines induce recruitment of immune cells while proteins, endothelin-1 and CTGF (connective tissue growth factor) lead to proliferation of vascular smooth muscle cells. Increased numbers of vascular smooth muscle cells and accumulation of

ECM proteins result in stiffness of vessels, eventually causing narrowed capillaries, oxidative stress and hypoxia [134]. In other words, vascular dysfunction and abnormalities, early symptoms of SSc, are the result of activated vascular smooth muscle cells.

Endothelial and epithelial cells mostly contribute to inflammation and early symptoms of systemic sclerosis whereas fibroblasts generally play role on development of fibrosis. Fibroblast to myofibroblast transformation is the main event that leads accumulation of extracellular matrix proteins and development of fibrotic tissue, and proteins produced by cells in the same environment drive this shift [135]. However, an interesting study revealed myofibroblasts is not only originated from fibroblasts but also from adiponectin-positive progenitors in intradermal adipose tissue [136].

1.4.3 Current and Prospective Treatment Options in SSc

Systemic sclerosis has no curative treatment because disease symptoms vary among patients as well as response to applied drug therapies [137]. Therefore, treatment options approved for SSc are only useful to relieve specific symptoms such as interstitial lung disease and pulmonary arterial hypertension. Similar to other autoimmune diseases, immunosuppressive therapies are widely used in treatment of systemic sclerosis. However, long-term effects of immunosuppressive drugs have not been well-established. A recent study in 2018 investigated the long-term effects of cyclophosphamide therapy [138]. In this study, 30 patients received cyclophosphamide (CYC) for 2 years and were followed for 5 years after CYC therapy. Results showed that CYC therapy for two years failed to cure interstitial lung disease and did not improve lung functions, it only stopped disease progression.

European League of Rheumatism (EULAR) have published recommendation for treatment of SSc at 2017 [139]. According to this, vasodilators including nifedipine, sildenafil and tadalafil are most recommended drugs against digital ulcers and Raynaud phenomenon seen in SSc patients. Furthermore, immunomodulatory drugs such as methotrexate and cyclophosphamide are suggested to treat progressive skin disease. Therapeutics that are currently under development focus on four different targets; fibrosis, inflammation, immune and vascular system.

TGF β pathway is one of the therapeutic targets. For instance, fresolimumab, a humanized antibody for all 3 isoforms of TGF β was failed in phase I trial. Although treatment improved clinical symptoms by decreasing myofibroblast infiltration to skin [140], complete blockade of TGF β pathway might have side-effects on wound healing, immunity and tumor suppression [110]. Receptors such as integrin α V β 6 on epithelial cells, α V β on epithelial cells and α V β 8 on

dendritic cells also play role on TGF β activation and are targeted for treatment of SSc. A humanized antibody targeting integrin α V β 6 which is upregulated during injury and fibrosis is currently in phase II with patients having idiopathic pulmonary fibrosis (IPF) [141]. Furthermore, therapies targeting various tyrosine kinases are under investigation since blockade of tyrosine kinase function abolished progression of fibrosis. In this regard, nintedanib draw the attention for becoming the first antifibrotic agent used in clinics. Nintedanib is a small molecule that diminishes the activity of multiple receptor tyrosine kinases such as FGF, VEGF and PDGF inhibiting these signaling pathways and fibroblast proliferation [142]. It is now approved by FDA for the use in patients with idiopathic pulmonary fibrosis, and phase III trials was successfully completed with 580 SSc patients having lung fibrosis [143].

Inflammation is the main driver of fibrosis as it is outlined before. IL-6 is the main inflammatory cytokine which signals through JAK-STAT pathway and IL-6 receptor. Since mice cannot produce IL-6 or given IL-6R antibody were protected from bleomycin-induced inflammation and fibrosis, therapies targeting IL-6 signaling pathway are currently under investigation. IL-6R antagonist tocilizumab has finished phase II trials in patients with SSc [144]. Although patients treated with either tocilizumab for 96 weeks or with placebo for 48 weeks and with tocilizumab for another 48 weeks showed improvements in Rodnan skin score and lung function, it led to serious infections in some of the patients [145]. Small molecules that inhibit TLR4 signaling and activation has been also studied. T5342126, a molecule competing with MD2, TLR4 co-receptor, abrogated collagen synthesis and myofibroblast differentiation in fibroblasts treated with different TLR4 agonists [146]. Furthermore, Pfizer recently developed and patented an IRAK4 inhibitor (PF-06650833), a kinase in downstream of TLR4, and its safety and efficiency has been tested in patients having rheumatoid arthritis; however, results has not been reported yet [147].

Chemokines are another set of targets for the treatment in autoimmune diseases; however, most of them had failed. For instance, serum levels of CCL2 was surprisingly higher in anti-CCL2 antibody given rheumatoid arthritis patients, in as dose-dependent manner, and no improvement was observed in clinical symptoms [148]. The reason of this unexpected result might be due to compensatory mechanisms regulating chemokine secretion and signaling.

Vascular injury, abnormalities and Raynaud's phenomenon are early symptoms of SSc. Pulmonary arterial disease is one of the vascular dysfunctions that arises in SSc patients. Selexipag, a prostacyclin agonist leading to vasodilation, was tested against Raynaud's phenomenon attacks in 78 SSc patients; however, no significant difference in symptoms was detected between placebo and treatment groups [149, 150]. Statins have been shown to have beneficial effects on treatment of vascular diseases in SSc. A small study indicated lower

Raynaud's condition score and decrease in the expressions of vascular/endothelial activation and injury markers in addition to angiogenic factors [151].

Consequently, search for development of a curative therapy is still a continued effort although few promising drugs are on trials.



1.5. Aim and Outline of the Study

Systemic sclerosis is an autoimmune disease affecting one or many organs in body. Vasodilators and immunomodulators are frequently used to treat SSc. However, none of these have succeeded to fully alleviate the symptoms of the disease. Our main goal is to explore therapeutic potential of an immunosuppressive oligodeoxynucleotide called A151 ODN on mouse model of systemic sclerosis established by intratracheal bleomycin instillation. A151 ODN is patterned after the repeat sequence motif of the telomeric DNA, and it is expressing 4 repeats of “TTAGGG” motif. To further boost its *in vivo* immunosuppressive capacity and protect it from premature degradation, we aimed to entrap this ODN within anionic liposomes.

Firstly, modulation of A151 ODN on cytokine production mediated by TLR1/2, TLR4 and TLR9 will be assessed in splenocytes culture. Then, we will focus on two cell types that have critical roles on pathogenesis of the systemic sclerosis; macrophages and fibroblasts. Initially, bone marrow-derived macrophages (BMDMs) will be activated with TLR ligands in absence or presence of A151 ODN, and surface markers that mediate macrophage activation, adhesion and migration will be analyzed using flow cytometry. Inhibitory effects of A151 ODN on IL-1 β secretion upon AIM2 and NLRP3 inflammasome pathways activation will be evaluated using ELISA. Conditioned media (CM) will be collected from BMDMs that will be stimulated with TLR1/2, TLR4 and TLR9 agonists in absence or presence of A151 ODN, and this will be included to the mouse fibroblast cell line NIH3T3, culture and cytokine secretions of NIH3T3 and BMDMs will be measured using ELISA. Therefore, suppression of A151 ODN on cytokine production as well as chemokine and inflammatory mediator secretion will be explored. Fibroblasts will be utilized to identify anti-fibrotic potential of A151 ODN. To this end, NIH3T3 cells will be incubated with bleomycin plus TLR ligands and TGF β , and how A151 ODN modulates Tgf β and Col1a1 gene expressions will be analyzed using RT-qPCR.

To test the therapeutic potential of A151 ODN *in vivo*, we will first investigate if pre-A151 treatment prevents inflammation in early phase of SSc following establishment of systemic sclerosis model in mice. Mice will be treated with A151 ODN 3 days before bleomycin administration and will be sacrificed at day 3 following bleomycin injection. In another experiment, mice will be treated with either A151 ODN alone or within anionic liposomes at 3rd and 5th days following bleomycin application and will be killed at 7th day. We will attempt to unearth the anti-inflammatory capacity of immunosuppressive A151 ODN on lung inflammation. Lastly, to determine the anti-fibrotic and therapeutic effects of A151 ODN on established lung fibrosis and systemic sclerosis, mice will receive liposome-encapsulated A151

ODN at day 5, day 10 or at day 15 following bleomycin instillation and will be sacrificed at day 20 to investigate the anti-fibrotic cues mediated by the liposome encapsulating A151 ODN.



CHAPTER 2

2. Materials and Methods

2.1 Materials

2.1.1 TLR Ligands and Antigens

For *in vitro* experiments; TLR1/2 ligand Pam3csk4, AIM2 inflammasome activator p(dA/dT) and NLRP3 inflammasome activator Nigericin were purchased from Invivogen (San Diego, USA). TLR4 ligand LPS (isolated from *E. coli*) was obtained from Sigma (St. Louis, USA). Phosphorothioate backbone modified TLR9 ligand K23 ODN, control ODN 1471, and immunosuppressive A151 ODN were purchased from Alpha DNA (Montreal, Canada), and their base sequences are provided below.

K23 CpG ODN (12-mer): 5'-TCGAGCGTTCT-3'

1471 Control ODN (10-mer): 5'-TCAAGCTTGA-3'

A151 Suppressive ODN (24-mer): 5'-TTAGGGTTAGGGTTAGGGTTAGGG-3'

2.1.2 Lipids

Phosphatidylcholine (PC), 1,2-dioleoyl-sn glycerol-3-phosphoethanolamine (DOPE) and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabama, USA).

2.1.3 Cell Culture Media and Standard Buffer Solutions

DNase/RNase free water, DPBS (Dulbecco's Phosphate Buffered Saline), trypsin 2.5% 10X, RPMI-1640 (Roswell Park Memorial Institute Medium), high glucose DMEM (Dulbecco's Modified Eagle Medium), penicillin/streptomycin, non-essential amino acids, L-glutamine and FBS (fetal bovine serum) were from Gibco (Waltham, USA). HEPES (N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid) were obtained from Biological Industries (Cromwell, USA).

2.1.4 Antibodies

Cytokine antibody pairs (monoclonal unlabeled antibodies for coating and their biotinylated conjugates for detecting) were given in Table 2.1, and the recombinant proteins used to construct ELISA standard curves were given in Table 2.2. ELISA antibodies and recombinant proteins were either purchased from Biolegend (San Diego, USA) or Mabtech (Nacka, Sweden).

Table 2.1. Mouse-specific monoclonal antibody pairs (unlabeled and biotinylated) used throughout the ELISA studies.

Product	Catalog No.	Clone	Company	Working Concentration
Anti-mouse IL-6	504502	MP5-20F3	Biolegend	2 µg/ml
Anti-mouse IL-1β	503502	B122	Biolegend	6 µg/ml
Anti-mouse IL-12	3451-1A-20	C15.6	Mabtech	2 µg/ml
Anti-mouse IFNγ	3321-1A-20	AN18	Mabtech	1 µg/ml
Anti-mouse TNFα	3511-1A-20	MT1C8/23C9	Mabtech	2 µg/ml
Biotin anti-mouse IL-6	504602	MP5-32C11	Biolegend	1:1000
Biotin anti-mouse IL-1 β	515801	Poly5158	Biolegend	1:1000
Biotin anti-mouse IL-12	3451-1A-20	C17.8	Mabtech	1:1000
Biotin anti-mouse IFNγ	3321-1A-20	R4-6A2	Mabtech	1:1000
Biotin anti-mouse TNFα	3511-1A-20	MT11B10	Mabtech	1:1000

Table 2.2. Standard recombinant proteins and their respective initial concentrations used throughout the ELISA studies.

Recombinant Cytokine Product	Catalog No.	Company	Working Concentration
IL-6	575702	Biolegend	100 ng /ml
IL-1β	575102	Biolegend	250 ng/ml
IL-12	3451-1A-20	Mabtech	10 ng/ml
IFNγ	3321-1A-20	Mabtech	20-40 ng/ml
TNFα	3511-1A-20	Mabtech	25-50 ng/ml
TGFβ	580702	Biolegend	50 ng/ml

Streptavidin-alkaline phosphatase was purchased from Mabtech, while p-nitrophenyl phosphate disodium hexahydrate (PNPP) was obtained from Thermo Fisher Scientific (Waltham, USA).

Specificities of fluorochrome-conjugated antibodies for flow cytometry experiments were listed in Table 2.3. Fluorochrome-conjugated antibodies were from Biolegend, Tonbo Biosciences (San Diego, USA) and BD (Franklin Lakes, USA).

Table 2.3 Fluorochrome-conjugated mouse-specific antibodies used throughout flow cytometry studies.

Antibody	Catalog No.	Clone	Company
CD11b-FITC	N/A	N/A	Tonbo
CD11b-PE	101208	M1/70	Biolegend
CD11c-Pacific Blue	117322	N418	Biolegend
CD40-PE/Cy5	124618	3/23	Biolegend
CD45-PE/Cy5	103110	30-F11	Biolegend
CD45R(B220)-FITC	103205	RA3-6B2	Biolegend
CD49b-PE	108908	DX5	Biolegend
CD54 (ICAM1)-PE	116108	YN1/1.7.4	Biolegend
CD80-FITC	104706	16-10A1	Biolegend
CD86-APC/Cy7	105030	GL-1	Biolegend
CD117-FITC	553354	2B8	BD
CD182 (CXCR2)-AF647	149604	SA045E1	Biolegend
F4/80-APC	123116	BM8	Biolegend
F4/80-PE/Cy7	123114	BM8	Biolegend
F4/80-FITC	123108	BM8	Biolegend
Gr-1-PE	108408	RB6/8C5	Biolegend
MHCII(I/A-I/E)-BV650	107641	M5/114.15.2	Biolegend
Ly6C-BV785	128041	HK1.4	Biolegend
Ly6G-PE	127608	1A8	Biolegend
Ly6G-PE/Cy7	127618	1A8	Biolegend

Antibodies that were purchased from Abcam (Cambridge, UK) and Cell Signaling Technology (Danvers, USA) and used for immunoblotting experiments were given below.

Table 2.4. Primary and HRP-conjugated secondary antibodies used throughout Western blotting experiments.

Antibody Name	Catalog No.	Company	Working Concentration
Anti-alpha smooth muscle actin	ab5694	Abcam	1:2000 in 5% BSA
Anti-Phospho-Smad2 (Ser465/467)	138D4	Cell Signaling Technology	1:1000 in 5% BSA
Anti-Smad2/3	8685	Cell Signaling Technology	1:1000 in 5% BSA
Anti-β-actin	3700	Cell Signaling Technology	1:2000 in 5% non-fat milk powder
Anti-rabbit IgG-HRP Linked	7074	Cell Signaling Technology	1:5000 in 5% BSA
Anti-mouse IgG-HRP Linked	7076	Cell Signaling Technology	1:5000 in 5% non-fat milk powder

2.2 Methods

2.2.1 Liposome Preparation

Lipid stocks were prepared by dissolving in chloroform, and 10-20 mg/ml stocks were stored at -20°C until use. For anionic liposome preparation, 10 μ mole of PC:PS:DOPE were mixed in 1.0:0.25:0.5 molar ratio in a round bottom flask [152]. The flask was hooked to a rotary evaporator (ILMVAC, Ilmenau, Germany) and immersed into a water bath that was adjusted to 37°C, and the solvent was completely evaporated at 260 rpm for approximately 15 minutes of rotation. The solvent-free dry lipid film was purged with nitrogen for 2-3 minutes to eliminate residual chloroform and replace oxygen, thereby preventing lipid peroxidation process. Multi-lamellar vesicles were generated by adding glass beads to flask, and 1 ml PBS was added for 10 μ mole dried lipid film. Flask was gently swirled until all lipid layer is removed from the inner surface of flask. Solution was collected and put into a glass tube and then was sonicated with 10 cycles of 30 seconds “on” and 30 seconds “off” on ice using Vibra Cell Sonicator (Sonics and Materials, Newton, USA) to generate small unilamellar vesicles (SUV).

2.2.1.1 A151 ODN Encapsulation within Liposomes

SUV were mixed with A151 ODN, and the mixture was immediately snap-frozen in liquid nitrogen. For encapsulation within 2.5 μ mole of liposomes, 100 μ g of A151 ODN was used. Following overnight freeze-drying by Virtis Benchtop K (SP Industries, Warminster, USA), liquid free powder liposome-A151 mixture can be stored at 4°C for years or rehydrated for encapsulation. For encapsulation, sterile DNase/RNase free water was added 1/10 of total initial snap frozen volume, and mixture was vigorously vortexed for 15 sec. in every 5 minutes for total 30 minutes at room temperature. At the end of 30 minutes, 1/10 of total volume of sterile PBS was added to the mixture, vortexed gently and waited for 10 minutes at room temperature to allow equilibration of tonicity. Remaining 8/10 of total volume was completed with PBS. Formulations were stored at 4°C until further use.

2.2.1.2 Size Measurement of Liposomes using Dynamic Light Scattering

Anionic liposomes were diluted with PBS and was adjusted to 1 μ M concentration. The size measurements were performed in triplicates using Zetasizer Nano ZS instrument (Malvern, UK).

2.2.2 Cell Culture

2.2.2.1 Splenocyte Isolation

Splenocytes were isolated from surgically taken spleen of 6-8 weeks old male BALB/c or C57BL/6 mice. Back of the sterile plunger of a 2 ml syringe was used to obtain single cell suspension from spleen. Cells were washed twice with 2% FBS containing complete RPMI medium, centrifuged at 300 x g for 5 minutes and suspended in 5% FBS containing complete RPMI for cell counting and stimulation. Cell counting was performed by flow cytometry.

2.2.2.2 Bone Marrow-Derived Macrophages (BMDM) Preparation

6-8 weeks old male C57BL/6 mice were euthanized by cervical dislocation, femurs and tibias were removed. Following immersion of the bones into 70% ethanol for a short while, both ends of bones were cut with a scissor and bone marrow was flushed out using a syringe. Cells were washed with 2% RPMI, centrifuged at 300 x g for 5 minutes. After supernatant was removed,

cells were suspended in 100 µl of ACK Lysis Buffer (Lonza, Basel, Switzerland) to lyse red blood for 30 seconds, and cells were washed with 2% RPMI. After centrifugation, cells were counted by flow cytometry.

30% (v/v) L-929 conditioned medium containing 10% RPMI was prepared to seed the cells (for preparation of L-929 conditioned medium, please refer to section 2.2.2.4.1). Cells were seeded as 5×10^5 cells/1 ml media/well in 24-well ultra-low adherent plates which were purchased from Corning (New York, USA). 300 µl of conditioned media was supplemented to the cells 3 days after seeding. At the end of 7 days culture, cells were harvested by adding 10 mM EDTA dissolved in sterile PBS and incubating at 4°C for 20 minutes. F4/80-APC and CD11b-FITC conjugated antibodies were used to characterize cells as bone marrow derived macrophages [153] using Novocyte flow cytometer (ACEA Biosciences, San Diego, USA).

2.2.2.3 Single Cell Isolation from Lung Tissue

Mouse (C57Bl/6, female, 6-8wks old) lungs were cut into small pieces and incubated with 1.5 Wünsch Liberase DL (Roche, Basel, Switzerland) diluted in 2% RPMI for 1 hour at 37°C by vigorous vortexing every 15 minutes. Lung pieces containing Liberase solution was then transferred to 70 µm cell strainers and washed with 1 ml, 2% fresh RPMI media. Cells were precipitated at 500 x g for 5 minutes. Supernatant was removed, and cells were washed again with fresh cold media. Cell pellet was dissolved in 2% RPMI and cells were stained for determination of immune cell population (please see section 2.2.6.2 for details).

2.2.2.4 Cell Lines

2.2.2.4.1 L-929

L-929 (NCTC clone 929 [L cell, L-929, derivative of strain L] (ATCC® CCL-1™)) is a mouse fibroblast cell line. The suggested growth medium was high glucose DMEM with 10% FBS. When cell confluency reaches 80%, medium is refreshed in every 2-3 days. For cell detachment, cells are washed with PBS, and incubated for 3-5 minutes in 1X trypsin at 37°C. Following washing the cells with fresh medium, they were sub-cultured either in 1:4 or 1:8 ratio.

To obtain conditioned medium necessary for bone marrow derived macrophage experiments, 5×10^5 cells in 50 ml of 10% RPMI were transferred into T175 flasks [154]. After 6-7 days, supernatant was collected from confluent flask. Medium was centrifuged at 300 x g for 5 minutes, filtered with 0.22 µm filter and stored at -20°C until further use.

2.2.2.4.2 NIH3T3

NIH3T3 (ATCC® CRL-1658™) is a mouse fibroblast cell line. When cell confluency reaches 80%, medium is refreshed in every 2-3 days. For cell detachment, cells are washed with PBS and incubated for 3-5 minutes in 1X trypsin at 37°C. Following washing the cells with fresh medium, they were sub-cultured either in 1:6 or 1:8 ratio. 10% FBS containing high glucose DMEM was used for cell growth.

2.2.2.5 Cell Counting and Layering

After washing the cells, they were suspended in 1 ml of respective cell culture medium. 10 µl of cell aliquots were taken and 1:10 diluted with fresh medium. Then, it was 1:2 diluted with trypan blue. 10 µl of 1:20 diluted cell suspension was added on hemocytometer, and cells in red areas were counted in Figure 2.1.

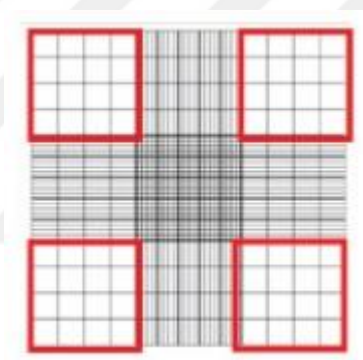


Figure 2.1 Representative hemocytometer grids under the light microscope.

White and bright dots show alive cells while blue and dim dots represent dead cells. Volume of the grids were 0.1 mm³, and total cell number was calculated using the formula below:

Number of viable cells/ml = (sum of bright cell # in red grids/4) x 10⁴ x 20 (dilution factor) x 1 (ml)

To count viable cells using Novocyte flow cytometer, primary or cell lines were suspended in 1 ml warm culture media. Suspension was diluted using an isotonic solution such as PBS and counted. In the case of primary cells Zap-O-globin solution (1 drop for every 2ml solution) was added to remove the RBCs. After cell debris and apoptotic cells were gated-out in FSC-H vs SSC-H plot, singlet cell population was taken in FSC-H vs FSC-A plot. Cell count was multiplied by dilution factor, and number of cells in the suspension was calculated.

2.2.3 *In Vitro* Stimulation of Cells with Ligands

During *in vitro* assays of the immune cells varying sensor ligands or stimulants were used throughout the study. These were listed in Table 2.5.

Table 2.5. Stimulants and their concentrations used during *in vitro* stimulation assays.

Ligand	Concentration
CpG ODN-K23 (TLR9L)	1.0 μ M (3.6 μ g/ml)
Control ODN-1471	1.0 μ M (3.0 μ g/ml)
Suppressive ODN-A151	3.0 μ M (21.6 μ g/ml)
Pam3csk4 (TLR1/2L)	1 μ g/ml
LPS (TLR4L)	1 μ g/ml
Nigericin (NLRP3L)	10 μ M
Poly (dA/dT) (AIM2L)	1.5 μ g/ml
TGF β	50 ng/ml
Bleomycin	0.1 μ g/ml

For stimulation of splenocytes, 2×10^5 cells/250 μ l/well were inoculated into 96-well plate. After 24 hours of incubation with TLR ligands and A151 ODN, supernatants were collected, and ELISA was performed.

For stimulation of NIH3T3 cells, 8×10^5 cells/1.5 ml/well were seeded into 6 well plate. After 8 hours of incubation with ligands, cells were harvested, centrifuged and collected. The RNA isolation, cDNA synthesis and RT-qPCR were performed from each treated group of cells.

Conditioned media (CM) from BMDMs were obtained by 24 hours of stimulation of cells that were inoculated as 8×10^5 cells/1 ml/well in 12-well plate. Supernatants were collected, centrifuged and filtered with 0.22 μ m filter and stored at -20°C until further use. When culturing NIH3T3 cells, CM was mixed with fresh cell culture media in a ratio of 1:4 (v/v) and added on NIH3T3 cells that were seeded as 1×10^5 cells/250 μ l/well in 96 well plate for 24 hours of stimulation [155].

In some experiments, BMDMs were plated as 1.5×10^5 cell/250 μ l/well in 96 well plate for inflammasome activation and were primed with 0.1 μ g/ml LPS for 2 hours. Poly(dA/dT) or nigericin was then added on cells to activate AIM2 and NLRP3 inflammasome, respectively. Following 24 hours of incubation, cell supernatants were collected for ELISA. For stimulation with TLR ligands, 4×10^5 cells/700 μ l/well was inoculated in 24-well plate. Surface marker expressions of cells were analyzed with flow cytometry after 24 hours of stimulation.

2.2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

At the end of cell stimulation, plates were centrifuged at 300 x g for 5 minutes and supernatants were transferred into a new plate. Immulon 2HB plates (Thermo Fisher Scientific) were coated 50 µl/well with anti-cytokine antibodies dissolved in sterile filtered PBS and incubated at 4°C overnight or at RT for 4 hours [156]. After coating, antibody solution was removed from the plates, and 200 µl/well blocking solution (Appendix B1.3) was added into wells. Following 2 hours of incubation with blocking buffer, plates were washed with washing buffer (Appendix B1.4) 5 times for 1-minute intervals and rinsed once with dH₂O. Supernatants and 1:2 serially diluted recombinant standard proteins were added 50 µl/well and incubated at 4°C overnight or at RT for 4 hours. After the washing step as described above, plates were incubated with 50 µl/well of 1:1000 diluted biotinylated anti-cytokine for 2 hours at RT. Plates were washed, rinsed, and streptavidin-alkaline phosphatase were 1:1000 diluted in blocking buffer, added 50 µl/well. Cells were incubated for 1 hour at RT, followed by washing and 50 µl/well of PNPP addition. Several OD readings at 405 nm were taken until the recombinant cytokine standard curve forms a 4-parameter S-shaped curve by Microplate Reader (Molecular Devices, San Jose, USA). The concentrations of the unknown treatments were calculated from the best fitted curve using Softmax ProV5 software (Molecular Devices).

2.2.5 Determination of Gene Expression

2.2.5.1 Total RNA Isolation

Total RNA was isolated with Trizol reagent (Thermo Fisher Scientific) according to manufacturer's protocol. All work must be done under chemical hood and centrifugation steps must be performed at 4°C. Briefly, cells were suspended in 1 ml of Trizol, and 200 µl chloroform was added. Lysate were vigorously shaken, incubated at RT for 3 minutes and centrifuged at 16000 x g for 15 minutes. Upper clear phase was collected carefully by not interfering with the other layers. 500 µl of isopropanol was added to precipitate RNA, solution was mixed and incubated for 10 minutes at RT. Following 10 minutes of centrifugation at 16000 x g, supernatant was removed. Pellet was washed once with 1 ml of 75% ethanol and once with 1 ml of 99.8% ethanol by centrifuging at 6000 x g for 5 minutes. After ethanol was removed carefully by not disrupting the pellet, centrifuge tubes were tilted to evaporate the remaining ethanol under the lamin-air hood. Purified RNA was dissolved in DNase/RNase free water, concentration and purity were determined by Nanodrop (Thermo Fisher Scientific). RNA samples can be stored at -80°C for further use.

2.2.5.2 cDNA Synthesis

cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's protocol. Briefly, 1 µl of random hexamer and 1000 ng of RNA were mixed, and solution was completed to 12 µl by adding DNase/RNase free water. Samples were pre-denatured at 65°C for 5 minutes in MJ Mini Thermocycler (Bio-Rad, Hercules, USA). 4 µl of Reaction Buffer, 2 µl of dNTP, 1 µl of RiboLock and 1 µl of RevertAid which were supplied with the kit were mixed and added onto each sample. Tubes were incubated at 42°C for 60 minutes and 70°C for 5 minutes for enzyme deactivation. cDNAs can be stored at -20°C for further use.

2.2.5.3 RT-qPCR

The details of the in house designed PCR primers, their expected product sizes, and optimum melting temperatures were provided in Table 2.6. All primers were designed in house using Primer-Blast Software (NCBI, Bethesda, USA).

Table 2.6. RT-qPCR primers, expected product sizes and efficiencies.

Name	Primer	Sequence	Size (bp)	Tm	Efficiency
α-SMA	Forward	ATGCAGAAGGAGATCACAGC	150	55	2.057
	Reverse	GTATTCCTGTTTGCTGATCCAC			
β-actin	Forward	TTCGTTGCCGGTCCACACCC	90	55	1.844
	Reverse	GCTTTGCACATGCCGGAGCC			
Col1a1	Forward	AGCCGCAAAGAGTCTACATG	144	55	2.125
	Reverse	CTTAGGCCATTGTGTATGCAG			
Col1a2	Forward	CCGTGCTTCTCAGAACATCA	193	55	2.031
	Reverse	CTTGCCCCATTCAATTTGTCT			
Smad2	Forward	ATGTCGTCCATCTTGCCATTC	173	58	1.83
	Reverse	AACCGTCCTGTTTTCTTTAGCTT			
Tgfβ	Forward	CCTGAGTGGCTGTCTTTTGA	70	55	2.076
	Reverse	CCTGTATTCCGTCTCCTTGG			
Timp1	Forward	CCCAGAAATCAACGAGACCAC	136	62	1.954
	Reverse	TCCACAGAGGCTTTCCATGAC			

1 µl of cDNA, 3 µl of nuclease-free water, 5 µl of SYBR Green (Roche), 0.5 µl of forward primer (10 pmole) and 0.5 µl of reverse primer (10 pmole) were mixed per reaction in 96-well PCR plates. After a brief centrifugation, PCR reaction was performed by the LightCycler® 480 Real-Time PCR System (Roche). RT-qPCR conditions were provided in Table 2.7.

Table 2.7. RT-qPCR conditions used in experiments.

PCR Steps	Conditions
Initial denaturation	95°C 5'
Denaturation	95°C 15''
Annealing	20'' Refer to table 2.5 for temperatures
Extension	72°C 20''
Cycles	45

2.2.6 Flow Cytometry Analysis

2.2.6.1 Cell Fixation

Cells were fixed with Fix & Perm Medium A (Invitrogen, Carlsbad, USA) for further use in flow cytometry analysis. 100 µl of Fix & Perm Medium A was added for approximately 5×10^6 cells while vortexing, and cells were incubated at RT for 15 minutes. 1 ml FACS Buffer (Appendix B3) was added on cells and centrifuged at 300 x g for 5 minutes to remove fixation buffer. The supernatants were then aspirated, cells were suspended in 1 ml FACS Buffer and stored at 4°C up to 1 week.

2.2.6.2 Surface Marker Staining of Cells

Staining of fixed cells was performed in total volume of 100 µl. 1 µg/ml of fluorochrome conjugated antibody cocktail was prepared in 50 µl FACS buffer for each sample. Before adding the antibody cocktail, cells were incubated in 50 µl FACS buffer containing 0.5 µl TruStain fcX™ (Biolegend) for 1-2 minutes, then antibody cocktail was added. Incubation was done in dark at RT for 30 minutes. Cells were washed with FACS buffer and centrifuged at 300 x g for 5 minutes. Stained cells were resuspended in FACS buffer and analyzed using Novocyte flow cytometer instrument. Of important note, either PBS or medium was used for staining of living cells instead of FACS buffer. Moreover, incubation of living cells with dye cocktail was done at 4°C.

2.2.7 Western Blotting

2.2.7.1 Protein Isolation and Determination of Protein Concentration via BCA Assay

Lung tissue was homogenized using homogenizer (OMNI Inc., Marietta, USA) in RIPA buffer (see Appendix B2.8). Cells were incubated on ice and vortexed vigorously in every 10 minutes for 1 hour and then centrifuged at 10000 x g for 20 minutes at 4°C. Supernatants were transferred into a new microfuge tubes to measure the protein concentrations.

Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) according to manufacturer's protocol with minor modifications. Briefly, BSA protein standards were prepared with 1X PBS by diluting 10 times in 1:2 ratio, starting from 2 mg/ml. 5 µl of protein samples and 25 µl of each standard were pipetted on flat bottom 96-well plate. 200 µl of working reagent prepared with Reagent A:Reagent B in 1:50 ratio was added to wells. Following 30 minutes incubation in dark at 37°C, absorbance values were measured via Synergy Microplate Reader HT (Biotek, Winooski, USA) at 562 nm.

20 µg of protein was transferred from each sample to prepare loading mix, and remaining protein was aliquoted and stored at -80°C for further use. 2.5 µl of 4X Laemmli (Appendix B2.5) and 20 µg of protein were used to prepare the mix for loading into a well. Volume of mixture was completed with RIPA up to 10 µl. Mixture was heated to 95°C for 5 minutes.

2.2.7.2 SDS-PAGE

10% separating gels and 5% stacking gels (see Appendix B2.6, and B2.7) were prepared. Bio-Rad Mini Protean Tetra Cell (Bio-Rad, USA) equipment was used to run proteins through gel. Proteins were loaded in wells, they were run at 60 V. After they move to separating gel, voltage was increased to 100 V. Page ruler prestained protein ladder (Thermo Fisher Scientific) was used as protein ladder.

2.2.7.3 Wet Transfer

Transfer was performed using Mini Transblot Cell system (Bio-Rad). 1X transfer buffer (Appendix B2.4) that has been freshly prepared and stored at 4°C was poured into tank. A 0.45 µm PVDF membrane (Thermo Fisher Scientific) and Whatman papers (GE Healthcare, Chicago, USA) were cut as the same size of gel. To activate PVDF membrane, it was first

soaked in methanol for 5 minutes and then was equilibrated in 1X transfer buffer. Two sponges and four Whatman papers were used to prepare a sandwich in transfer apparatus and placed into tank. Transfer was done at 100 V for 90 minutes.

2.2.7.4 Blocking and Incubation with Antibodies

Blocking was done in 0.1% Tween-20 and 5% BSA (for phosphorylated proteins) or 5% non-fat milk (for other proteins) containing 1X PBS for 2 hours of incubation at RT. Primary antibodies were prepared in 0.05% Tween-20 and 5% BSA/milk containing 1X PBS, and membranes were incubated with primary antibodies overnight at 4°C [157]. Membranes were washed 3X for 10 minutes each with 1X PBS-T and then incubated with respective secondary antibodies for 1 hour at RT. Following three times of washing with 1X PBS-T, membranes were put in 1X PBS solution to obtain gel images.

2.2.7.5 Gel Imaging

ECL mix was prepared mixing detection reagent 1 (peroxide solution) and detection reagent 2 (luminol enhancer solution) in 1:1 ratio (Thermo Fisher Scientific). Gel images were taken by Amersham Imager 600 (GE Healthcare) accessorized with an inbuilt image analysis software.

2.2.8 Animal Experiments

2.2.8.1 Subcutaneous Bleomycin Administration

Back of 8-12 week-old male BALB/c mice was shaved, and a 1 cm² area was drawn with a marker. Each animal received subcutaneous (s.c.) bleomycin injection (100 µg/100 µl/mouse, a gift from Koçak Farma, Ankara, Turkey) that was dissolved in saline every other day for 35 days. The four sides of the 1cm² square in a clockwise manner was used for successive injections [158]. The animals were followed daily, their postures and weight losses were recorded.

2.2.8.2 Intratracheal Bleomycin Instillation

To develop lung inflammation and fibrosis, a method known as intratracheal instillation was applied to 8-12 weeks-old female C57BL/6 mice. After the animals were anesthetized with

ketamine, 50 µg/mouse bleomycin that was dissolved in 50 µl saline was slowly delivered through the lungs via larynx [159]. Animals were investigated until they wake up and were followed until they were confirmed that they behave normally in the cages.

2.2.8.3 Free and Liposomal A151 ODN Treatment

In order to explore preventive effects of A151 ODN on bleomycin induced lung inflammation, mice received intraperitoneal (i.p.) injections of 100 µg free or liposome encapsulating A151 ODN at day 0. Bleomycin was given 3 days later, and mice were sacrificed at day 6 after A151 ODN injection.

In some experiments where we sought to treat the lung inflammation following bleomycin insult, mice received two i.p. injections of 100 µg either free or liposomal A151 ODN at day 3 and day 5 following bleomycin instillation and mice were sacrificed at day 7.

To analyze A151 ODN mediated anti-fibrotic outcomes in another experiment, a group of mice received i.p. 100 µg of liposomal A151 ODN at 5th, 10th and 15th days following bleomycin instillation. All animals were then sacrificed at day 20. Cells were harvested and analyzed as described below.

2.2.8.4 Bronchoalveolar Lavage Fluid (BALF) Collection

Mouse was sacrificed via excess dose of anesthetics at the end of the experiment. Neck skin, salivary glands and muscles were removed to expose the trachea. Small incision was made by a scissor, and 22G catheter was placed in trachea. Catheter and trachea were tied together firmly by a nylon string [160]. 1 ml of fresh sterile PBS was flushed into alveolar space three times, and fluid was collected each time. Cells were centrifuged at 400 x g for 5 minutes, supernatant was collected and stored to perform ELISA while cells were used for flow cytometry analysis.

2.2.9 Statistical Analysis

Statistically significant differences between two groups were determined by Student's t test, more than two groups were analyzed by one-way ANOVA using GraphPad Prism Software version 6 (San Diego, USA). $p < 0.05$ was considered as significant.

CHAPTER 3

3. Results

3.1 Physicochemical properties of liposomes

3.1.1 Size and polydispersity index determined by DLS

Physicochemical characterization of liposomes was performed in aqueous dispersed state by the use of a dynamic light scattering instrument. The vesicular size and the polydispersity index were obtained from these studies.

	Size (d.n...	% Number:	St Dev (d.n...
Z-Average (d.nm): 111.2	Peak 1: 22.46	100.0	7.504
Pdl: 0.393	Peak 2: 0.000	0.0	0.000
Intercept: 0.940	Peak 3: 0.000	0.0	0.000
Result quality Good			

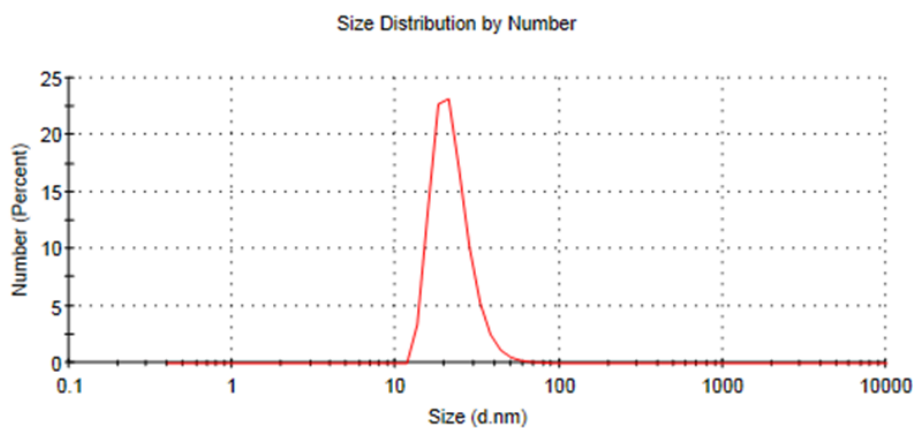


Figure 3.1 Size distribution analyses of anionic liposomes by DLS.

1 μM of anionic liposomes were measured via dynamic light scattering to analyze size, polydispersity index and size distribution. Upper part shows size in nm with standard deviation and polydispersity index (PDI), histogram in lower panel demonstrates size distribution. Data is a representative finding of 3 independent analyses with similar results.

Average size of anionic liposomes was measured as 22.5 ± 7.5 nm (Figure 3.1). Sonication was performed to generate small unilamellar vesicles (SUVs) whose size was below 100 nm [161]. Polydispersity index (PDI) basically indicates how broad the size distribution of sample is. Materials with a polydispersity index larger than 0.7 are polydisperse and unfavorable for nano-delivery while PDI values smaller than 0.7 are accepted as monodisperse, hence can be used as drug carriers [162]. Polydispersity index of anionic liposomes was found as 0.373 ± 0.027 . Consequently, anionic liposomes that were prepared for A151 encapsulation were appropriate to use as drug carriers due to their size and polydispersity index.

3.2 Determination of free and liposomal A151 ODN's immuno-suppressive capacity *in vitro*

3.2.1 Studies with splenocytes involving free or liposome encapsulating A151 ODN

To explore the general suppressive capacity of A151 ODN on immune cells, splenocytes were utilized since they represent a heterogenous immune cell population. None of control ODN, anionic liposomes, free A151 and A151-encapsulated liposomes have a significant effect on basal cytokine production from splenocytes (Figure 3.2). In this experiment, liposomes were used as $432 \mu\text{M}$ for the splenocytes stimulation, and that was the required amount to encapsulate $3 \mu\text{M}$ A151.

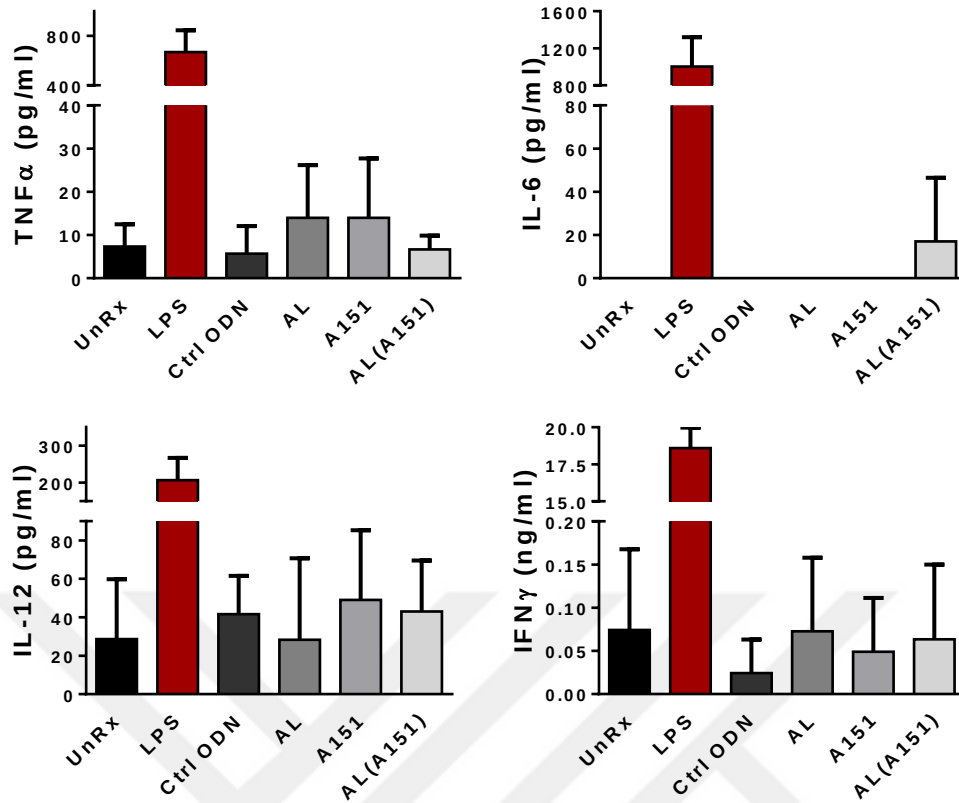


Figure 3. 2 TNF α , IL-6, IL-12 and IFN γ production from murine splenocytes in response to control ODN (Ctrl ODN), anionic liposomes (AL), A151 or liposome-encapsulated A151 (AL(A151)) stimulation. Splenocytes (8×10^5 cells/ml) were stimulated with LPS (1 μ g/ml), control ODN 1471 (1 μ M), anionic liposomes (432 μ M), A151 ODN (3 μ M) and 3 μ M equivalent of A151 ODN-loaded anionic liposomes for 24 hours in culture. Cytokine levels from cell supernatants were determined by ELISA. $p = ns$ (non-significant) for all comparisons between untreated (UnRx) vs treatment groups either analyses by ANOVA. Representative results of at least 3 independent experiments run in triplicate are shown.

Although the underlying causes of systemic sclerosis remains elusive, TLR2, TLR4 and TLR9 signaling were thought to play a crucial role during the maintenance of persistent inflammation and non-resolving fibrosis [82]. In that regard, we planned to explore down-modulatory effects of A151 ODN on these TLR-driven signaling pathways and investigated whether A151 encapsulation within liposomes further represses TLR ligand triggered cytokine productions from splenocytes.

While unencapsulated A151 ODN did not decrease pro-inflammatory cytokine production in response to Pam3csk4 stimulation, encapsulation of ODN within liposomes; however, significantly reduced both IL-6 and TNF α secretion capacity in a dose dependent manner (Figure 3.3A). A151 ODN in both free and liposomal forms did not abolish TNF α and IL-6 production upon stimulation with TLR4 ligand LPS (Figure 3.3B). Confirming our previous observations, A151 ODN was found to be very effective against suppressing K23 type CpG ODN induced pro-inflammatory cytokine production [23]. Although the dose of A151 ODN

was as low as 0.3 μ M within the culture fluid, there were ~50% inhibition of CpG ODN mediated IL-6 and TNF α secretions. Of note, encapsulation of A151 ODN within liposomes did not further enhance this suppressive capacity (Figure 3.3C). Of special interest, these suppressions were of motif dependent, a control ODN devoid of a G-run motif did not yield any appreciable levels of suppressive effect on cytokine types investigated whereas A151 ODN (a G-run expressing ODN) as expected elicited this inhibitory effect.

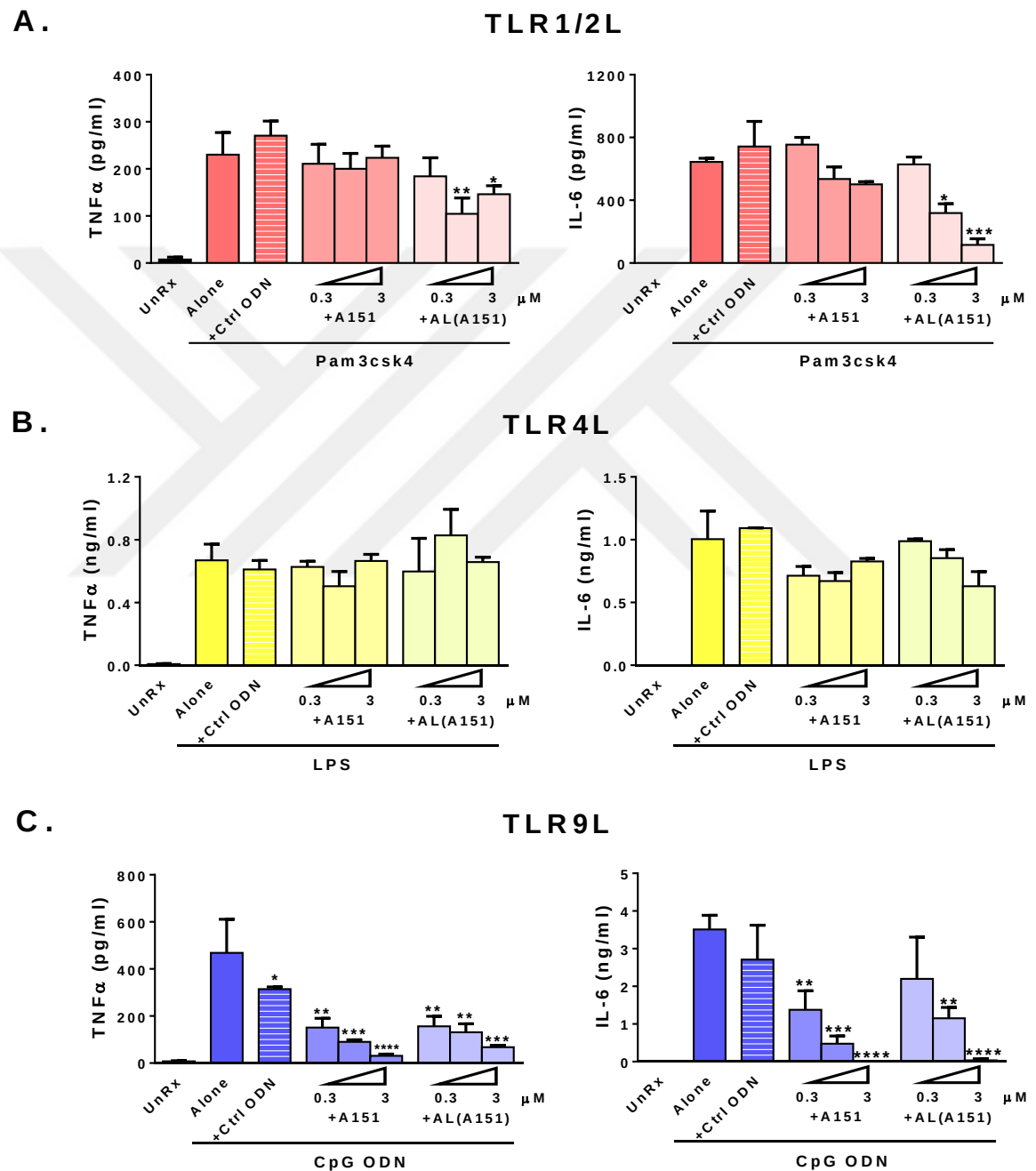


Figure 3.3 Effects of free and liposome-encapsulated A151 on TNF α and IL-6 production from splenocytes stimulated with TLR1/2, TLR4 and TLR9 ligands. Splenocytes (8×10^5 cells/ml) were stimulated with TLR1/2 ligand Pam3csk4 (1 μ g/ml), TLR4 ligand LPS (1 μ g/ml) and TLR9 ligand CpG ODN (1 μ M) for 24 hours. Immunosuppressive A151 ODN with three different doses (0.3, 1.0 and 3.0 μ M) of free and liposome-encapsulated forms (AL(A151)) were

co-incubated with the cells during this period. The cell supernatants were recovered to perform cytokine ELISA, and changes in $\text{TNF}\alpha$ and IL-6 production levels were determined. Comparisons were made according to ligand alone groups, and statistical significances were determined using one-way ANOVA. Representative results of at least 3 independent experiments run in triplicate are shown. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

In addition to pro-inflammatory cytokines such as $\text{TNF}\alpha$ and IL-6, levels of Th1-biased inflammatory cytokines such as IL-12 and $\text{IFN}\gamma$ in the presence or absence of A151 ODN were also checked by ELISA from the same supernatants (Figure 3.4).



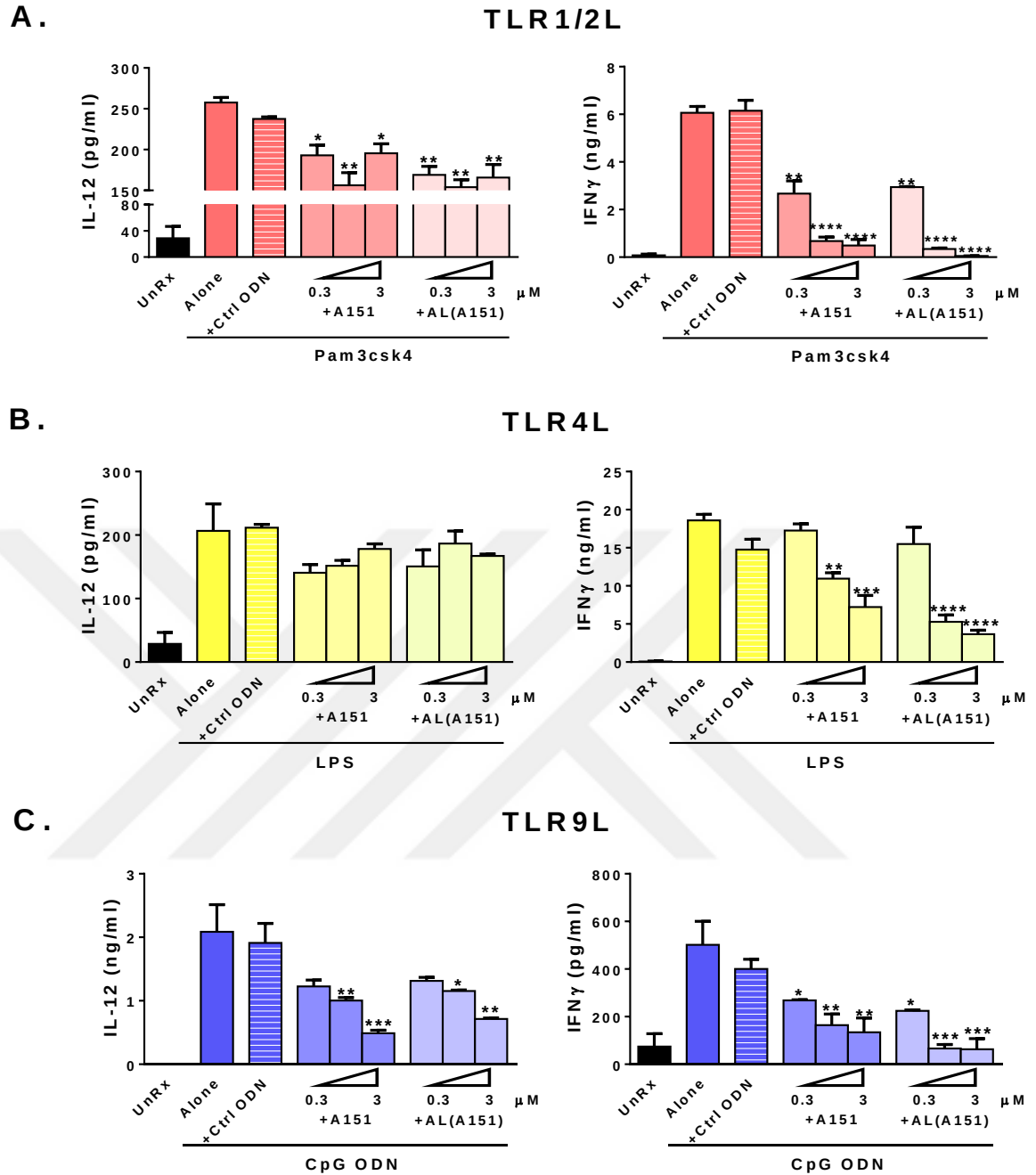


Figure 3.4 Effects of free and liposome-encapsulated A151 on IL-12 and IFN γ production from splenocytes stimulated with TLR1/2, TLR4 and TLR9 ligands. Splenocytes (8×10^5 cells/ml) were stimulated with TLR1/2 ligand Pam3csk4 (1 μ g/ml), TLR4 ligand LPS (1 μ g/ml) and TLR9 ligand CpG ODN (1 μ M) for 24 hours. Immunosuppressive A151 ODN with three different doses (0.3, 1.0 and 3.0 μ M) of free and anionic liposome-encapsulated forms (AL(A151)) were also co-incubated with the cells. The cell supernatants were recovered to perform cytokine ELISA and changes in IL-12 and IFN γ production levels were determined. Comparisons were made according to ligand alone groups, and statistical significances were determined using one-way ANOVA. Representative results of at least 3 independent experiments run in triplicate are shown. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Data revealed that free form of A151 ODN reduced IL-12 and IFN γ levels in a dose dependent manner in response TLR1/2 ligand stimulation. Suppression of IL-12 and IFN γ was further improved when liposome encapsulating A151 ODN was used (Figure 3.4A). Although A151 ODN (either alone or within liposomes) was ineffective to reduce IL-12 levels, it significantly inhibited IFN γ production in response to LPS (Figure 3.4B). This suppression was dose dependent and liposome inclusion boosted the inhibitory capacity. In response to TLR9 stimulation, consistently with previous observations, we detected that A151 ODN (free or liposome encapsulating counterpart) was a potent suppressor of both IL-12 and IFN γ (Figure 3.4C). It was noteworthy to mention that A151 encapsulation within liposomes induced stronger IFN γ suppression for all tested ligands (Figure 3.4A-C right panels). Lastly, as detected in Figure 3.3 and 3.4, control ODN did not elicit any inhibition, suggesting that the suppression is motif specific.

Taken together, these results implied that A151 ODN could act as a promising candidate in prevention of TLR-driven inflammation in diseases such as SSc.

3.2.2 Studies with macrophages and fibroblasts

Macrophages and fibroblasts are two important cell types contributing inflammation and fibrosis by both themselves and regulating each other in specific tissues. Therefore, we aimed to investigate how A151 ODN alter these cells during the course of inflicted non-sterile inflammation.

3.2.2.1 Suppressive effects of A151 ODN on cytokine production and surface activation markers in BMDMs

Macrophages react to danger signals by producing cytokines and chemokines as well as increasing phagocytic and antigen presenting capacities [163]. Therefore, surface marker expressions such as CD86 or CD80 co-stimulatory molecules and MHC class II molecule (MHC-II) expressions are indication of activation-maturation and increase in APC function. Figure 3.5 and Figure 3.6 illustrate changes of surface marker expressions in response to TLR1/2, TLR4 and TLR9 triggering and how incubation with A151 ODN alters the expression levels of these molecules.

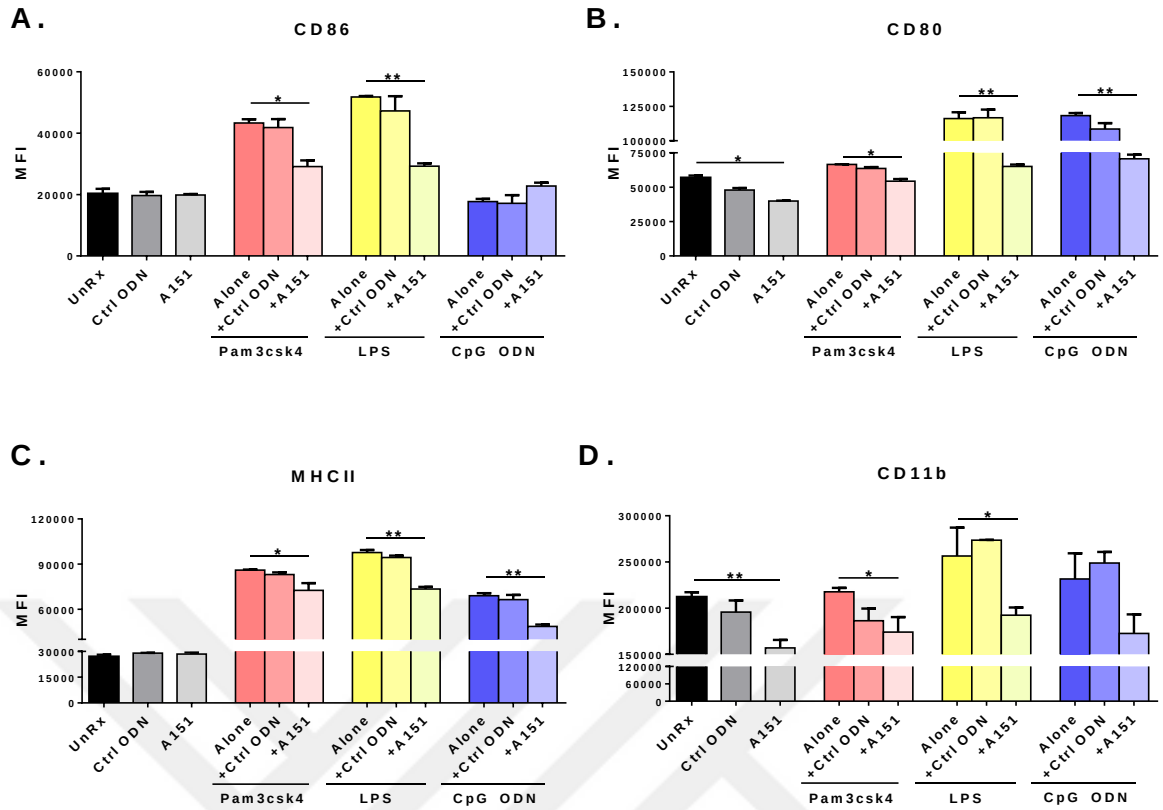


Figure 3.5 Effect of A151 ODN on expressions of co-stimulatory molecules in bone marrow derived macrophages (BMDM) stimulated with TLR1/2, TLR4 and TLR9 ligands. BMDMs (4×10^5 cells) were stimulated with TLR1/2 ligand, Pam3csk4 (1 μ g/ml), TLR4 ligand, LPS (1 μ g/ml) and TLR9 ligand CpG ODN (1 μ M) for 24 hours, cells were collected, and A) CD86, B) CD80 C) MHC-II and D) CD11b expressions were analyzed via flow cytometry. BMDMs were characterized by their F4/80 and CD11b expressions following staining with a-F4/80-APC and a-CD11b-FITC antibody staining. Statistical significances were determined using one-way ANOVA. Representative results of at least 3 independent experiments run in duplicate are shown. (Control ODN: 1 μ M, A151: 3 μ M, MFI: Mean fluorescent intensity, * $p < 0.05$, ** $p < 0.01$).

BMDMs were generated from mouse bone marrow cells according to the protocol by Ying et al. with minor modifications [164], flow cytometric assessment confirmed their characterization as well as their cell purity which was ranged between 95% to 98%. Then, BMDMs were harvested, transferred to 24-well plates and incubated with Pam3csk4 (1 μ g/ml), LPS (1 μ g/ml) and CpG ODN (1 μ M) for 24 hours. In addition, the stimulation media was supplemented either with control or A151 ODN (1 and 3 μ M, respectively). The cells were harvested, fixed and stained against CD80, CD86, MHC-II and CD11b molecules and analyzed by Novocyte flow cytometer.

Data revealed that Pam3csk4 and LPS but not CpG ODN upregulated CD86 expression (Figure 3.5A). LPS and CpG ODN but not Pam3csk4 upregulated CD80 expression level (Figure 3.5B). A151 ODN but not control ODN treatment; however, reduced the levels of both CD80 and

CD86 back to baseline levels (Figure 3.4A and 3.5B). While all three TLR ligands enhanced the antigen presenting capacity of BMDMs as evidenced by elevation of MHC-II expression, addition of A151 ODN significantly reduced the surface expression independent of ligand stimulations (Figure 3.5C).

Lastly, A151 ODN downregulated the expression of a chief marker of mouse macrophages, CD11b, even lower than baseline levels while incubation with LPS and CpG ODN elevated its levels (Figure 3.5D). Notably, CD11b forms the integrin Mac-1 ($\alpha_M\beta_2$) with CD18 which is expressed on macrophages/monocytes and neutrophils, and this complex regulates migration and adhesion [165]. Additionally, A151 did not lead a decrease in CD11b⁺ population in BMDMs (data not shown).

Next, we analyzed expressions of CD40, CXCR2 and ICAM1 levels from BMDMs, since these markers correlate well with the macrophage activation and recruitment.

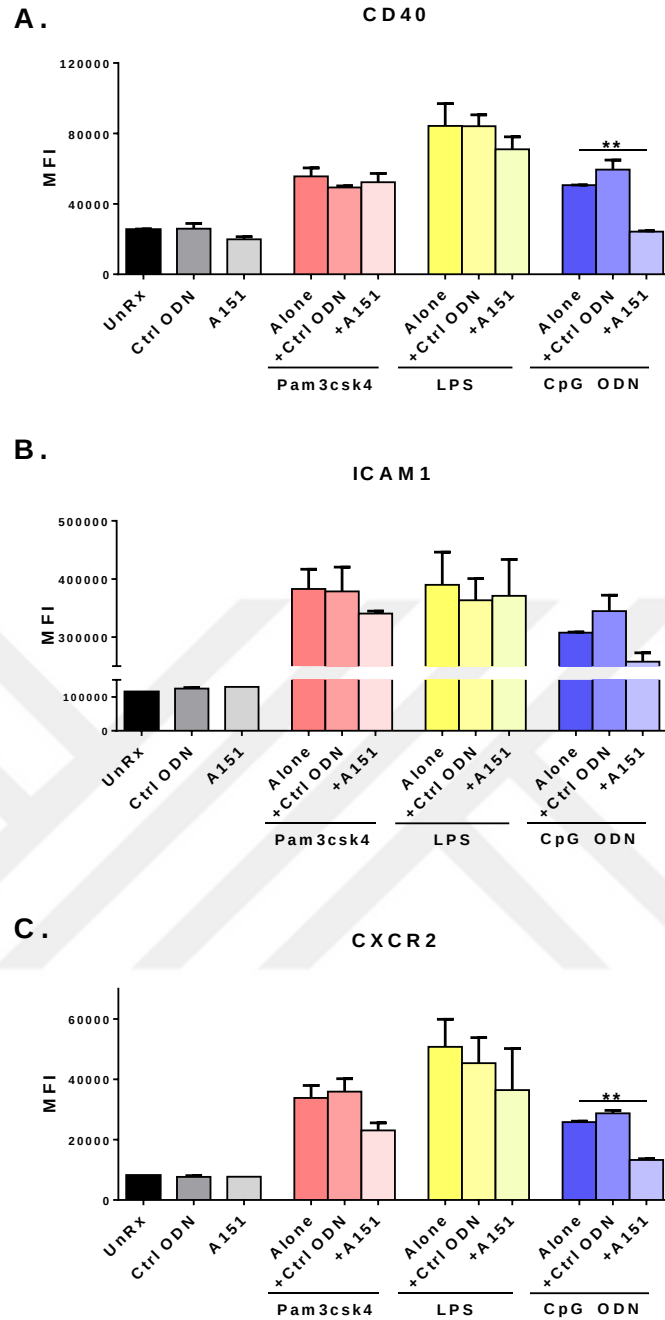


Figure 3. 6 A151 ODN dependent inhibition of BMDM-specific recruitment and activation marker expressions following stimulation with TLR1/2, TLR4 and TLR9 ligands. As explained in Fig. 4.5 legend, BMDMs (4×10^5 cells) were stimulated with indicated concentrations of Pam3csk4, LPS and CpG ODN for 24 hours. Cells were collected, fixed and stained with fluorochrome-conjugated antibodies against A) CD40, B) ICAM1 and C) CXCR2 and changes in the expression patterns of these markers in response to either Control ODN or A151 ODN co-incubation were determined using flow cytometry. Statistical significances were determined using one-way ANOVA. Representative results of at least 3 independent experiments run in triplicate are shown. (Control ODN: 1 μ M, and A151 ODN: 3 μ M; MFI: Mean fluorescent intensity, ** $p < 0.01$).

CD40 molecule on macrophages interact with CD40L on T cells; therefore, it leads to both activation and proliferation of T cells and induction of macrophages to produce chemokines and cytokines [166]. ICAM1 (CD54) is a transmembrane glycoprotein which confers macrophage adhesion with endothelial cells and initiates intracellular signaling that results in gene expression and activation [167]. CXCR2 is a chemokine receptor whose best-known ligand is KC (CXCL1). It modulates the recruitment of neutrophils and macrophages to inflamed areas [168]. A151 ODN significantly decreased elevated CD40 levels only after TLR9 activation (Figure 3.6A). Moreover, although not significant, ICAM1 surface expression on BMDMs were slightly decreased by A151 ODN following Pam3csk4 and CpG ODN stimulation (Figure 3.6B). Lastly, A151 ODN reduced CXCR2 levels that were elevated after stimulation with all three TLR ligands; however, only CpG ODN mediated upregulation was significantly downregulated by A151 ODN (Figure 3.6C).

In summary, A151 ODN is a promising candidate inhibiting overexuberant macrophage activation that was initiated by certain TLR ligands.

Next, different doses of bleomycin (BLM) were incubated with BMDMs in culture. The inflammatory consequences along with any possible *in vitro* detrimental effects induced by BLM was sought to be explored. Initial experiments were aimed to estimate the BLM dose that does not initiate macrophage death. Doses higher than 0.1 $\mu\text{g/ml}$ were found to be toxic on macrophages (data not shown); therefore, lower doses were used in subsequent experiments. Following 24 hours of incubation with bleomycin and A151 ODN, supernatants were used to determine IL-6 levels by ELISA (Figure 3.7).

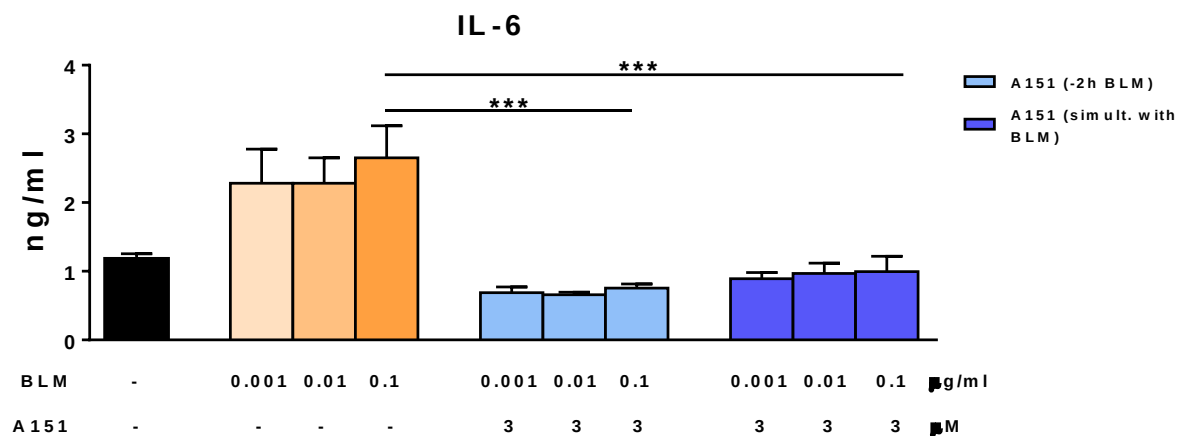


Figure 3.7 IL-6 production from BMDMs stimulated with bleomycin in the presence or absence of A151 ODN. BMDMs (1.5×10^5 cells) were stimulated with three different doses of bleomycin (0.001, 0.01 and 0.1 $\mu\text{g/ml}$) for 24 hours. A151 ODN was added to culture medium either 2 hours before or at the time of bleomycin addition. Extracellular IL-6 was determined by ELISA from 24 hours culture supernatants. Significances were determined using one-way ANOVA. Representative results of at least 3 independent experiments run in triplicate are shown. (BLM: Bleomycin, *** $p < 0.001$)

Bleomycin treatment in a dose-dependent manner increased IL-6 production (Figure 3.7). A151 ODN addition significantly down-regulated BLM induced IL-6 production from BMDMs. Of note, bleomycin did not lead to any elevation either in TNF α or IL-1 β secretion from BMDMs (data not shown).

In conclusion, A151 could efficiently reduce one of the cardinal pro-inflammatory cytokines, namely IL-6 production following bleomycin stimulation *in vitro*. This finding implied that A151 ODN might be utilized as a preventive drug against bleomycin-triggered *in vivo* inflammatory and probably fibrotic processes in SSc.

3.2.2.2 Inflammasome studies

As it was stated earlier, inflammasome activation and augmented IL-1 β serum level is one of the major sources of inflammation in systemic sclerosis patients. To investigate how A151 ODN modulates inflammasome signaling cascade and subsequent IL-1 β secretion from macrophages, AIM2 and NLRP3 inflammasome pathway inducing ligands were tested on BMDMs in the presence or absence of A151 ODN therapy (Figure 3.8).

A151 ODN was known to inhibit AIM2 inflammasome activation and IL-1 β secretion by inhibiting ligands to bind its receptor, thereby preventing inflammasome complex formation [37]. Herein, we have shown that if A151 ODN is included in the culture media 2 hours after p(dA/dT) addition, a sharp reduction in IL-1 β level was seen (Figure 3.8). Even in the lowest A151 ODN dose, which is 0.3 μ M, significant reduction in IL-1 β levels from BMDMs was obtained. Of important note, the p(dA/dT) concentration used in these experiments was 5-fold more than what was used in the previous study [37].

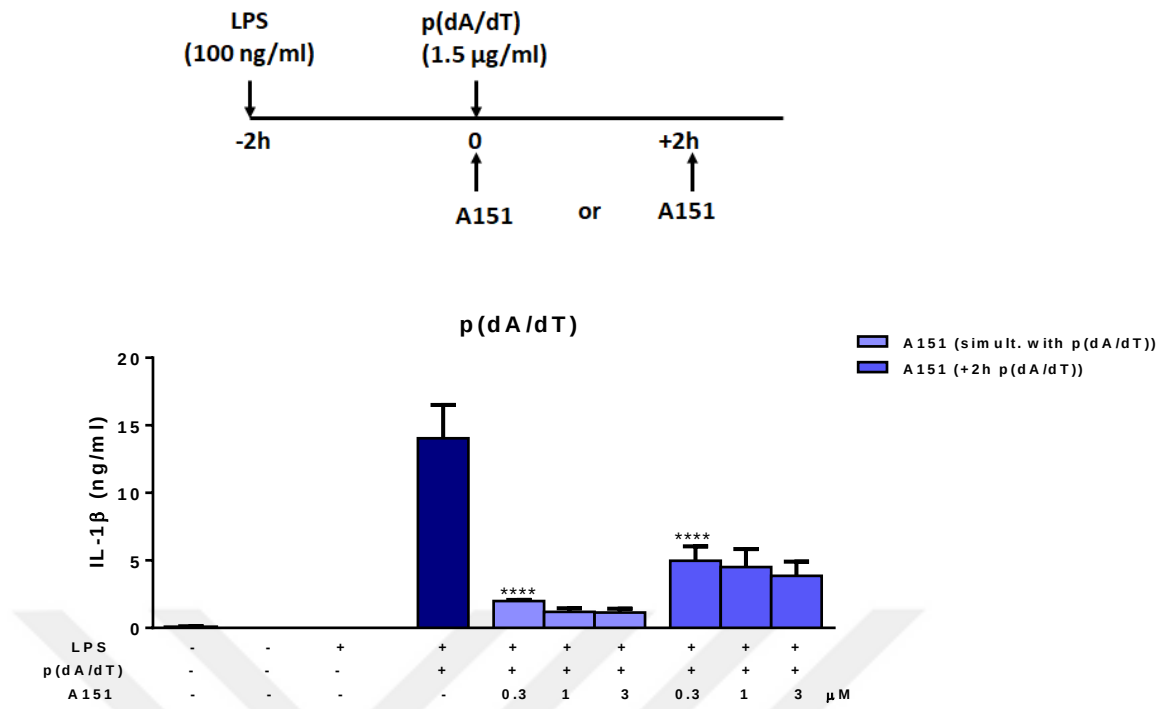


Figure 3.8 Altered IL-1 β production in response to dose dependent A151 ODN administration in BMDMs activated with p(dA/dT). BMDMs (1.5×10^5 cells) were first primed with LPS (100 ng/ml) for 2 hours, then AIM2 inflammasome activator poly (dA/dT) (1.5 μ g/ml) was added. A151 ODN was used in different doses (0.3, 1.0 and 3.0 μ M) either at the same time with or 2 hours after p(dA/dT) stimulation. IL-1 β levels were measured using cell supernatants by ELISA following 24 hours of stimulation. Experiment design was given on the upper panel while IL-1 β productions were shown at the bottom. Significances were determined using one-way ANOVA, and comparisons were made according to LPS+p(dA/dT) group. Representative results of at least 3 independent experiments run in triplicate are shown. (**** $p < 0.0001$)

Even though A151 ODN cannot inhibit NLRP3 inflammasome in contrast to AIM2, another study that was done with rats claimed A151 ODN decreased i) IL- β production, ii) Nlrp3 gene and iii) the protein expressions upon LPS stimulation [169].

Therefore, we hypothesized that A151 ODN might decrease NLRP3 activation by reducing its expression when it was added even before priming with LPS. To this end, A151 ODN was added 2 hours before priming, together with LPS-priming or 2 hours after priming, and nigericin was used as NLRP3 activator. However, A151 ODN slightly decreased IL-1 β production in response to NLRP3 triggering upon nigericin treatment even when it was administered in high dose 2 hours before priming (Figure 3.9), which is in contrast to our hypothesis.

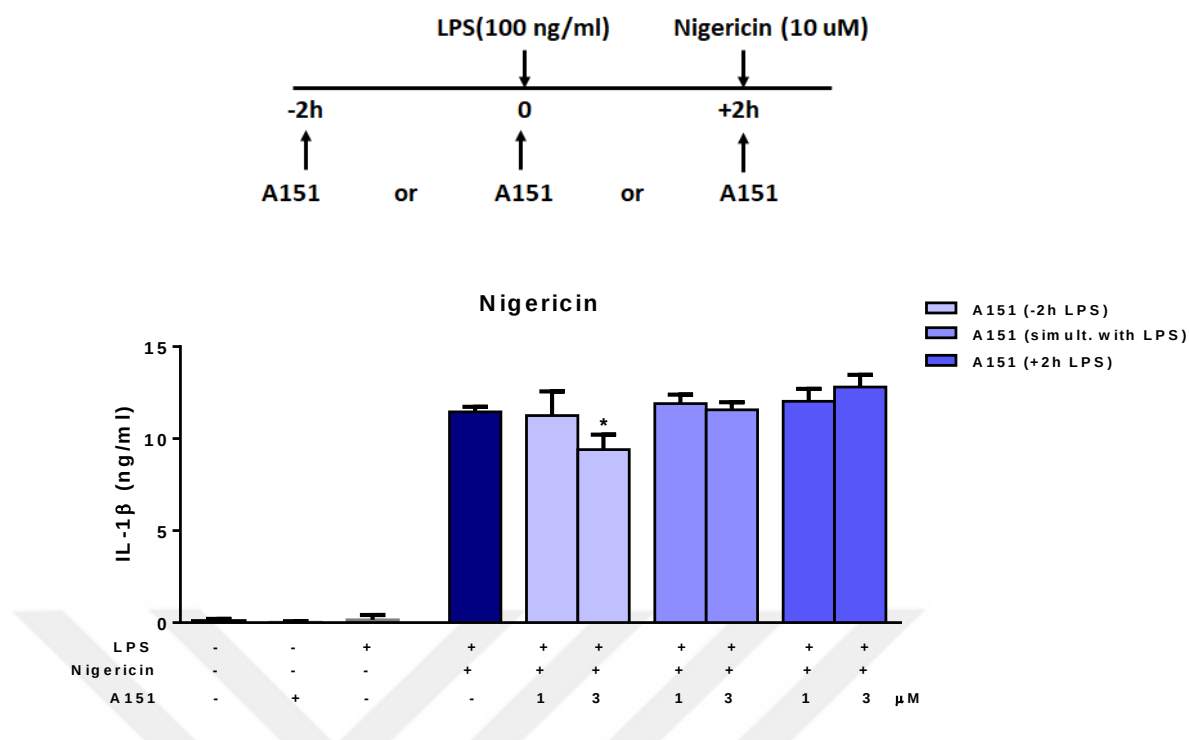


Figure 3.9 Effect of A151 ODN on nigericin induced NLRP3 inflammasome activation in BMDMs. BMDMs (1.5×10^5 cells) were primed with 100 ng/ml LPS for 2 hours, then 10 μ M nigericin was used to activate NLRP3 inflammasome pathway. A151 ODN was used in 2 different doses (1 μ M and 3 μ M) either 2 hours before, at the time of or 2 hours after LPS priming of the BMDMs. IL-1 β levels were determined by ELISA from 24 hours of culture supernatants. Scheme showing experiment flow was presented on the upper panel while IL-1 β productions were shown in bottom. Significances were determined using one-way ANOVA, and comparisons were made according to LPS+nigericin group. Representative results of at least 3 independent experiments run in triplicate are shown. (*p<0.05)

Our findings pointed that immunosuppressive A151 ODN significantly inhibits AIM2 but not NLRP3 inflammasome activation. Also, A151 ODN is capable of reducing IL-1 β production even after AIM2 inflammasome pathway is activated from cultured BMDMs.

3.2.2.3 Conditioned media experiments

Fibroblasts and macrophages are two cell populations interacting with each other in health and disease conditions. When there is an injury or inflammation in tissue, chemokines and cytokines produced from damaged epithelial cells recruit and activate macrophages while activated macrophages further enhance inflammatory response [83]. Recruited immune cells while promoting tissue damage, they also activate fibroblasts and help to increase their fibrotic gene expressions, thereby contributing their excess extracellular matrix protein production. Following accumulation of fibrotic proteins, such as collagen and fibronectin, inflammation subsides, thus fibrosis and wound healing process are initiated [83].

To mimic this process and simulate the intricate interaction of macrophages and fibroblasts, we designed a series of experiments where the conditioned medium of one cell was introduced to the culture medium of the second cell type. This would allow us to observe how, for example, BMDM activation would influence fibroblasts' specific cytokine production and furthermore to what extent A151 ODN could regulate this inflammatory process. To this end, BMDMs were first stimulated in presence of TLR1/2, TLR4 or TLR9 agonists with or without A151 ODN addition for 6 hours. Then, cells were washed with fresh media, and they were then suspended in a ligand free fresh warm media for another 24 hours. Supernatants obtained from BMDMs following 24 hours of incubation were filtered and this "conditioned media" (CM hereafter) was added onto mouse fibroblast cell line, NIH3T3 cells in 1:4 (v/v) ratio for 24 hours. Cytokine production in BMDMs and NIH3T3 cells were measured separately via ELISA.

We measured TNF α , IL-6 and IL-12 levels from both cell culture supernatants. In theory, if nothing is produced or consumed by fibroblasts, the macrophage culture conditioned medium is 4X higher in terms of cytokine concentration compared to fibroblast culture medium. In Figure 3.10, the workflow of this experiment is depicted as a separate scheme. Moreover, the figure summarizes findings concerning TNF α , IL-6 and IL-12 secretion profiles from BMDMs (left panels) that was stimulated for 6h +24h with TLR ligands. Next to these plots, fibroblast cell line "NIH3T3" dependent cytokine production levels were presented. These cells were incubated with 25% of conditioned media obtained from BMDMs.

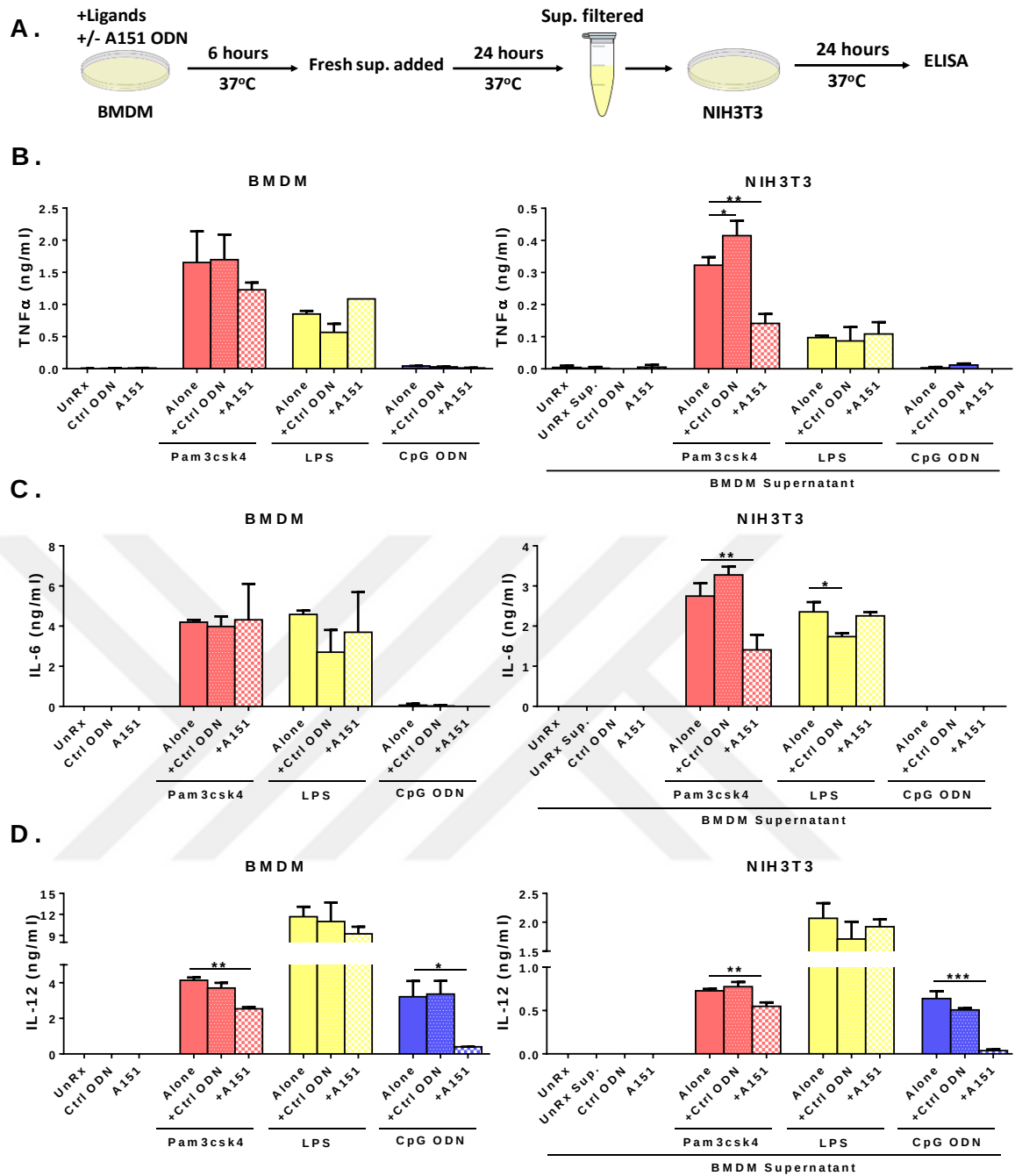


Figure 3.10 TNFα, IL-6 and IL-12 production from BMDMs stimulated with TLR ligands and NIH3T3 cell line stimulated with conditioned media obtained from BMDMs. A) Workflow of conditioned media experiments. BMDMs (8x10⁵ cells/ml) were stimulated with TLR1/2 (Pam3csk4: 1 µg/ml), TLR4 (LPS: 1 µg/ml) and TLR9 (CpG ODN: 1 µM) ligands for 6 hours. Supernatant was removed, fresh medium was added, and cells were incubated for further 24 hours. Collected supernatants were labelled as “CM”, saved for ELISA and filtered to add on NIH3T3 cells in 1:4 (v/v) ratio. Following 24 hour-incubation with NIH3T3 cells, the supernatants were collected from CM treated NIH3T3 cells and ELISA was performed for both cell supernatants and concentrations were plotted for B) TNFα, C) IL-6 and D) IL-12 levels. Left panels represent BMDMs while right panels show cytokine productions from NIH3T3 cells. Significances were determined using one-way ANOVA, and comparisons were made among each TLR ligand group. Representative results of at least 3 independent experiments run in triplicate are shown. (*p<0.05, **p<0.01, ***p<0.001, Ctrl ODN: 3 µM, A151 ODN: 3 µM).

The results of this experiment, even though seems complicated to assess, is very straightforward. CM of BMDMs when stimulated with a 1 µg/ml Pam3csk4 (a TLR1/2 ligand), is the most influential media composition on fibroblasts. As seen in Figure 3.10C, Pam3csk4 induced ~4.0 ng/ml of IL-6. A quarter of this amount was included onto fibroblast culture media within CM. IL-6 secreted from fibroblasts; however, contrary to other cytokines, was similar when the right panels of Figure 3.10 is compared to the left panels were examined. If we consider the produced and consumed amounts of IL-6 in the culture at the end of 24 hours of incubation, it is obvious that only the Pam3csk4 stimulated CM significantly boosted IL-6 production from NIH3T3. Simply put, this data implied that TLR1/2 triggered CM is the most suitable environment to aggravate inflammatory response from fibroblasts in culture. Lastly, since not only cytokines but also several chemokines and different metabolites contribute to immune response, reduced amounts of these mediators within the BMDM CM could attenuated the response obtained from NIH3T3 cells following exposure to Pam3csk4 treated CM. Different studies reported that cells produced KC (CXCL1), MIP1 α (CCL3), MIP1 β (CCL4), MIP2 (CXCL2) and nitric oxide (NO) upon TLR1/2 induction with Pam3csk4 [170, 171]. Therefore, one or more of these chemokines/metabolites might be strongly downregulated by A151 ODN treatment throughout BMDM culture; thus, induction of either TNF α or IL-12 was not promoted.

The other noteworthy observation from this experiment is the following. Although for 6 hours of A151 ODN treatment was partially inhibitory on BMDMs, the CM harvested from this culture was highly inhibitory on NIH3T3 cells. Especially Pam3csk4 and CpG ODN treated and A151 incubated treatment groups for all cytokines measured gave more pronounced inhibitory response on NIH3T3 cells compared to BMDM cells (compare the red and blue bars on the left and right side of each graph in Figure 3.10B-D). The only exception to this was the LPS treated group for all cytokines (compare yellow bars on the left- and right-hand side of each graph).

The TLR9 ligand CpG ODN is a well-known producer of pro-inflammatory cytokines such as TNF α , IL-6 and IL-12 [172]; however, at the end of 6 hours of pre-incubation with BMDMs it did not induce detectable levels of TNF α and IL-6. As expected, A151 ODN downregulated CpG ODN mediated IL-12 production in both BMDMs and NIH3T3 cells (Figure 3.10D). It is important to note that IL-12 concentrations measured from NIH3T3 supernatants in response to CM of TLR9 culturing were approximately $\frac{1}{4}$ of the BMDMs produced IL-12 amount; therefore, it is fair to conclude that fibroblast inflammatory response is less likely to be influenced by TLR9 triggering than TLR1/2 triggering (Figure 3.10D).

3.3 Determination of A151 ODN's anti-fibrotic effect *in vitro*

Besides A151 ODN's well-known anti-inflammatory effects, revealing its anti-fibrotic potential is crucial for its use in fibrotic diseases. Therefore, mouse fibroblast cell line NIH3T3 was used, and modulation of fibrotic genes by A151 was investigated.

3.3.1 Studies with fibroblasts

It was reported that TLR signaling activates fibroblasts to i) produce molecules such as IL-6 cytokine, MCP-1 (CCL2) and RANTES (CCL5) chemokines ii) increase the expression of α -SMA and extracellular matrix proteins which are collagens, fibronectin, hyaluronan and tenascin [173-175]. In order to document that A151 ODN could directly alter the fibrotic responses of fibroblasts, TLR1/2, TLR4 and TLR9 ligands were used to stimulate NIH3T3 cells, and Col1a1 gene expression upon 8 hours of incubation with or without A151 was determined via RT-qPCR (Figure 3.11).

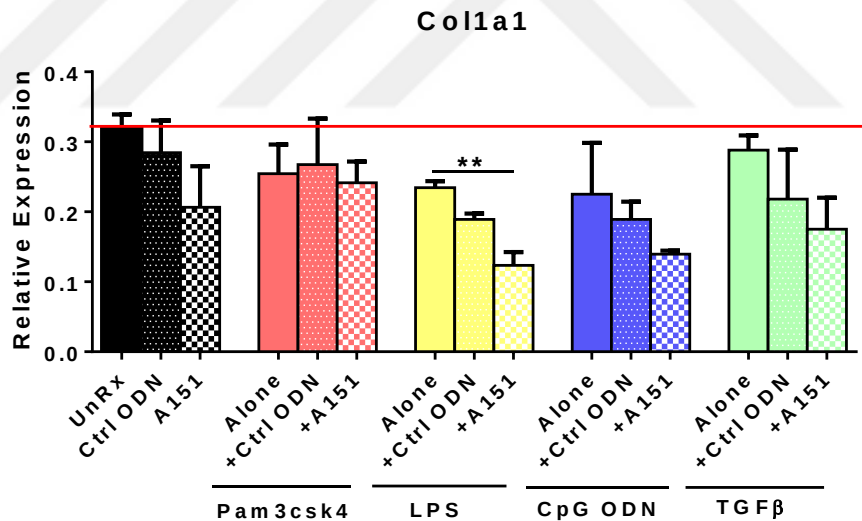


Figure 3.11 Col1a1 expression in NIH3T3 cells in response to TLR ligand and TGFβ stimulation. NIH3T3 cells (8×10^5 cells) were stimulated with TLR1/2 ligand (Pam3csk4, 1μg/ml), TLR4 ligand (LPS, 1μg/ml) TLR9 ligand (CpG ODN, 1μM) and TGFβ, (50 ng/ml) as a positive control in presence or absence of A151 (3μM) for 8 hours. Col1a1 gene expression levels were determined from the total RNA of the treated cells by qRT-PCR and normalized to β-actin expression. Significances were determined using one-way ANOVA, and comparisons were made among each ligand group, **p<0.01. Representative results of at least 3 independent experiments run in duplicate are shown. (Ctrl ODN: 3 μM)

TLR signaling was reported to activate fibroblasts and lead upregulation of fibrotic genes such as Col1a1, α -SMA and Tgfβ. Especially, TLR4 signaling has very well-defined role on

development of fibrosis [176]. Although in this assay we did not detect upregulation of Col1a1 gene expression in response to stimulation with three types of the TLR ligands and surprisingly even after 50 ng/ml TGF β treatment that was used as a positive control, strikingly and significantly A151 ODN treatment substantially but not significantly down-regulated Pam3csk4 and CpG ODN mediated Col1a1 gene expression (Figure 3.11). Nonetheless, A151 ODN significantly downregulated Col1a1 expression upon stimulation with LPS (yellow bars in Figure 3.11). It also important to note that all TLR ligands in this assay reduced the baseline Col1a1, and TGF β could not boosted beyond untreated baseline expression level.

A151 ODN, however, reduced Col1a1 gene levels from untreated fibroblasts, TLR ligand and TGF β treated fibroblasts. Control ODN, as expected did not induce or inhibited Col1a1 expression.

Next, because bleomycin is a widely used agent to induce fibrosis in different mouse models, NIH3T3 cells were stimulated with two different doses of bleomycin to determine its fibrotic insult. Similarly, we co-incubated NIH3T3 cells with A151 ODN and expected to observe the preventive effect on fibrotic gene upregulation. RT-qPCR was utilized to reveal Tgf β gene expressions upon stimulation with bleomycin in the absence or presence of A151 ODN (Figure 3.12).

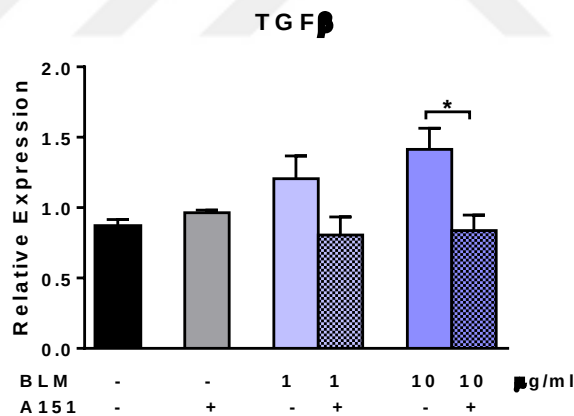


Figure 3.12 TGF β gene expression in response to bleomycin in NIH3T3 cells. NIH3T3 cells (8×10^5 cells) were stimulated with bleomycin (1 μ g/ml and 10 μ g/ml) and A151 ODN (3 μ M) for 8 hours. TGF β gene expression level was determined from the total RNA of the samples by qRT-PCR. Significances were determined using Student's t test. Representative results of at least 3 independent experiments run in duplicate are shown. (* $p < 0.05$)

Bleomycin increased Tgf β expression in a dose dependent manner while A151 ODN reduced the gene level to its untreated basal level (Figure 3.12).

Overall, A151 has the potential for being used as anti-fibrotic agent even though it is more powerful as a immunosuppressor.

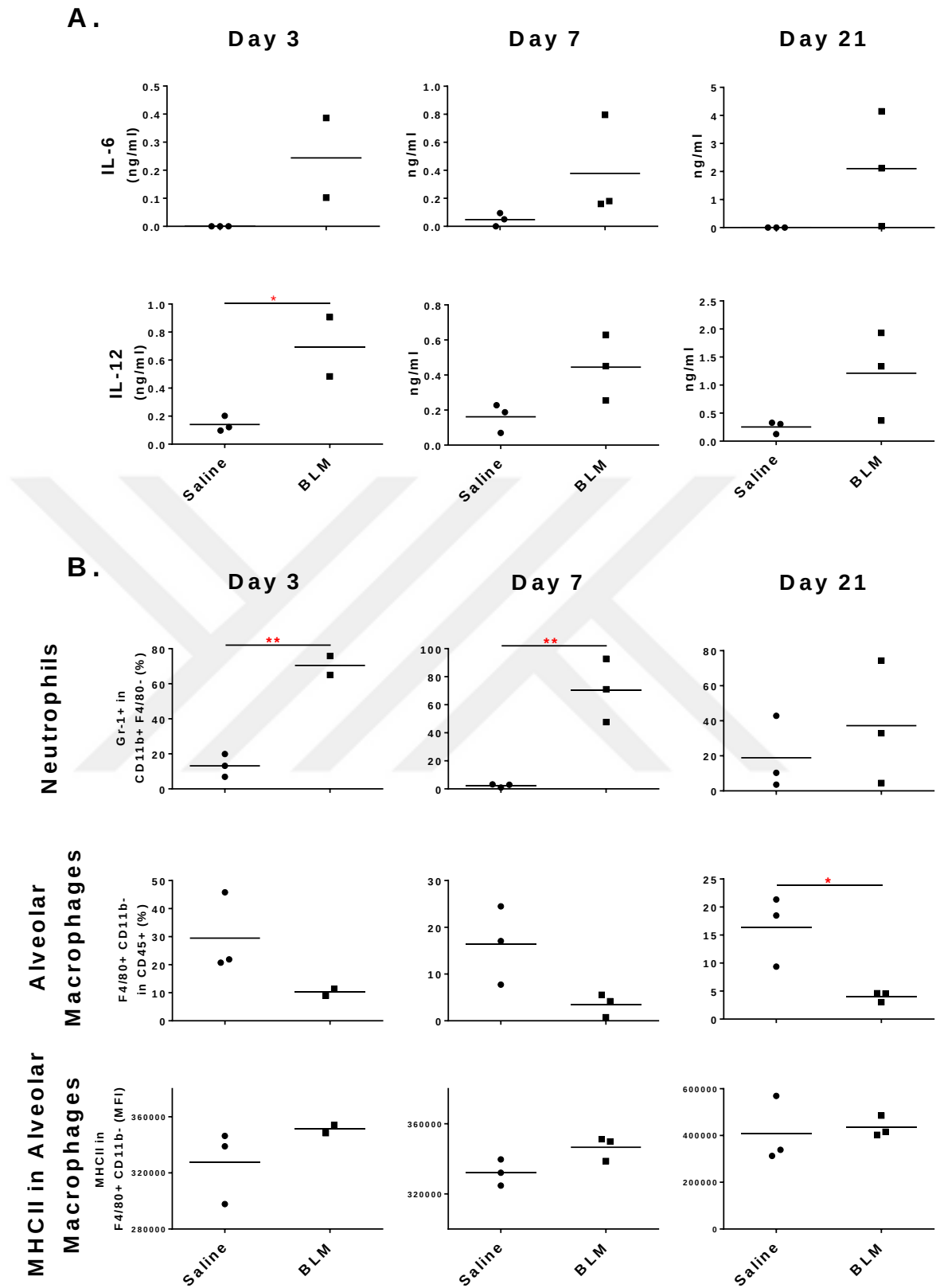
3.4 Establishment of bleomycin induced scleroderma with intratracheal instillation

Bleomycin is a mixture of cytotoxic glycopeptides that is isolated from a bacterium named *Streptomyces verticillus*. After its discovery in 1969 by a Japanese scientist, it was approved by FDA in 1973 and widely used in treatment of cancer. Testicular and ovarian cancers, Hodgkins' and non-Hodgkins' lymphoma are some of the cancer types that bleomycin is utilized as a chemotherapeutic agent. Its mechanism of action is defined as forming double strand breaks in DNA [177]. Fever, weight loss and vomiting are few of its frequently seen side effects. 6-10% of patients received bleomycin therapy develop pulmonary fibrosis. Thus, bleomycin became a widely used strategy to develop lung injury, skin and lung fibrosis and systemic sclerosis models in mice.

We first attempted to develop skin fibrosis on mice by subcutaneous bleomycin injections every other day for 35 days. A fresh bleomycin solution in PBS was prepared (100µg BLM/100µl PBS/mouse) and then was injected on the back of each animal. A group of mice were sacrificed at day 21, day 28 and day 35 to assess the status and stage of fibrosis. To this end, fibrotic gene expressions in skin were analyzed via RT-qPCR, immune cell populations accumulated at the scar region were determined by flow cytometry, and cytokines either in plasma or produced from splenocytes in culture were measured by ELISA. Unfortunately, expressions of fibrotic genes, such as type I collagen or Tgfβ were not higher in bleomycin-injected mice compared to PBS (control) group (Figure A.1). Furthermore, immune cell populations obtained from skin samples were not consistent throughout the experiment period, and macrophages, DCs, B- and T-cells were not significantly different between control and bleomycin treated groups (Figure A.2). Although IL-6 levels in blood serum was higher in bleomycin-instilled group in all time-points, their amounts were very low, and TNFα in serum was inconsistent between the groups (Figure A.3). Overall, none of the markers used to analyze the progression of bleomycin-induced skin fibrosis were reproducibly confirmed. We repeated the in vivo experiments twice with a limited success. From these daunting in vivo experiences, we concluded that the BALB/c mice strain could be the main underlying reason of our limited success. Indeed, there are conflicting reports between mouse strains and according to literature findings, BALB/c background mice is considered as "bleomycin-resistant strain", especially from the works that Yamamoto et al. [158].

After we have failed to establish bleomycin induced-systemic sclerosis by subcutaneous injections, we decided to modify our strategy and sought to develop lung inflammation and lung fibrosis model via a single bolus dose of intratracheal delivery of bleomycin. In contrast to multiple injections in establishment of skin fibrosis, a single instillation of bleomycin to trachea was also reported to lead lung inflammation that may last approximately 14 days [159]. After that, inflammation regresses and scar is established as fibrosis. Fibrotic phase begins 10-14 days after bleomycin administration and remains until it starts to dissolve at 35th to 40th day. Notably, most of the studies focused on bleomycin-induced lung inflammation and fibrosis utilized C57BL/6 mice; therefore, we employed this strain for the BLM instillation experiments.

We intratracheally instilled bleomycin to a group of mice (50µg/50µl/mouse) and saline was administered as a control group. A group of mice (n=2 or 3) was killed at 3rd, 7th and 21st days following bleomycin injection, BALF and lung samples were retrieved and investigated in detail both at cellular and at molecular levels. Figure 3.13 summarizes these extensive *in vivo* findings based on bronchoalveolar lavage fluid samples and lung cells as well as lung-resident immune cells.



C.

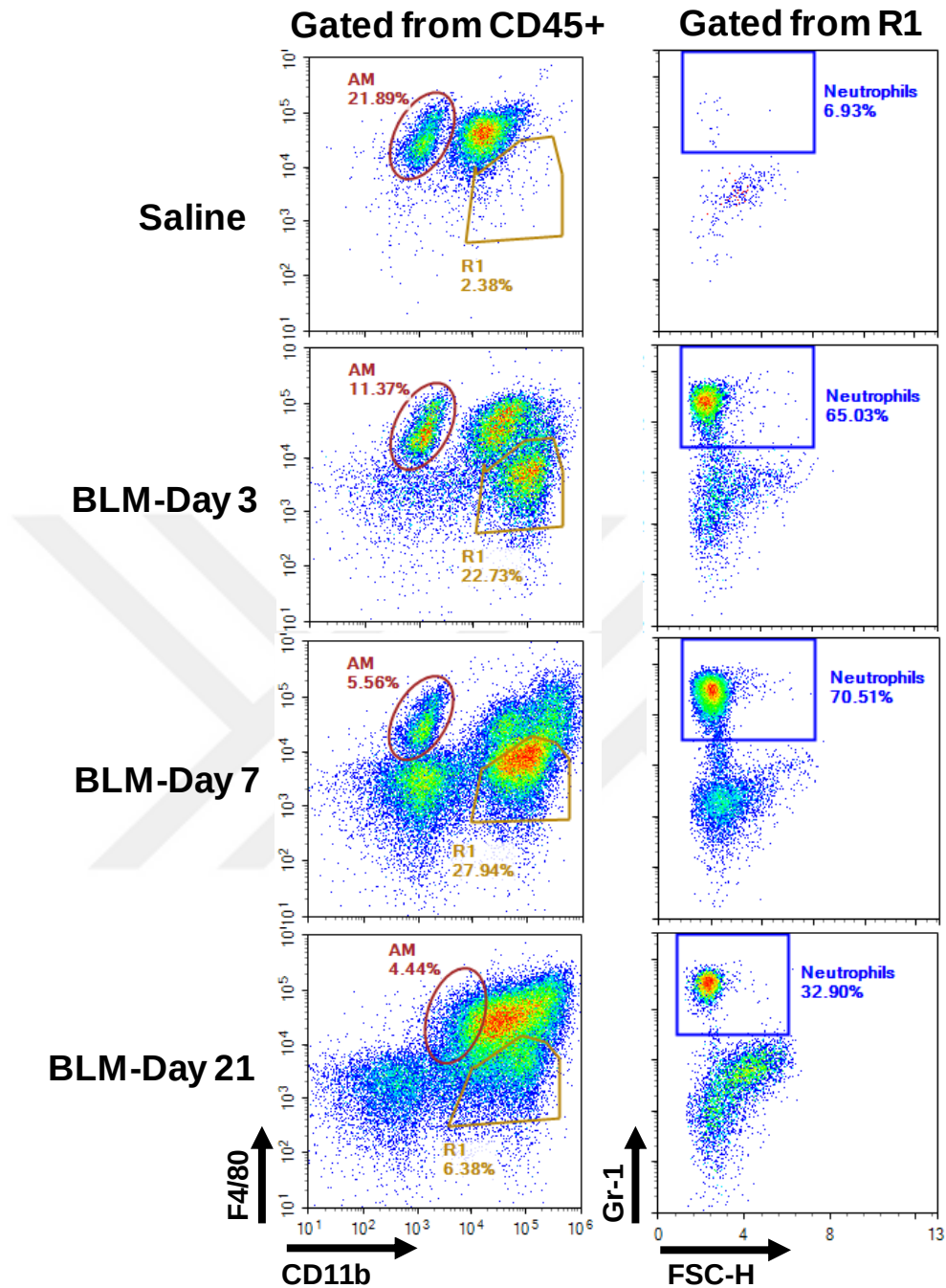


Figure 3.13 Changes in cytokine levels and cell populations in BALF at day 3, 7 and 21 following bleomycin administration. A) IL-6 and IL-12 levels obtained from BALF samples of mice (n=2 or 3) were analyzed via ELISA. B) Cell populations of alveolar macrophages, neutrophils and MHC-II expression in alveolar macrophages following bleomycin instillation were determined by flow cytometry. C) Representative dot plots showing changes in cell populations over time. Alveolar macrophages were characterized as CD45⁺,F4/80⁺,CD11b^{low} and neutrophils were CD45⁺,F4/80⁻,CD11b⁺,Gr-1⁺. R1 gate indicates polymorphonuclear cells which are characterized by F4/80⁻,CD11b⁺. Statistical differences were determined performing Student's t test. Representative results of at least 2 independent experiments are shown. (AM: Alveolar macrophages, MFI: Mean fluorescent intensity, BLM: Bleomycin, *p<0.05, **p<0.001).

BALF cytokine levels and cell populations were the most important indicators of an inflammation in single dose bleomycin-instilled lungs [178, 179]. ELISA of BALF samples revealed that concentrations of IL-6 and IL-12 were higher in bleomycin instilled animals throughout 3 weeks of follow-up period (Figure 3.13A). Both IL-6 and IL-12 levels in the BALF were increased following instillation and peaked at day 21. IL-6 rose from ~0.25 ng/ml to ~2.25 ng/ml by day 21, a 10-fold increase, whereas, during the same period, a 2-fold increase in BALF IL-12 was detected (an upward movement from 0.7 ng/ml to 1.4 ng/ml). Furthermore, as a sign of elicited early inflammatory response, Gr-1⁺ neutrophils were recruited to alveolar space where it constituted >70% of CD11b⁺, F4/80⁻ population at day 3, thereafter it gradually subsided down to nearly 40% by day 21 (Figure 3.13B, upper panel). In contrast to neutrophils, alveolar macrophages decreased in BALF upon bleomycin instillation (from day 3 to day 21 period) which indicates that bleomycin might have toxic effects on macrophages (Figure 3.13B and C). This was a contradictory finding to that of published literature which states that F4/80⁺/CD11b⁻ BALF resident alveolar macrophages increases following intratracheal bleomycin instillation where it peaks at day 7 [159, 178, 180]. In our hands, mice alveolar macrophages were ~10% by day 3 and gradually dropped down to ~4% at day 21. Lastly, MHCII expression in alveolar macrophages increased, although not significantly, demonstrating macrophages in bleomycin instilled mice slightly increased their antigen presentation capacity. We attempted to deduce the changes in the cardinal gene levels that reveal the fibrotic effects of bleomycin by investigating RNA of lung samples with RT-qPCR and protein extracts with Western blotting. These results are detailed in Figure 3.14.

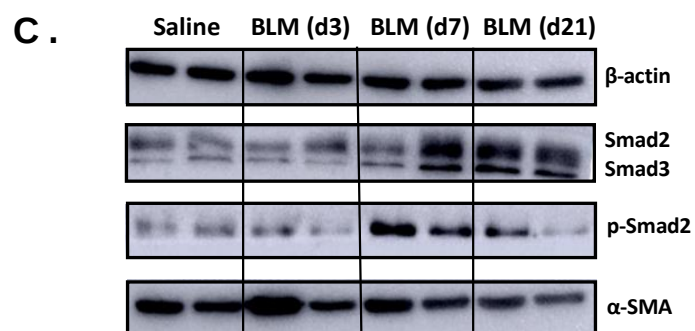
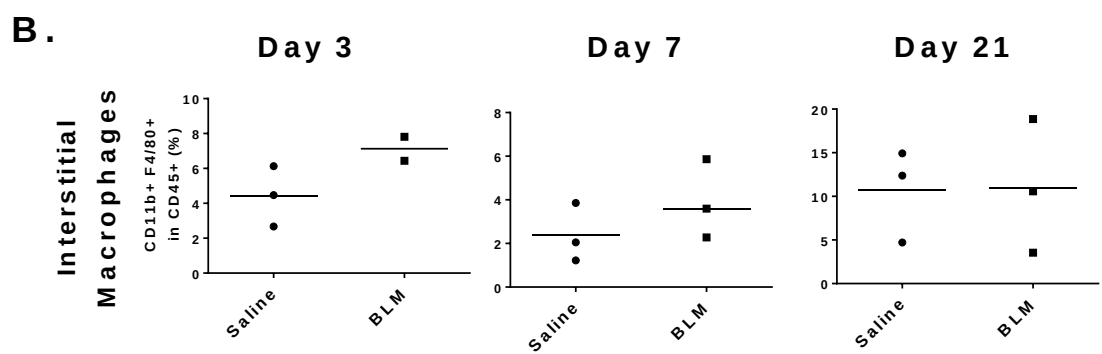
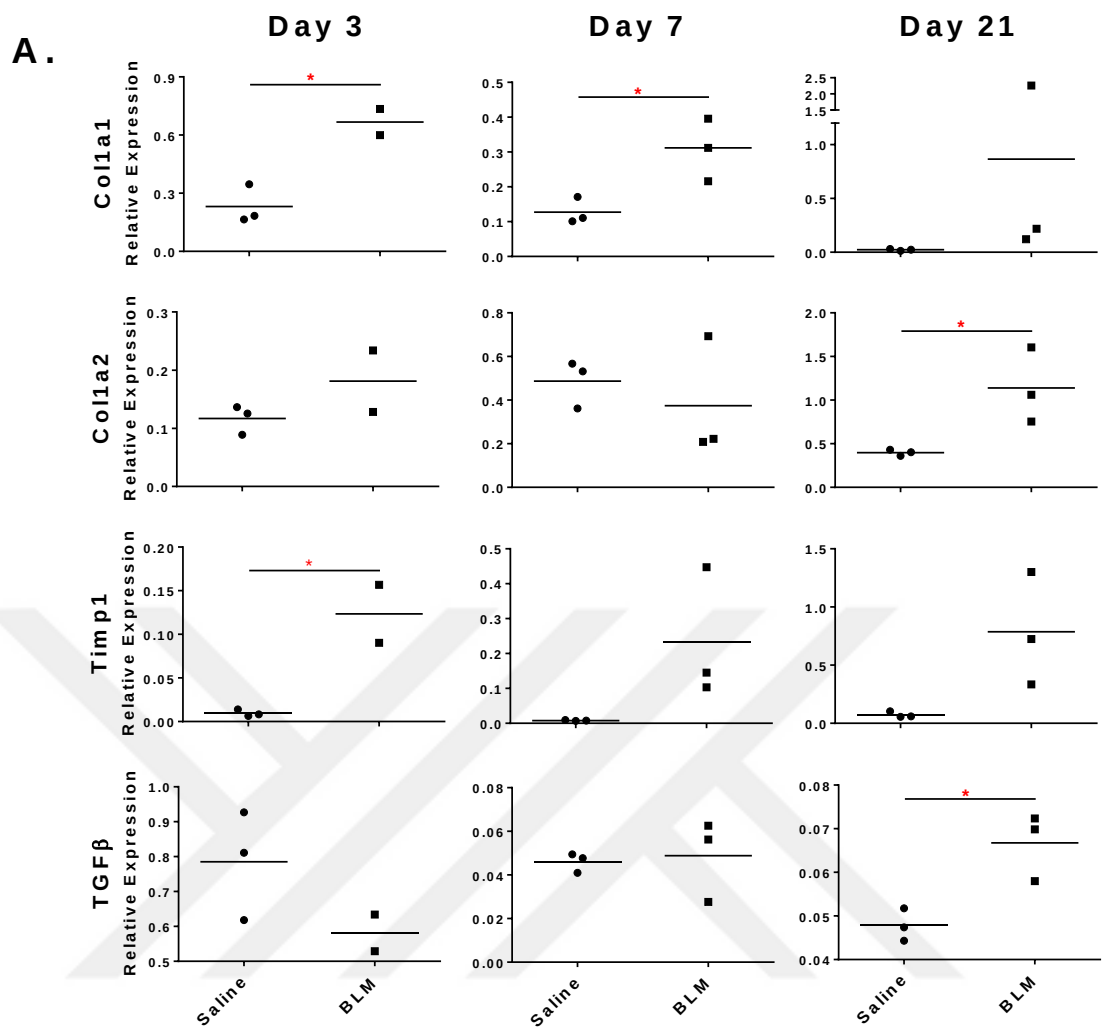


Figure 3.14 Changes in gene expression levels, macrophage cell population and protein expressions in lung tissue after 3, 7 and 21 days upon bleomycin administration. A) Col1a1, Col1a2, Timp1 and Tgf β gene expressions were normalized to expression of β -actin and determined by RT-qPCR. B) Lung tissue was digested with liberase, and single-cell suspension was obtained. Interstitial macrophage population was analyzed via flow cytometry and characterized as CD45⁺, CD11b⁺, F4/80⁺. C) Smad2/3, p-Smad2 and α -SMA protein levels were determined performing Western blotting. Significances were determined by performing Student's t test. Representative results of at least 2 independent experiments are shown. (n=2 or 3) (BLM: Bleomycin, *p<0.05)

Col1a1 gene expression started to rise even in 3rd day which is positively correlated with the increase in Timp1 gene level. Timp1 (tissue inhibitor of matrix metalloproteinases-1) inhibits Mmp1 (matrix metalloproteinase-1) which decreases type I collagen in extracellular matrix by degrading it. Except the downmodulation in Col1a2 levels at day 7, type I collagen and Timp1 expressions were higher in bleomycin-instilled mice in all time points. Elevation in Tgf β expression in lung tissue started after 3rd day (Figure 3.14A). Flow cytometry analysis demonstrated that interstitial macrophage population in lung tissue did not significantly changed by bleomycin instillation throughout the test period of the experiment (Figure 3.14B). Lastly, immunoblotting revealed that Smad2/3 protein levels increased from day 3 and onwards until day 21. Phospho-Smad2 level was found to be at the highest level at day 7 (Figure 3.14C). Since Smad2 and Smad3 are positive regulators of TGF β signaling, upregulation in Tgf β gene expression at day 7 is parallel to the increased levels of Smad2/3 proteins and Smad2 phosphorylation levels. We did not, however, observe any significant change in α -SMA levels, which was defined as a fibrosis-related protein that is supposed to be positively correlated with Tgf β expression (compare Fig 3.14A bottom panel vs Fig3.14C) [181]. It is worth mentioning that, considering this protein as one of the fibrosis markers is debatable, as recently reported, α -SMA expression is not directly regulated with the degree of fibrosis as shown for various animal models [182].

Consequently, bleomycin not only caused inflammation in lung by elevating IL-6 and IL-12 serum levels and accumulation of neutrophils in BALF, it also induced higher expression levels of several cardinal fibrotic genes and their proteins in the lung tissue. Therefore, we successfully established bleomycin-induced lung inflammation and acute scleroderma model in mouse.

3.4.1 Preventive effect of free A151 ODN on early stage of bleomycin-induced scleroderma

We next investigated whether A151 ODN has a preventive role on the development of lung inflammation in mice. To this end, we first administered 100 μ g of free A151 ODN

intraperitoneally. Three days later, 50 μ g bleomycin was instilled, and mice were sacrificed after 3 more days (Figure 3.15A).

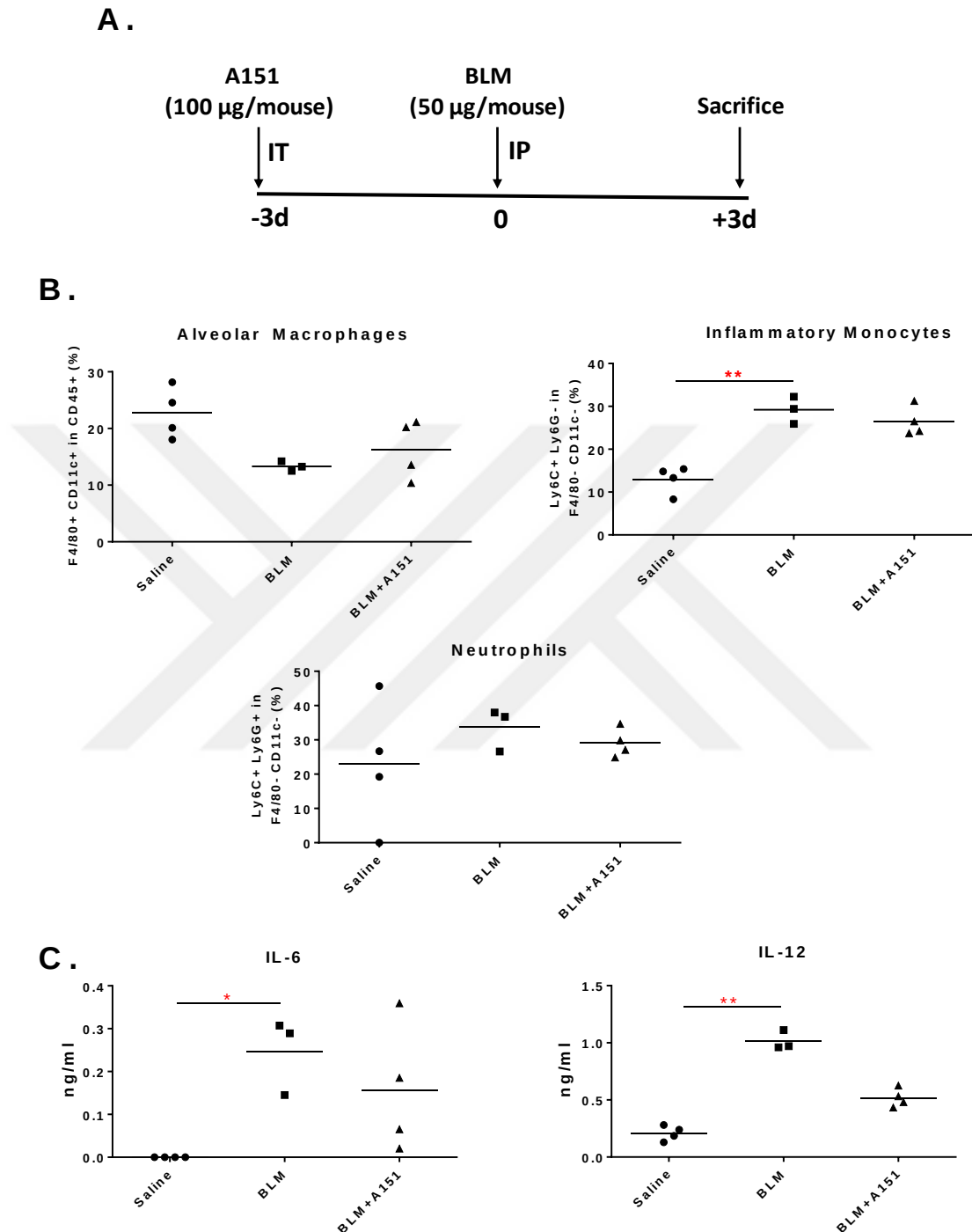


Figure 3.15 Analysis of BALF cell populations and cytokine levels in mice that were pre-treated with A151 ODN for 3 days, then injected bleomycin. A) Experimental design involving A151 ODN pretreatment of mice (n=3 or 4). B) Determination of alveolar macrophages (CD45⁺, F4/80⁺, CD11c⁺), inflammatory monocytes (CD45⁺, F4/80⁻, CD11c⁻, Ly6G⁻, Ly6C⁺) and neutrophils (CD45⁺, F4/80⁻, CD11c⁻, Ly6G⁺, Ly6C⁺) from BALF using flow cytometry. C) BALF resident IL-6 and IL-12 cytokine levels were measured using ELISA. Significances were determined by performing one-way ANOVA. (IT: Intratracheal, IP: Intraperitoneal, BLM: Bleomycin, **p<0.01)

As seen in Figure 3.15A-C, A151 ODN pre-treatment reduced cell accumulation to the BALF as well as IL-6 and IL-12 proinflammatory cytokine levels. We observed that while BLM instillation, as shown previously (please see Fig. 3.13), decreased alveolar macrophage population in the BALF, A151 ODN pre-treatment of animals reduced numbers of neutrophils and inflammatory monocytes compared to only bleomycin administered mice group, although not significant (Figure 3.15B). Similarly, BLM instillation resulted in significant elevation of IL-6 and IL-12 in BALFs, and when mice were pre-treated with A151 ODN, we detected substantially less amounts of these cytokines (Figure 3.15C).

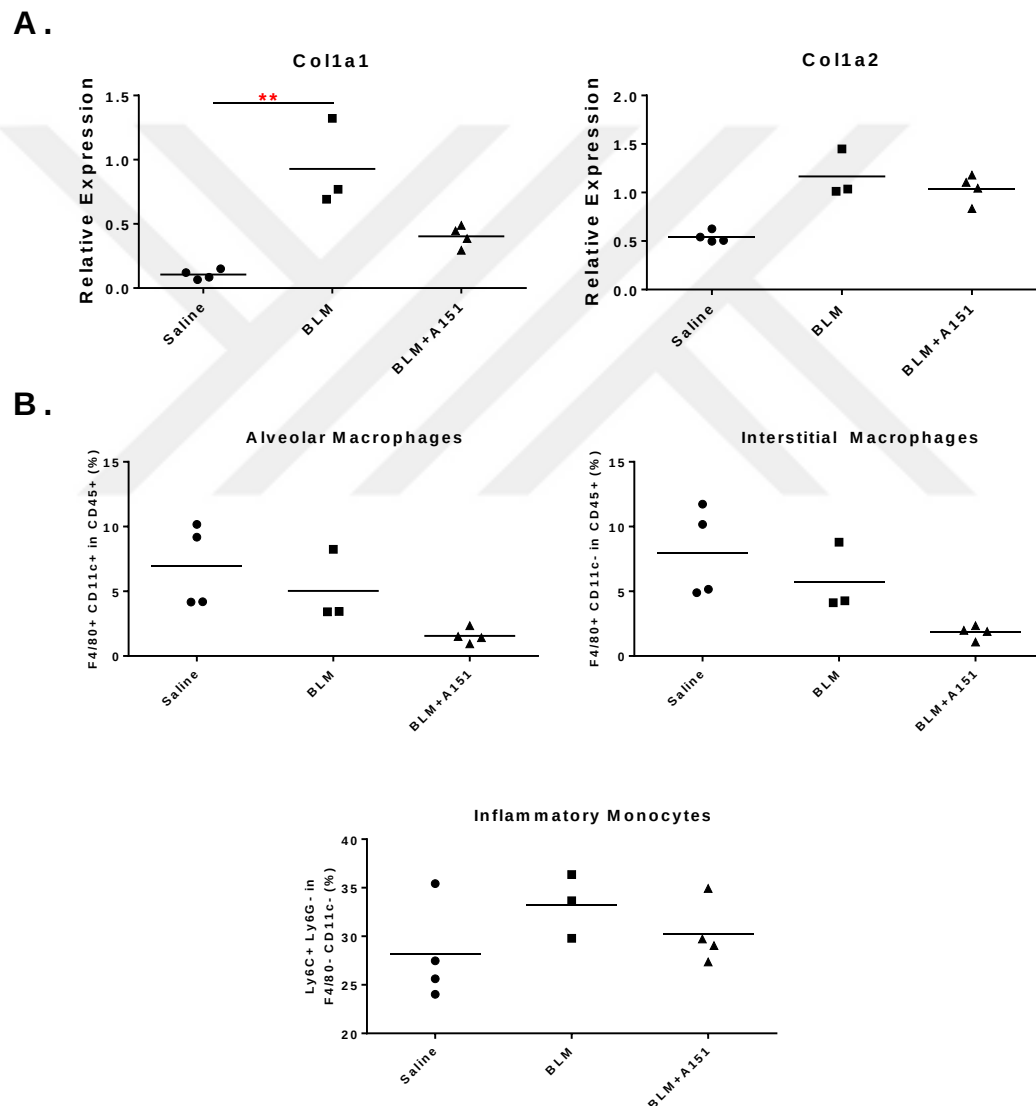


Figure 3.16 Analysis of lung cell populations and collagen gene expressions in mice that were pre-treated with A151 ODN for 3 days, then injected with bleomycin and followed for three days before sacrificing. A) Col1a1 and Col1a2 gene expressions in lung were analyzed using RT-qPCR. B) Alveolar macrophages (CD45⁺, F4/80⁺, CD11c⁺), interstitial macrophages (CD45⁺, F4/80⁺, CD11c⁻) and inflammatory monocytes (CD45⁺, F4/80⁻, CD11c⁻, Ly6G⁻, Ly6G⁺) in lung tissue digested with liberase were determined using flow cytometry.

Significances were determined by performing one-way ANOVA. (IT: Intratracheal, IP: Intraperitoneal, BLM: Bleomycin, **p<0.01)

RT-qPCR analyses of the lung tissues revealed that A151 ODN downregulated Col1a1 expression from these tissues whereas as observed previously it did not affect Col1a2 expression (Figure 3.16A). Contrary to the increase in alveolar macrophages obtained from BALF, interstitial and alveolar macrophage populations in BLM instilled lungs further decreased with A151 ODN pre-treatment (Figure 3.16B). Moreover, inflammatory monocytes in lung tissues which was gated as Ly6C⁺ Ly6G⁻ cells, although not statistically significant, reduced upon A151 ODN intraperitoneal pre-treatment of animals.

In conclusion, these preliminary experiments established that A151 ODN led to a reduction of inflammatory immune cell populations in lung and in BALF. Moreover, pre-treatment of animals 3 days before bleomycin instillation with A151 ODN decreased BALF cytokine levels and downmodulated collagen expression. Despite the fact that none of these alterations were significant, we speculated that this was due to the low sample size.

3.4.2 Therapeutic implications of free and liposome-encapsulated A151 ODN formulations on bleomycin induced lung inflammation

After demonstrating that A151 ODN has promising effects on prevention of lung inflammation, we next investigated whether it could be used as a therapeutic agent in lung inflammation. In this experimental setup, mice were given free or liposome-encapsulated A151 ODN at 3rd and 5th days following intratracheal bleomycin instillation and sacrificed at 7th day (Fig 3.17A).

We observed that 2 days after BLM administration, mice started to lose at least 10% of its original weight (Figure 3.17B, red square plot). However, while saline treated group sustained their weights at stable levels over the course of the experiment, BLM treated animals continued to lose their body weights and from day 4 and onwards this reached nearly 20% loss of their original weights. A151 ODN treated mice groups also got leaner but the loss level never reached to ~10% of their body weights. In fact, at day 4, animals were indistinguishable from saline treated counterparts, however, unexpectedly, A151 ODN (either free or liposome loaded counterpart) treatment did not have any significant effect on preventing weight loss mediated by bleomycin instillation.

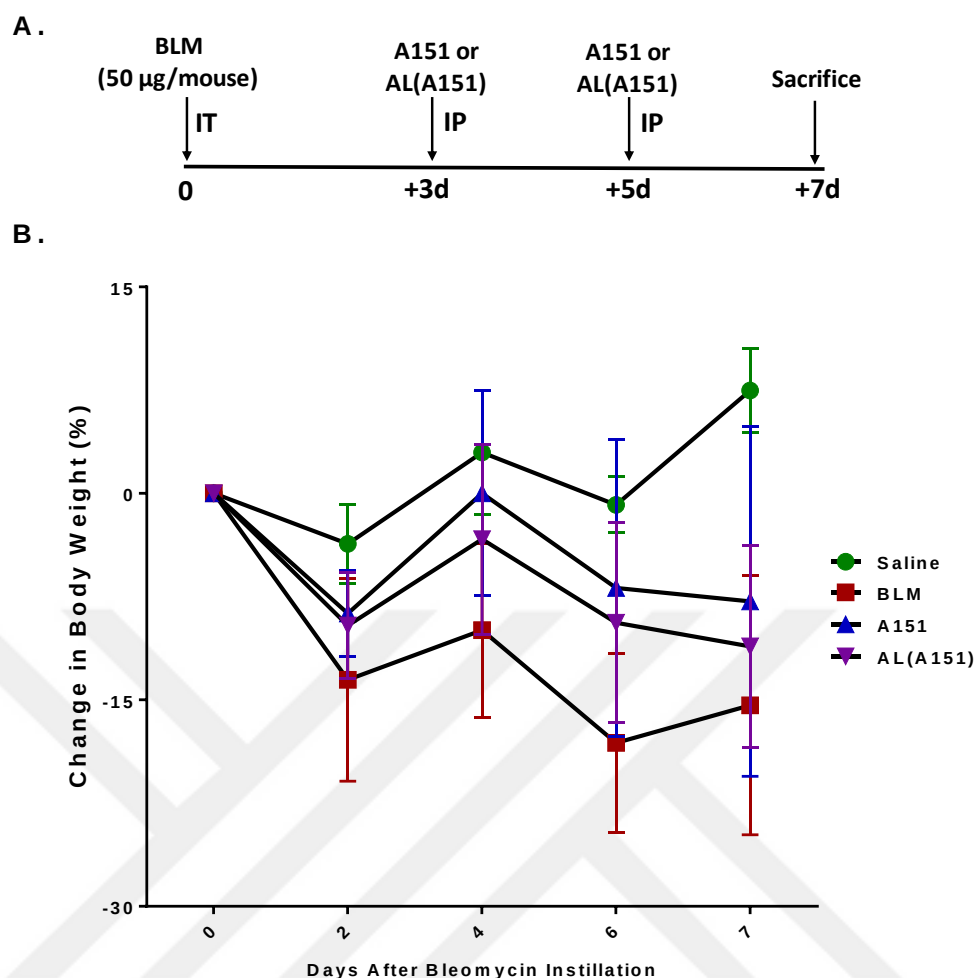
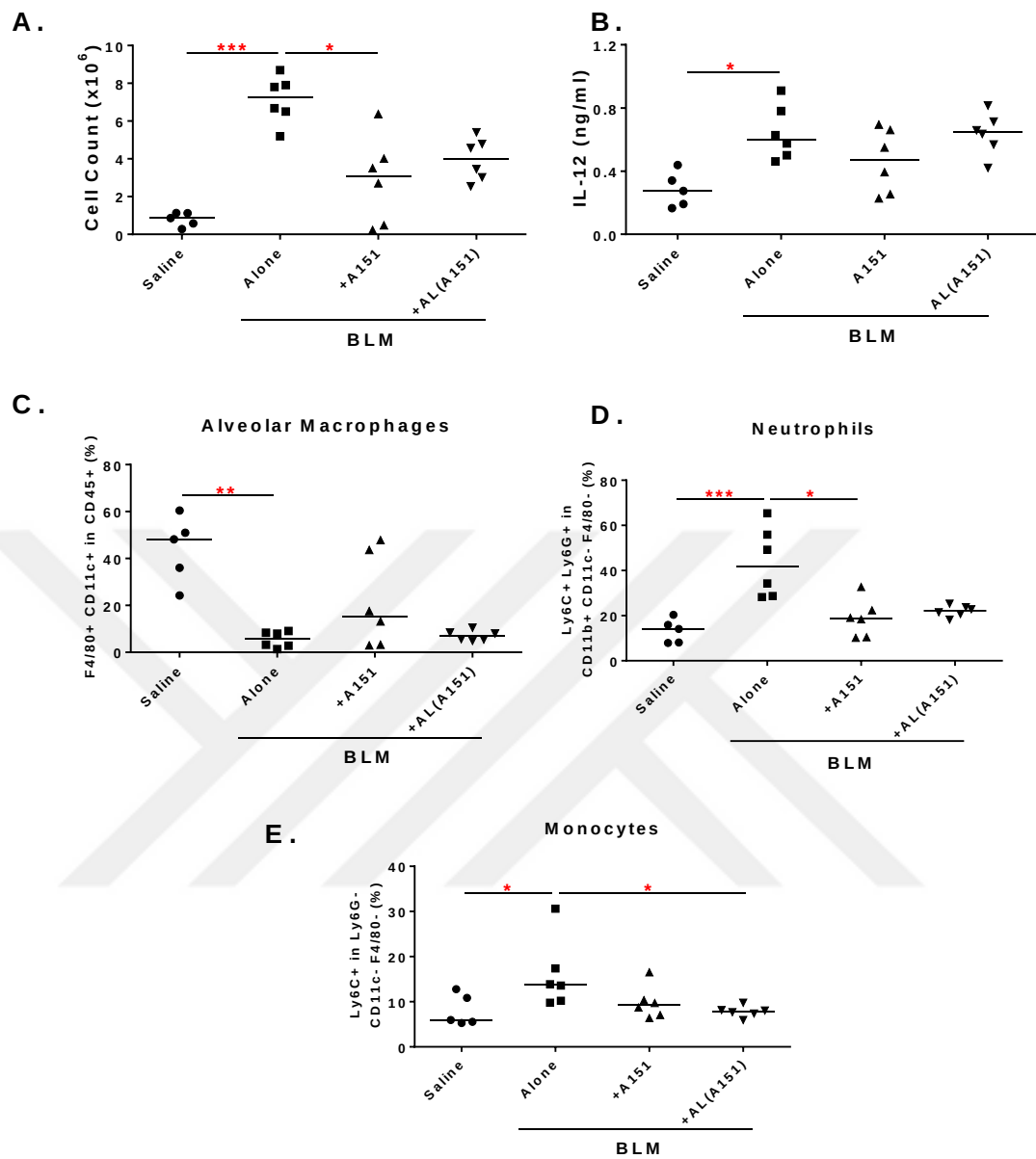


Figure 3.17 Experimental design of bleomycin induced lung inflammation and change in body weights of mice. A) Experiment design demonstrating A151 ODN and BLM administrations (n=6). B) Percent changes in body weights of mice throughout the experiment. (IT: Intratracheal, IP: Intraperitoneal, BLM: Bleomycin, AL(A151): Anionic liposome-encapsulating A151)

Following bleomycin administration, number of cells retrieved from BALF significantly increased, as expected. Down-modulatory effect of A151 ODN treatment on BALF content upon bleomycin delivery was presented in the Figure 3.18.

BALF



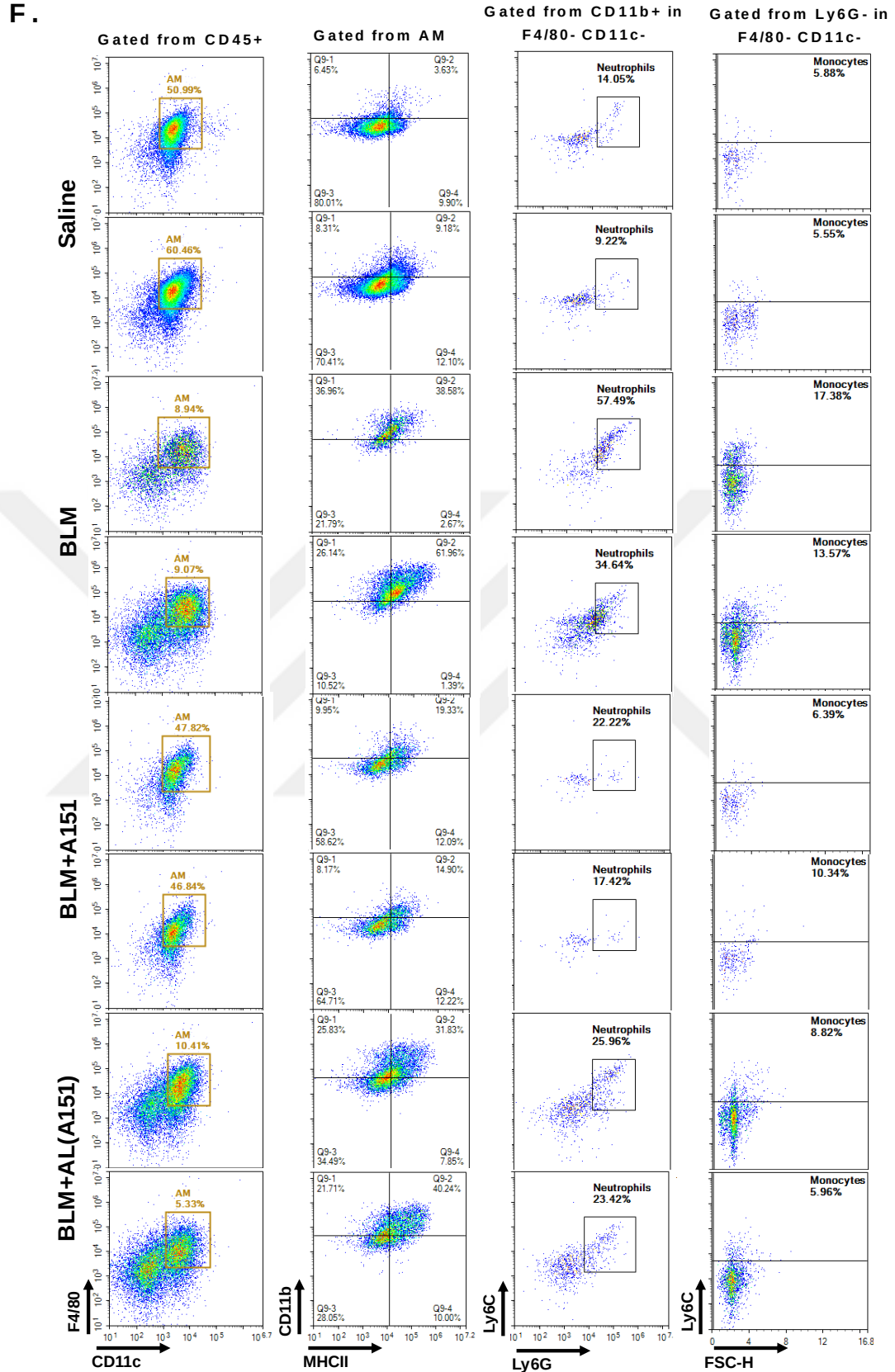


Figure 3.18 Changes in BALF cytokine levels and cell population in response to bleomycin instillation and A151 ODN treatment. A) Total cell numbers accumulated in BALF samples were counted using flow cytometry. B) IL-12 levels from BALF samples was determined using ELISA. C) Percent of alveolar macrophages, D) neutrophils and E) monocytes within the BALF

samples were accounted using flow cytometry. F) Representative dot plots of alveolar macrophages, CD11b/MHCII expression in alveolar macrophages and neutrophils. Alveolar macrophages were identified as CD45⁺ F4/80⁺ CD11c⁺, while neutrophils were Ly6C⁺ Ly6G⁺ CD11b⁺ F4/80⁻ CD11c⁻ and monocytes were Ly6C⁺ Ly6G⁻ F4/80⁻ CD11c⁻. Results of two animals were shown for each group as representative dot-plot images. Significances were determined using one-way ANOVA comparing BLM alone group with others. (BLM: Bleomycin, AL(A151): Anionic liposome encapsulated A151, AM: Alveolar macrophages, *p<0.05, **p<0.01).

Total cell numbers in BALF increased from basal levels more than 4-fold in response to bleomycin infusion (a rise from ~1.0 million cells to over 7 million cells) whereas A151 ODN significantly decreased total cell number (a drop to about 3 million cells, Figure 3.18A). Unfortunately, loading A151 ODN within anionic liposomes (AL) did not confer any additional advantage either to suppress IL-12 secretion (while free A151 ODN treatment partly decreased IL-12 levels in response to bleomycin) or to decrease further total BALF resident immune cell numbers (Figure 3.18A and 3.18B). Interestingly, delivery of free A151 ODN had a partial effect on restoration of alveolar macrophages (Figure 3.18C). We found that while 2 animals returned to saline treated animal levels and another 2 mice only partially responded to A151 ODN treatment; however, of 6 mice, 2 animals did not respond to A151 therapy, leaving the general outlook as being non-significant macrophages restoration following A151 ODN therapy (Fig 3.18C). When we investigated the BALF resident neutrophil population that was accumulated in the alveolar space, we noticed that compared to saline instilled mice, there were more than 2-fold more neutrophils in the BALF following BLM instillation. This level almost reduced to baseline level in animals that received free A151 ODN therapy. This reduction was not further improved when A151 was encapsulated within anionic liposomes (Figure 3.18D). Our findings are in accord with the findings that was demonstrated in mice that was investigating the effect of A151 ODN on silica-induced pulmonary inflammation [43]. However, the mechanism behind this still remains elusive. We also observed that F4/80⁺ CD11c⁻ population increased upon bleomycin administration and this population disappeared with free A151 treatment (Figure 3.18F left panel). Further investigation revealed that a part of this population were monocytes. Monocytes were identified as being Ly6C⁺ and Ly6G⁻, and they were nearly 2-fold more upon bleomycin instillation while A151 treatment significantly decreased their accumulation (Figure 4.18E). Liposomal encapsulation seemed to be more effective to reduce monocyte population since one mouse did not respond free A151 treatment. It is possible that double-negative population could be of T- and B-cells or non-immune cell origin, too. Therefore, it is obvious that we need to study this population more extensively.

Alveolar macrophages are known to express low levels of CD11b and MHCII compared to other macrophage subsets in mouse [183]. As it can be seen in Figure 3.18F (middle panel), alveolar macrophages of naïve animals that received only saline expressed low levels of CD11b and

MHCII, as expected. However, bleomycin administration upregulated CD11b expression levels, unexpectedly. Therefore, we wished to analyze the CD11b and MHCII expression levels of alveolar macrophages as well as CD11c level that was monitored as one of the macrophage activation markers in some studies [184]. Lastly, co-stimulatory molecule CD86 was also included to the analysis since it is an essential activation marker for macrophages.

We observed that, CD11b, CD11c and MHCII (Figure 3.19A, 3.19B, 3.19D, respectively) expressions significantly elevated in alveolar macrophages upon BLM instillation, while CD86 expression remained almost the same (Figure 3.19C). Treatment with free A151 if not significantly, definitely substantially, reduced surface marker levels of all these 4 molecules (Figure 3.19).



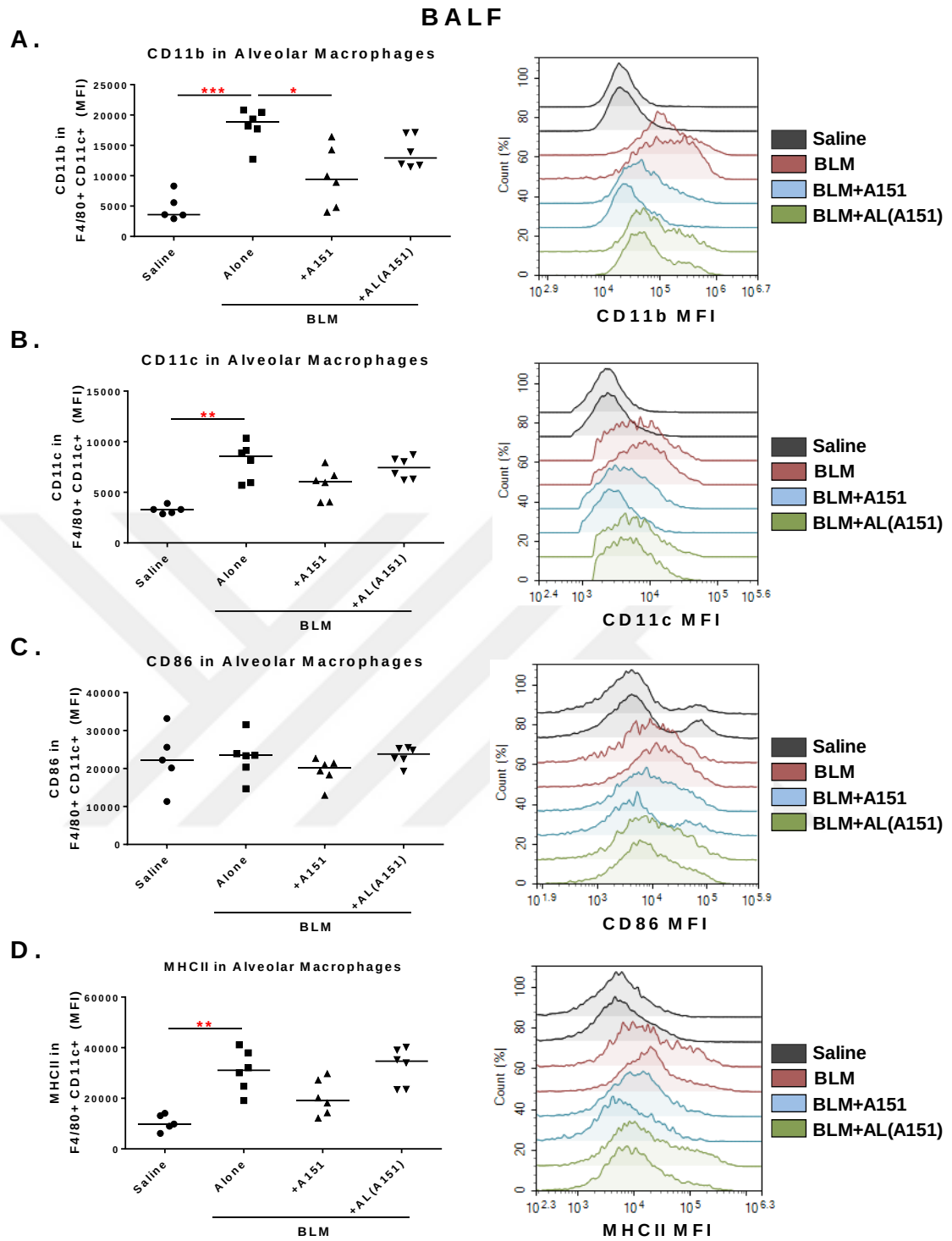


Figure 3.19 Surface marker expressions of alveolar macrophages obtained from BALF. A) CD11b, B) CD11c, C) CD86 and D) MHCII expressions were analyzed from alveolar macrophages in BALF using flow cytometry. Graphs with MFI values were shown in left panels while representative histograms for two mice in each group were given in right panels. Alveolar macrophages were identified as CD45⁺ F4/80⁺ CD11c⁺. Significances were determined using one-way ANOVA comparing BLM alone group with other treatments. (BLM: Bleomycin, AL(A151): Anionic liposome-encapsulated A151, MFI: Mean fluorescent intensity, **p<0.01).

The status of the surface markers was checked in alveolar macrophages that was obtained from the lung samples (Figure 3.20). Cells were recovered by lavage are identified as alveolar cells while cells obtained by enzymatic digestion after lavage of lungs were defined as interstitial cells, not all of the alveolar macrophages could be recovered from alveolar space by performing lavage [183]. Therefore, we determined alveolar macrophage population from enzymatically digested lung tissues and analyzed their CD11b, CD11c, MHCII and CD86 expression levels using flow cytometry (Figure 3.20).

Similar results were observed when surface expressions of lung resident macrophages were stained against CD11b, CD11c, CD86 and MHCII from bleomycin treated mice (Figure 3.20). Although not significant, unencapsulated A151 ODN reduced the expressions of these markers to a greater extent, compared to liposome-encapsulated A151 ODN group. Overall, alveolar macrophages in mice administered with bleomycin elevated antigen presenting capacities as evidenced by increased MHCII surface expressions and magnified co-stimulatory molecule expressions (i.e. CD86 levels) while unencapsulated form of A151 ODN downregulated the expressions of all these surface markers better than liposomal A151 ODN.

LUNG

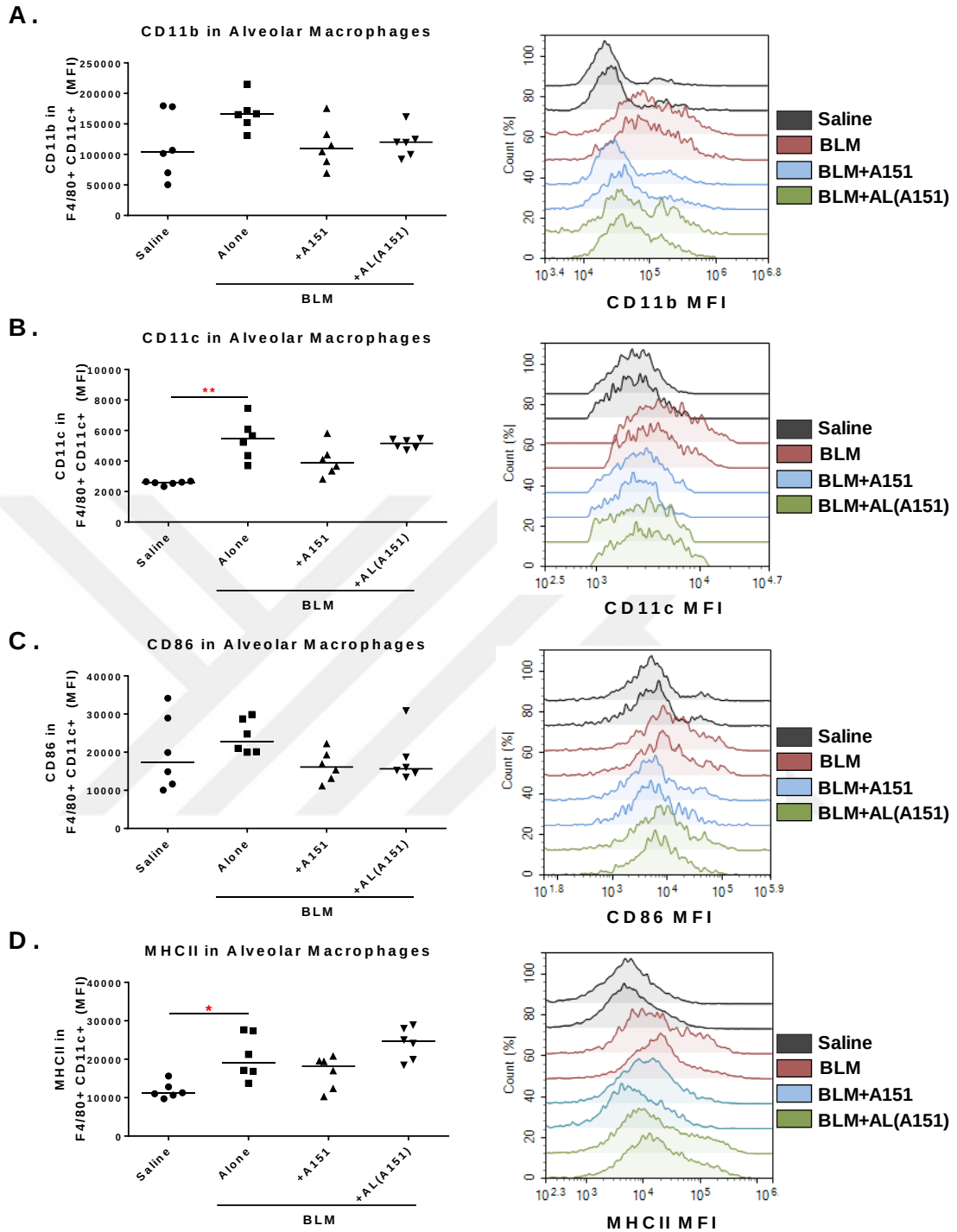


Figure 3.20 Cell surface expressions of alveolar macrophages obtained from lung following bleomycin instillation. A) CD11b, B) CD11c C) CD86 and D) MHCII expressions were analyzed from alveolar macrophages in lung using flow cytometry. Lungs were enzymatically digested and single cell suspensions were obtained and to perform staining. The MFI plots of individual animals were presented in left panels while representative histograms of two animals/treatment group were given in the right panels. Alveolar macrophages were identified as CD45⁺ F4/80⁺ CD11c⁺. Significances were determined using one-way ANOVA comparing BLM alone group with other treatments. (BLM: Bleomycin, AL(A151): Anionic liposome-encapsulated A151, MFI: Mean fluorescent intensity, **p<0.01).

Lastly, fibrotic gene expressions such as; Col1a1, Col1a2, Tgf β and Timp1 were analyzed from lung tissue purified total RNAs using RT-qPCR (Figure 3.21A). Furthermore, protein levels of Smad2/3 and α -SMA were determined via immunoblotting (Figure 3.21B).

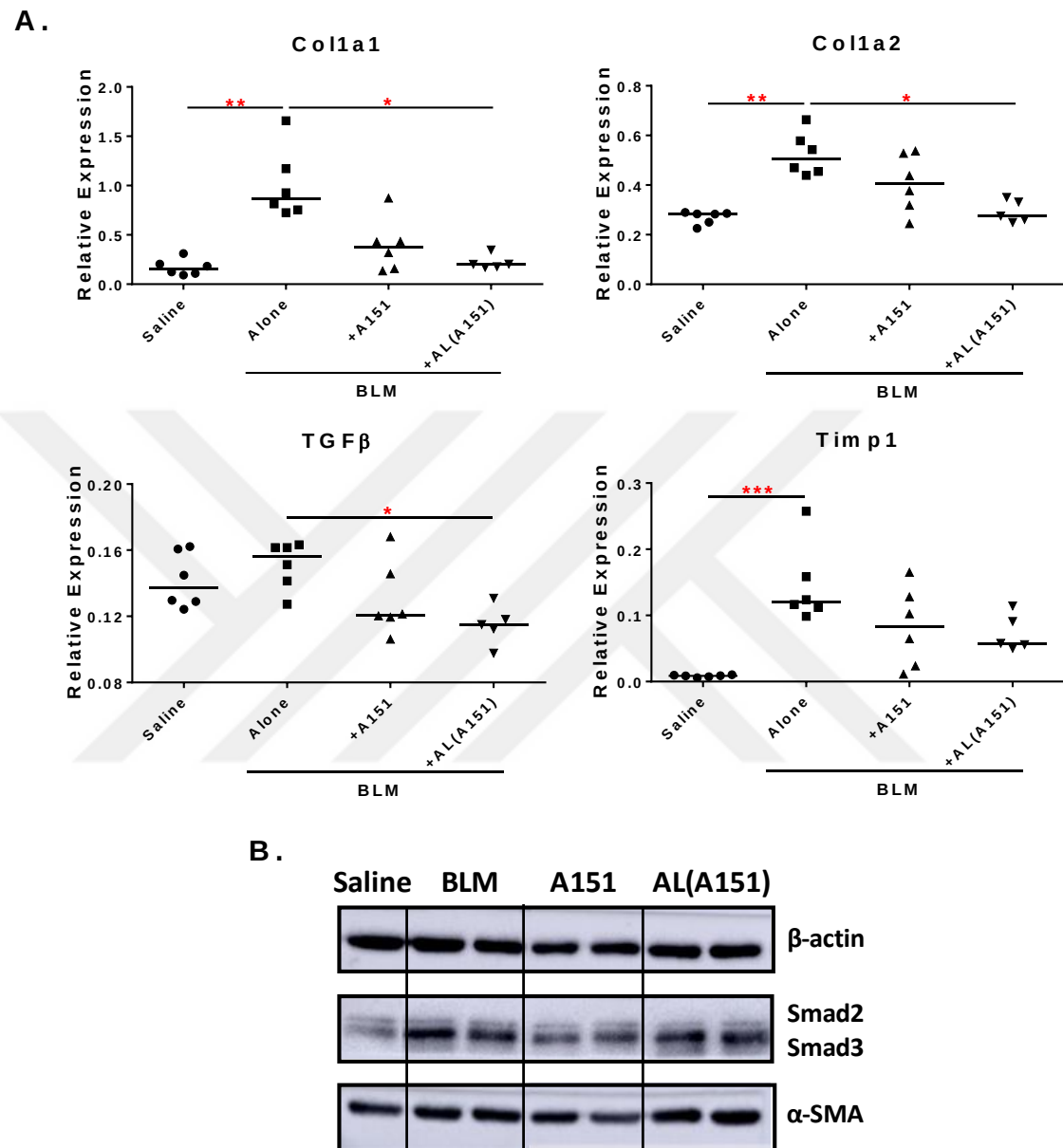


Figure 3.21 Gene and protein expressions of lung samples from bleomycin and A151 delivered mice. A) Col1a1, Col1a2, TGF β and Timp1 gene expressions were analyzed by RT-qPCR. Expressions were normalized to β -actin. B) Smad2/3 and α -SMA protein levels were determined by Western blotting. Significances were determined using one-way ANOVA comparing BLM alone group with others. (BLM: Bleomycin, AL(A151): Anionic liposome-encapsulated A151, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Significant increase was observed in Col1a1, Col1a2 and Timp1 gene levels in bleomycin-instilled mice compared to saline group. Of note, Tgf β gene level was also elevated, although not significantly (Figure 3.21A). Judging by the reduction of the mRNA levels of the

abovementioned four genes, our data revealed that entrapment of A151 ODN within anionic liposomes further enhanced the anti-fibrotic potential of this immunosuppressive ODN. Following BLM treatment, when mice were intraperitoneally injected with A151 ODN, gene expressions significantly reduced in Col1a1, Col1a2 and Tgf β . A151 treatment in either free or liposomal formulation also decreased Timp1 gene levels; however, it was not significant. Of note, establishment of fibrosis is not completed by day 7 following bleomycin administration [159]; however, in our hands, upregulation of fibrotic gene expressions indicated that tissue damage caused by elicited severe inflammation initiated elevation in these fibrotic gene transcription, thereby leading to “a transitional phase” through fibrosis.

During the course of the establishment of lung fibrosis, Smad2 and Smad3 are the most dominant proteins downstream of TGF β signaling pathway and our investigation of lung samples revealed that these proteins were upregulated upon bleomycin administration, and when treated with the A151 ODN (either unloaded or liposome loaded counterparts), their levels were reduced in the lungs (Figure 3.21B). While anionic liposome loaded A151 ODN failed to reproduce the effect of free A151 ODN treatment, α -SMA levels of the lungs were substantially reduced by unloaded A151 ODN treatment (Fig. 3.21B, lower immunoblot panel).

Overall, A151 ODN attenuated lung inflammation induced by bleomycin which our data demonstrated that it decreased neutrophil infiltration to alveolar space and reduced elevated activation markers of macrophages and also the production of IL-12 from cells in alveolar space. A151 ODN encapsulation within liposomes provided advantage to reduce the transcription levels of several fibrotic genes. Down modulation of fibrotic gene expressions, such as type I collagen, Tgf β and fibrotic protein levels such as Smad2/3 were thought to be indirect consequences of attenuated inflammation.

3.4.3 Therapeutic effects of liposome-encapsulated A151 ODN formulation on bleomycin induced fibrosis

We next investigated anti-fibrotic effects of liposome-encapsulated A151 ODN in bleomycin-induced scleroderma model. Since liposome-encapsulated A151 ODN was shown to be more effective specifically downregulation of fibrotic gene mRNA expressions, we sought to determine the therapeutic potential of single injection of liposome-encapsulated A151 ODN at different times points following bleomycin instillation in the next experiment. For this, mice received 100 μ g of anionic liposome-encapsulated A151 at 5th, 10th or 15th days following 50 μ g of intratracheal bleomycin instillation (Figure 3.22).

IL-12 and TNF α levels were detected to be elevated in BALF of bleomycin-instilled mice. Liposomal A151 ODN given at 10th and 15th day significantly reduced TNF α levels. Strikingly

IL-12 production was not statistically suppressed albeit there was a substantial suppression at day 10 and 15 (Figure 3.22B).

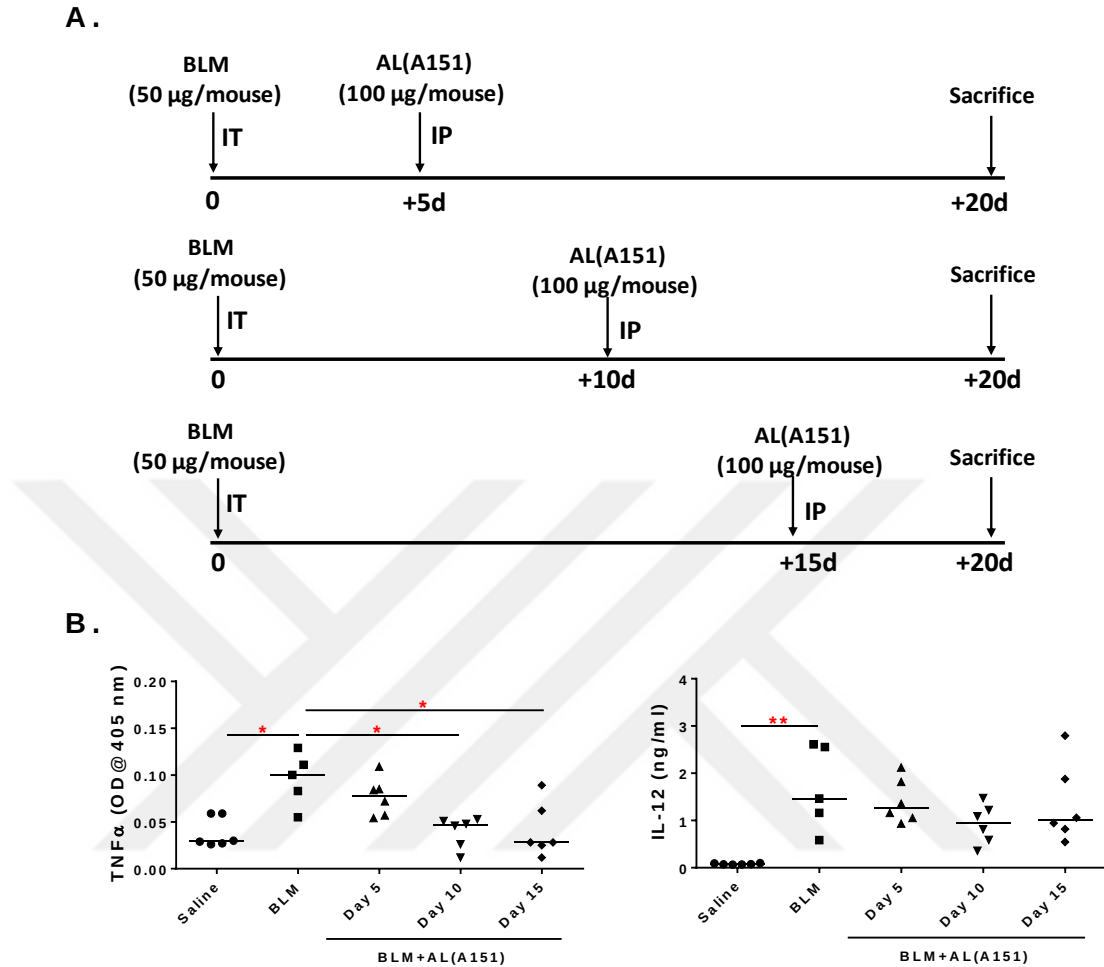


Figure 3.22 Liposomal A151 ODN treatment in bleomycin induced scleroderma model and cytokine levels in BALF. A) Experimental design (n=6). B) TNF α and IL-12 cytokine levels in BALF were measured by ELISA. Statistical significances were determined using one-way ANOVA comparing BLM alone group with other groups. (IT: Intratracheal, IP: Intraperitoneal, BLM: Bleomycin, AL(A151): Anionic liposome-encapsulated A151, *p<0.05, **p<0.01)

Similar to the *in vivo* experiment that immunosuppressive capacity of A151 ODN in lung inflammation was investigated, we next checked the alteration of the percent alveolar macrophage levels in lungs of these animals following bleomycin administration. As expected, these cells were significantly decreased in BALF even though it contradicts with the literature.

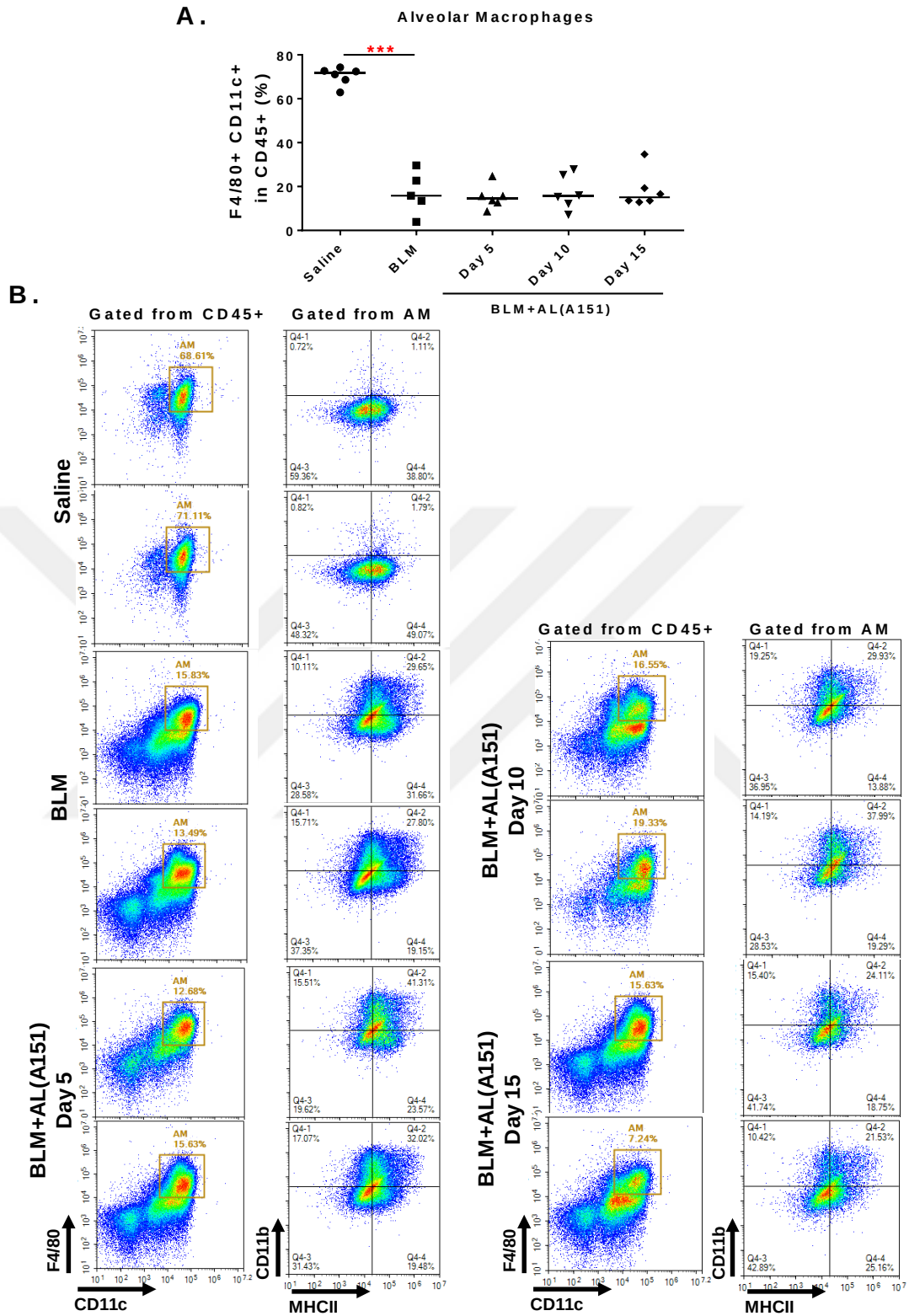


Figure 3.23 Alveolar macrophages in BALF samples after a single intraperitoneal liposomal A151 ODN injection into bleomycin-induced scleroderma in mice. A) Alveolar macrophages were analyzed from BALF samples via flow cytometry. B) Representative dot plots of two mice from each group showing alveolar macrophage population and CD11b/MHCII expressions. Alveolar macrophages were identified as CD45⁺ F4/80⁺ CD11c⁺. Significances were determined using one-way ANOVA comparing BLM alone group with others. (BLM:

Bleomycin, AL(A151): Anionic liposome-encapsulated A151, AM: Alveolar macrophages, *** $p < 0.001$).

Administration of liposome-encapsulated A151 ODN; however, did not restore this population in the lung BALFs (Figure 3.23A). CD11b⁺ MHCII⁺ population in alveolar macrophages increased with bleomycin (Figure 3.23B).

Besides the elevation of CD11b and MHCII on alveolar macrophages isolated from BALF, expression of CD80 were also significantly increased (Figure 3.24A-C). Treatment with liposomal A151 ODN, when given at day 15 following bleomycin administration slightly reduced MHCII expression. A151 injection did not influenced the expression levels of CD11b and CD80 on alveolar macrophages (Figure 3.24A-C).



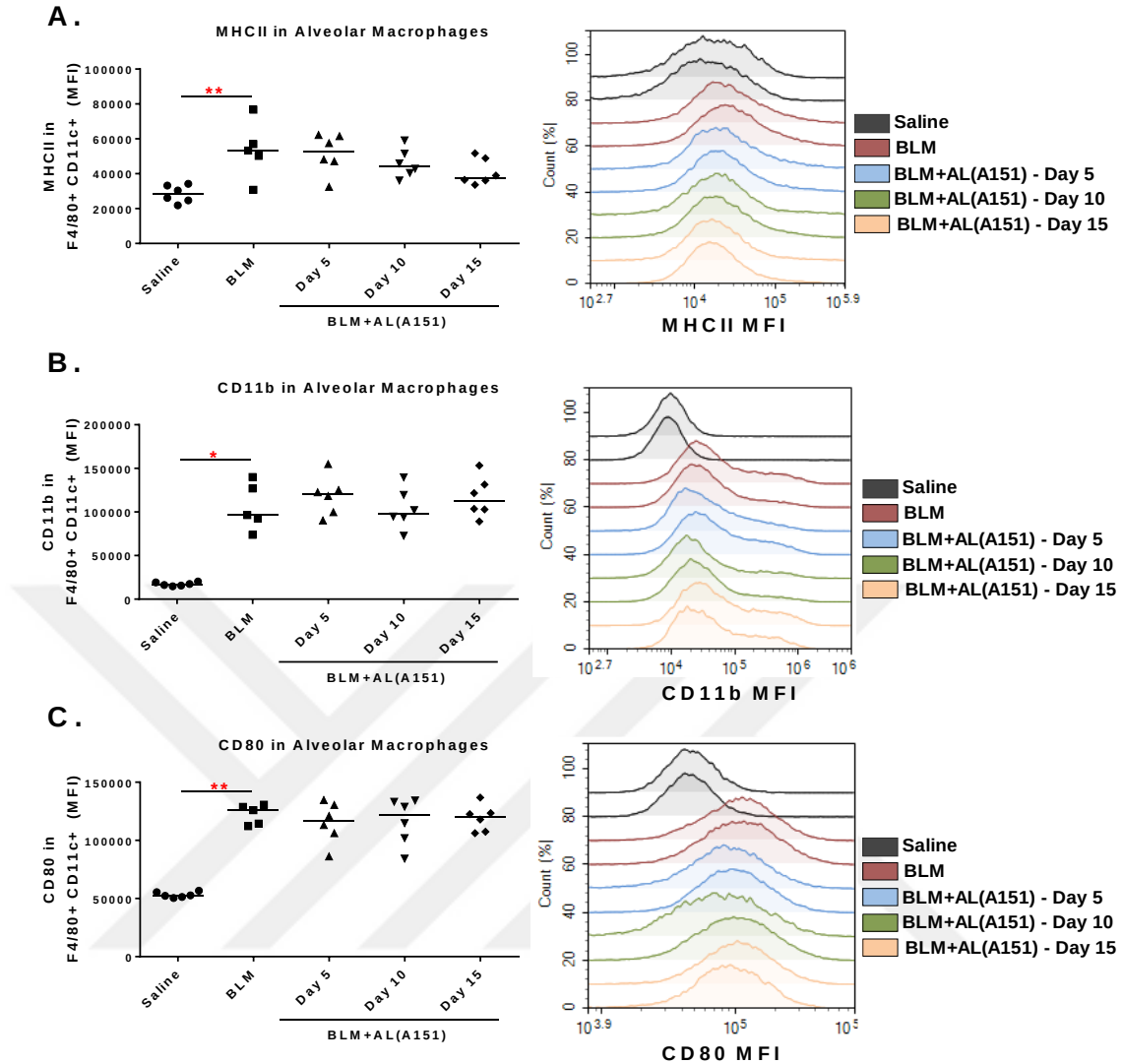


Figure 3. 24 MHCII, CD11b and CD80 expressions in BALF of liposome-encapsulated A151 ODN treated mice following bleomycin instillation. A) MHCII, B) CD11b and C) CD80 expressions were analyzed from alveolar macrophages in BALF using flow cytometry. Plots of MFI values were shown on left panel while representative histograms from two mice for each group were presented on right panel. Alveolar macrophages were identified as CD45⁺, F4/80⁺ and CD11c⁺. Significances were determined using one-way ANOVA comparing BLM alone group with other treatments. (BLM: Bleomycin, AL(A151): Anionic liposome-encapsulated A151, MFI: Mean fluorescent intensity, **p<0.01, ***p<0.001)

Gene expression analysis via RT-qPCR revealed significant increase in Col1a1 and Col1a2 levels following BLM instillation of the mice group compared to saline group (Figure 3.25). Liposomal A151 ODN given at day 15 partly reduced Col1a2 expression; however, the change was not significant.

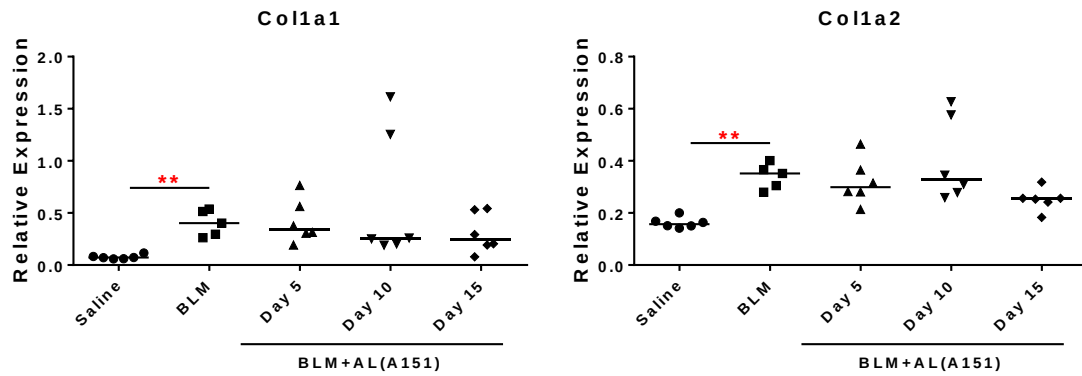


Figure 3. 25 Collagen gene expressions in lungs of mice treated with liposomal A151 ODN following bleomycin instillation.

Overall, single injection of liposome-encapsulated A151 ODN at a later stage following BLM instillation neither reduced surface marker expressions of alveolar macrophages nor decreased fibrotic gene mRNA expressions in BLM-induced lungs representing fibrosis model. Because bleomycin is a very powerful and potent agent as a drug for human and agent to develop disease model on mice, the protective role of A151 ODN could be improved if it was decided to be injected to animals more than once following BLM instillation.

CHAPTER 4

DISCUSSION

Immune system is evolved to protect hosts from infections. However, aberrant regulation of immune responses leads to two pathologic conditions, i) immune deficiencies which result in inefficient immune response against pathogens and ii) autoimmune diseases that are characterized by failure to discriminate self from non-self [185]. Autoimmune diseases are seen in ~7-9% of population with a greater risk for affecting women, and to date, more than 80 different types have been identified [186]. These diseases could be either organ-specific such as type I diabetes or systemic such as rheumatoid arthritis or systemic lupus erythematosus. Etiology of autoimmune diseases remain elusive.

Systemic sclerosis (SSc) is not commonly seen worldwide, although mortality rate is very high due to being heterogenous and complex disease [73]. The three hallmarks used in SSc diagnosis are i) Raynaud's phenomenon, ii) presence of anti-nuclear antibodies and iii) fibrosis on skin or internal organs. Making accurate diagnosis of this disease as important as applying effective treatment. Unfortunately, a curative therapy for systemic sclerosis has not been developed. Approved treatment options; however, targets only specific symptoms and relieve disease manifestations during the course of therapy; therefore, combinational and sequential treatment is generally preferred [187, 188].

A151 ODN is a single stranded oligonucleotide sequence mimicking the natural telomeric repeat motifs in human genome. While it was discovered to inhibit downstream of TLR9 signaling activated by CpG DNA, its wide range of immunosuppressive ability on different immune cells have been characterized later. We and others have tested A151 ODN in different inflammation models such as endotoxic shock, atherosclerosis and uveitis on mice and proved that A151 ODN could be a potential DNA-based therapeutic option for different inflammatory diseases [32, 41, 44]. Furthermore, the findings that A151 ODN impeded the onset of genetic model of systemic lupus erythematosus (SLE) and prevented the development of rheumatoid arthritis implied that it might be also used in the treatment of autoimmune diseases [39, 40]. Besides, immunomodulatory agents including immunosuppressants have been used in treatment of systemic sclerosis [189]. Based on these previous experiences and encouraging findings, we aimed to investigate whether A151 ODN could also be used for the therapy of systemic sclerosis

and if so, we attempted to explore the anti-fibrotic potential which has not been determined to date. In this thesis, we utilized A151 ODN in two different forms, either unencapsulated or anionic liposome-encapsulated forms of the A151 ODN throughout this study. Liposome encapsulation was done in order to protect the ODN from DNase activity and eliminate non-specific adsorption to serum proteins upon injection. In this regard, following our initial optimization experiments with 5 different liposome constructs, anionic liposomes were chosen as the most potent drug carrier depot system to load A151 ODN.

Following the findings on importance of TLRs in initial course of systemic sclerosis, we first investigated how A151 ODN alters immune activation mediated by different TLRs. In addition to the verifying results on well-known suppressive effects of A151 in TLR9 signaling, we also found that this molecule not only capable of abolishing IFN γ production upon TLR1/2 and TLR4 activation but it also downregulated IL-12 secretion after TLR1/2 stimulation from splenocytes (Figure 3.4). We proposed that modulation of immune responses mediated by TLRs other than TLR9 might be due to A151 ODN ability to inhibit STAT3 and STAT1 phosphorylation [33]. It is a well-known feature that Pam3csk4 and LPS immediately initiate immune cell signaling and pro-inflammatory cytokine production, even before A151 ODN is started to be internalized by the cells. Especially, LPS is very potent to upregulate TNF α gene expression and its production even after 15 minutes and 2 hours of stimulation, respectively [190]. Furthermore, LPS sharply elevated phosphotyrosine kinase activity in cells indicating that it leads to phosphorylation and activation of several proteins related to immune cell activation. Our unpublished data suggested that A151 ODN abolished STAT3 phosphorylation following 5 hours of stimulation. TNF α and IL-6 are initially produced cytokines in immune cells upon activation; thus, A151 ODN did not alter the secretion of these cytokines through inhibition of STAT3 activity. However, it is possible that A151 ODN interferes with production of cytokines, IL-12 and IFN γ which are produced in later stages, as a result of abrogated STAT3 activation since STAT3 activation in cells induces NF κ B activation, hence cytokine production [191]. Of note, STAT (signal transducer and activator of transcription) proteins are master transcription factors playing role on transcription of many genes related to cytokine signaling and immune response [192]. These proteins both modulate immune cell differentiation and effector functions and are regulated by cytokine signaling.

Conditioned media (CM) experiments was designed to helped us to simulate interactions between macrophages and fibroblasts either during health or pathologic conditions in lung, and we aimed to analyze how A151 ODN modulates deleterious inflammatory processes via direct or indirect interactions. CM experiments further demonstrated that A151 ODN besides inhibition of IL-6 and IL-12 production that was induced by TLR1/2 signaling in macrophages, it also down-modulated inflammatory secretome of macrophages; hence, it led to decreased

cytokine production from fibroblasts (Figure 3.10). Notably, TLR2 is essential in bleomycin induced pulmonary inflammation and fibrosis since it has been shown to promote immune cell recruitment to lungs as well as MCP1 and IP10 production [193]. It would also be interesting to analyze how A151 ODN directly modulates secretome profile of NIH3T3 fibroblast cells and how incubation with CM from these fibroblasts alter cytokine production of BMDMs. Our previous studies showed that NIH3T3 cells express all TLR genes; however, we could not detect any pro-inflammatory or inflammatory cytokine production following induction of TLR1/2, TLR4 and TLR9 signaling. As a result, we concluded that NIH3T3 cells are irresponsive to TLR stimulation, or one or more TLRs in NIH3T3 are not functional, at least for the doses that we used throughout those studies; therefore, we decided to omit studies involving analyses of fibroblast CM on BMDMs. Finally, to analyze the alterations in secretome of NIH3T3 and BMDMs due to A151 ODN incubation, proteomics analyses using mass spectrometry or Nanostring experiments designed to evaluate either BMDM or NIH3T3 cell derived mRNA could be performed in the future studies.

We initially planned to analyze A151 ODN's therapeutic effects on bleomycin-induced skin fibrosis which is another approach used to study systemic sclerosis in mice; however, contrary to published literature, we failed to establish this model even though bleomycin was injected either at different doses or given at different frequencies (daily or every other day) to both BALB/c and C57BL/6 background mice [194-196]. We were confident that our bleomycin was potent enough to establish fibrosis, since same batch was employed during lung instillation experiments. While BALB/c strain might be resistant to develop skin fibrosis using bleomycin, as it was also suggested by Yamamoto et al. [158], we have no supporting data to explain how we failed with C57BL/6 background animals.

After revisiting the literature, we embarked on to establish bleomycin-induced lung inflammation and fibrosis by a single intratracheal delivery of 50 µg BLM/mouse to female C57BL/6 mice [197]. Although most of the studies utilizes single injection of bleomycin for development of systemic sclerosis on mice, there are few studies claiming that lung fibrosis due to repetitive injections of bleomycin similar to pulmonary lung fibrosis and interstitial lung disease in humans [198, 199]. However, we preferred single injection likewise most of the studies have done since intratracheal delivery is an invasive method and quickly affects weight and general health of mice. We analyzed the cellular and molecular changes in BALF and extracted lung tissues following 3rd, 7th and 21st days of bleomycin instillation and compared the results with the other relevant studies. Detailed microarray analysis from lung tissues obtained after different time points following bleomycin instilled mice revealed that gene expressions are differentially regulated in inflammatory and fibrotic stages of the disease [200]. For instance, Timp2 and Timp3 were upregulated in fibrotic phase, whereas Timp1 expression increased

starting from day 1 and stayed high throughout the course of the disease. Moreover, gene expression of most of the collagen isoforms elevated 3 days after bleomycin instillation, peaked at day 21 and started to decrease thereafter. Lastly, Smad2 and Smad3 expressions were higher while Smad7 was lower during first 21 days compared to naive mice. Our findings on gene expressions were in accord with the study of Ji et al., where they identified that upon bleomycin administration, immune cells in alveolar space increased 2-fold after 3 days, 4-fold after 7 days, and their number remained elevated compared to saline-injected animals even after 21st day [178]. Furthermore, number of neutrophils peaked at 3rd day as an indication of early inflammation while number of macrophages peaked at 7th day in BALF samples of bleomycin-treated mice. We reported that highest number of neutrophils recruited to alveolar space at day 7 (Figure 3.13B). Although different studies stated that macrophages are recruited to alveolar space in response to bleomycin administration [159, 178], several *in vivo* experiments performed by us, consistently showed that BALF macrophage numbers significantly decreased following bleomycin instillation compared to control mice. In some studies, additional markers such as CD64 and Siglec-F [201, 202] were used to gate BALF resident macrophages, we however, identified alveolar macrophages based on their F4/80⁺ CD11c⁺ CD11b⁻ expressions, similar to the strategy followed by others [178, 203, 204]. Therefore, we are convinced that there is a reduction on macrophage population upon bleomycin instillation. Since cell viability analysis revealed that bleomycin that is higher than 0.1 µg/ml causes cell death on macrophages *in vitro*, intratracheal delivery of 50 µg bleomycin/mouse might be causing cell death on lung macrophages *in vivo*. Strikingly, none of the relevant studies in the literature have reported this observation. Of note, we have not tested the lethal dose of bleomycin on other cells.

In bleomycin-induced mouse model of systemic sclerosis, due to overexuberant inflammatory response there is a tissue damage followed by fibrosis; therefore, one could dissect and investigate different phases of this disease with the use of this animal model. We terminated *in vivo* experiments 3, 7 and 21 days after bleomycin administration; thus, we could investigate stages such as early inflammation, transition between inflammation to fibrosis and established fibrosis separately. Unfortunately, single injection of A151 ODN at either day 5, 10 or 15 following bleomycin instillation did not ameliorate fibrotic development (Figures 3.22-3.25). We hypothesized that A151 ODN is not potent enough to be delivered in single dose against a powerful inflammatory agent like bleomycin. Conversely, treatment with A151 ODN twice at day 3 and 5 gave promising results to abolish inflammation and prevent fibrosis (Figure 3.17-21). It is important to note that liposome encapsulation, to our surprise, seem to decline the immunosuppressive capacity of A151 ODN, perhaps due to limited availability to relevant immune cells at the inflamed site. Additionally, it might have caused reduced accumulation of A151 ODN at the scar tissue in the lungs.

Study conducted by Sato and colleagues have shown that A151 ODN but not control ODN impaired neutrophil infiltration in silica induced pulmonary inflammation [43] which supported our findings on decreased neutrophil migration. Moreover, we demonstrated reduced infiltration of monocytes into alveolar space. It is highly possible that CD11c⁺ F4/80⁺ cells other than monocytes recovered from BALF were B- and T-lymphocytes since they are reported to infiltrate into alveolar space by day 6 [159]. However, we could not stain B- and T-cells due to limitations in fluorochrome-conjugated antibodies, and isolated cell numbers were not plenty to conduct multiple staining for the analysis of two different dye panels. Based on the *in vitro* studies of A151 ODN, it has been demonstrated that B-cell activation [36] as well as Th1 biased T-cell differentiation could be decreased [33]. Based on these previous observations, we concluded that A151 ODN could also led to a reduced lymphocyte migration and activation. Therefore, we would like to propose that decreased lymphocyte numbers in BALF could cause low level of IL-12 production in those animals that had free A151 ODN.

BALF yielded $\sim 1 \times 10^6$ cells from healthy mice while inflammation due to bleomycin increased total cell number at least 4-fold. Even though we reported that bleomycin diminished alveolar macrophage population in contrast to the published findings from the literature, remaining macrophages highly upregulated their CD11b, MHCII and CD11c expressions, thereby we assume that they are activated. Alveolar macrophages are resident cells found on the inner surface of alveolar epithelium and mainly engages in phagocytosis [205]. They are unique among mouse macrophages and characterized by their low expression of MHCII and CD11b and high expression of CD11c. To our knowledge, there is no report on CD11b upregulation in alveolar macrophages upon bleomycin administration whereas alveolar macrophages were shown to increase CD11b expressions following LPS or viral infection [206]. Moreover, A151 downregulated elevated CD11b expression level in those alveolar macrophages that we retrieved from BALF. In our opinion, these observations are novel findings originated from this study. *In vitro* studies provided additional support to the *in vivo* findings concerning reduced CD11b expression levels following A151 ODN treatment (Figure 3.5D). A recent study published in 2018 by McCubbrey et.al. reported that elimination of CD11b^{hi} macrophages significantly attenuated the development of lung fibrosis [207]. Notably, macrophages expressing high levels of CD11b was defined as mixed population of macrophages with migrated monocytes in that study. Taken together, A151 ODN could diminish lung inflammation by decreasing macrophage activity in addition to the decreased migration of neutrophils and monocytes as well as decreased CD11b and even MHCII expression levels.

It is a known fact that infiltration of myeloid and lymphoid cells is one of the key contributing factors in development of fibrosis while cell death and senescence in alveolar epithelial cells caused by instilled bleomycin has been identified as another element in fibrotic progression in

lung [208, 209]. Particularly, cell death induced by bleomycin could be observed after 48 hours of bleomycin stimulation in *in vitro* culture condition and it leads to fibroblast activation. Furthermore, bleomycin has been shown to drive A549 alveolar epithelial cell line to epithelial-to-mesenchymal transition (EMT) that was characterized by the increased activity of TGF β -Smad2/3 pathway, elevated vimentin expression and decreased E-cadherin levels [210]. We have not identified how A151 ODN treatment modulates these changes in alveolar epithelial cells following bleomycin administration. Although A151 ODN treatment that is applied 3 days after bleomycin instillation might not rescue alveolar epithelial cell death, investigating effects of A151 ODN on EMT would be particularly pertinent to our study.

A recent study conducted by our group, indicated that A151 ODN down-regulates mTOR pathway activity via PI3K/Akt axis (Yazar et.al., under revision). Although we have not tested how A151 ODN induced alterations of mTOR and pathway elements in case of bleomycin-induced systemic sclerosis in mice, based on those findings by Yazar et.al., it is plausible to hypothesize that one of the mechanisms of action of A151 ODN could be to abolish disease progression might be through regulating the mTOR pathway. Indeed, therapeutic effects of well-known mTOR inhibitor, rapamycin (sirolimus), has been investigated on systemic sclerosis patients. A case study reported that one month of treatment with rapamycin decreased skin stiffness, autoantibody production from B cells and collagen deposition on patient having systemic sclerosis [211]. Furthermore, a pilot study confirmed the safety and efficacy of rapamycin on SSc [212], and phase II trial of rapamycin will be initiated on a larger cohort for a detailed investigation [213]. Since rapamycin gives promising results on treatment of SSc, and earlier studies demonstrating that psoriasis could be eliminated by the use of an immunosuppressive motif, we suggest that A151 ODN could be considered as another option in the management of SSc.

Studies proved that PI3K signaling enhances bleomycin-induced lung fibrosis by promoting production of both inflammatory and pro-fibrotic mediators [214]. A recent study employed PI3K inhibitor, CL27c, to mice that was instilled together with intratracheal bleomycin administration every other day starting from day 7 until day 21 [215]. Data revealed that inhaled PI3K inhibitor reduced macrophage, lymphocyte and neutrophil accumulation in addition to the downregulated Col1a1, Tgf β , Ctgf (connective tissue growth factor) and Mmp2. Histological examination by H&E and Masson's trichrome staining of lung sections depicted a decrease in inflammation and fibrosis, respectively. Another target of A151 ODN might be STAT3 in the context of lung inflammation and fibrosis. Chakraborty et al. demonstrated that TGF β -induced p-STAT3 accumulation on skin of SSc patients, and furthermore fibroblast-specific STAT3 knockout mice was resistant to bleomycin-induced skin fibrosis. Moreover, attenuation of bleomycin-induced skin fibrosis by STAT3 inhibitor was shown by reduced dermal thickness,

collagen deposition and collagen gene expressions [216]. In another study by Papaioannou et.al., it was reported that STAT3 controls Col1a2 gene transcription and has a crucial role on myofibroblast differentiation in lung [217]. Consequently, it is probable that mechanism of action of A151 ODN on lung inflammation is through mTOR pathway inhibition as well as inhibition of STAT3 phosphorylation. However, in order to prove the mechanism of action of A151 ODN upon bleomycin induction, phosphorylation and protein levels of STAT3 and mTOR pathway elements are needed to be analyzed within the context of BLM-induced lung inflammation.

Overall, our *in vitro* and *in vivo* mouse data suggested that A151 ODN has a promising therapeutic potential on preventing and abolishing established lung inflammation induced by a very potent chemotherapeutic agent, bleomycin. Even though the anti-fibrotic capacity as well as the molecular mechanism of action needs further investigation, the findings of this thesis merits to conclude that A151 ODN is a potent DNA -based immunosuppressor, and it is capable to impede fibrotic scar formation. It is fair to propose that these findings could be translated to prevent hepatic dysfunction mediated by chronic fibrosis, a prerequisite step before cirrhosis, that subsequently leads to hepatocellular carcinoma.

APPENDICES

APPENDIX A

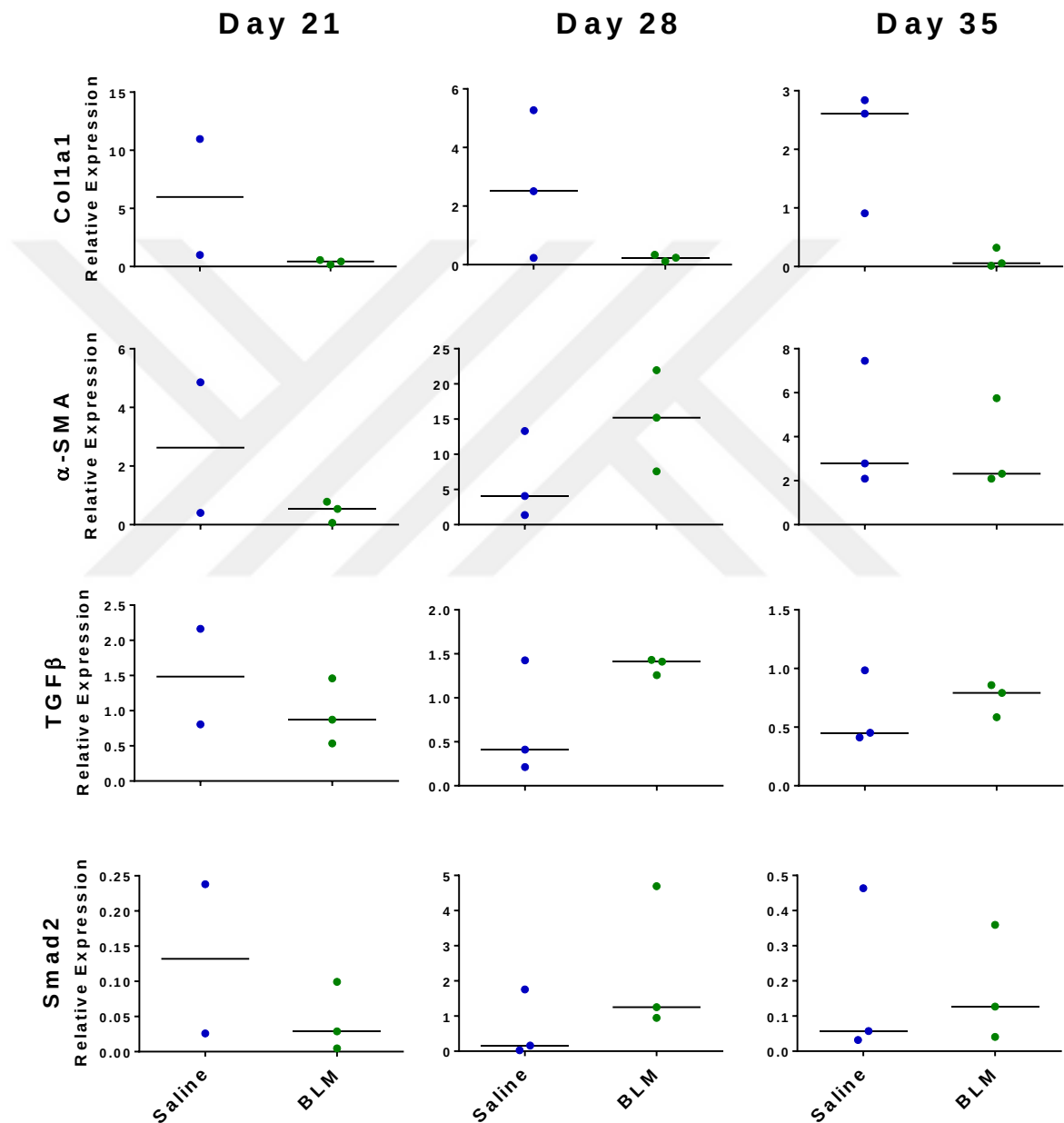


Figure A1. Gene expressions of skin samples obtained from subcutaneous saline and bleomycin-given mice. Saline or 100 μ g/mouse bleomycin was subcutaneously injected to back skin of mice (n=2 or 3). A group of mice was sacrificed at 21st, 28th and 35th days following first injection, Col1a1, α -SMA, TGF β and Smad2 gene expressions were determined by RT-qPCR. (BLM: Bleomycin).

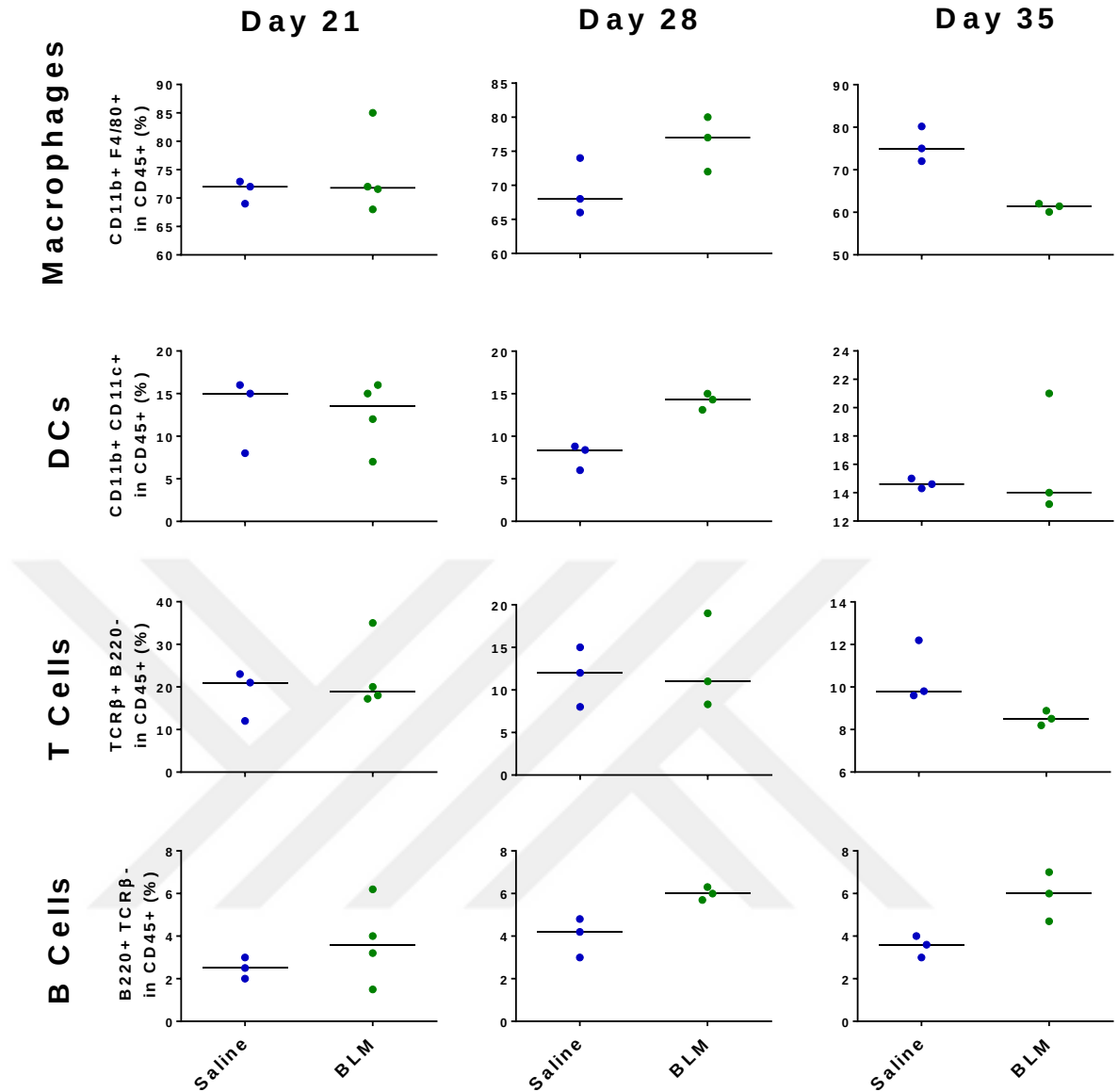


Figure A2. Immune cell populations of skin samples obtained from subcutaneously saline and bleomycin-given mice. Saline or 100 µg/mouse bleomycin was subcutaneously injected to back skin of mice. A group of mice was sacrificed at 21st, 28th and 35th days following first injection, single cell suspension was obtained after enzymatic digestion of skin samples. Macrophages (CD45⁺ CD11b⁺ F4/80⁺), dendritic cells (CD45⁺ CD11b⁺ CD11c⁺), T cells (CD45⁺ TCRβ⁺ B220⁻) and B cells (CD45⁺ B220⁺ TCRβ⁻) were identified via flow cytometry. (BLM: Bleomycin).

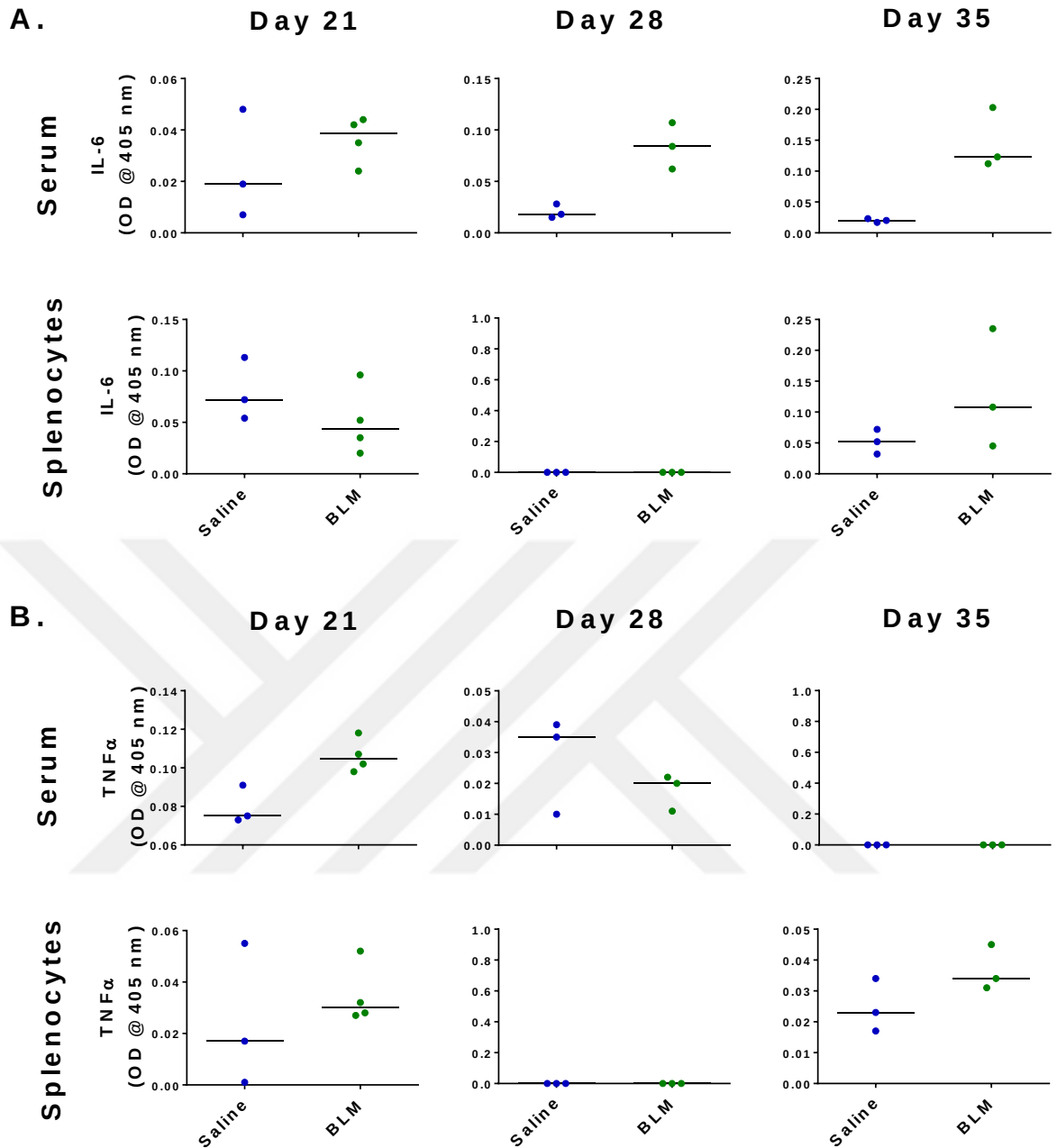


Figure A3. Cytokines produced by splenocytes and in blood serum from mice subcutaneously received either saline or bleomycin. Saline or 100 $\mu\text{g}/\text{mouse}$ bleomycin was subcutaneously injected to back skin of mice. A group of mice was sacrificed at 21st, 28th and 35th days following first injection. Blood serum was obtained after incubation for 90 minutes at 37°C while splenocytes were incubated for 24 hours without treatment. A) IL-6 and B) TNF α levels in blood serum and cell supernatants collected from splenocytes were measured by ELISA. (BLM: Bleomycin).

APPENDIX B

Appendix B1 – ELISA

B1.1-10X Phosphate Buffered Saline (10X PBS):

- 80 g NaCl
- 2 g KCl
- 8.01 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$
- 2 g KH_2PO_4
- 1 L dH_2O^*

*(complete to 1 liter after dissolving all the dry solutes)

pH must be set to 6.8 and autoclaved.

B1.2-1X PBS:

- 100 ml 10X PBS
- 900 ml dH_2O

pH must be set to 7.4.

B1.3-Blocking Buffer:

- 500 ml 1X PBS
- 25 g BSA (bovine serum albumin)
- 250 μl Tween-20

B1.4Washing Buffer (ELISA):

- 500 ml 10X PBS
- 2.5 ml Tween-20
- 4.5 L dH_2O

Appendix B2 - Immunoblotting

B2.1-1X PBS-T:

- 100 ml 10X PBS
- 900 ml dH₂O
- 1 ml Tween-20

B2.2-10X Running Buffer:

- 30.285 g Tris-Base
- 144.134 g glycine
- 100 ml SDS (10%)
- 800 ml dH₂O*

*(complete to 0.8 liter after dissolving all the dry solutes and adding SDS soln.)

B2.3-10X Transfer Buffer:

- 30.285 g Tris-Base
- 144.134 g glycine
- 900 ml dH₂O

*(complete to 0.9 liter after dissolving all the dry solutes)

B2.4-1X Transfer Buffer:

- 100 ml 10X transfer buffer
- 200 ml methanol
- 700 ml dH₂O

B2.5-4X Laemmli Buffer:

- 4 ml 100% glycerol
- 2.4 ml 1M Tris-HCl (pH=6.8)
- 0.8 g SDS

- 4 mg bromophenol blue
- 0.5 ml β -mercaptoethanol
- 3.1 ml dH₂O

B2.6-5% Stacking Gel:

- 3.4 ml dH₂O
- 600 μ l 1M Tris (pH=6.8)
- 100 μ l 5% SDS
- 800 μ l 30% acrylamide mix
- 50 μ l 10% APS
- 5 μ l TEMED

B2.7-10% Separating Gel:

- 6.15 ml dH₂O
- 3.75 ml 1.5M Tris (pH=8.8)
- 300 μ l 5% SDS
- 4.95 ml 30% acrylamide mix
- 125 μ l 10% APS
- 10 μ l TEMED

B2.8-RIPA Buffer:

- 72 μ l 2M NaCl (1.688 g NaCl in 10 ml dH₂O)
- 50 μ l 1M Tris (pH=8)
- 10 μ l NP-40
- 10 μ l 10% SDS
- 815 μ l dH₂O
- 10 μ l protease inhibitor (100X)
- 100 μ l phosphatase inhibitor (10X)

Appendix B3- Flow Cytometry

FACS Buffer (PBS-BSA-Na Azide Buffer):

- 500 ml 1X PBS
- 5 g BSA
- 125 mg Na-Azide



REFERENCES

1. Parkin, J. and B. Cohen, *An overview of the immune system*. Lancet, 2001. **357**(9270): p. 1777-89.
2. Delves, P.J. and I.M. Roitt, *The immune system. First of two parts*. N Engl J Med, 2000. **343**(1): p. 37-49.
3. Medzhitov, R. and C.A. Janeway, Jr., *Innate immunity: impact on the adaptive immune response*. Curr Opin Immunol, 1997. **9**(1): p. 4-9.
4. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition*. Annu Rev Immunol, 2002. **20**: p. 197-216.
5. Chaplin, D.D., *Overview of the immune response*. J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S3-23.
6. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity*. J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S33-40.
7. Netea, M.G., et al., *Trained immunity: A program of innate immune memory in health and disease*. Science, 2016. **352**(6284): p. aaf1098.
8. Arts, R.J.W., et al., *BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity*. Cell Host Microbe, 2018. **23**(1): p. 89-100 e5.
9. Mulder, W.J.M., et al., *Therapeutic targeting of trained immunity*. Nat Rev Drug Discov, 2019.
10. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors*. Nat Immunol, 2010. **11**(5): p. 373-84.
11. Levine, B., N. Mizushima, and H.W. Virgin, *Autophagy in immunity and inflammation*. Nature, 2011. **469**(7330): p. 323-35.
12. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. Cell, 2006. **124**(4): p. 783-801.
13. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation*. Cell, 2010. **140**(6): p. 805-20.

14. Lemaitre, B., et al., *The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults*. Cell, 1996. **86**(6): p. 973-83.
15. Lemaitre, B., *The road to Toll*. Nat Rev Immunol, 2004. **4**(7): p. 521-7.
16. Rock, F.L., et al., *A family of human receptors structurally related to Drosophila Toll*. Proc Natl Acad Sci U S A, 1998. **95**(2): p. 588-93.
17. Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr., *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity*. Nature, 1997. **388**(6640): p. 394-7.
18. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity*. Int Immunol, 2005. **17**(1): p. 1-14.
19. O'Neill, L.A., D. Golenbock, and A.G. Bowie, *The history of Toll-like receptors - redefining innate immunity*. Nat Rev Immunol, 2013. **13**(6): p. 453-60.
20. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors*. Annu Rev Immunol, 2003. **21**: p. 335-76.
21. Akira, S., *Pathogen recognition by innate immunity and its signaling*. Proc Jpn Acad Ser B Phys Biol Sci, 2009. **85**(4): p. 143-56.
22. Akira, S. and K. Takeda, *Toll-like receptor signalling*. Nat Rev Immunol, 2004. **4**(7): p. 499-511.
23. Yamada, H., et al., *Effect of suppressive DNA on CpG-induced immune activation*. J Immunol, 2002. **169**(10): p. 5590-4.
24. Krieg, A.M., et al., *Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs*. Proc Natl Acad Sci U S A, 1998. **95**(21): p. 12631-6.
25. Gursel, I., et al., *Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation*. J Immunol, 2003. **171**(3): p. 1393-400.
26. Avalos, A.M. and H.L. Ploegh, *Competition by inhibitory oligonucleotides prevents binding of CpG to C-terminal TLR9*. Eur J Immunol, 2011. **41**(10): p. 2820-7.
27. Peter, M., et al., *Characterization of suppressive oligodeoxynucleotides that inhibit Toll-like receptor-9-mediated activation of innate immunity*. Immunology, 2008. **123**(1): p. 118-28.

28. Patole, P.S., et al., *Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells*. J Am Soc Nephrol, 2005. **16**(5): p. 1326-38.
29. Bayik, D., I. Gursel, and D.M. Klinman, *Structure, mechanism and therapeutic utility of immunosuppressive oligonucleotides*. Pharmacol Res, 2016. **105**: p. 216-25.
30. Stacey, K.J., et al., *Phosphorothioate Backbone Modification Changes the Pattern of Responses to CpG, in Microbial DNA and Host Immunity*, E. Raz, Editor. 2002, Humana Press: Totowa, NJ. p. 63-77.
31. Araie, Y., et al., *Enhanced Activity of Immunosuppressive Oligodeoxynucleotides by Incorporating Them into Hexapod-Like Nanostructured DNA*. Biol Pharm Bull, 2018. **41**(4): p. 564-569.
32. Shirota, H., et al., *Suppressive oligodeoxynucleotides protect mice from lethal endotoxic shock*. J Immunol, 2005. **174**(8): p. 4579-83.
33. Shirota, H., M. Gursel, and D.M. Klinman, *Suppressive oligodeoxynucleotides inhibit Th1 differentiation by blocking IFN-gamma- and IL-12-mediated signaling*. J Immunol, 2004. **173**(8): p. 5002-7.
34. Bode, C., J. Wang, and D.M. Klinman, *Suppressive oligodeoxynucleotides promote the generation of regulatory T cells by inhibiting STAT1 phosphorylation*. Int Immunopharmacol, 2014. **23**(2): p. 516-22.
35. Bode, C., et al., *Suppressive oligodeoxynucleotides promote the development of Th17 cells*. PLoS One, 2013. **8**(7): p. e67991.
36. Sackesen, C., et al., *Suppression of B-cell activation and IgE, IgA, IgG1 and IgG4 production by mammalian telomeric oligonucleotides*. Allergy, 2013. **68**(5): p. 593-603.
37. Kaminski, J.J., et al., *Synthetic oligodeoxynucleotides containing suppressive TTAGGG motifs inhibit AIM2 inflammasome activation*. J Immunol, 2013. **191**(7): p. 3876-83.
38. Steinhagen, F., et al., *Suppressive oligodeoxynucleotides containing TTAGGG motifs inhibit cGAS activation in human monocytes*. Eur J Immunol, 2018. **48**(4): p. 605-611.

39. Dong, L., et al., *Suppressive oligonucleotides protect against collagen-induced arthritis in mice*. Arthritis Rheum, 2004. **50**(5): p. 1686-9.
40. Dong, L., et al., *Suppressive oligodeoxynucleotides delay the onset of glomerulonephritis and prolong survival in lupus-prone NZB x NZW mice*. Arthritis Rheum, 2005. **52**(2): p. 651-8.
41. Cheng, X., et al., *Suppressive oligodeoxynucleotides inhibit atherosclerosis in ApoE(-/-) mice through modulation of Th1/Th2 balance*. J Mol Cell Cardiol, 2008. **45**(2): p. 168-75.
42. Mallat, Z., *Suppressive oligodeoxynucleotides inhibit atherosclerosis*. J Mol Cell Cardiol, 2008. **45**(2): p. 156-8.
43. Sato, T., et al., *Suppressive oligodeoxynucleotides inhibit silica-induced pulmonary inflammation*. J Immunol, 2008. **180**(11): p. 7648-54.
44. Yagci, F.C., et al., *Mammalian telomeric DNA suppresses endotoxin-induced uveitis*. J Biol Chem, 2010. **285**(37): p. 28806-11.
45. Bangham, A.D. and R.W. Horne, *Negative Staining Of Phospholipids And Their Structural Modification By Surface-Active Agents As Observed In The Electron Microscope*. J Mol Biol, 1964. **8**: p. 660-8.
46. Gregoriadis, G., *The carrier potential of liposomes in biology and medicine (first of two parts)*. N Engl J Med, 1976. **295**(13): p. 704-10.
47. Gregoriadis, G., *The carrier potential of liposomes in biology and medicine (second of two parts)*. N Engl J Med, 1976. **295**(14): p. 765-70.
48. Gregoriadis, G., et al., *Drug-carrier potential of liposomes in cancer chemotherapy*. Lancet, 1974. **1**(7870): p. 1313-6.
49. Gregoriadis, G., *Drug entrapment in liposomes*. FEBS Lett, 1973. **36**(3): p. 292-6.
50. Akbarzadeh, A., et al., *Liposome: classification, preparation, and applications*. Nanoscale Res Lett, 2013. **8**(1): p. 102.
51. Allen, T.M., *Liposomes. Opportunities in drug delivery*. Drugs, 1997. **54 Suppl 4**: p. 8-14.
52. Himanshu, A., P. Sitasharan, and A.J.I. Singhai, *Liposomes as drug carriers*. 2011. **2**(7): p. 945-951.
53. Etheridge, M.L., et al., *The big picture on nanomedicine: the state of investigational and approved nanomedicine products*. Nanomedicine, 2013. **9**(1): p. 1-14.

54. Nagayasu, A., K. Uchiyama, and H. Kiwada, *The size of liposomes: a factor which affects their targeting efficiency to tumors and therapeutic activity of liposomal antitumor drugs*. Adv Drug Deliv Rev, 1999. **40**(1-2): p. 75-87.
55. Betageri, G. and D.J.I.j.o.p. Parsons, *Drug encapsulation and release from multilamellar and unilamellar liposomes*. 1992. **81**(2-3): p. 235-241.
56. Bozzuto, G. and A. Molinari, *Liposomes as nanomedical devices*. Int J Nanomedicine, 2015. **10**: p. 975-99.
57. Immordino, M.L., F. Dosio, and L. Cattel, *Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential*. Int J Nanomedicine, 2006. **1**(3): p. 297-315.
58. Gabizon, A.A., *Stealth liposomes and tumor targeting: one step further in the quest for the magic bullet*. Clin Cancer Res, 2001. **7**(2): p. 223-5.
59. Ferreira Ddos, S., et al., *pH-sensitive liposomes for drug delivery in cancer treatment*. Ther Deliv, 2013. **4**(9): p. 1099-123.
60. Bailey, A.L. and S.M. Sullivan, *Efficient encapsulation of DNA plasmids in small neutral liposomes induced by ethanol and calcium*. Biochim Biophys Acta, 2000. **1468**(1-2): p. 239-52.
61. Angelini, G., et al., *Neutral liposomes containing crown ether-lipids as potential DNA vectors*. Biochim Biophys Acta, 2013. **1828**(11): p. 2506-12.
62. Christensen, D., et al., *Cationic liposomes as vaccine adjuvants*. Expert Rev Vaccines, 2011. **10**(4): p. 513-21.
63. Fillion, M.C. and N.C. Phillips, *Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells*. Biochim Biophys Acta, 1997. **1329**(2): p. 345-56.
64. Krasnici, S., et al., *Effect of the surface charge of liposomes on their uptake by angiogenic tumor vessels*. Int J Cancer, 2003. **105**(4): p. 561-7.
65. Kelly, C., C. Jefferies, and S.A. Cryan, *Targeted liposomal drug delivery to monocytes and macrophages*. J Drug Deliv, 2011. **2011**: p. 727241.
66. Patil, S.D. and D.G. Rhodes, *Conformation of oligodeoxynucleotides associated with anionic liposomes*. Nucleic Acids Res, 2000. **28**(21): p. 4125-9.

67. Antimisiaris, S.G., *Preparation of DRV liposomes*. Methods Mol Biol, 2010. **605**: p. 51-75.
68. Barenholz, Y., *Doxil(R)--the first FDA-approved nano-drug: lessons learned*. J Control Release, 2012. **160**(2): p. 117-34.
69. Gluck, R., et al., *Immunopotentiating reconstituted influenza virus virosome vaccine delivery system for immunization against hepatitis A*. J Clin Invest, 1992. **90**(6): p. 2491-5.
70. Stegmann, T., et al., *Functional reconstitution of influenza virus envelopes*. EMBO J, 1987. **6**(9): p. 2651-9.
71. Bulbake, U., et al., *Liposomal Formulations in Clinical Use: An Updated Review*. Pharmaceutics, 2017. **9**(2).
72. Diro, E., et al., *A randomized trial of AmBisome monotherapy and AmBisome and miltefosine combination to treat visceral leishmaniasis in HIV co-infected patients in Ethiopia*. PLoS Negl Trop Dis, 2019. **13**(1): p. e0006988.
73. Denton, C.P. and D. Khanna, *Systemic sclerosis*. Lancet, 2017. **390**(10103): p. 1685-1699.
74. Chiffot, H., et al., *Incidence and prevalence of systemic sclerosis: a systematic literature review*. Semin Arthritis Rheum, 2008. **37**(4): p. 223-35.
75. van den Hoogen, F., et al., *2013 classification criteria for systemic sclerosis: an American College of Rheumatology/European League against Rheumatism collaborative initiative*. Arthritis Rheum, 2013. **65**(11): p. 2737-47.
76. Ratchford, E.V. and N.S. Evans, *Raynaud's phenomenon*. Vasc Med, 2015. **20**(3): p. 269-71.
77. Kiely, D.G., et al., *Pulmonary hypertension: diagnosis and management*. BMJ, 2013. **346**: p. f2028.
78. Gabrielli, A., E.V. Avvedimento, and T. Krieg, *Scleroderma*. N Engl J Med, 2009. **360**(19): p. 1989-2003.
79. Herrick, A.L., et al., *Treatment outcome in early diffuse cutaneous systemic sclerosis: the European Scleroderma Observational Study (ESOS)*. Ann Rheum Dis, 2017. **76**(7): p. 1207-1218.

80. Lee, S.B. and R. Kalluri, *Mechanistic connection between inflammation and fibrosis*. *Kidney Int Suppl*, 2010(119): p. S22-6.
81. Allanore, Y., et al., *Systemic sclerosis*. *Nat Rev Dis Primers*, 2015. **1**: p. 15002.
82. Ciechomska, M., et al., *Role of toll-like receptors in systemic sclerosis*. *Expert Rev Mol Med*, 2013. **15**: p. e9.
83. Mack, M., *Inflammation and fibrosis*. *Matrix Biol*, 2018. **68-69**: p. 106-121.
84. Ogawa, F., et al., *Serum levels of heat shock protein 70, a biomarker of cellular stress, are elevated in patients with systemic sclerosis: association with fibrosis and vascular damage*. *Clin Exp Rheumatol*, 2008. **26**(4): p. 659-62.
85. Yoshizaki, A., et al., *Clinical significance of serum HMGB-1 and sRAGE levels in systemic sclerosis: association with disease severity*. *J Clin Immunol*, 2009. **29**(2): p. 180-9.
86. Gasse, P., et al., *Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis*. *Am J Respir Crit Care Med*, 2009. **179**(10): p. 903-13.
87. O'Reilly, S., et al., *Serum amyloid A induces interleukin-6 in dermal fibroblasts via Toll-like receptor 2, interleukin-1 receptor-associated kinase 4 and nuclear factor-kappaB*. *Immunology*, 2014. **143**(3): p. 331-40.
88. Bhattacharyya, S., et al., *Toll-like receptor 4 signaling augments transforming growth factor-beta responses: a novel mechanism for maintaining and amplifying fibrosis in scleroderma*. *Am J Pathol*, 2013. **182**(1): p. 192-205.
89. Piccinini, A.M., et al., *Distinct microenvironmental cues stimulate divergent TLR4-mediated signaling pathways in macrophages*. *Sci Signal*, 2016. **9**(443): p. ra86.
90. Park, J.S., et al., *High mobility group box 1 protein interacts with multiple Toll-like receptors*. *Am J Physiol Cell Physiol*, 2006. **290**(3): p. C917-24.
91. Fang, F., et al., *Toll-like Receptor 9 Signaling Is Augmented in Systemic Sclerosis and Elicits Transforming Growth Factor beta-Dependent Fibroblast Activation*. *Arthritis Rheumatol*, 2016. **68**(8): p. 1989-2002.

92. Yoshizaki, A., et al., *Nucleosome in patients with systemic sclerosis: possible association with immunological abnormalities via abnormal activation of T and B cells*. Ann Rheum Dis, 2016. **75**(10): p. 1858-65.
93. Henderson, J., et al., *Targeting TLRs and the inflammasome in systemic sclerosis*. Pharmacol Ther, 2018.
94. Artlett, C.M., et al., *The inflammasome activating caspase 1 mediates fibrosis and myofibroblast differentiation in systemic sclerosis*. Arthritis Rheum, 2011. **63**(11): p. 3563-74.
95. Papayannopoulos, V., *Neutrophil extracellular traps in immunity and disease*. Nat Rev Immunol, 2018. **18**(2): p. 134-147.
96. Kolaczowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation*. Nat Rev Immunol, 2013. **13**(3): p. 159-75.
97. Ermert, D., et al., *Mouse neutrophil extracellular traps in microbial infections*. J Innate Immun, 2009. **1**(3): p. 181-93.
98. Grommes, J. and O. Soehnlein, *Contribution of neutrophils to acute lung injury*. Mol Med, 2011. **17**(3-4): p. 293-307.
99. Chrysanthopoulou, A., et al., *Neutrophil extracellular traps promote differentiation and function of fibroblasts*. J Pathol, 2014. **233**(3): p. 294-307.
100. Dwivedi, N., et al., *Felty's syndrome autoantibodies bind to deiminated histones and neutrophil extracellular chromatin traps*. Arthritis Rheum, 2012. **64**(4): p. 982-92.
101. Dwivedi, N., et al., *Deimination of linker histones links neutrophil extracellular trap release with autoantibodies in systemic autoimmunity*. FASEB J, 2014. **28**(7): p. 2840-51.
102. Guerriero, J.L., *Macrophages: The Road Less Traveled, Changing Anticancer Therapy*. Trends Mol Med, 2018. **24**(5): p. 472-489.
103. Epelman, S., K.J. Lavine, and G.J. Randolph, *Origin and functions of tissue macrophages*. Immunity, 2014. **41**(1): p. 21-35.
104. Moreno-Moral, A., et al., *Changes in macrophage transcriptome associate with systemic sclerosis and mediate GSDMA contribution to disease risk*. Ann Rheum Dis, 2018. **77**(4): p. 596-601.

105. Trombetta, A.C., et al., *A circulating cell population showing both M1 and M2 monocyte/macrophage surface markers characterizes systemic sclerosis patients with lung involvement*. Respir Res, 2018. **19**(1): p. 186.
106. Stifano, G. and R.B. Christmann, *Macrophage Involvement in Systemic Sclerosis: Do We Need More Evidence?* Curr Rheumatol Rep, 2016. **18**(1): p. 2.
107. Wynn, T.A. and K.M. Vannella, *Macrophages in Tissue Repair, Regeneration, and Fibrosis*. Immunity, 2016. **44**(3): p. 450-462.
108. Mathes, A.L., et al., *Global chemokine expression in systemic sclerosis (SSc): CCL19 expression correlates with vascular inflammation in SSc skin*. Ann Rheum Dis, 2014. **73**(10): p. 1864-72.
109. Distler, O., et al., *Expression of RANTES in biopsies of skin and upper gastrointestinal tract from patients with systemic sclerosis*. Rheumatol Int, 1999. **19**(1-2): p. 39-46.
110. Varga, J. and B. Pasche, *Transforming growth factor beta as a therapeutic target in systemic sclerosis*. Nat Rev Rheumatol, 2009. **5**(4): p. 200-6.
111. Biswas, S.K. and A. Mantovani, *Orchestration of metabolism by macrophages*. Cell Metab, 2012. **15**(4): p. 432-7.
112. Stunault, M.I., et al., *Metabolism Plays a Key Role during Macrophage Activation*. Mediators Inflamm, 2018. **2018**: p. 2426138.
113. Diskin, C. and E.M. Palsson-McDermott, *Metabolic Modulation in Macrophage Effector Function*. Front Immunol, 2018. **9**: p. 270.
114. Zhou, D., et al., *Macrophage polarization and function: new prospects for fibrotic disease*. Immunol Cell Biol, 2017. **95**(10): p. 864-869.
115. Murray, L.A., et al., *Serum amyloid P therapeutically attenuates murine bleomycin-induced pulmonary fibrosis via its effects on macrophages*. PLoS One, 2010. **5**(3): p. e9683.
116. Luzina, I.G., et al., *Occurrence of an activated, profibrotic pattern of gene expression in lung CD8+ T cells from scleroderma patients*. Arthritis Rheum, 2003. **48**(8): p. 2262-74.
117. Fuschiotti, P., et al., *Interleukin-13-producing CD8+ T cells mediate dermal fibrosis in patients with systemic sclerosis*. Arthritis Rheum, 2013. **65**(1): p. 236-46.

118. Atamas, S.P., et al., *Production of type 2 cytokines by CD8+ lung cells is associated with greater decline in pulmonary function in patients with systemic sclerosis*. 1999. **42**(6): p. 1168-1178.
119. Fuschiotti, P., *Current perspectives on the role of CD8+ T cells in systemic sclerosis*. Immunol Lett, 2018. **195**: p. 55-60.
120. Klein, M., et al., *Contribution of CD8+ T cells to inflammatory cytokine production in systemic sclerosis (SSc)*. Autoimmunity, 2016. **49**(8): p. 532-546.
121. Liu, M., et al., *New insights into CD4(+) T cell abnormalities in systemic sclerosis*. Cytokine Growth Factor Rev, 2016. **28**: p. 31-6.
122. Brembilla, N.C., et al., *Th17 cells favor inflammatory responses while inhibiting type I collagen deposition by dermal fibroblasts: differential effects in healthy and systemic sclerosis fibroblasts*. Arthritis Res Ther, 2013. **15**(5): p. R151.
123. Klein, S., et al., *Reduction of regulatory T cells in skin lesions but not in peripheral blood of patients with systemic scleroderma*. Ann Rheum Dis, 2011. **70**(8): p. 1475-81.
124. Antiga, E., et al., *Regulatory T cells in the skin lesions and blood of patients with systemic sclerosis and morphoea*. Br J Dermatol, 2010. **162**(5): p. 1056-63.
125. Jiang, N., M. Li, and X. Zeng, *Correlation of Th17 cells and CD4(+)CD25(+) regulatory T cells with clinical parameters in patients with systemic sclerosis*. Chin Med J (Engl), 2014. **127**(20): p. 3557-61.
126. Lafyatis, R., et al., *B cell infiltration in systemic sclerosis-associated interstitial lung disease*. Arthritis Rheum, 2007. **56**(9): p. 3167-8.
127. Whitfield, M.L., et al., *Systemic and cell type-specific gene expression patterns in scleroderma skin*. Proc Natl Acad Sci U S A, 2003. **100**(21): p. 12319-24.
128. Sakkas, L.I. and D.P. Bogdanos, *Systemic sclerosis: New evidence re-enforces the role of B cells*. Autoimmun Rev, 2016. **15**(2): p. 155-61.
129. Mavropoulos, A., et al., *Breg Cells Are Numerically Decreased and Functionally Impaired in Patients With Systemic Sclerosis*. Arthritis Rheumatol, 2016. **68**(2): p. 494-504.

130. Henault, J., et al., *DNA topoisomerase I binding to fibroblasts induces monocyte adhesion and activation in the presence of anti-topoisomerase I autoantibodies from systemic sclerosis patients*. Arthritis Rheum, 2006. **54**(3): p. 963-73.
131. Villalta, D., et al., *Diagnostic accuracy and predictive value of extended autoantibody profile in systemic sclerosis*. Autoimmun Rev, 2012. **12**(2): p. 114-20.
132. Laurent, P., et al., *Innate Immunity in Systemic Sclerosis Fibrosis: Recent Advances*. Front Immunol, 2018. **9**: p. 1702.
133. Fuschiotti, P., *Current perspectives on the immunopathogenesis of systemic sclerosis*. Immunotargets Ther, 2016. **5**: p. 21-35.
134. Trojanowska, M., *Cellular and molecular aspects of vascular dysfunction in systemic sclerosis*. Nat Rev Rheumatol, 2010. **6**(8): p. 453-60.
135. Gilbane, A.J., C.P. Denton, and A.M. Holmes, *Scleroderma pathogenesis: a pivotal role for fibroblasts as effector cells*. Arthritis Res Ther, 2013. **15**(3): p. 215.
136. Marangoni, R.G., et al., *Myofibroblasts in murine cutaneous fibrosis originate from adiponectin-positive intradermal progenitors*. Arthritis Rheumatol, 2015. **67**(4): p. 1062-73.
137. Volkmann, E.R. and J. Varga, *Emerging targets of disease-modifying therapy for systemic sclerosis*. Nat Rev Rheumatol, 2019. **15**(4): p. 208-224.
138. Pavlov-Dolijanovic, S., et al., *Long-term effects of immunosuppressive therapy on lung function in scleroderma patients*. Clin Rheumatol, 2018. **37**(11): p. 3043-3050.
139. Kowal-Bielecka, O., et al., *Update of EULAR recommendations for the treatment of systemic sclerosis*. Ann Rheum Dis, 2017. **76**(8): p. 1327-1339.
140. Rice, L.M., et al., *Fresolimumab treatment decreases biomarkers and improves clinical symptoms in systemic sclerosis patients*. J Clin Invest, 2015. **125**(7): p. 2795-807.
141. *A Trial to Evaluate the Efficacy of PRM-151 in Subjects With Idiopathic Pulmonary Fibrosis (IPF)*.

142. Fala, L., *Ofev (Nintedanib): First Tyrosine Kinase Inhibitor Approved for the Treatment of Patients with Idiopathic Pulmonary Fibrosis*. Am Health Drug Benefits, 2015. **8**(Spec Feature): p. 101-4.
143. *A Trial to Compare Nintedanib With Placebo for Patients With Scleroderma Related Lung Fibrosis*.
144. *A Study of the Efficacy and Safety of Tocilizumab in Participants With Systemic Sclerosis (SSc)*.
145. Khanna, D., et al., *Safety and efficacy of subcutaneous tocilizumab in systemic sclerosis: results from the open-label period of a phase II randomised controlled trial (faSScinate)*. Ann Rheum Dis, 2018. **77**(2): p. 212-220.
146. Bhattacharyya, S. and J. Varga, *Endogenous ligands of TLR4 promote unresolving tissue fibrosis: Implications for systemic sclerosis and its targeted therapy*. Immunol Lett, 2018. **195**: p. 9-17.
147. *Safety and Efficacy of Pf-06650833 In Subjects With Rheumatoid Arthritis, With An Inadequate Response To Methotrexate*.
148. Haringman, J.J., et al., *A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis*. Arthritis Rheum, 2006. **54**(8): p. 2387-92.
149. Denton, C.P., et al., *Efficacy and Safety of Selexipag in Adults With Raynaud's Phenomenon Secondary to Systemic Sclerosis: A Randomized, Placebo-Controlled, Phase II Study*. Arthritis Rheumatol, 2017. **69**(12): p. 2370-2379.
150. *Effects of Selexipag in Adults With Raynaud's Phenomenon Secondary to Systemic Sclerosis*. <https://ClinicalTrials.gov/show/NCT02260557>.
151. Kuwana, M., Y. Okazaki, and J. Kaburaki, *Long-term beneficial effects of statins on vascular manifestations in patients with systemic sclerosis*. Mod Rheumatol, 2009. **19**(5): p. 530-5.
152. Gursel, I., et al., *Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides*. J Immunol, 2001. **167**(6): p. 3324-8.
153. Zhang, X., R. Goncalves, and D.M. Mosser, *The isolation and characterization of murine macrophages*. Curr Protoc Immunol, 2008. **Chapter 14**: p. Unit 14 1.

154. Trouplin, V., et al., *Bone marrow-derived macrophage production*. J Vis Exp, 2013(81): p. e50966.
155. Holt, D.J., L.M. Chamberlain, and D.W. Grainger, *Cell-cell signaling in co-cultures of macrophages and fibroblasts*. Biomaterials, 2010. **31**(36): p. 9382-94.
156. Tincer, G., et al., *Immunostimulatory activity of polysaccharide-poly(I:C) nanoparticles*. Biomaterials, 2011. **32**(18): p. 4275-82.
157. Kahraman, T., et al., *Circulating LL37 targets plasma extracellular vesicles to immune cells and intensifies Behcet's disease severity*. J Extracell Vesicles, 2017. **6**(1): p. 1284449.
158. Yamamoto, T., et al., *Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma*. J Invest Dermatol, 1999. **112**(4): p. 456-62.
159. Izbicki, G., et al., *Time course of bleomycin-induced lung fibrosis*. Int J Exp Pathol, 2002. **83**(3): p. 111-9.
160. Han, H. and S.F. Ziegler, *Bronchoalveolar Lavage and Lung Tissue Digestion*. Bio Protoc, 2013. **3**(16).
161. Lin, C.M., et al., *Size-Dependent Properties of Small Unilamellar Vesicles Formed by Model Lipids*. Langmuir, 2012. **28**(1): p. 689-700.
162. Danaei, M., et al., *Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems*. Pharmaceutics, 2018. **10**(2).
163. Mosser, D.M. and X. Zhang, *Activation of murine macrophages*. Curr Protoc Immunol, 2008. **Chapter 14**: p. Unit 14 2.
164. Ying, W., et al., *Investigation of macrophage polarization using bone marrow derived macrophages*. J Vis Exp, 2013(76).
165. Podolnikova, N.P., et al., *The Role of Integrins alphaMbeta2 (Mac-1, CD11b/CD18) and alphaDbeta2 (CD11d/CD18) in Macrophage Fusion*. Am J Pathol, 2016. **186**(8): p. 2105-2116.
166. Elgueta, R., et al., *Molecular mechanism and function of CD40/CD40L engagement in the immune system*. Immunol Rev, 2009. **229**(1): p. 152-72.
167. Hubbard, A.K. and C. Giardina, *Regulation of ICAM-1 expression in mouse macrophages*. Inflammation, 2000. **24**(2): p. 115-25.

168. Jacobs, J.P., et al., *Deficiency of CXCR2, but not other chemokine receptors, attenuates autoantibody-mediated arthritis in a murine model*. Arthritis Rheum, 2010. **62**(7): p. 1921-32.
169. Zhao, J., et al., *Synthetic Oligodeoxynucleotides Containing Multiple Telemeric TTAGGG Motifs Suppress Inflammasome Activity in Macrophages Subjected to Oxygen and Glucose Deprivation and Reduce Ischemic Brain Injury in Stroke-Prone Spontaneously Hypertensive Rats*. Plos One, 2015. **10**(10).
170. Dearman, R.J., et al., *Toll-like receptor ligand activation of murine bone marrow-derived dendritic cells*. Immunology, 2009. **126**(4): p. 475-84.
171. Kakutani, R., et al., *Essential role of Toll-like receptor 2 in macrophage activation by glycogen*. Glycobiology, 2012. **22**(1): p. 146-59.
172. Klinman, D.M., *Immunotherapeutic uses of CpG oligodeoxynucleotides*. Nat Rev Immunol, 2004. **4**(4): p. 249-58.
173. Kurt-Jones, E.A., et al., *Use of murine embryonic fibroblasts to define Toll-like receptor activation and specificity*. J Endotoxin Res, 2004. **10**(6): p. 419-24.
174. Satta, N., et al., *The role of TLR2 in the inflammatory activation of mouse fibroblasts by human antiphospholipid antibodies*. Blood, 2007. **109**(4): p. 1507-14.
175. Hartupée, J. and D.L. Mann, *Role of inflammatory cells in fibroblast activation*. J Mol Cell Cardiol, 2016. **93**: p. 143-8.
176. Li, X.X., et al., *Toll-like receptor 4 promotes fibrosis in bleomycin-induced lung injury in mice*. Genetics and Molecular Research, 2015. **14**(4): p. 17391-17398.
177. Hecht, S.M., *Bleomycin: New perspectives on the mechanism of action*. Journal of Natural Products, 2000. **63**(1): p. 158-168.
178. Ji, W.J., et al., *Temporal and spatial characterization of mononuclear phagocytes in circulating, lung alveolar and interstitial compartments in a mouse model of bleomycin-induced pulmonary injury*. J Immunol Methods, 2014. **403**(1-2): p. 7-16.
179. Gur, I., et al., *Lymphokines in bleomycin-induced lung injury in bleomycin-sensitive C57BL/6 and -resistant BALB/c mice*. Experimental Lung Research, 2000. **26**(7): p. 521-534.

180. Kikuchi, N., et al., *Aggravation of bleomycin-induced pulmonary inflammation and fibrosis in mice lacking peroxiredoxin I*. Am J Respir Cell Mol Biol, 2011. **45**(3): p. 600-9.
181. Zhang, H.Y., et al., *Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis*. Am J Pathol, 1996. **148**(2): p. 527-37.
182. Sun, K.H., et al., *alpha-Smooth muscle actin is an inconsistent marker of fibroblasts responsible for force-dependent TGFbeta activation or collagen production across multiple models of organ fibrosis*. Am J Physiol Lung Cell Mol Physiol, 2016. **310**(9): p. L824-36.
183. Misharin, A.V., et al., *Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung*. Am J Respir Cell Mol Biol, 2013. **49**(4): p. 503-10.
184. Hume, D.A., *Macrophages as APC and the dendritic cell myth*. J Immunol, 2008. **181**(9): p. 5829-35.
185. Wang, L., F.S. Wang, and M.E. Gershwin, *Human autoimmune diseases: a comprehensive update*. J Intern Med, 2015. **278**(4): p. 369-95.
186. Theofilopoulos, A.N., D.H. Kono, and R. Baccala, *The multiple pathways to autoimmunity*. Nat Immunol, 2017. **18**(7): p. 716-724.
187. Pestana-Fernandez, M., et al., *Long-term Efficacy and Safety of Monotherapy Versus Combination Therapy in Systemic Sclerosis-associated Pulmonary Arterial Hypertension: A Retrospective Cohort Study from the Nationwide Spanish Scleroderma Registry (RESCLE)*. J Rheumatol, 2019.
188. Hassoun, P.M., et al., *Ambrisentan and Tadalafil Up-front Combination Therapy in Scleroderma-associated Pulmonary Arterial Hypertension*. Am J Respir Crit Care Med, 2015. **192**(9): p. 1102-10.
189. Manno, R. and F. Boin, *Immunotherapy of systemic sclerosis*. Immunotherapy, 2010. **2**(6): p. 863-78.
190. Amura, C.R., et al., *Differential regulation of lipopolysaccharide (LPS) activation pathways in mouse macrophages by LPS-binding proteins*. J Immunol, 1998. **161**(5): p. 2552-60.

191. Lam, L.T., et al., *Cooperative signaling through the signal transducer and activator of transcription 3 and nuclear factor- κ B pathways in subtypes of diffuse large B-cell lymphoma*. Blood, 2008. **111**(7): p. 3701-13.
192. Ihle, J.N., *STATs: signal transducers and activators of transcription*. Cell, 1996. **84**(3): p. 331-4.
193. Kim, H.S., et al., *TLR2-mediated production of IL-27 and chemokines by respiratory epithelial cells promotes bleomycin-induced pulmonary fibrosis in mice*. J Immunol, 2011. **187**(8): p. 4007-17.
194. Koca, S.S., et al., *The Protective Effects of Bevacizumab in Bleomycin-Induced Experimental Scleroderma*. Adv Clin Exp Med, 2016. **25**(2): p. 249-53.
195. Ponsoye, M., et al., *Treatment with abatacept prevents experimental dermal fibrosis and induces regression of established inflammation-driven fibrosis*. Ann Rheum Dis, 2016. **75**(12): p. 2142-2149.
196. Yan, Q., et al., *Targeting miR-155 to Treat Experimental Scleroderma*. Sci Rep, 2016. **6**: p. 20314.
197. Wang, K., et al., *Identification of ANXA2 (annexin A2) as a specific bleomycin target to induce pulmonary fibrosis by impeding TFEB-mediated autophagic flux*. Autophagy, 2018. **14**(2): p. 269-282.
198. Degryse, A.L., et al., *Repetitive intratracheal bleomycin models several features of idiopathic pulmonary fibrosis*. Am J Physiol Lung Cell Mol Physiol, 2010. **299**(4): p. L442-52.
199. Pinart, M., et al., *Single and repeated bleomycin intratracheal instillations lead to different biomechanical changes in lung tissue*. Respir Physiol Neurobiol, 2009. **166**(1): p. 41-6.
200. Peng, R., et al., *Bleomycin induces molecular changes directly relevant to idiopathic pulmonary fibrosis: a model for "active" disease*. PLoS One, 2013. **8**(4): p. e59348.
201. Svedberg, F.R., et al., *The lung environment controls alveolar macrophage metabolism and responsiveness in type 2 inflammation*. Nat Immunol, 2019. **20**(5): p. 571-580.

202. Ishida, Y., et al., *Essential involvement of the CX3CL1-CX3CR1 axis in bleomycin-induced pulmonary fibrosis via regulation of fibrocyte and M2 macrophage migration*. Sci Rep, 2017. **7**(1): p. 16833.
203. Gibbons, M.A., et al., *Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis*. Am J Respir Crit Care Med, 2011. **184**(5): p. 569-81.
204. Yue, H., et al., *Anti-fibrosis effect for Hirsutella sinensis mycelium based on inhibition of mTOR p70S6K phosphorylation*. Innate Immun, 2017. **23**(7): p. 615-624.
205. Morales-Nebreda, L., et al., *The heterogeneity of lung macrophages in the susceptibility to disease*. Eur Respir Rev, 2015. **24**(137): p. 505-9.
206. Duan, M., et al., *CD11b immunophenotyping identifies inflammatory profiles in the mouse and human lungs*. Mucosal Immunol, 2016. **9**(2): p. 550-63.
207. McCubbrey, A.L., et al., *Deletion of c-FLIP from CD11b(hi) Macrophages Prevents Development of Bleomycin-induced Lung Fibrosis*. Am J Respir Cell Mol Biol, 2018. **58**(1): p. 66-78.
208. Aoshiba, K., T. Tsuji, and A. Nagai, *Bleomycin induces cellular senescence in alveolar epithelial cells*. Eur Respir J, 2003. **22**(3): p. 436-43.
209. Lee, V.Y., et al., *Bleomycin induces alveolar epithelial cell death through JNK-dependent activation of the mitochondrial death pathway*. Am J Physiol Lung Cell Mol Physiol, 2005. **289**(4): p. L521-8.
210. Chen, K.J., et al., *Bleomycin (BLM) Induces Epithelial-to-Mesenchymal Transition in Cultured A549 Cells via the TGF-beta/Smad Signaling Pathway*. J Cancer, 2016. **7**(11): p. 1557-64.
211. Fried, L., et al., *Efficacy of rapamycin in scleroderma: a case study*. Lymphat Res Biol, 2008. **6**(3-4): p. 217-9.
212. Su, T.I., et al., *Rapamycin versus methotrexate in early diffuse systemic sclerosis: results from a randomized, single-blind pilot study*. Arthritis Rheum, 2009. **60**(12): p. 3821-30.
213. *A Pilot-Study of Sirolimus for the Treatment of Systemic Sclerosis*. <https://ClinicalTrials.gov/show/NCT03365869>.

214. Kral, J.B., et al., *Sustained PI3K Activation exacerbates BLM-induced Lung Fibrosis via activation of pro-inflammatory and pro-fibrotic pathways*. Sci Rep, 2016. **6**: p. 23034.
215. Campa, C.C., et al., *Inhalation of the prodrug PI3K inhibitor CL27c improves lung function in asthma and fibrosis*. Nat Commun, 2018. **9**(1): p. 5232.
216. Chakraborty, D., et al., *Activation of STAT3 integrates common profibrotic pathways to promote fibroblast activation and tissue fibrosis*. Nat Commun, 2017. **8**(1): p. 1130.
217. Papaioannou, I., et al., *STAT3 controls COL1A2 enhancer activation cooperatively with JunB, regulates type I collagen synthesis posttranscriptionally, and is essential for lung myofibroblast differentiation*. Mol Biol Cell, 2018. **29**(2): p. 84-95.

Copyright Permissions

Copyright permission for Figure 1.1

Order Details	
Nature Reviews Immunology	
Billing Status: N/A	
Order detail ID: 71902018	Permission Status: Granted
ISSN: 1474-1733	Permission type: Republish or display content
Publication Type: Journal	Type of use: Thesis/Dissertation
Volume:	Order License Id: 4591310399565
Issue:	☐ Hide details
Start page:	Requestor type: Publisher, not-for-profit
Publisher: NATURE PUBLISHING GROUP	Format: Print, Electronic
	Portion: Image/photo
	Number of images/photos requested: 1
	The requesting person/organization: Gizem Kilic
	Title or numeric reference of the portion(s): Figure 1.1
	Title of the article or chapter the portion is from: Chapter 1.1.1.1
	Editor of portion(s): N/A
	Author of portion(s): N/A
	Volume of serial or monograph: N/A
	Page range of portion: 4
	Publication date of portion: May 2019
	Rights for: Main product
	Duration of use: Life of current edition
	Creation of copies for the disabled: no
	With minor editing privileges: no
	For distribution to: Worldwide
	In the following language(s): Original language of publication
	With incidental promotional use: no
	Lifetime unit quantity of new product: Up to 499
	Title: THERAPEUTIC POTENTIAL OF AN IMMUNOSUPPRESSIVE OLIGODEOXYNUCLEOTIDE ENCAPSULATED WITHIN LIPOSOMES ON BLEOMYCIN-INDUCED MOUSE MODEL OF LUNG INFLAMMATION AND FIBROSIS
	Institution name: Bilkent University
	Expected presentation date: May 2019
Note: This item was invoiced separately through our RightsLink service. More info	
\$ 0.00	

Copyright permission for Figure 1.2

Order Details	
Pharmacological research : the official journal of the Italian Pharmacological Society	
Billing Status: N/A	
Order detail ID: 71902026	Permission Status: ✔ Granted
ISSN: 1043-6618	Permission type: Republish or display content
Publication Type: Journal	Type of use: Thesis/Dissertation
Volume:	Order License Id: 4591330421565
Issue:	
Start page:	☐ Hide details
Publisher: ACADEMIC PRESS	Requestor type: Publisher, not-for-profit
Author/Editor: SOCIETA ITALIANA DI FARMACOLOGIA	Format: Print, Electronic
	Portion: Image/photo
	Number of images/photos requested: 1
	The requesting person/organization: Gizem Kilic
	Title or numeric reference of the portion(s): Figure 1.2
	Title of the article or chapter the portion is from: Chapter 1.2.1.2
	Editor of portion(s): N/A
	Author of portion(s): N/A
	Volume of serial or monograph: N/A
	Page range of portion: 7
	Publication date of portion: May 2019
	Rights for: Main product
	Duration of use: Life of current edition
	Creation of copies for the disabled: no
	With minor editing privileges: no
	For distribution to: Worldwide
	In the following language(s): Original language of publication
	With incidental promotional use: no
	Lifetime unit quantity of new product: Up to 499
	Title: THERAPEUTIC POTENTIAL OF AN IMMUNOSUPPRESSIVE OLIGODEOXYNUCLEOTIDE ENCAPSULATED WITHIN LIPOSOMES ON BLEOMYCIN-INDUCED MOUSE MODEL OF LUNG INFLAMMATION AND FIBROSIS
	Institution name: Bilkent University
	Expected presentation date: May 2019
Note: This item was invoiced separately through our RightsLink service. More info	
\$ 0.00	

Copyright permission for Figure 1.3

Order Details		Billing Status: N/A	
Nature Reviews Disease Primers			
Order detail ID:	71902804	Permission Status:	✔ Granted
ISSN:	2056-676X	Permission type:	Republish or display content
Publication Type:	e-Journal	Type of use:	Thesis/Dissertation
Volume:		Order License Id:	4592020906333
Issue:		☐ Hide details	
Start page:		Requestor type	Publisher, not-for-profit
Publisher:	Nature Publishing Group	Format	Print, Electronic
Author/Editor:	Nature Publishing Group	Portion	Image/photo
		Number of images/photos requested	1
		The requesting person/organization	Gizem Kilic
		Title or numeric reference of the portion(s)	Figure 1.3
		Title of the article or chapter the portion is from	Chapter 1.4.2
		Editor of portion(s)	N/A
		Author of portion(s)	N/A
		Volume of serial or monograph	N/A
		Page range of portion	13
		Publication date of portion	May 2019
		Rights for	Main product
		Duration of use	Life of current edition
		Creation of copies for the disabled	no
		With minor editing privileges	no
		For distribution to	Worldwide
		In the following language(s)	Original language of publication
		With incidental promotional use	no
		Lifetime unit quantity of new product	Up to 499
		Title	THERAPEUTIC POTENTIAL OF AN IMMUNOSUPPRESSIVE OLIGODEOXYNUCLEOTIDE ENCAPSULATED WITHIN LIPOSOMES ON BLEOMYCIN-INDUCED MOUSE MODEL OF LUNG INFLAMMATION AND FIBROSIS
		Institution name	Bilkent University
		Expected presentation date	May 2019
Note: This item was invoiced separately through our RightsLink service . More info			
\$ 0.00			