TWO FACES OF ASC:
CENTRAL INNATE IMMUNITY ADAPTOR ASC TURNS INTO MELANOMA ALLY
AND
NOVEL MECHANISM DEMISTIFYING dsRNA INDUCED NLRP3 INFLAMMASOME ACTIVATION THROUGH BAX/BAK MEDIATED CELL DEATH

by

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B.S., Electrical and Electronic Engineering, Boğaziçi University, 2007

Submitted to the Institute for Graduate Studies in
Science and Engineering in partial fulfillment of
the requirements for the degree of
Master of Science

Graduate Program in Molecular Biology and Genetics
Boğaziçi University

2011

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DATE OF APPROVAL: 30.09.2011

To Mustafa Kemal Atatürk & Charles Darwin

ACKNOWLEDGEMENTS

I would like to express my love, respect and appreciation for Mustafa Kemal Atatürk for making this possible. I would like to thank Charles Darwin for giving me the greatest inspiration with his brilliant idea of evolution by natural selection.

Assoc. Prof. Nesrin Özören had all that it needed to make me and others thrive as graduate students. Besides for providing more than one would expect from a supervisor, I would like to thank her for letting me be a part of this exquisite experience, for having the vision and courage to support an engineer with no biology background. I hope I was able to show my appreciation by doing the best job I could for our project, for our lab, and for science. I would like to thank Prof. Gabriel Nunez and Dr. Luigi Franchi for their mentoring and support. I was lucky enough to work with them during the first years of my scientific journey. I would like to thank Dr. Anj Dlugosz and Dr. Monique Verhaugen for kindly providing primary melanocytes and melanoma cells. I would like to thank Dr. Robert Weinberg for kindly providing answers to my basic questions. I would like to thank Dr. Batu Erman for kindly providing packaging cells for retrovirus production. I would like to thank Dr. Tamer Önder for kindly providing plasmids for melanocyte transformation. I would like to thank Assist. Prof. Necla Birgül İyison and Assoc. Prof. Arzu Karabay for evaluating my study. I would like to thank Assoc. Prof. Nesrin Özören, Assist. Prof. Arzu Çelik, Assist. Prof. Stefan Fuss, Assoc. Prof. Esra Battaloğlu, Prof. Nazlı Başak, Prof. Aslı Tolun, Assoc. Prof. Müge Türet Sayar, Prof. Hande Çağlayan and Prof. Kuyaş Buğra for teaching me biology. I would like to thank past and present AKİL lab members Dr. Şahru Yüksel, Damla Erdoğan, Burcu Sümer, Metehan Çifdalöz, Serkan Uğurlu, Chara Charsou, Duygu Demiröz, Elif Eren, Yetiş Gültekin for their collaboration.

I would like to thank my biologist friends Çağdaş Bulut, İzzet Akiva, Yeliz Yılmaz, Duygu Dağlıkoca, Neslihan Zohrap, Gamze Küser and Funda Ejder. I would like to thank Burçak Özeş for being Burçak Özeş. I would like to thank Boğaziçi University for being the best University.

ABSTRACT

TWO FACES OF ASC: CENTRAL INNATE IMMUNITY ADAPTOR ASC TURNS INTO MELANOMA ALLY AND NOVEL MECHANISM DEMISTIFYING dsRNA INDUCED NLRP3 INFLAMMASOME ACTIVATION THROUGH BAX/BAK MEDIATED CELL DEATH

In this study, by specifically focusing on the role of ASC in melanoma and NLRP3 activation in response to double stranded RNA, we try to provide answers to more general but basic questions: “How and why would cancer cells alter the function of inflammatory proteins?” and “How can NLRP3 inflammasome recognize varying PAMPs and DAMPs with totally different natures and molecular structures?”, respectively. Although having mRNA expression for all necessary NLRP3 inflammasome components, healthy primary melanocytes showed no response to NLRP3 activating signals as measured by IL-1 beta secretion via ELISA. Whereas ASC was silenced at the transcription level, the protein was present and functional in certain melanoma cell lines. Moreover, knock-down of Asc in these cell lines by shRNA resulted in diminished NLRP3 activation. Overall, our observations support the idea that cancer cells may activate the nonfunctional inflammasome machinery, supposedly to increase cytokine secretion which may help angiogenesis and subsequently metastasis. In the second part of our study, we investigate dsRNA recognition mechanism and pathway leading to the NLRP3 inflammasome activation. Treating LPS primed Ips-1/Trif DKO macrophages we observed that Ips-1 is required for intracellular pIC recognition. Inflammasome activity analysis of Bax/Bak DKO macrophages and immunofluorescence assay for active Bax showed that, Ips-1 acts on apoptotic Bcl2 family members Bax/Bak triggering programmed cell death mechanism. Resulting membrane permeabilization causes loss of cytosolic K+ ion which is sensed by NLRP3 leading to NLRP3 inflammasome activation. Our data provides the lacking unifying model of NLRP3 activation mechanism: Any pathogenic organism or danger signal causes loss of membrane integrity. Resulting damage disturbs the active regulation of cytosolic ion balance, causing loss of K+ from cytosol, which is sensed by NLRP3 inflammasome resulting in NLRP3 inflammasome activation.

ÖZET

ASC'IN İKİ YÜZÜ: DOĞAL BAĞIŞIKLIK SİSTEMİ TEMEL ADAPTÖRÜ ASC MELANOMA MÜTTEFİKİNE DÖNÜŞÜYOR VE BAX/BAK TEMELLİ ÖLÜM YOLU ÜZERİNDEN ÇALIŞAN YENİ BİR MEKANİZMA, ÇİFT SIRALI RNA'NIN NLRP3 INFLAMAZOMUNU NASIL ÇALIŞTIRDIĞINI AÇIKLIYOR

Bu çalışmada, iki genel soruya cevap arıyoruz: "Kanser hücreleri inflamazomla ilgili proteinlerin işleyişini neden ve nasıl değiştirir?" ve "NLRP3 inflamazomu nasıl tamamen farklı doğadaki PAMP ve DAMP moleküllerini tanıyabilir?". Bu soruların cevaplarını sırasıyla ASC'ın melanomadaki rolünü ve NLRP3 inflamazomunun çift sıralı RNA tarafından nasıl aktive edildiğini araştırarak arıyoruz. Sağlıklı melanositler NLRP3 inflamazomu için gerekli tüm proteinleri mRNA seviyesinde üretiyor olmalarına rağmen, NLRP3 inflamazomunu çalıştıran uygulamalara yanıt vermediler. Öte yandan, ASC bazı melanoma hatlarında mRNA düzeyinde baskılansa da, bu hatlarda NLRP3 inflamazom makinesi çalışır durumda. Dahası, shRNA kullanarak ASC'ı susturduğumuzda NLRP3 çalışması azalıyor. Bütün olarak bakıldığında, gözlemlerimiz, kanser hücrelerinin inflamazom makinesini aktive edebiliyor görüşünü destekliyor. Bu durum muhtemelen, sitokin salımını artırarak yeni damar oluşumuna ve kanserin yayılmasına yardım ediyor. Çalışmamızın ikinci bölümünde, çift sıralı RNA'nın tanınması ve bu yolla NLRP3 inflamazomunun çalışması işlevlerinin nasıl gerçekleştiğini araştırdık. LPS ile dürtülenmiş Ips-1/Trif DKO makrofajları pIC ile uyararak Ips-1 proteininin hücre içi pIC tanınmasında gerekli olduğunu gördük. Bax/Bak DKO makrofajlarda Bax proteininin çalışmasını izleyerek, Ips-1 proteininin Bcl-2 ailesi üyeleri Bax/Bak üzerinden hücre ölüm mekanizmalarını tetiklediğini gördük. Verilerimiz, NLRP3 inflamazomunun nasıl çalışmaya başladığını açıklayacak bütünleştirici modeli sağıyor: Zararlı organizmalar veya tehlike sinyalleri sırasıyla hücre zarı geçirgenliğini artırarak K+ iyonlarının hücre dışına kaçmasına, böylelikle hücre içi iyon dengesinin bozulmasına yol açıyor. İyon dengesindeki bu bozulma NLRP3 tarafından algılanıyor ve bu NLRP3 inflamazom makinesini tetikliyor.

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LIST OF ACRONYMS / ABBREVIATIONS

APS	Ammonium per sulfate
ATP	Adenosine triphosphate
BCC	Basal Cell Carcinoma
Bcl-2	B-cell Lymphoma 2
bp	Base pair
DMEM	Dulbecco Modified Eagle's Minimal Essential Medium
BMDM	Bone Marrow Derived Macrophage
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
EtBr	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
E. coli	Escherichia coli
FBS	Fetal Bovine Serum
FG12	Lentiviral vector
GFP	Green Fluorescent Protein
HBS	HEPES Balanced Salt
HEK	Human Embryonic Kidney
HMGS	Human Melanocyte Growth Supplement
IL	Interleukin

kb	Kilo Base
kDa	Kilo Dalton
LPS	Lipopolysaccharide
mA	Milliamper
mM	Millimolar
ml	Milliliter
µl	Microliter
MGM	Melanocyte Growth Medium 254 CF
mRNA	Messenger ribonucleic acid
NO	Nitric Oxide
PAGE	Polyacrylamide gel electrophoresis
pIC	polycytidylic acid (Poly(I:C))
PBS	Phosphate buffered saline
rpm	Revolutions per minute
PVDF	Polyvinyl difluoride
RNA	Ribonucleic Acid
RNAi	RNA interference
SDS	Sodium dodecylsulfate
shRNA	Short Hairpin Ribonucleic Acid
siRNA	Small Interference Ribonucleic Acid
TAE	Tris-Acetate-EDTA
TBS	Tris buffered saline

TBST	Tris buffered saline with Tween 20
TEMED	N,N,N’,N’,-tetramethylethylenediamine
V	Volt

1. INTRODUCTION

1.1. Skin

Skin is the outer cover of the body and it is continuous with the mucous membranes lining the body's orifices. Skin is evolved to serve in different conditions in different organisms such as amphibians, reptiles, birds (Alibardi, 2003). Human skin is similar to other mammals, but it does not have a pelt and seems hairless besides being covered by hair follicles.

Main function of the skin is to protect body against external physical, chemical and biological threats (Butnaru and Kanitakis, 2002). Sensation is another function. Embedded nerve ends enable reaction to heat, pressure, touch, etc.

Human skin consists of stratified, cellular epidermis and an underlying dermis of connective tissue. Epidermis ('epi' meaning 'over' or 'upon' in Greek) is the outermost layer of the skin. There are various cell populations in the epidermis: keratinocytes, melanocytes, Langerhans' cells, and Merkel cells.

The barrier function of the skin is mostly held by epidermis. This layer is maintained by epidermal stem cells which reside in the basal layer and generate daughter cells which move upward toward the surface of the skin, forming keratinocytes (Koster, 2009). Keratinocytes are the majority of the cells forming epidermis. Melanocytes are also located in the epidermis.

The dermis consists of fibroblasts, mast cells, monocytes, macrophages, and two kinds protein fibers: collagen which has a great tensile strength and forms the major constituent of the dermis, and elastin which makes up only a small proportion of the bulk. The dermis has a capacity to retain water. It also has a very rich blood supply; however, no vessels pass through the dermal-epidermal junction.

1.2. Melanocytes

Melanocytes are located in the bottom layer of the epidermis. However, skin is not the only host of melanocytes, they are also found in the eye, meninges, bones, and heart. In vertebrates, melanocytes are well known for their role in skin pigmentation, hair and feather coloration, and for their ability to produce and distribute melanin to surrounding keratinocytes (Sulaimon and Kitchell, 2003). Humans have around 1000 melanocytes per millimeter square of their skin.

Production and distribution of melanin seem to be the main function of melanocytes. Melanin is the pigment which can protect the cells from the effects of UV radiation. Production of melanin is called melanogenesis and it occurs in melanosomes, the organelles originating from the endoplasmic reticulum and located in melanocytes.

However, production of melanin may not be the only function of melanocytes. It was shown that melanocytes can produce some signaling molecules such as cytokines, melanocortin peptides, catecholamines, serotonin, eicosanoids, and NO (Tsatmali *et al.*, 2002).

Considering, the variety signaling molecules melanocytes can secrete and the interaction between melanocytes and other cell types, it was proposed that melanocytes act as local “stress sensors” in the epidermis and provide communicatory links with several different systems (Slominski *et al.*, 1993).

1.3. Melanoma

Melanoma is a cancer that stems from melanocytes. Because most of these cells make melanin, melanoma tumors are often brown or black. But this is not always the case, and melanomas can also have no color. Melanoma most often starts on the trunk of fair-skinned men and on the lower legs of fair-skinned woman, but it can start in other places too. Having dark skin lowers the risk of melanoma but it does not eliminate it.

Skin cancer is the most common of all cancers. Melanoma accounts for less than 5% of skin cancer cases, however, it causes the majority of skin cancer deaths. The American Cancer Society estimates that each year there are 68720 new cases of melanoma, 8650 of which result in death. The lifetime risk of getting melanoma is about 1 in 50 for whites, 1 in 1000 for blacks, and 1 in 200 for Hispanics in the United States.

The exact cause of melanoma is unknown, but certain risk factors are linked to melanoma. Exposure to UV radiation is the biggest risk factor. Certain types of moles increase the chance of getting melanoma. A mole (nevus) is a benign skin tumor. A person with many moles is more likely to develop melanoma. Fair skin increases the risk of melanoma 10 times compared to dark skin. Heredity also effects melanoma risk. Around 10% of people with melanoma have a close relative with the disease. Having had melanoma already increases the risk of getting another melanoma. Weakened immune system increases the risk of developing melanoma. People who have been treated with medicines that suppress immune systems are more vulnerable to melanoma. Melanoma incidence increases with age. Men have higher rate of developing melanoma compared to women. People with Xeroderma pigmentosum – a rare inherited condition that causes decreased ability to repair damage caused by sunlight – are at greater risk of melanoma.

The development of melanoma requires a series of transitions. Initially, aberrant proliferation of melanocytes produces melanocytic nevi in the epidermis. Most of these do not cause malignant disease. However, a few may start to spread usually laterally. This is called Radial Growth Phase (RGP). The next phase is Vertical Growth Phase (VGP), which shows pronounced invasion into the dermis. In early VGP, the majority of melanocytes remain in cluster while only a few isolated cells break away from the bulk of the melanoma. Transition into VGP is characterized by acquisition of metastatic potential. VGP, then progresses to more aggressive forms of melanoma with extensive vascularization and invasion. To understand the cause of melanoma invasion, one should determine the molecular changes that underpin the RGP to VGP transition and the subsequent motility and intravasation of melanoma cells (Gaggioli and Sahai, 2007).

Various genetic changes have been related to melanoma. These include mutations in cell cycle regulators such as CDKN2A and signaling molecules such as NRAS, BRAF,

PTEN and amplification of AKT3 (Gaggioli C, 2007). While there are no strong candidates to mediate the transition from RGP to VGP, microarray and immunohistochemical analysis have identified numerous genes whose expression level is altered as melanoma become invasive (Bittner *et al.*, 2000).

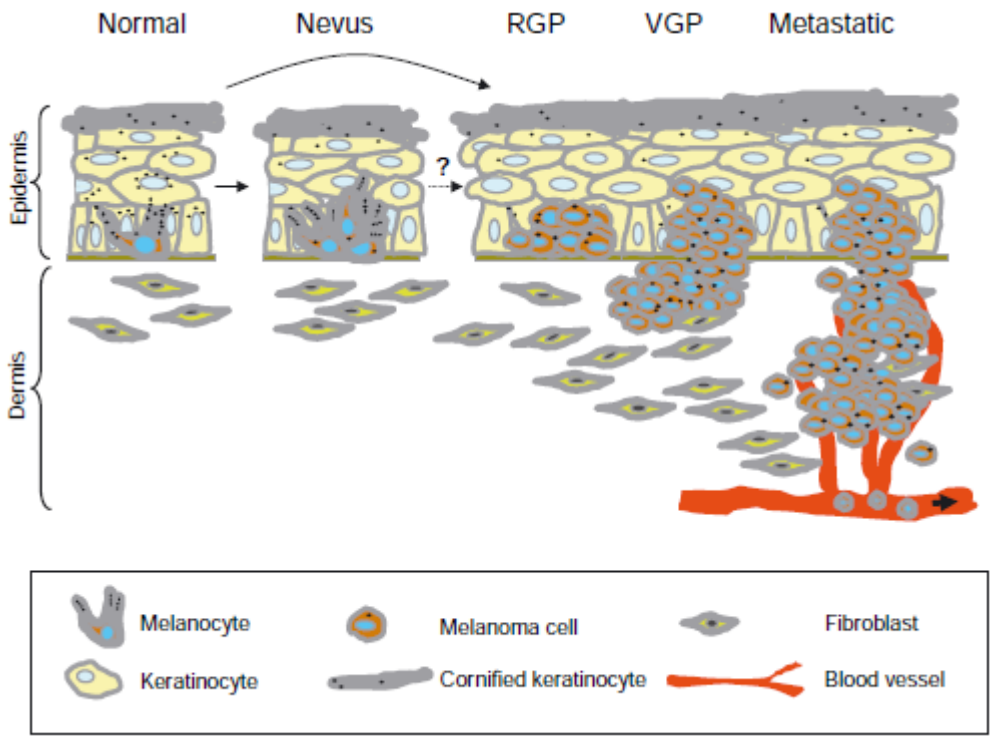

Figure 2.1. Developmental phases of metastatic melanoma (adapted from Gaggioli and Sahai, 2007)

Various genetic changes have been related to melanoma. These include mutations in cell cycle regulators such as CDKN2A and signaling molecules such as NRAS, BRAF, PTEN and amplification of AKT3 (Gaggioli C, 2007). While there are no strong candidates to mediate the transition from RGP to VGP, microarray and immunohistochemical analysis have identified numerous genes whose expression level is altered as melanoma become invasive (Bittner *et al.*, 2000).

Reduced E-cadherin levels are observed during melanoma development (Li *et al.*, 2001). It was suggested that this change may help melanocytes escape from the control of keratinocytes and disseminate. Increased N-cadherin levels suggested that this may help

melanoma cells make interactions with stromal fibroblasts, facilitating survival outside the epidermis (Smalley *et al.*, 2005).

Production of VEGF-A and VEGF-C by melanoma is crucial for the development of blood and lymphatic vasculature which generates a route for metastatic dissemination. Tumor vasculature is usually comprised of endothelial cells. Melanoma cells can also form part of the vasculature (Maniotis *et al.*, 1999). This phenomenon is called vascular mimicry and thought to be realized by expression of endothelial specific genes in melanoma cells such as ESM-1 and VE-Cadherin.

Changes in cell cycle and apoptotic regulators are also observed and thought to be crucial for melanocyte survival outside the epidermis. Gain of Ras together with loss of TP53 function can drive invasion, probably due to increased survival of invasive cells (Chudnovsky *et al.*, 2005).

1.4. ASC

ASC is a 42 kDa protein containing two protein interaction domains, a pyrin domain (PYD) and a caspase recruitment domain (CARD) (McConnell and Vertino, 2000).

ASC was identified by two independent labs using two different approaches (i) as an apoptosis associated speck-like protein (Masumoto *et al.*, 2001) and (ii) as a methylation target in cancer cells (Conway *et al.*, 2000).

1.4.1. ASC as an apoptosis associated speck-like protein

In an effort to discover proteins responsible for morphological changes associated with differentiation, Masumoto *et al.* developed monoclonal antibodies against proteins that partitioned into an insoluble cytoskeletal fraction upon differentiation of HL-60 cells with retinoic acid. The corresponding genes were then identified through an expression cloning approach. One of the genes isolated in this screen was named ASC (Apoptosis-associated Speck-like protein containing a CARD) based on the observation that the protein, which was located in the cytosol of HL-60 (human promyelocytic leukemia cells),

localized to perinuclear aggregates or “specks” upon treatment with retinoic acid (RA) or etoposide (Masumoto *et al.*, 1999).

The cDNA of ASC was shown to encode a 195 amino acids peptide. Further investigation revealed a significant homology between 87 amino acid residues in the C terminus of ASC and mammalian cIAP1, cIAP2, and CARD of the human death receptor caspase-2. So it was proposed that ASC may be involved in the signaling pathway of apoptosis through its CARD domain, as some other proteins with CARD domain do. The other end of ASC, namely N terminus, showed a high degree homology to PYRIN. These findings resulted in the proposed structure of ASC as a containing a C-terminal CARD domain and an N-terminal pyrin domain.

The CARD (Caspase Recruitment Domain), the PYRIN domain, the death domain, and the death effector domain subfamilies constitute the Death Domain Fold superfamily. Interaction of Death Domain Fold containing proteins modulates the activity of several downstream effectors, such as caspases and transcription factors (Stehlik, 2007).

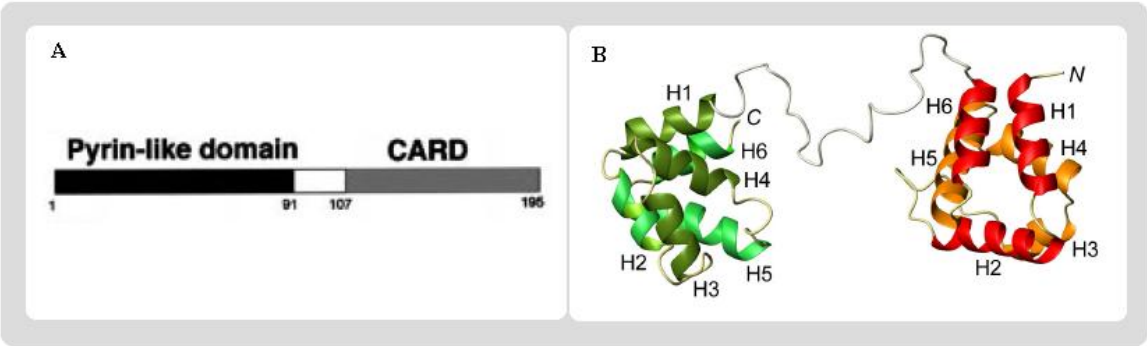

Figure 2.2. ASC protein. A. Schematic representation of ASC (Masumoto J T. S., 1999) B. Ribbon representation of the solution structure of full length human ASC. Helices of the CARD are colored in green, helices of PYD is colored in red.

The CARD proteins act in three major pathways: regulation of caspase activation in the context of apoptosis, regulation of caspase activation in the context of inflammation, and regulation of NF-KB in the context of innate or adaptive immune responses. To generalize, it can be stated that, all of the CARD proteins have a role in host defense

against infection, environmental stress, or cellular damage (Bouchier-Hayes, and Martin, 2002). So ASC, having a CARD domain, is suitable for a role in these pathways.

PYRIN domain-mediated signal transduction has role in various cellular functions including innate immunity, inflammation, differentiation, apoptosis, and cancer (Stehlik, 2007). Mutations in the Mefv gene encoding PYRIN cause familial Mediterranean fever, an inherited disorder that is characterized by episodes of fever and serosal inflammation (Bertin, and DiStefano, 2000).

ASC, with its bipartite domain structure consisting of an N-terminal PYRIN domain and a C-terminal CARD domain, may function as a FADD-like adaptor protein that binds multiple proteins via its N- and C- terminal domains (Bertin, and DiStefano, 2000). Indeed, ASC has a critical role in inflammation and innate immunity as well as in apoptosis and cancer development, probably due to its dual character containing both domains (Shiohara *et al.,* 2002).

Using FISH and Stanford G3 radiation hybrid mapping panel, the chromosomal location of ASC was found to be 16p11.2-11 (Masumoto *et al.,* 1999).

Part of the study regarding tissue and cellular distribution of ASC suggested that the level of ASC expression may vary according to the cell lineage, maturation stage, or cell transformation. Later, in 2001, two years after his original paper regarding the discovery of ASC, Masumoto detailed his study on expression of ASC in different tissues. ASC expression was observed in anterior horn cells of the spinal cord, trophoblasts of the placental villi, tubule epithelium of the kidney, seminiferous tubules and Leydig cells of the testis, hepatocytes and interlobular bile ducts in the liver, squamous epithelial cells of the tonsil and skin, hair follicle, sebaceous and eccrine glands of the skin, and peripheral blood leukocytes. In the colon, ASC was detected in mature epithelial cells facing the luminal side rather than immature cells located deeper in the crypts. Authors concluded that, high levels of ASC are abundantly expressed in epithelial cells and leukocytes, which are involved in host defense against external pathogens and in well-differentiated cells, the proliferation of which is regulated (Masumoto *et al.,* 2001)

Masumoto also observed the effect of ASC on etoposide-induced apoptosis of HL-60. Cells transfected by antisense oligonucleotides, which express 57% less ASC than the controls, showed a significant decrease in the percentage of apoptosis compared to control cells. This suggested that ASC may have pro-apoptotic stimuli by anticancer drugs such as etoposide or vincristine (Masumoto *et al.*, 1999).

Further studies showed a strong correlation between the redistribution of TMS1/ASC into the speck-like aggregates and phenotypic markers of apoptosis induced by chemotherapeutic agents.

1.4.2. ASC as a target of methylation-induced silencing

In another study, ASC was identified in a screen for downstream targets of methylation-associated gene silencing, so the gene was named as TMS1 – Target of Methylation-Induced Silencing. For the cell lines which do not express ASC ASC expression was restored after treatment with DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine. The absence of ASC expression in these cell lines was therefore not attributable to abnormalities at the gene level or to the inability to express TMS1, but rather was directly related to the methylation (Conway *et al.*, 2000).

The presence of the CARD domain made Conway *et al.* suggest that TMS1 plays a role in apoptosis. To test this hypothesis, they observed the effect of ectopic TMS1 expression in 293 cells. Cells transfected with TMS1 became condensed, rounded, and eventually lifted off the culture surface, indicating apoptosis. Increase in apoptosis was 6 fold. Moreover, the apoptotic activity was blocked by the general caspase inhibitor zVAD, indicating that TMS1-induced cell death requires caspase activation. Another experiment on breast cancer cells showed that overexpression of TMS1 had similar growth inhibitory effects on both TMS1-negative and TMS1-positive breast cancer cells. So, the group concluded that TMS1 functions in the promotion of caspase-dependent apoptosis and overexpression of TMS1 inhibits the growth of breast cancer cells (Conway *et al.*, 2000).

Methylation and silencing of TMS1 in human breast cancer cells suggested that TMS1 may have a tumor suppressor function. TMS1/ASC expression was shown to be

decreased by methylation induced gene silencing in breast cancer, gastric cancer (Moriai R, 2002), lung cancer (Virmani *et al.,* 2003), and melanoma (Guan *et al.,* 2003). This observation implies involvement of ASC in suppression of tumor development including melanoma.

1.4.3. ASC and Inflammasomes

ASC also has a role in innate immunity. CARD domain of ASC strongly and specifically interacts with caspase-1, leading to caspase-1 activation. Similarly, overexpression of FADD specifically induced activation of caspase-8. Thus, it's suggested that analogous to the adaptor protein FADD that links the DED of caspase-8 to the DD of Fas, ASC may connect the CARD of caspase-1 to the PYD of NLRs. Experiments in cell-free system showed that proinflammatory caspase activation and ptoIL-1B processing is lost upon prior immunodepletion of ASC, showing that ASC has a role in IL-1B secretion (Martinon *et al.,* 2002).

In a study questioning the role of ASC in the host defense system, using macrophages from WT and ASC knockout mice, Ozoren *et al*, (2006) showed that ASC is essential for secretion of IL-1B/IL-18, but dispensable for IL-6, TNF-alpha, and IFN-beta production, in macrophages infected with Lysteria.

1.4.4. ASC and Apoptosis

There is accumulating data indicating the role of ASC in apoptosis. It was reported that over-expression of ASC induced apoptosis in a caspase-dependent manner in HEK293 cells (McConnell and Vertino, 2000). Moreover, suppression of ASC expression in HL-60 cells decrease the response of cells to apoptotic stimuli (Masumoto *et al.,* 1999). ASC triggers apoptosis when co-expressed with NLRP3 in HEK293 cells (Dowds *et al.,* 2003).

Because ASC was reported to be silenced in a significant proportion of primary breast tumors and breast cancer cell lines, it was proposed that lack of ASC may provide advantage to cancer cells due to its pro-apoptotic function. Cells must override number of apoptotic signals during their progression to cancer. These signals include DNA damage,

hypoxia, and the absence of critical survival factors (Lowe and Lin, 2000). Moreover, in order to metastasize, tumor cells must survive through the blood stream and must be able to colonize foreign sites. These events are normally prevented by apoptosis of epithelial cells when deprived of substratum interactions (Frisch and Ruoslahti, 1997). So, cells must acquire alterations that disrupt apoptotic signaling to make tumor progression possible. According to the idea that ASC has tumor suppressive function, loss of ASC expression by methylation-mediated silencing may contribute to escape from apoptosis.

1.4.5. ASC and Cancer

ASC was discovered as a methylation target in human breast cancer as presented in section 1.4.2. Aberrant methylation of ASC in breast cancer was confirmed by another study which reported methylation in 12 out of 26 (46%) of breast cancer cell lines, and 20 out of 63 (32%) of breast tumor tissues, suggesting that methylation of ASC may play a role in the pathogenesis of breast cancer (Virmani *et al.,* 2003). Large-scale gene expression profiling studies implied that ASC is down regulated by more than 2-fold in 27 out of 117 primary breast tumors.

Epigenetic silencing of ASC was also observed in other human cancers such as gastric cancer (Moriai R, 2002), small lung cancer (Virmani *et al.,* 2003), colorectal cancer (Yokoyama *et al.,* 2003), and prostate cancer (Das *et al.,* 2003). ASC was reported to be suppressed in also melanoma (50-60%) by aberrant methylation (Guan *et al.,* 2003). It should be noted that, all reported ASC down-regulation data presented here is in fact representative for transcription of the mRNA only. The studies involve ASC mRNA level measurements using rtPCR technique and effect of methylation on mRNA expression.

Even though accumulating data suggests a role of down regulation of ASC in cancer cells, the corresponding functional evidence is absent. In colorectal cancer, no statistical differences were seen in the extent of differentiation and invasion, lymph node metastasis, and pathologic type between the methylated and unmethylated tissues (Liu *et al.,* 2006).

This intriguing observation, combined with the fact that all ASC down-regulation data relays on rtPCR, puts the assumed role Asc methylation in cancer jeopardy, implying either inefficiency of methylation in significantly decreasing ASC protein levels or independency of ASC expression from tumorigenesis. Making the picture even more complicated, a recent study showed that melanoma cells benefit from ASC to induce inflammation signaling which was suggested to facilitate melanoma development (Okomato *et al.*, 2010).

1.5. NLRP3 Inflammasome

The concept of 'inflammasome' stands for a hypothetical structure formed by collection of inflammatory proteins in order to cleave caspase-1, a cysteine protease, in response to various activating stimuli with the common attribute of being sensed through cytosolic receptors (Ozkurede *et al.*, 2012). Caspase-1 is activated by cleavage and, in turn, cleaves the cytokines IL-1B and IL-18 into their active form to be secreted (Bauernfeind *et al.*, 2009). Hence, the 'inflamamsomes' control the production and secretion of two main inflammatory cytokines.

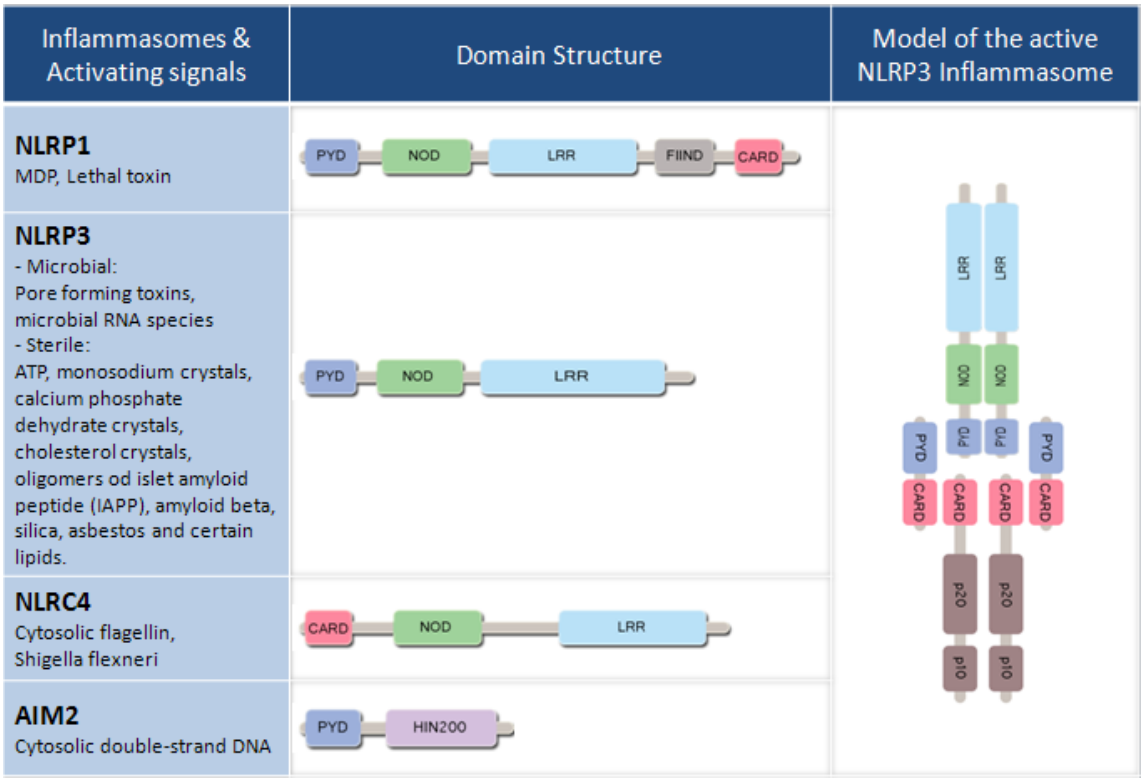

Figure 2.3. NLRP1, NLRP3, NLRC4 and AIM inflammasomes and corresponding stimuli.

In theory, any cytosolic receptor protein that has a role in activation of caspase-1 has potential to form an inflammasome upon stimulation. However, to date, there are four well-defined and generally accepted inflammasomes (Franchi *et al.,* 2009). They are named by their 'sensing' protein which is usually a member of NLR family – an exception is AIM2.

The NLRP1 inflammasome is formed by NLRP1, ASC and CASPASE-1 in response to MDP and Anthrax lethal toxin from Bacillus anthracis (Faustin *et al.,* 2007).

The NLRC4 inflammasome is formed by NLRC4, ASC and CASPASE-1 in response to cytosolic flagellin from Salmonella typhimurium (Franchi *et al.,* 2006), Pseudomonas aeruginosa (Franchi *et al.,* 2007), and Leginella pneumorphia (Amer *et al.,* 2006). ASC is involved in but is not required for NLRC4 inflammasome activation.

AIM2 inflammasome is formed by AIM2, ASC and CASPASE-1 in response to cytosolic double stranded DNA which is possessed by vaccinia virus mCMV, Francisella tularensis and also probably viral and bacterial pathogens (Alnemri, 2010).

The mostly studied and well characterized one is NLRP3 inflammasome, or definitively NLRP3-ASC-CASPASE-1 inflammasome. This inflammasome will be introduced in detail in the rest of this section.

1.5.1. Activation of NLRP3 Inflammasome

The activation of NLRP3 inflammasome requires two signals designated as first or 'priming signal' and second or 'activating signal' (Ozkurede *et al.,* 2012). As the name implies the second signal is the real activator (Figure 1.4). However, without the first signal which primes the cell by increasing expression of inflammatory proteins and cytokines, the activation is inefficient. So, both signals are required.

The priming signal is provided by PAMPs through TLRs or NLRs which subsequently lead to the activation of transcription factor NF-KB (Bauernfeind *et al.,* 2009). Recognition of PAMPs by TLRs causes upregulation of both NLRP3 itself and the

cytokine IL-1B. So, when the NLRP3 is over-expressed, the NLRP3 inflammasome can be activated without priming just by supplying the activating signal alone to activate CASPASE-1. However, since the substrate of CASPASE-1, namely pro-IL-1B is not upregulated, IL-1B secretion is absent in this case. So, it's reasonable to state that NLRP3 mediated IL-1B secretion is controlled at three levels: (i) upregulation of NLRP3, (ii) upregulation of IL-1B and (iii) activation of the inflammasome complex.

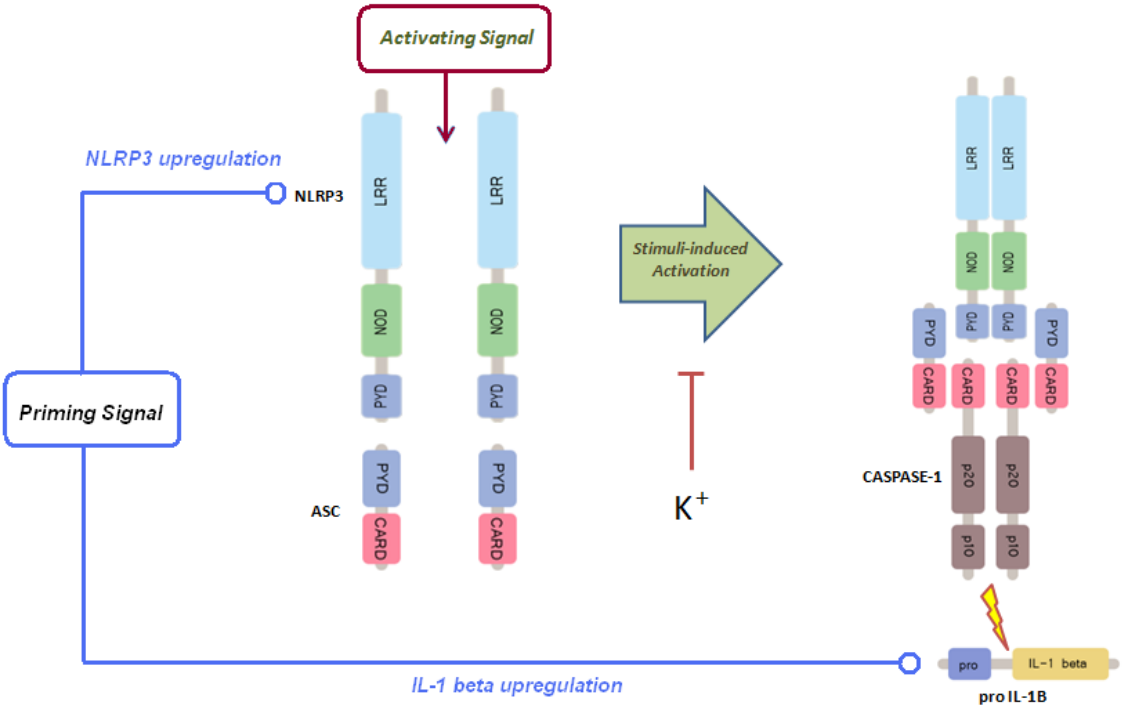

Figure 2.4. NLRP3 Inflammasome Activation

Extracellular ATP applied on primed macrophages causes a robust CASPASE-1 activation and IL-1B secretion. ATP acts by opening the P2X7 ion channels (Ferrari *et al.*, 2006). It was shown that in the case of freshly isolated monocytes, even the release of ATP from the stimulated cells themselves, can help activation of NLRP3 inflammasome, providing autocrine signaling (Piccini *et al.*, 2008).

1.5.1.1. Non-microbial activators & sterile inflammation

Interestingly, it has been shown that when priming is provided, the NLRP3 inflammasome can also be activated by non-microbial stimuli such as monosodium

crystals, beta-amyloid and ATP (Cassel and Sutterwala, 2010). Moreover, without involvement of pathogen associated molecular patterns, in a sterile environment, NLRP3 inflammasome can be still activated. In this case, priming signal can be efficiently supplied by inflammatory cytokines such as TNF-α, IL-1α and IL-1β for activation of macrophages and dendritic cells in response to danger signals and particulate matter (Franchi *et al.*, 2009). The main activating signals in a sterile environment are ATP, monosodium crystals and calcium pyrophosphate dehydrate crystals (Martinon *et al.*, 2006), cholesterol crystals (Duewell *et al.*, 2010), and oligomers of islet amyloid polypeptide (Masters *et al.*, 2010).

1.5.1.2. The mystery of NLRP3 activation

Whereas it widely considered to be well-characterized, one simple question about NLRP3 activation makes the scientists curious: “How can various stimuli with different natures be sensed by the same inflammasome, NLRP3?”, “Is there a common upstream element that every one of these signals converge on?”. The unifying theory of NLRP3 inflammasome activation is still lacking.

Three main models emerged during the struggle to answer this question, each with its own shortcomings.

The first suggestion is that NLRP3 senses changes in cytosolic potassium ion concentration. This argument is supported by the observations that activating signals such as ATP and pore forming toxins cause K+ efflux, and consistently, when the cells are supplied with media with high concentration of K+, blocking K+ efflux, NLRP3 inflammation is blocked (Franchi *et al.*, 2007).

Another model focuses on lysosomal damage, suggesting that particulate matter such as silica crystals and aluminum salts impairs lysosomal integrity causing release of lysosomal molecules which are in turn recognized by NRLP3 leading to activation of the inflammasome (Hornung *et al.*, 2008).

The third model places ROS between activating signal and NLRP3 inflammasome. According to this idea, varying stimuli cause mitochondrial malfunctioning and this causes

exceeding ROS production, which is, in turn, is sensed by NLRP3. The weak point of this model is the unpublished observation (of ours and other groups) that blocking ROS is not sufficient to block NLRP3 inflammasome activation for all stimuli.

1.5.2. IL-1B secretion and function

When activated, NLRP3 inflammasome results in activation of Caspase-1, which in turn cleaves IL-1B into its active form and IL-1B is secreted from the cell. IL-1B does not have the leader sequence which is generally present in secreted proteins. The mechanism of IL-1B secretion is not clear yet. Suggested mechanisms include exosome shedding (Qu *et al.*, 2009), shedding of plasma membrane micro vesicles (MacKenzie *et al.*, 2001), and lysosomal secretion (Andrei *et al.*, 1999).

Extracellular IL-1B binds to IL-1R, the receptor protein for IL-1B and IL-1alpha. IL-1Ra acts as an antagonist for this receptor with a higher binding affinity compared to IL-1B. This makes it good regulator of IL-1B signaling.

IL-1B is a powerful mediator of inflammatory signaling (Dinarello, 1996). It can induce fever and hepatic acute phase response which involves C-reactive protein and serum amyloid-A. Locally, IL-1B signaling induces cell adhesion molecules ICAM-1, VCAM-1, P-selectin and E-selectin (Bochner *et al.*, 1991), promoting neutrophil recruitment to inflamed tissues. Upon IL-1B recognition, neutrophils and other leukocytes produce additional cytokines including TNF-alpha and IL-6 (Dinarello, 2011).

1.5.3. IL-1B and Cancer

IL-1B was observed to be secreted by various tumor cells. Further investigation showed that tumor-derived IL-1β has role in tumor growth, immunosuppression, and chemoresistance.

Pancreatic cancer cell chemoresistance, both intrinsic and gained upon chemotherapeutic treatment, was shown to be related to IL-1B secretion by these cancer cells. Consistently, IL-1R antagonist treatment successfully reverses chemoresistance in

pancreatic cancer cells and increases chemotherapy related apoptosis (Muerkoster *et al.*, 2006).

IL-1B was also suggested to contribute to tumor growth by facilitating angiogenesis. Accordingly, tumor related angiogenesis was inhibited by IL-1R antagonist treatment in a model of fibrosarcoma which involves IL-1B secretion. Blocking IL-1 signaling resulted in less and thinner blood capillaries (Bar *et al.*, 2004).

Since IL-1B secretion increases macrophage recruitment and activation, resulting innate immune response activity causes production of endothelial cell activating factors such as VEGF by infiltrating immune cells (Carmi *et al.*, 2009). Hence, IL-1B secretion subsequently triggers angiogenesis, helping cancer cells reach blood vessels, providing a necessary step for migration.

1.6. Double Stranded RNA as an activator of NLRP3 inflammasome

Double stranded RNA (dsRNA) constitutes an important PAMP which serves to recognize RNA viruses, DNA viruses and bacterial RNA.

Double stranded RNA can be sensed by multiple receptors. Extracellularly it is recognized by TLR3 on the cell membrane and additionally by TLR7 in the endosomes, which signal through TRIF and MyD88 respectively.

Intracellularly two retinoic acid inducible gene-1-like receptors (RLRs) sense dsRNA. Of these, RIG-I is preferentially activated by short dsRNA, whereas, MDA-5 recognizes long dsRNAs and synthetic dsRNA analog pIC with more than a thousand nucleotides (Rehwinkeland Sousa, 2010).

Rig-I was shown to be involved in detection of paramyxoviruses, orthomyxoviruses, rhabdoviruses and flaviviruses. Mda-1 is required for retection of picornaviruses, reoviruses and flaviviruses, suggesting cooperation of two receptors. Consistently, West Nile virus and Dengue virus are recognized by both Rig-I and Mda-5 (Fujita, 2009).

IPS-1, also named MAVS or CARD adaptor inducing ILN-beta (CARDIF), provides downstream signaling for RIG-I and MDA5. Both receptors activate IPS-1 located on mitochondrial membrane through CARD-CARD interaction (Saito and Gale, 2008). The activated IPS-1 induces upregulation in the activity of IRF3/7 and NF-kappa B, increasing expression of Type I IFNs and pro form of inflammatory cytokines IL-1 beta and IL-18. IPS-1 activity also triggers tumor-suppressive behavior by inducing apoptosis. Apoptotic activity of IPS-1 signaling can be suppressed by anti-apoptotic Bcl-2 family member Bcl-$x_{L,}$ suggesting involvement of pro-apoptotic Bcl-2 family members in dsRNA mediated cell death (Poeck *et al.*, 2008).

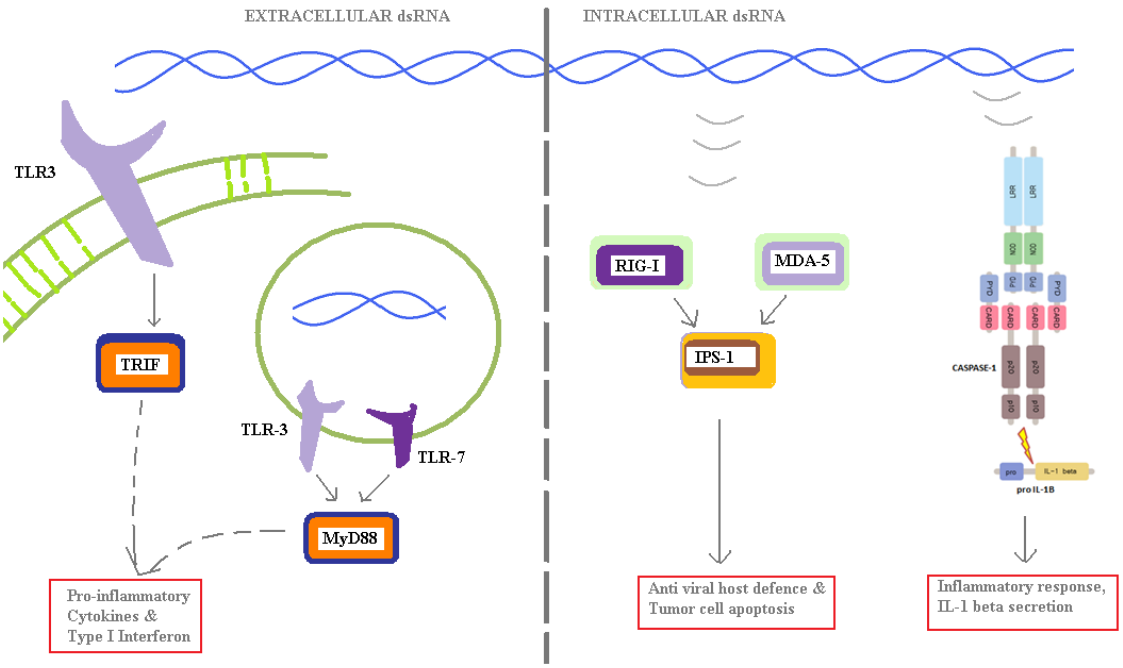

Figure 1.5. Double stranded RNA activates NLRP3 inflammasome and induces inflammatory response as seen in the cases of viral infection including adenovirus and influenza virus infections. Although NLRP3 involvement was clearly shown, explanation on how can be dsRNA recognized or sensed by NLRP3 is lacking since there is no evidence on direct interaction of NLRP3 to dsRNA.

1.7. Summarizing remarks

Inflammation is in the heart of survival mechanisms against internal and external treats an organism faces. Protection from pathogenic microorganisms such as viruses,

bacteria, fungi is just a part of its various acts. Inflammation is involved in cancer, infection, diabetes, obesity, Alzheimer's disease, organ rejection, aging, and so on – virtually in any kind of abnormality in an organism. Although we can describe the organism's reaction against a pathogen in molecular level with astonishing detail and precision in certain specific cases, we still lack the main understanding regarding the basics of activation mechanisms of innate immune response and its role in cancer development.

Clarifying possible mechanisms of inflammasome involvement in cancer development would provide us with new targets for cancer prevention and treatment.

Elucidation of NLRP3 activation mechanism to explain how different stimuli can activate this special inflamasome has been one of the main interests in the field of innate immunity since NLRP3 inflammasome was first described. Understanding of a unifying mechanism would enable us to develop treatments to alter inflammatory pathways in order to improve treatment of a wide range of diseases including genetic autoimmflammatory disease such as Cryopyrin-Associated Periodic Syndromes, Familial Mediterranean Fever, aging related abnormalities such as Alzheimer's disease and aggressive mortal diseases such as cancer.

2. AIM OF THE STUDY

In this study, we aimed to answer two basic questions to provide a better understanding of the mechanism of NLRP3 inflammasome activation and its role in cancer development.

In the first part of our study we planned to examine the role of NLRP3 inflammasome activity, a central regulator of innate immune responses, in melanoma growth and development. We study expressional changes in Nlrp3, Asc and Cascpase-1 mRNA and protein levels in metastatic melanoma cells in comparison to primary human melanocytes. In order to link mRNA and/or protein levels with functional inflammasome activity we monitor cytokine release profiles of both cancer and healthy cells in response to stimuli including various pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPSs). We aimed not only to list the alterations in proteins constituting NLRP3 inflammasome but also to relate these changes to functional relevance and to provide underlying reasoning on why and how would such alterations provide advantage to cancer cells. Secondly, our study intended to delineate the unknown pathways linking cytosolic double stranded RNA to NLRP3 inflammasome activation. By elucidating these pathways, we strived to understand how infection of DNA viruses, RNA viruses and some bacteria is sensed by the innate immune system.

Employing bone marrow derived macrophages from mice deficient in extracellular pathogen signaling pathway components Tlr3, MyD88, Trif as well as intracellular pathogen signaling pathway components Mda5, Ips-1, Nlrp3, Caspase-1, we try to understand how cytosolic dsRNA is sensed. We monitor NLRP3 inflammasome activity via Caspase-1 cleavage through western blotting analysis and IL-1 beta secretion through ELISA, in BMDMs transfected with pIC (dsRNA).

By providing the missing mechanism of NLRP3 activation in response to dsRNA we expect to get clues on the general mechanism of NLRP3 activation and explain the bigger question of how NLRP3 can sense varying PAMPs and DAMPs with different nature and characteristics.

3. MATERIALS AND METHODS

3.1. Chemicals, Plastic and Glassware

The chemicals used through the experiments were purchased from Sigma-Aldrich (USA) or Merck (Germany) with a few exceptions that are stated in the text. The cell culture media for melanocytes and keratinocytes were purchased from Cascade Biologics (Invitrogen, USA). Other cell culture media and supplements were acquired from Gibco (Invitrogen, USA). The plasticware used in the cell culture procedures were purchased from TPP (Switzerland) in sterile packages. The glassware and the laboratory-prepared buffers were sterilized by autoclaving at 121°C and/or by filter sterilization using 0.22 um filters (Millipore, Ireland).

3.2. Cell Lines, Primary Cells, Cell Culture

The human embryonic kidney cell lines HEK 293FT / HEK 293T (gift from M.S. Soengas) and HEK 293T (gift from Mehmet Öztürk) and human melanoma cells lines (gift from Monique Verhaugen from Dr. Dlugosz's Lab at University of Michigan) were grown in DMEM supplemented with 10 % FBS, 1 mM sodium pyruvate, 1X L-glutamine (Gibco, Invitrogen, USA), 50 U/ml penicillin, 50 µl/ml streptomycin and 1X MEM NNA ($DMEM^+$).

Primary human melanocytes (gift from Monique Verhaugen from Dr. Dlugosz's Lab at University of Michigan) were grown in Melanocyte Growth Medium 254 CF (MGM) supplemented with Human Melanocyte Growth Supplement (HMGS).

Cell lines and primary cells were grown at 37°C and under 5 % CO_2. The cells were passaged when necessary and the passage number were recorded. The cell lines were stored in 7 % DMSO at -70°C, melanocytes were stored in 38 % MGM, 11 % DMSO and 51 % FBS and 62 % KGM, 8 % DMSO, and 30 % FBS, respectively at -70°C.

3.3. Bone Marrow Derived Macrophage Preparation

Tibiae and femurs from wild-type (gifts from Dr. Lisa Borghesi, University of Pittsburgh, PA, USA), Nlrp3 -/-, Casp-1 -/-, Tlr3 -/-, Mda-5 -/-, Trif -/-, Ips-1 -/-, Trif -/- Ips-1 -/-, Bax -/-, Bax +/- Bak -/- and Bax -/- Bak -/- mice were collected. BM cells were extracted using a 27-gauge needle and passed through a 21-gauge needle to obtain a homogenous mixture. Cells were plated in petri dishes (Day 1) in DMEM (Mediatech, Inc., Herndon, VA, USA), supplemented with 20% Gemcell™ FBS (Gemini Bio-Products, West Sacramento, CA, USA), 2 mM L-glutamine, 500 U penicillin/500 ug streptomycin (Lonza, Inc., Walkersville, MD, USA), 1 mM sodium pyruvate (MP Biomedicals, Solon, OH, USA), and 30% L cell supernatant. Differentiation media for BMDM were changed every 3–4 days, and BMDM were used for experiments on Days 8–22. For experiments, BMDM were cultured in IMDM (Lonza, Inc.) containing 10% Gemcell™ FBS, 2 mM L-glutamine, and 500 U penicillin/500 ug streptomycin.

3.4. L cell supernatant

L cell fibroblasts (CCL-1 from American Type Culture Collection, Manassas, VA, USA) were incubated in DMEM, supplemented with 10% Gemcell™ FBS, 2 mM L-glutamine, 500 U penicillin/500 ug streptomycin, 1X nonessential amino acids (Irvine Scientific, Santa Ana, CA, USA), and 1 mM sodium pyruvate until confluent. Supernatant was collected and filtered through a 0.22-um filter.

3.5. Plasmids

3.5.1. Plasmids for lentiviral transduction for oncogene expression, tumor-suppressor silencing and ASC knock-down

For lentiviral gene transductions a four plasmid system was used. The pHCMV-G, pRSV-Rev and pMDLg/pRRE plasmids are part of the general mechanism of the lentiviral transduction system, whereas the FG12 and H1 plasmids are the carriers of the genes of interests (gifts from M.S. Soengas). KH1-ASCi-GFP was designed and generated by Burcu Sumer in our laboratory (Sumer, 2006).

Table 3.1. Plasmids used in lentiviral transduction.

Vector	**Source**	**Gene of Interest**	**Function**
pHCMV-G	Gift from M.S. Soengas	VSV-G gene (viral entry into the cell) HCMV promoter, ampicillin resistance gene	These three vectors constitute required proteins for production of infectious virus production. Their roles in the order listed are infection, gene integration and viral packaging.
pRSV-Rev		reverse transcriptase gene	
pMDLg/pRRE		gag, pol genes	

3.6. Lentiviral Transduction

Vector carrying shRNA for ASC were infected into primary human melanocytes or melanoma cells using the two-step Lentiviral transduction system. In step one, either via lipofectamine (Lipofectamine™ 2000 Transfection Reagent from Invitrogen) transfection or via calcium phosphate transfection plasmids were transfected into 293T packaging cells to produce virus of interest. In the second step the generated viruses were used to infect the melanoma cells. CaPO4 transfection is explained in this section, whereas lipofectamine transfected is covered in a separate section since it was employed both for virus production on 293T cells and pIC stimulation on BMDMs, melanoma lines and human primary melanocytes.

3.6.1. Calcium Phosphate Transfection of HEK 293T Packaging Cell Line

One day before the transfection $6x10^6$ Human embryonic kidney 293T (HEK 293T) viral packaging cells were plated into a 10 cm^2 cell culture dish for each sample. On the second day, the medium of the cells was replenished with nine ml $DMEM^+$ containing 25µM chloroquine. The plates were put into the incubator until the plasmid/calcium phosphate colloids were prepared. To prepare the plasmid/ calcium phosphate colloids firstly a calcium chloride solution was prepared containing 250 mM calcium chloride, 4 µg of helper plasmids pHCMV-G, pRSV rev, pMDLg, and 4 µg of plasmid with the gene of

interest in a final volume of 500 µl. This plasmid containing calcium chloride solution was added onto 500 µl 2X HEPES Buffered Saline (HBS) solution (280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4 • $2H_2O$, 12 mM dextrose (D-glucose) and 50 mM HEPES with a pH adjusted to seven with 0.5 N NaOH (filter sterilized with a 0.22 µm filter) slowly. The 1 ml micropipette was used to produce bubbles in the HEPES solution to increase the colloidal precipitation. The solution was incubated for 5 minutes at room temperature and added dropwise onto the HEK 293T cells. Cells were transferred into the CO_2 incubator, their medium was refreshed after 8 hours. On the third day, the viral particles were ready for infection. From this point on, biosafety level two rules were followed strictly while handling anything that came into contact with virus particles.

3.6.2. Lentiviral Infection of melanoma cells

On the third day of the lentiviral transduction procedure, $5x10^6$ melanoma cells were plated into 10 cm^2 dishes for each infection condition. On the fourth day, the supernatants of the transfected HEK 293T cells were collected using a 10 ml syringe. Onto the tip of the syringe, polybreen with the final concentration of 8 µg/ml had been added. The supernatants was filtered through a 0.45 um filter and 4 ml of the filtered supernatant was added directly onto the cells after aspirating their medium. After one hour 5 ml fresh medium was added. Six ml $DMEM^+$ was added to the HEK 293T cells. After 4 hours the supernatant of the HEK 293T cells were collected again and the melanoma cells were infected for a second time following the procedure. The cells were incubated for four additional hours and after a total of eight hours the media of the melanocytes plates were refreshed to remove the polybreen. GFP production could be monitored after 48 hours. For the melanoma cells infected with shRNA plasmids, the knock-down of the particular protein could be monitored after 72 to 96 hours.

Due to biosafety level 2 regulations, plastic and glassware were bleached (10 % bleach in water) and the trash was autoclaved before disposal. Bleach was also used to kill the packaging HEK 293T cells and they were discarded after additional UV exposure. The surfaces of the hood were cleaned with bleach solution and 80 % ethanol and exposed to UV for at least for 20 minutes. Disposable coats and double gloves were used during cell culture.

3.7. Lipofectamine transfection

Lipofectamine transfection was used for lentivirus production, retrovirus production and intracellular pIC stimulation. All the lipofectamine transfections were done with Lipofectamine™ 2000 Transfection Reagent from Invitrogen and the following procedure suggested by the company. One day before the transfection cells were plated on 10cm plates (for virus production), 48 well plate (pIC stimulation for cytokine secretion) or 6 well plates (pIC stimulation for western blot analysis). On the day of transfection, the plates were 50-90% confluent depending on cell type and purpose.

For pIC infection, Lipofectamine in the same amount (by mass) as the plasmid of interest was used. For virus production the Lipofectamine amount was three fold of the plasmid to be transfected. Plasmid/pIC and lipofectamine were incubated separately in equal volume of Opti-MEM Reduced Serum Medium for 5 minutes. Then two solutions were added together and this new plasmid-lipofectamine or pIC-lipofectamine mixture was incubated for 20 minutes. Then the solution was applied on the cells. Cells were incubated in the incubator for 2, 4, 6 hours or 48 hours depending on experiment.

3.8. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

Cells were harvested, washed, and lysed or lysed directly in wells with Nonidet P-40 lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich). Bradford assay (Bio-Rad, Hercules, CA, USA) was conducted to determine protein concentration of lysates. The collected cell samples were boiled at 95°C for five minutes prior to electrophoresis. 20–50 ug protein lysate was run on SDS-PAGE gels at 150V for 80 minutes. A semi-dry transfer apparatus (BioRad, USA) was used for the transfer of proteins to the PVDF membrane (Millipore, Ireland). Thick blotting papers (Sigma-Aldrich, USA) and the membrane was cut to the size of the gel. The PVDF membrane was wetted in absolute methanol for 30 seconds and put into ice cold transfer buffer (0.293 % (w/V) glycine, 0.582 % (w/V) Tris-base, 25 % Methanol (pH 9.0)) together with blotting papers and the acrylamide gel. The transfer sandwich was prepared according to the manufacturer's instructions and the transfer was done at 20V for 2 hours.

The resulting membrane put into the blocking solution (5 % fat-free milk powder in 0.9 % (w/V) sodium chloride, 10% 1M Tris-HCl (pH 7.5), 1 % Tween (TBST) and incubated for one hour with mild shaking. The membrane was transferred to a sealed bag with five ml of primary antibody solution. Solution and dilution for primary antibody was prepared according to the manufacturer's suggestions. On the next day, the membrane was washed in TBST for 1x2', 3x15' and incubated with the 1:5000 diluted secondary antibody in same solution used for primary antibody exposure. The membrane is washed as previously. Western blotting luminol reagent (Santa Cruz Biotechnology) was applied, film exposed to membrane were developed and analyzed. In some cases, the same blot was stripped with Restore Plus Western blot stripping buffer (Thermo Scientific, Rockville, MD, USA), according to the manufacturer's protocol, and reprobed.

3.8.1. Antibodies

Human ASC antibody was collected as supernatant from ASC antibody producing hybridoma cells (gift from J. Masumoto). Cell supernatant was diluted with 3% milk in TBS in 1:1 ratio and was followed by anti-rat secondary antibody from Sigma-Aldrich, USA. Human Caspase-1 antibody was purchased from Cell-Signaling, US and was followed by anti-rabbit secondary antibody from Sigma-Aldrich, USA. Purified mouse anti-Bax, antibody for active Bax (anti-Bax clone 6A7), and antibody for Bak were purchased from Santa Cruz. Caspase-1 anti mouse was produced in Nunez Lab in rabbit and raised against the p20 subunit. Mouse anti IL-1 beta was purchased from R&D systems.

3.9. LDH Release Assay (Cell Membrane Permeabilization / Cell Death)

Triton X-100 (1%) was used as a control of complete cell lysis and maximal LDH release. Supernatants were collected and assayed in duplicate for LDH content using the LDH cytotoxicity assay kit from Cayman Chemical Co. (Ann Arbor, MI, USA). Percent cytotoxicity was calculated as follows:

$$\left(\frac{\text{mean OD value of experimental sample} - \text{mean blank OD}}{\text{mean OD value of Triton X-100 control sample} - \text{mean blank OD}}\right) \times 100$$

3.10. K^+ Depletion Assay

After supernatants were collected from 48 well plates for LDH or ELISA assays, the cells were incubated in 10% Nitric Acid (HNO3) in dH2O for 20 minutes. Using Yttrium (Y) as an internal control K+ concentration was measured by ICP-OES with emission lines of 371.029 for Y and 766.490 for K+.

3.11. Reverse transcription PCR

Total RNA was isolated using a total RNA isolation kit (Qiagen). cDNA was synthesized using the iScript cDNA synthesis kit (BioRad) and SYBRgreen master mix (Applied Biosystems) was used for QPCR. Actin was used for normalization. The following primer sequences (produced and supplied by Invitrogen, USA as designed) were used:

Table 3.2. Primer nucleotide sequences

Primer	Nucleotide sequence	Primer Length
NLRP3 forward	CGT GAG TCC CAT TAA GAT GGA GT	23
NLRP3 reverse	CCC GAC AGT GGA TAT AGA ACA GA	23
AIM2 forward	ACT TGC TGC ACC AAA ACT CT	20
AIM2 reverse	CTT GGG TCT CAA ACG TGA AGG	21
NLRC4 forward	TCA GAA GGA GAC TTG GAC GAT	21
NLRC4 reverse	GGA GGC CAT TCA GGG TCA G	19
ASC forward	TGG ATG CTC TGT ACG GGA AG	20
ASC reverse	CCA GGC TGG TGT GAA ACT GAA	21
CASPASE-1 forward	TTT CCG CAA GGT TCG ATT TTC A	22
CASPASE-1 reverse	GGC ATC TGC GCT CTA CCA TC	20
IL-1 beta forward	AGC TAC GAA TCT CCG ACC AC	20

Table 3.2. Primer nucleotide sequences (continued)

Primer	Nucleotide sequence	Primer Length
IL-1 beta reverse	CGT TAT CCC ATG TGT CGA AGA A	22
ACTIN forward	GTC TGC CTT GGT AGT GGA TAA TG	23
ACTIN reverse	TCG AGG ACG CCC TAT CAT GG	20

3.12. Immunofluoresence

The day before the stimulation, BMDMs were plated on 48 well dish, 200ul each well with concentration of 1 million cells per ml. On the next day, following LPS priming for 2 hours, cells were stimulated with transfected pIC or Staurosphorine for 2, 4 and 6 hours.

At the end of the stimulation, the supernatant was removed. Cells were washed once with PBS and then fixed with 4% formaldehyde at room temperature for 20 minutes. Formaldehyde was removed and the cells were washed with PBS.

The fixed cell samples were blocked and permeabilized with blocking buffer (1X PBS, 5% serum, 0.3% Triton X-100) for 2 hours at room temperature. The blocking buffer was removed and cells were washed once with PBS. Primary antibody for active form of BAX was diluted 1:1000 in blocking buffer and applied to cells for 2 hours at room temperature. Primary antibody solution was removed and the cells were washed with PBS 3 times. Secondary antibody was diluted 1:1000 in blocking buffer and applied for 2 hours at room temperature. The secondary antibody solution was removed and the cells were washed with PBS three times. Cells were observed under fluorescent microscope.

4. RESULTS

4.1. PART I : Role of ASC in melanoma

4.1.1. Inflammasome activation in primary melanocytes versus melanoma cells

ASC, the adaptor protein in NLRP3, NLRC4, AIM2 and NLRP1 inflammasomes was reported to be down-regulated in human melanoma cells, compared to human primary melanocytes (Conway KE, TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers ., 2000). This finding which reveals intact Asc mRNA expression in melanocytes brought the assumption that ASC protein was present in melanocytes. Although was not shown explicitly, we and others speculated that not only ASC but also other inflammasome components were produced by melanocytes whereas the melanoma cells were targeting this immune response ineffective by silencing ASC.

It was obvious that if the melanocytes really had required proteins constituting inflammasomes, then they should have responded to DAMPs and PAMPs by activating corresponding inflammasomes and secreting IL-1B. We started our study by testing this main assumption of the capability of melanocytes to fire their NLRP3 inflammasome.

We started stimulating primary melanocytes with various pathogen associated molecular patterns, as well as a cancer drug and ATP which were known to cause NLRP3 inflammasome activation and cell death and inflammasome activity. The purpose of the experiment was to see which pathways were intact and could be stimulated in primary melanocytes. Our expectation was to see robust IL-1B secretion and cell death when the cells were primed with LPS and stimulated with ATP, because this combination is the golden standard for testing NLRP3 inflammasome activation.

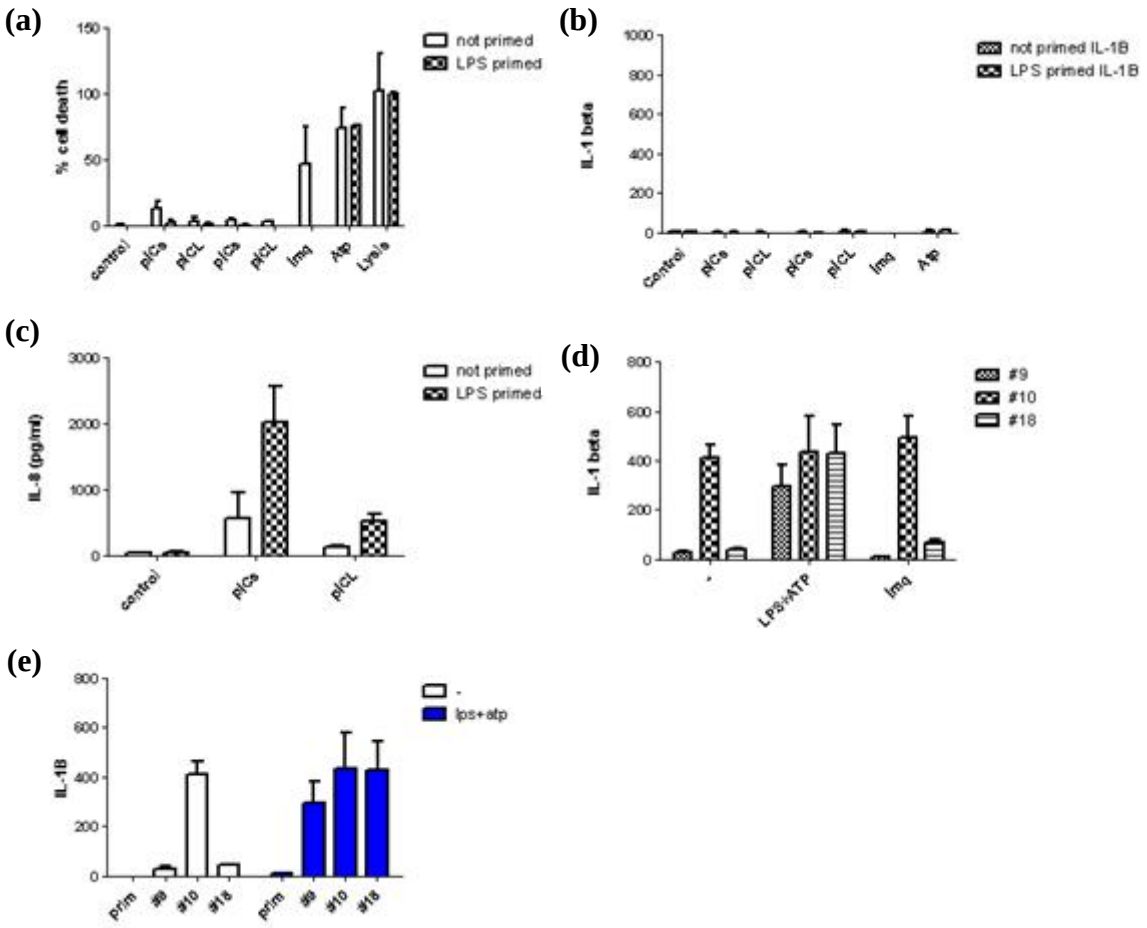

Figure 4.1. Response of primary melanocytes and melanoma cells to inflammatory stimuli. Human primary melanocyte (a) cell death, (b) IL-1 beta secretion in response to various inflammatory stimuli, (c) IL-8 secretion in response to cytosolic pIC when the cells were primed with LPS or without priming, (d) IL-1 beta secretion in response to ATP and imiquimod. (e) Comparison of human primary melanocytes and three melanoma cell lines.

The cells were primed for 2 hours with 1 µg/ml LPS prior to extracellular ATP stimulation. Then ATP was added to supernatant to have a final concentration of 5mM. Although change in cell morphology can be observed after 6 hours of pIC treatment and 30 minutes of ATP treatment, this time interval was not sufficient to cause any effect on melanocytes. So, cells were kept o/n and the next they the supernatant was collected for LDH assay and ELISA test.

As shown in Figure 4.1a, after 24 hours of stimulation, imiquimod and ATP caused robust cell death. It is worth noting that ATP causes cell death at 24 hours independent of

inflammasome activity due to continuous disruption of cytosolic ion concentrations. Intracellular, as well as extracellular pIC, did not cause plasma membrane disruption, even when the cells were primed with LPS.

The same samples were used for ELISA test in order to measure the concentration of IL-1B secreted in the supernatant. Transfected pIC or extracellular pIC, as well as Imiquimod resulted in no IL-1B secretion. What was totally unexpected was that, even though the LPS+ATP combination killed all the cells, IL-1B secretion was totally absent, which showed that the NLRP3 inflammasome was not activated (Figure 4.1b).

The observation that none of the stimuli resulting in IL-1B secretion was unexpected. The first trivial explanation would be that the melanocytes had no receptor for LPS at all. If the cells were not even recognizing the LPS, priming would be ineffective, and cells would not increase transcription of inflammasome components. However, it was already shown in the literature that the LPS receptor TLR4 was expressed by and functional in primary melanocytes. In order to clarify this point, we checked secretion of another cytokine, IL-8 which was secreted independent of inflammasome activity, in the cells treated with pIC, in the absence and presence of LPS priming to make sure that the LPS was recognized by the cells and that it was the NLRP3 inflammasome activation that was absent.

IL-8 secretion in response to intracellular short and long pIC increased three to four fold, reaching 2000pg/ml and 500pg/ml respectively, when the cells were primed with LPS (Figure 4.1c). Hence, the primary melanocytes express TLR4 and can recognize extracellular LPS.

This result showed that LPS priming was working in human primary melanocytes but NLRP3 inflammasome was not functional.

Next we checked whether the metastatic melanoma cell lines could activate the NLR3 inflammasome in response to LPS+ATP treatment. We have treated 3 different melanoma cell lines with LPS+ATP and also imiquimod. All the melanoma cells

responded LPS+ATP treatment by secreting IL-1B. Moreover, one of the melanoma lines, #10, was secreting IL-1B even in the absence of stimuli.

As we compared IL-1B secretion of primary melanocytes, we have seen that it's not the primary melanocytes but the melanoma cells that have intact NLRP3 inflammasome components, and can secrete IL-1B in response to stimuli.

As these results were seen in contradiction to the literature, which during the last decade agreed that, ASC was down regulated in melanoma, we wanted to check if this was really the case. The first report on ASC silencing was the result of a study comparing mRNA levels of a large set of proteins in melanoma. So we performed rtPCR to see the expression levels of main inflammasome components in primary melanocytes and in melanoma cells lines.

4.1.2. Expression of inflammasome components in primary melanocytes versus melanoma cells in the mRNA level

Since it was first identified as the target of methylation induced silencing, ASC was taken to be a protein that is expressed in human primary melanocytes and was considered to be down-regulated in melanoma lines. We were tempted to check the expression of NLRP3 inflammasome components in primary melanocytes and melanoma lines, because of our observation that, in terms of functionality, the phenomenon was opposite: NLRP3-ASC-CASPASE-1 inflammasome was activated in melanoma lines, however, did not respond to stimuli in human primary melanocytes, as measures by IL-1B cytokine secretion.

Primary melanocytes and two different metastatic melanoma lines were examined for the mRNA levels of NLRP3, NLRC4, AIM2, ASC and CASPASE-1 using real time PCR.

NLRP3 was upregulated more than a hundred fold in mRNA level in melanoma cell lines compared to primary melanocytes (Figure 4.2a). To our knowledge this is the first report on NLRP3 expression comparing melanoma cells to primary melanocytes. Increased level of NLRP3 expression was in line with IL-1B results.

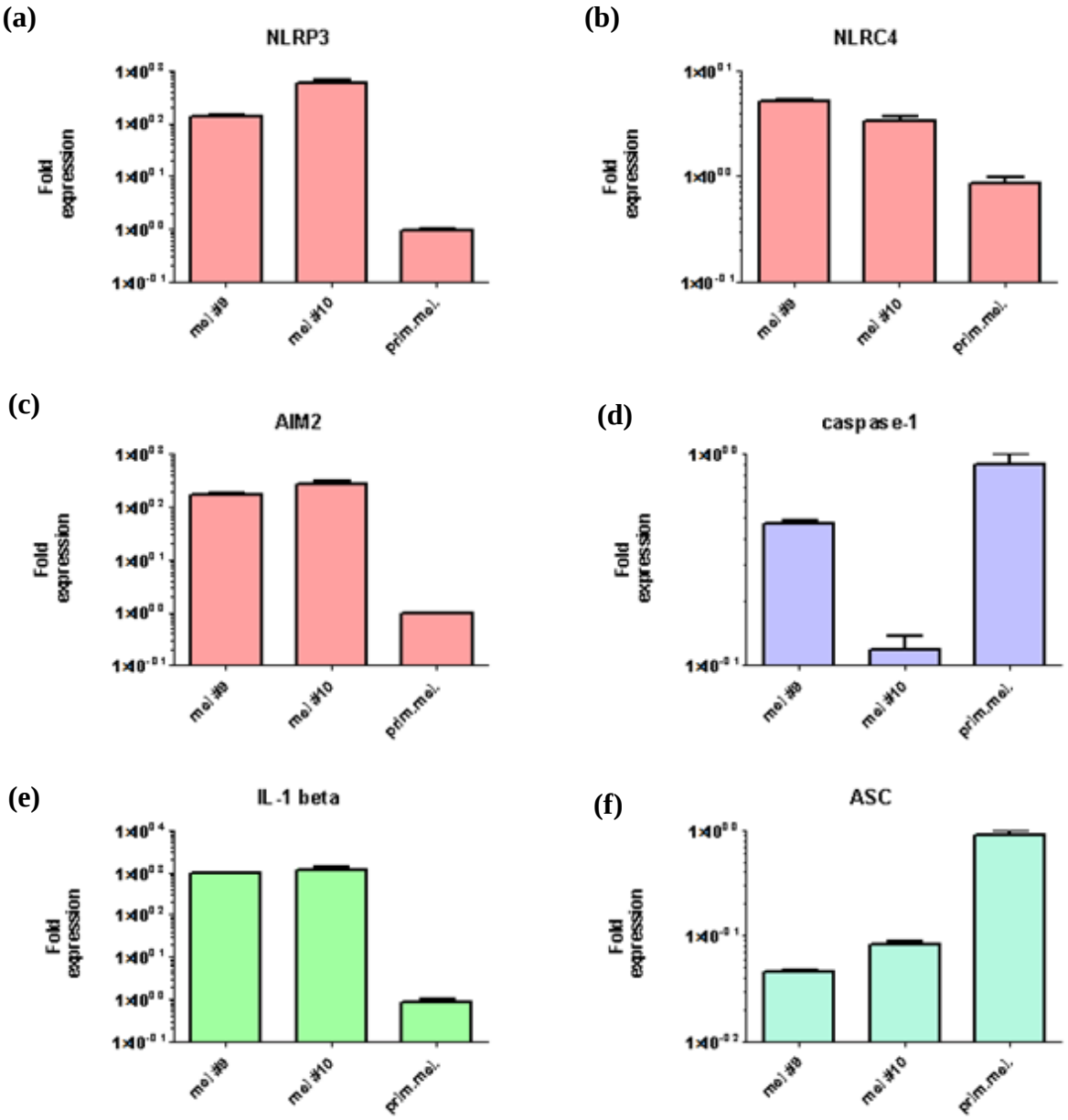

Figure 4.2. Expression levels of inflammasome proteins as determined by rtPCR. For each protein mRNA levels are shown for melanoma cell lines #9, melanoma cell line #10 and human primary melanocytes. (a). Nlrp3 expression. (b). Nlrc4 expression. (c). Aim2 expression. (d). Caspase-1 expression. (e). IL-1 beta (pro form) expression. (f). Asc expression.

The other receptor proteins of two different inflammasomes, NLRC4 and AIM2 were also found to be upregulated at the transcription level (Figure 4.2b and 4.2c).

Just like NLRP3, pro form of IL-1B, namely, pro-IL-1B expression was increased almost a thousand fold, making IL-1B secretion results strongly supported (Figure 4.2e).

The main role in regulation of IL-1B secretion belongs to Caspase-1, which cleaves the pro-IL-1B into its active form, to be secreted. Melanoma cell line #9 showed no dramatic difference, however, melanoma cell line showed 10 fold decrease in CASPASE-1 mRNA levels (Figure 4.2d).

Our main interest was ASC. Just as claimed in the literature it was down regulated at least ten fold in both melanoma lines (Figure 4.2f).

So, how was it possible to have less ASC and more inflammasome activity at the same time? As we reviewed the literature on the absence of ASC in melanoma, we have noticed that, all the published studies relied on mRNA levels, lacking protein level measurements.

The general picture of mRNA expression of inflammasome proteins at mRNA level suggests that the melanoma cells were already in a 'primed state' as they have both Nlrp3 and Il-1b upregulated enormously. Smartly, this hyper activated state was compensated by down-regulation of Asc and Caspase-1. Otherwise the cells would die due to continuous strong caspase activity. Overall, the cells are ready for a small activating signal to fire their inflammasomes, putting them in primed state by default.

4.1.3. ASC protein is available in melanoma cells but absent in human primary melanocytes

Although it is a low possibility to have less mRNA expression but more protein in a cell, we checked ASC protein amount in primary melanocytes and melanoma cells using western blotting. We have seen that none of the primary melanocytes from four different individuals had any detectable ASC protein (Figure 4.3 and data not shown). Being curious, this could be explained by many factors such as, inefficient western blotting, low

number of cells etc. However we should mention that, there are previous studies which report ASC expression in primary melanocytes in protein level.

To clarify the issue, we have tested ASC levels of 8 different melanoma cell lines and primary melanocytes, using the same number of cells. The result was that, the melanoma lines had the ASC protein, but the primary melanocytes did not (Figure 4.3). This was not totally in line with the first IL-1B results we got, but was also a novel finding stating that : though ASC expression was lowered at the transcription level, ASC protein levels were increased. Actually, it appears that it's the primary melanocytes but not the melanoma that lack ASC. In fact, melanomas had ASC available.

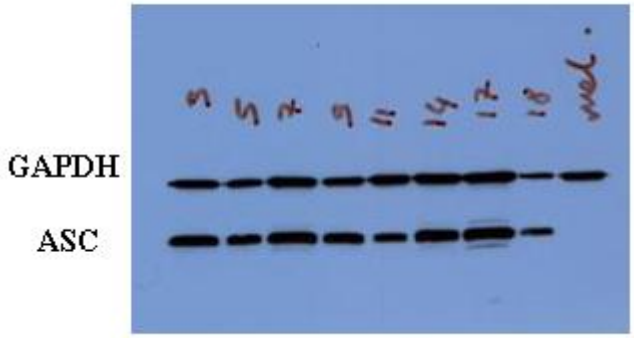

Figure 4.3. ASC protein is present in all eight melanoma cells lines examined, but not in primary melanocytes. First eight columns represent different melanoma cell lines labeled with their line numbers, the last column represents primary human melanocytes. GAPDH levels were shown as internal control.

4.1.4. Knock down of ASC in melanoma cells suppresses IL-1 beta secretion

In order the confirm the functionality and relevance of increased ASC protein levels in melanoma cell lines #9 and #10, these cells were lentivirally transduced to knock down ASC. Lentiviral transduction was employed to stably block ASC expression through RNA interference.

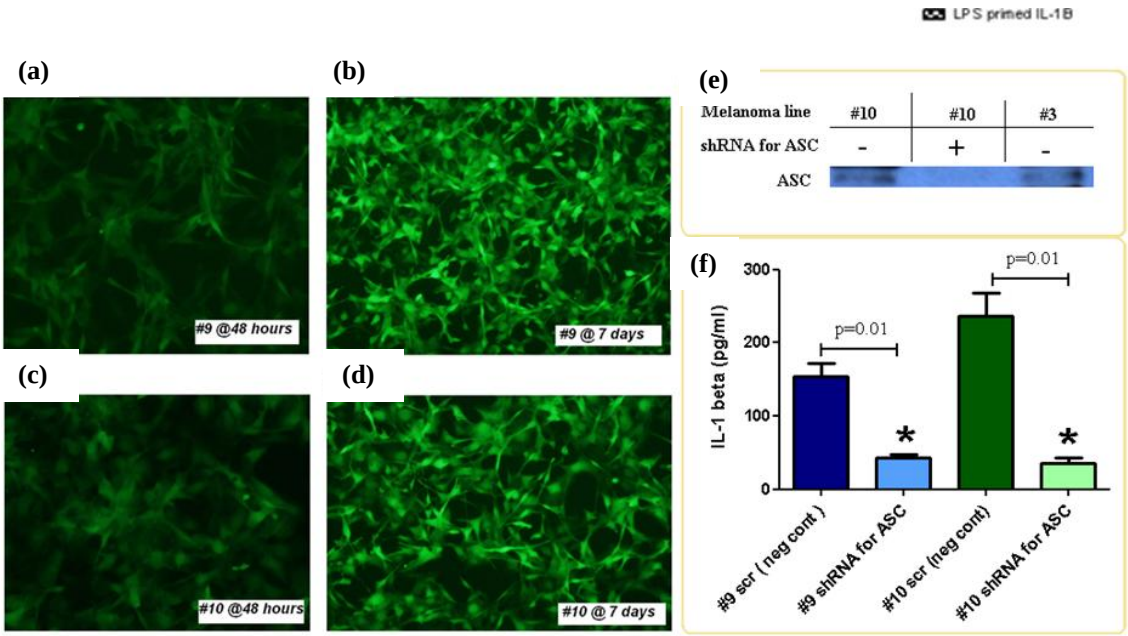

Figure 4.4. ASC knock-down in melanoma cells suppresses IL-1 beta secretion. Viral infection efficiency in melanoma line #9 at (a) 48 hours , (b) 7 days after infection and in melanoma line #10 at (c) 48 hours and (d) 7 days after infection. (e) ASC protein levels in melanoma cell line #10 when transfected with scrambled vector and shRNA for ASC. (f) ELISA results for two melanoma lines with or without ASC knock-down.

Using 293T cells as packaging cell line, we produced two lentivirus strains: one carrying shRNA for Asc, and one carrying scrambled sequence shRNA for Asc as negative control. Melanoma cell lines #9 and #10 were infected with lentivirus carrying shRNA for Asc and negative control lentiviruses. Efficiency of the infection was monitored at 48 hours and at seven days through GFP expression. After two weeks of incubation for stability, we measured ASC protein level by Western Blotting. ASC expression was diminished as expected (Figure 3.4, Panel E). Then we stimulated both ASC-expressing and ASC-knock-down strains of melanoma lines #9 and #10 with ATP after priming with LPS. We observed dramatic decrease in IL-1 beta secretion in both cell lines when their ASC expression was blocked. Melanoma line #9 IL-1 beta secretion decreased from 150pg/ml to 40pg/ml, melanoma line #10 IL-1 beta secretion decreased from 240pg/ml to 30pg/ml (p=0.01). This shows that ASC is not only present in these melanoma lines but also functional and required for IL-1 beta secretion.

4.2. PART II : NLRP3 inflammasome activation in response to dsRNA

4.2.1. Intracellular pIC and bacterial RNA activates caspase-1 through NLRP3 inflammasome

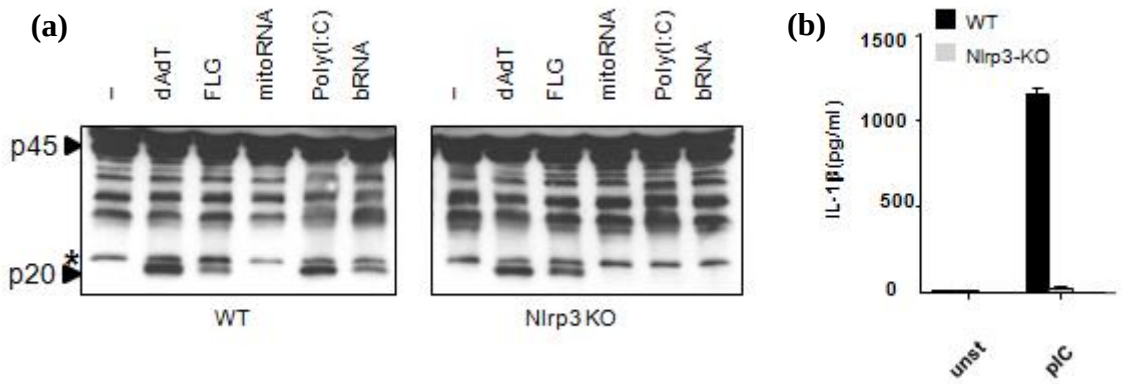

Figure 4.5. Activation of NLRP3 inflammasome by pIC and bacterial RNA. BMDMs were primed prior to stimulation. (a). Caspase-1 cleavage analysis for WT and Nlrp3-KO BMDMs treated with cytosolic dsDNA(dAdT), flagellin (FLG), mitochondrial RNA (miroRNA), pIC and bacterial RNA (bRNA). (b). Il-1 beta secretion in WT and Nlrp3-KO macrophages in response to cytosolic pIC.

First we confirmed that cytosolic bacterial dsRNA and cytosolic synthetic dsRNA analog pIC activates NLRP3 inflammasome. Bacterial dsRNA, as well as pIC, caused Caspase-1 cleavage (Figure 4.6a) and IL-1 beta secretion (Figure 4.6b) in an NLRP3 dependent manner. We also show that mitochondrial RNA does not cause inflammasome activation ruling out any role of mitochondrial RNA in observed activation.

Double stranded DNA (dAdT) and flagellin (FLG), which are sensed by AIM2 to and NLRC4 respectively, were used as controls. As expected, absence of NLRP3 had no effect on Caspase-1 cleavage in response to dsDNA and flagellin.

Our results confirmed that cytosolic synthetic dsRNA analog pIC activates NLRP3 inflammasome and NLRP3 inflammasome activity is required for Il-1 beta secretion.

4.2.2. IPS-1 is required for inflammasome activation by pIC

In order to figure out the pathway through which pIC is activating inflammasome through, we have used bone marrow derived macrophages from mice which do not express dsRNA receptors and downstream proteins.

First, we wanted to rule out the possibility that, even when we transfect the pIC into the cytoplasm, the effect might be due to the stimulation of extracellular pIC which is also available in the media. We have seen no decrease in secreted IL-1B in Tlr3-KO cells. This suggests that, there is no role of extracellular dsRNA receptor of TLR3 in the recognition of transfected pIC. Hence, the recognition was intracellular (Figure 4.7a).

Next, we have analyzed the cells not expressing MDA5 which is known to be a cytosolic receptor protein for long dsRNA. There was no abrogation in IL-1B secretion (Figure 4.7b).

The downstream proteins of extracellular and cytosolic dsRNA recognition pathways are TRIF and IPS-1 respectively. Seeing no effect of known receptor proteins, we have treated bone marrow derived macrophages form Trif/Ips-1 double KO mice with pIC. In this case IL-1B secretion was totally diminished (Figure 4.7e)

This result might lead to three different possibilities: Either TRIF of IPS-1 had the major role on signal transduction or they acted redundantly.

In order to clarify the point, we have performed the same experiments with bone marrow derived macrophages from Trif-KO and Ips-1-KO mice, separately. The results showed that it was IPS-1 that was required for inflammasome activation and TRIF had just a minor enhancing role, if any at all (Figure 4.7c and 4.7d).

Confirming our findings with IL-1B secretion, in western blot results, we saw cleaved form of CASPASE-1 in the lysates of WT cells. The cleavage was totally absent in the DKO samples, and was weak in Ips-1 KO samples.

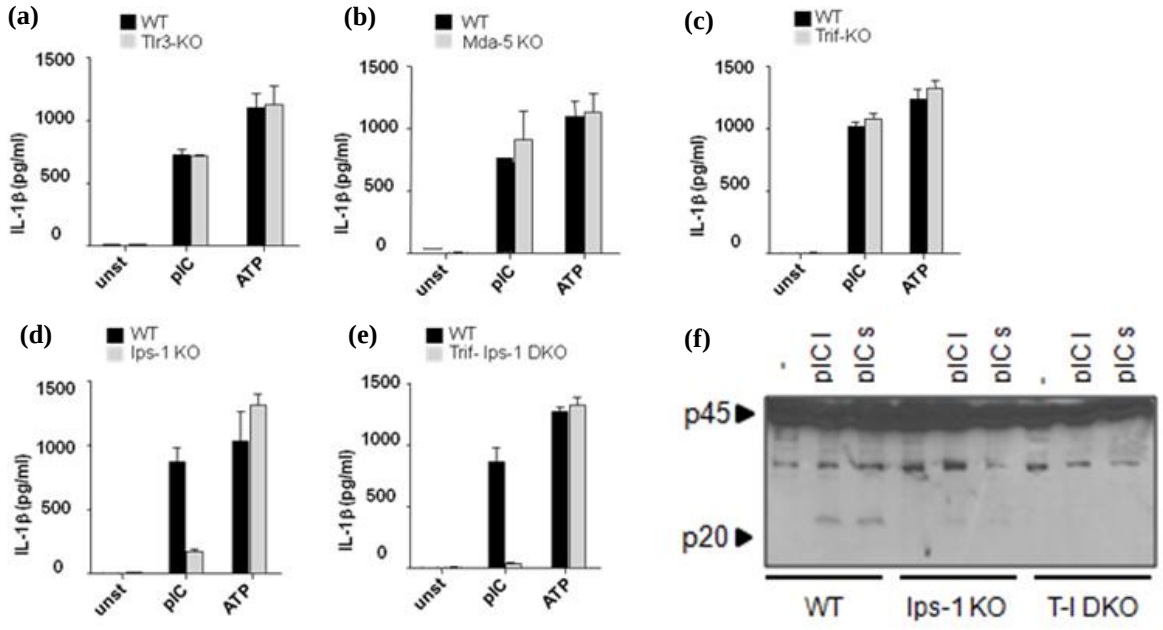

Figure 4.6. NLRP3 inflammasome activation in response cytosolic pIC requires Ips-1. Inflammasome activation in response to pIC and ATP in BMDMs from mice knocked-out for PAMP receptors. (a-e) IL-1 beta secretion in Tlr3-KO, Mda-5 KO, Trif-KO, IPs-1 KO, and Trif/Ips-1 DKO cells. (f) Western blot analysis for CASPASE-1 cleavage in response to pIC in Ips-1 KO and Trif/Ips-1 DKO cells.

One can sugegst that the Trif/Ips-1 KO cells do not have an intact NLRP3 inflammasome, hence the result we see here may be not due to the role of these two proteins in pIC dependent inflammasome activation, but it may be due to a side effect which renders NLRP3 inflammasome totally infunctional. To check his, we stimulated cells with nigericin which is known to activate the NLRP3 inflammasome as a positive control. Nigericin resulted in CASPASE-1 cleavage in all the WT, Ips1 KO and Trif/Ips-1 DKO cells (Figure 4.8). This results showed that the phenotype of the absence of Caspase-1 activation and Il-1 beta secretion in response to dsRNA is not a general unresponsiveness for any stimuli due to nonfunctional NLRP3 inflammasome, but it’s specific to dsRNA.

Our findings until this point demonstrate that IPS-1 is the major protein controlling the downstream pathways including, most importantly, NLRP3 inflammasome activation. The main question is then, how does IPS-1 activity cause NLRP3 inflammasome activation if these two mechanisms have no known interaction at all?

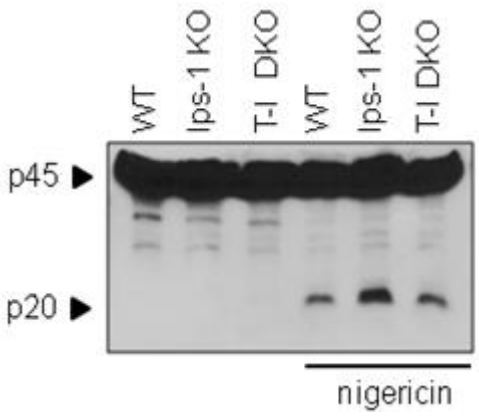

Figure 4.7. Ips-1 KO and Trif/Ips-1 DKO have functional NLRP3 inflammasome mechinary.

4.2.3. Membrane permeabilization correlates with NLRP3 activation

What we noticed was the expected fact that, in all the experiments we performed with Tlr3, Mda5, Trif, Ips1 and Trif/Ips1 KO cells, IL-1B secretion directly correlated with cell death as we saw by naked eye under the microscope. LDH assay confirmed our subjective observations. When BMDMs deficient in Tlr3, Mda5, Trif were treated with cytosolic pIC or when they were infected with salmonella (positive control), we observed around 60% and 80% cell death, respectively (Figure 4.8a-c). However pIC mediated cell death was decreased to 20% in Ips-1 KO cells and to 10% in Ips-1/Trif DKO cells (Figure 4.8d and 4.8e). Salmonella mediated cell death was not affected. Observed cell death showed the same pattern as Il-1 beta secretion results (compare Figure 4.6 to Figure 4.8).

Cell death was totally expected since it is already well established that NLRP3 activation causes cell death through active Caspase-1. So it's plausible to comment that cell death was an effect caused by inflammasome activation.

4.2.4. Membrane permeabilization is independent of NLRP3 inflammasome activation

As we checked this assumption, we made the central observation of our study: Cell death stemming from dsRNA stimulation was independent of Caspase-1 activity. BMDMs

from WT and Caspase-1 KO mice were treated with cytosolic PIC and were infected with Salmonella. Caspase-1 dependent Salmonella mediated cell death was totally abolished in BMDMs deficient in Caspase-1, however pIC dependent cell death was independent of Caspase-1 (Figure 4.9).

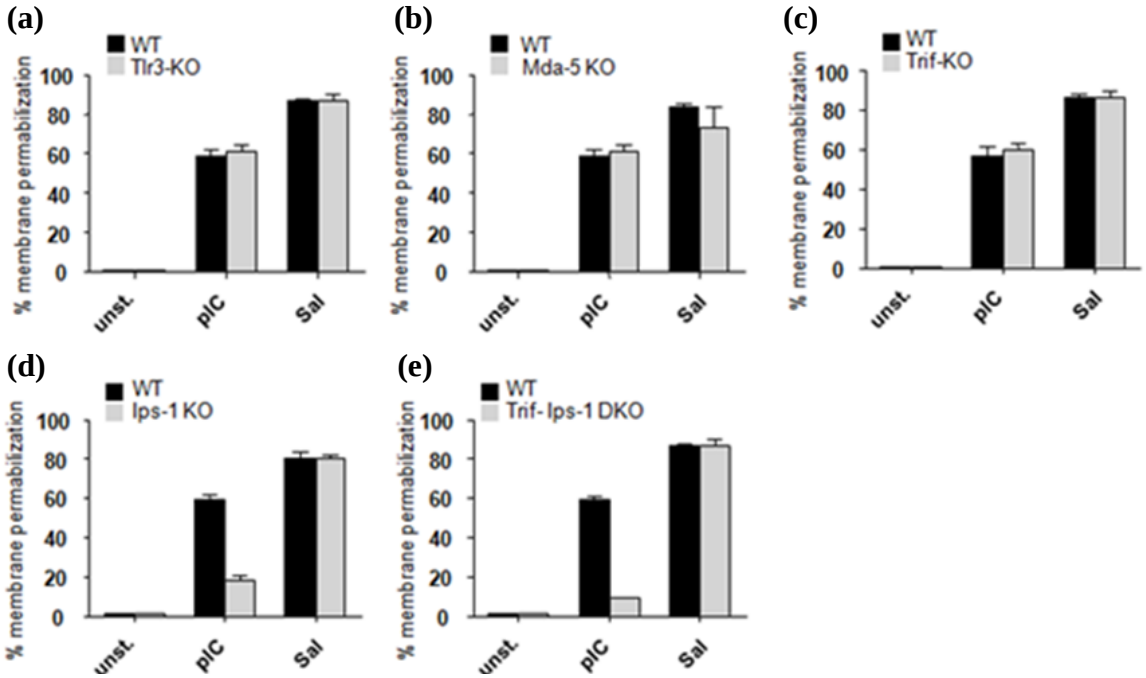

Figure 4.8. Membrane permeabilization requires Ips-1. A-E Membrane permeabilization in response to cytosolic pIC and Salmonella infection in Tlr3-KO, Mda-5 KO, Trif-KO, IPs-1 KO, and Trif/Ips-1 DKO cells. The cells were primed with LPS (1ug/ml) and treated with transfected pIC (10ug/ml) or were infected with Salmonella for six hours.

This result immediately gave rise to the hypothesis that if NLRP3 inflammasome activation is not the cause of cell death. The relation could be just the opposite; the same damage resulting in the form of cell death could be the activating signal for NLRP3 inflammasome.

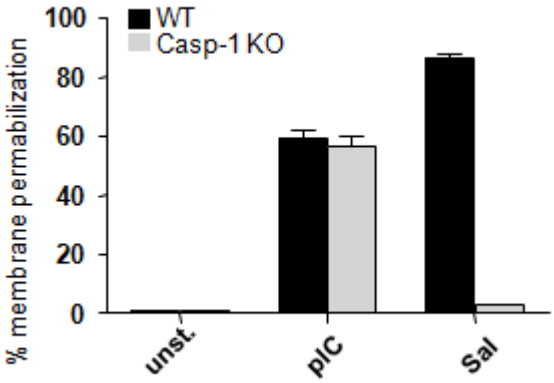

Figure 4.9. Membrane permeabilization in response to cytosolic pIC is independent of Caspase-1 activity. BMDMs were primed with LPS and treated with transfected pIC or were infected with Salmonella.

4.2.5. Cytosolic pIC activates BAX

It has been shown previously that IPS-1 can cause cell death can be inhibited by anti-apoptotic bcl-2 family member bcl-x_L (Besch *et al.*, 2009). So we checked whether cytosolic pIC triggers the activation of pro-apoptotic Bcl2 family members.

First, involvement of bcl-2 pro-apoptotic family member BAX was investigated. After BMDMs were treated with pIC or ATP following LPS priming, the supernatant was removed and the cells were fixed and incubated with antibody for active form of BAX as explained in the Methods section.

There was no signal with the samples treated with LPS alone or LPS and ATP. Samples primed with LPS and treated with pIC, on the other hand, had active form of BAX (Figure 4.10). Staurosphorine was used as a positive control to see activated BAX. As can be seen in higher magnification pictures, although not as strong as Staurosphorine, pIC treatment caused a comparable BAX activation.

Quantification of the observed BAX activation by counting the number of the cells with positive signal and calculating the ratio to all cells showed that pIC resulted in BAX

activation in 20% of cells whereas Staurosphorine treatment resulted in activation in around 50% of the cells.

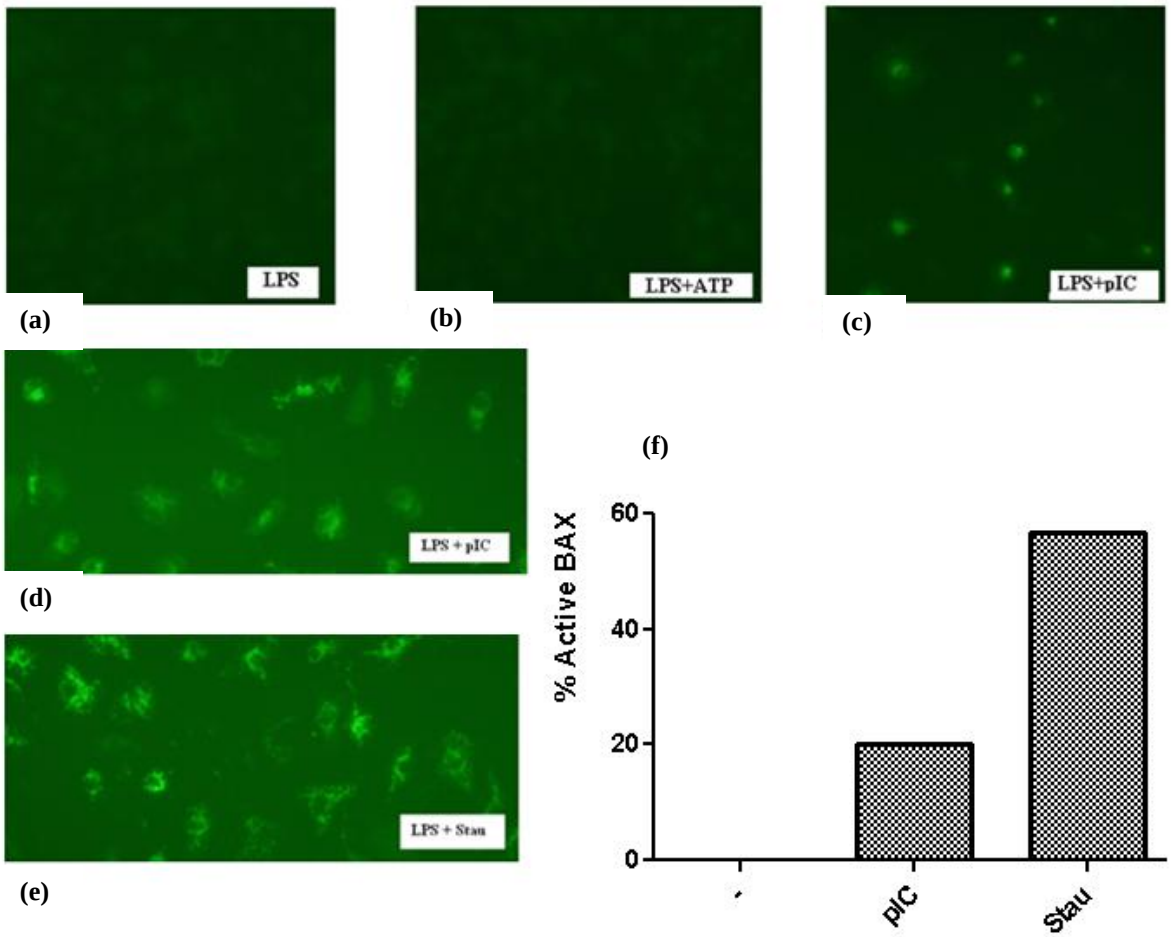

Figure 4.10. Cytosolic pIC induces BAX activation in BMDMs primed with LPS; ATP does not. Immunoflourescence for active BAX.ATP treatment is 30 mins, pIC and staurosphorine treatments are 6 hours. (a) No treatment. Cells treated (b) with ATP, (c) with pIC, (d) with pIC (high resolution) (e) with staurosphorin. (f) Ratio of active BAX flourescence positive cells to total popularion in cells treated with pIC and staurosphorine.

It's important to note that for the particular experiment having 20% of the cells with activated BAX does not explain the stronger phenotype we observed in terms of membrane permeabilization and IL-1B secretion. So, even though we had higher ratio in some other experiments, this suggests that there may be other players linking IPS-1 signaling to NLRP3 activation.

This result showed that pIC activates BAX as expected due its effect on IPS-1 signaling. However this does not mean that BAX activation is required for subsequent NLRP3 inflammasome activation and IL-1B secretion.

4.2.6. IPS-1 acts through Bax/Bak causing membrane permeabilization.

In the light of the observation that BAX is activated, we were curious whether this activation had any effect on signaling through NLRP3; in other words whether BAX is required for NLRP3 activation or not.

Bak, another apoptotic member of Bcl-2 family, has a similar function to Bax. Hence, with the idea that focusing on BAX alone would leave some gap between IPS-1 and cell death, we considered Bax/Bak together as probable linker of IPS-1 to NLRP3 activation.

To analyze whether Bax/Bak is required for pIC mediated NLRP3 inflammasome activation, we stimulated DMDMs from Bax+/- Bak-/- and Bax-/- Bak-/- knock-out mice with transfected pIC. The membrane permeabilization was measured for increasing doses of pIC; 2.5ug/ml, 5ug/ml and 10ug/ml.

Compared to wild type cells, cell membrane permeabilization decreased 40-60% in BMDMs from Bax/Bak double knockout mice (Figure 4.11a).

The diminished membrane permeabilization in Bax/Bak DKO cells correlated with Caspase-1 cleavage. Bax/Bak double knock-out cells had dramatically diminished CASPASE-1 cleavage in response to pIC treatment. This effect was specific to pIC, because when the cells were stimulated with ATP, Caspase-1 activation was kept intact even in the Bax/Bak DKO cells. On the other hand, absence of Bak, alone, did not block caspase cleavage (Figure 4.11c).

Confirming diminished CASPASE-1 cleavage, IL-1B secretion was decreased to half in the DKO cells. Partial effect of BAK can be seen in IL-1B secretion results. Moreover, when we stimulate with alpha-hemolysin which forms pores on the cell

membrane, absence of Bax and Bak had no effect on IL-1B secretion (Figure 4.11b). This shows that the phenotype is specific to intracellular pIC and the NLRP3 inflammasome is still intact and functional; it's the link between IPS-1 and NLRP3 activation that was rendered ineffective by knocking-out Bax/Bak.

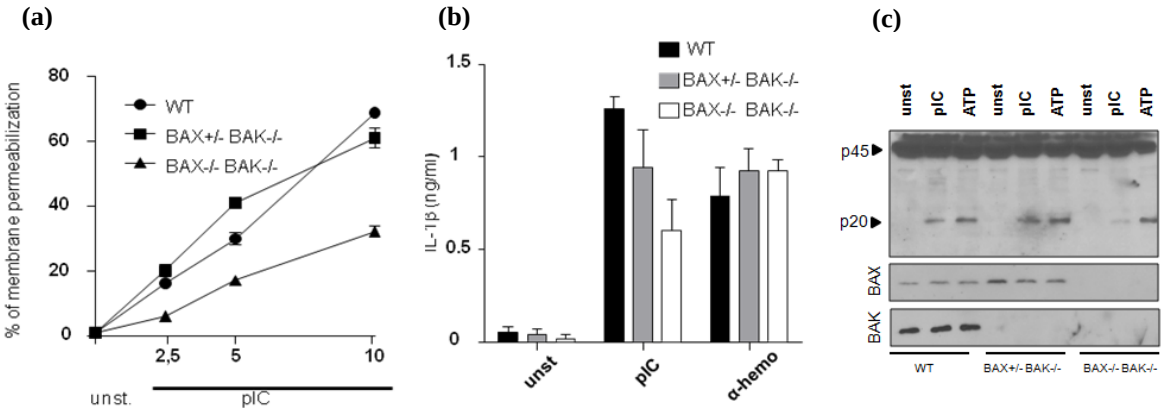

Figure 4.11. Bax/Bak has role in the membrane permeability in response to cytosolic pIC. (a) Membrane permeabilization of WT, Bak-KO and Bax/Bak DKO cells in response to pIC treatment for 2.5, 5 and 10 hours. (b) IL-1 beta secretion in WT, Bak-KO and Bax/Bak DKO cells in response to cytosolic pIC and alpha-hemolysin treatment. (c) Western blot analysis for CASPASE-1 cleavage in WT, Bak-KO and Bax/Bak DKO.

4.2.7. Cytosolic pIC causes K+ depletion

The important question was 'how the cell death can cause NLRP3 activation'. It's well established that whereas there are some cases that necrosis can induce inflammasome activation, another form of cell death, apoptosis, causes no inflammation or cytokine secretion at all. The phenotype observed during cell death by naked eye could be described as similar to necrotic cell death with some apoptosis taking place.

In terms of NLRP3 activation, the ultimate blocking agent was known to be the use of high K+ concentration media. In this condition, since the cells cannot get rid of the K+ inside the cytoplasm, NLRP activation is blocked. The only instance recorded in which NLRP3 activation occurs in the presence of high concentration of K+ is with the mutated

NLRP3, which is constitutively active probably due to a change in the protein structure stemming from the genetic mutations (unpublished observation).

In order to check if K+ efflux takes place in response to pIC treatment, we measured cytosolic K+ concentration in response to pIC stimulus. Both long and short pIC caused dramatic decrease in the amount of K+ in the cytoplasm, which points strong K+ efflux (Figure 4.12, panel A).

At this point it was not clear whether the K+ depletion is facilitated by K+ channels activated by the cell or it was a passive loss of K+ due to membrane permeabilization.

4.2.8. Activation of the Nlrp3-inflammasome requires K+ depletion, but membrane permeabilization does not.

As K+ efflux was taking place, we checked if blocking K+ efflux also blocks NLRP3 activation as expected. If this were the case, we could conclude that K+ efflux is required for NLRP3 inflammasome activation in response to cytosolic pIC.
Prior to the experiment, bone marrow derived macrophages were cultured either in normal media (5mM K+) or media with high concentration of potassium (70mM K+). The cells were primed with LPS and treated with transfected pIC and ATP, as described in previous experiments.

High concentration of K+ blocked Caspase-1 cleavage and IL-1B secretion in response to both pIC and ATP (Figure 4.12, panel B). As we checked membrane permeabilization under these conditions we observed that, high concentration of K+ had no effect on permeabilization (Figure 4.12, panel C). Hence, the cell membrane permeabilization is not enough for NLRP3 inflammasome activation, and it’s independent of K+ efflux. This lead us to hypothesize that membrane permeabilization occurs first and this causes passive loss of cytosolic K+ giving way to NLRP3 inflammasome activation.

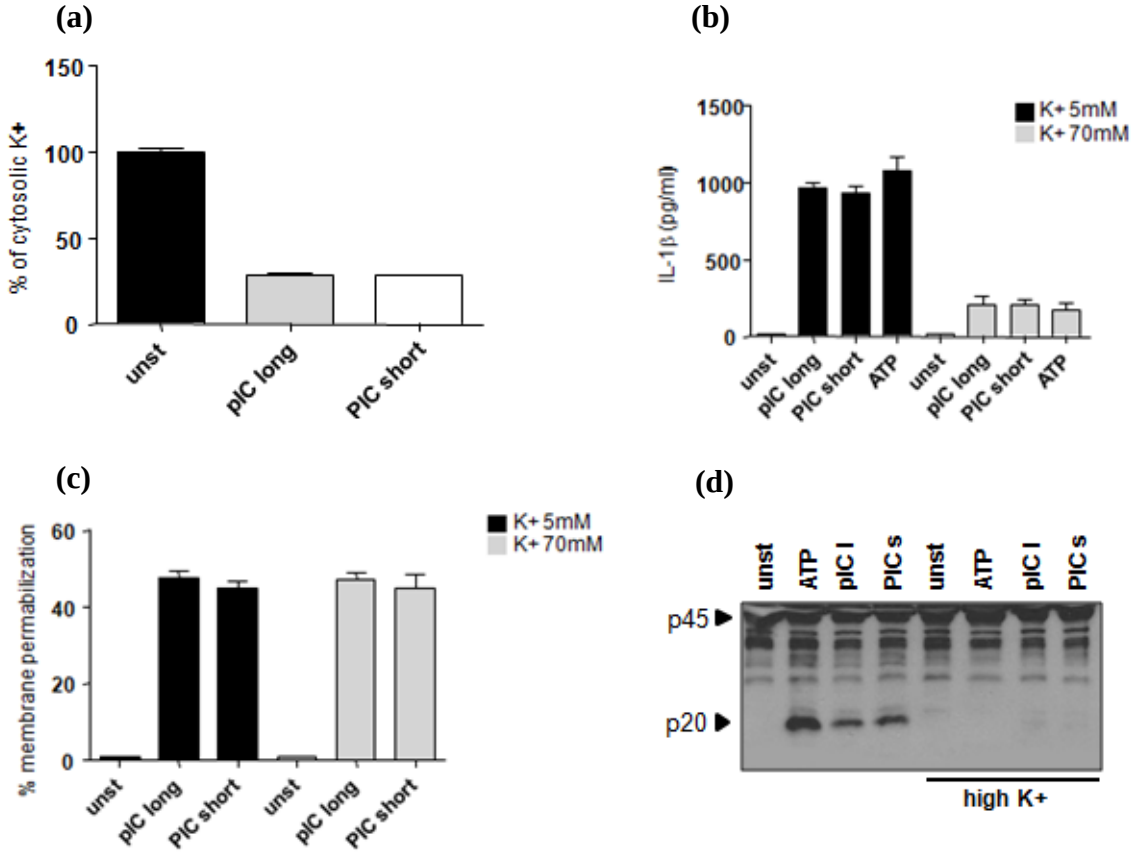

Figure 4.12. Cytosolic pIC activates NLRP3 inflammasome through cytosolic K+ depletion. (a) Cytosolic K+ concentration in BMDMs treated with cytosolic pIC. (b) IL-1 beta secretion in BMDMs treated with cytosolic pIC and ATP in normal media versus in media with high K+ concentration. (c) Membrane permeabilization in response to pIC in media with high K+ concentration. (d) Western blot analysis for cleaved caspase-1.

4.2.9. Blocking pIC induced membrane permeabilization blocks NLRP3 inflammasome activation by preventing K+ efflux

As we have previously shown Bax/Bak activity leads to membrane permeabilization. Moreover, IPS-1 which is located on mitochondria, is the main signaling protein in this pathway. These findings suggested mitochondria associated plasma membrane damage as a probable source of cytotoxic agents.

N-acetylcysteine (NAC) blocks reactive oxygen species and was shown to inhibit NLRP3 inflammasome activation in response to various stimuli. Whether NAC was directly interfering with NLRP3 inflammasome or it was blocking an intermediate mechanism was not clarified. Confirming the observation that NAC can inhibit mitochondria mediated cell damage, we observed that when BMDMs are treated with pIC in the presence of NAC, membrane permeabilization was blocked, leaving the membrane intact (Figure 4.13, panel A).

Moreover, as a consequence of the cell being saved from membrane damage, the K+ loss was abolished (Figure 4.13, panel B). This showed that, it's the membrane damage that causes K+ efflux and K+ depletion is blocked as long as the membrane efficiently functions as a barrier.

Clarifying that NAC does not block K+ efflux in every case, but only removes the intracellular cause of membrane damage, we performed the same experiment with ATP. In this case since ATP acts extracellularly to open specifically P2X7 ion channels, permitting ion translocation, NAC did not block K+ efflux (Figure 4.13, panel C). Overall, blocking intracellular agents that cause membrane permeabilization also blocks K+ efflux in response to cytosolic pIC.

Finally, we showed that inhibition of membrane permeabilization blocks K+ efflux and this subsequently results in inhibition of NLRP3 inflammasome activation (Figure 4.13, panel D). IL-1B secretion was abolished when the cells were stimulated in the presence of NAC and this result was confirmed by Western blot analysis showing this inhibition at CASPASE-1 cleavage level (Figure 4.13e).

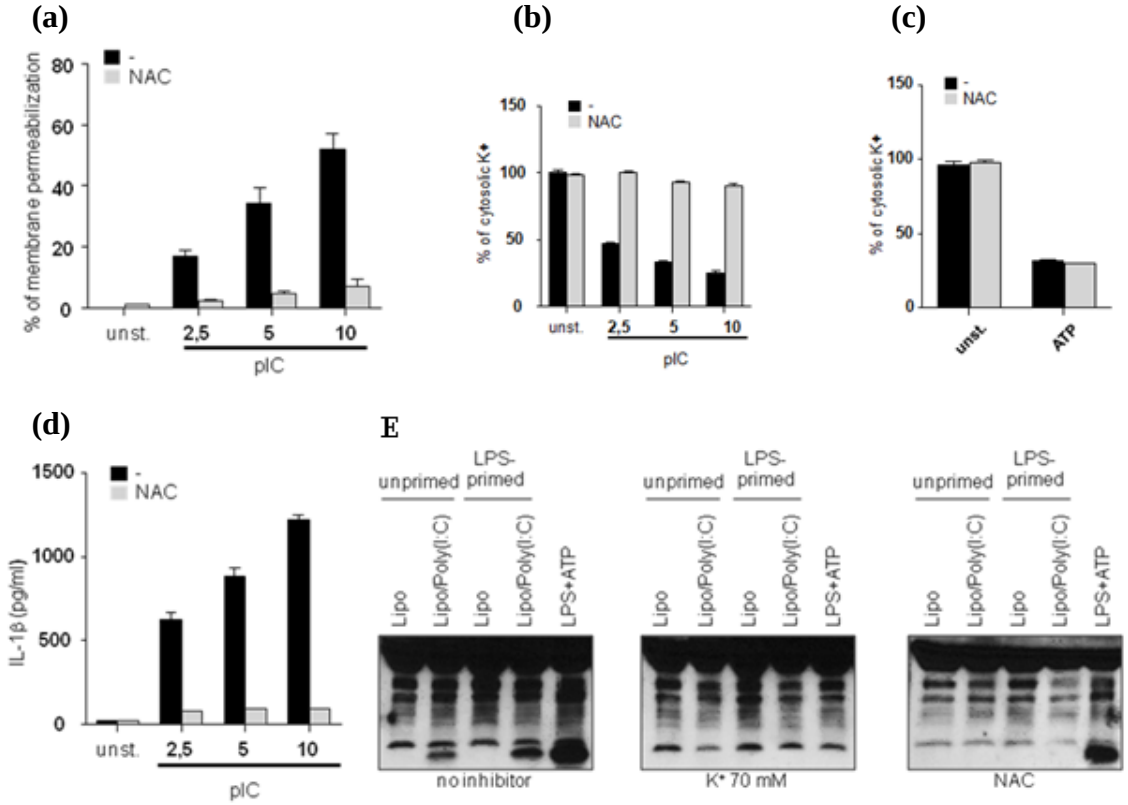

Figure 4.13. Blocking cell membrane permeabilization blocks pIC induced NLRP3 inflammasome activation by blocking K+ efflux. (a) NAC treatment blocks pIC induced membrane permeabilization. (b) NAC treatment blocks pIC induced K+ efflux. (c) Inhibition of K+ efflux is specific to intracellular pIC stimulation. BMDMs were stimulated with cytosolic pIC for six hours and ATP for 30 minutes after NAC treatment.

5. DISCUSSION

5.1. PART I : Role of ASC in melanoma

In this study we provide targeted analysis in an attempt to clarify ongoing controversy of last decade on the functionality and role of ASC in human melanoma cells versus healthy melanocytes.

Previous studies, including studies from our own group, have shown that ASC protein is lost in approximately 60% of melanoma cases including primary tumor sections and cell lines (Guan *et al.,* 2009) indicating a probable tumor-suppressive function for ASC. Our first project focused on elucidating tumor-suppressive effect of ASC expression in various melanoma cell lines lacking the expression of the protein. Considering the role of ASC in apoptosis pathways, we tested whether transduction of ASC would enhance apoptotic properties of metastatic melanoma cell lines (Erdogan, 2008). We also tested tumorigenicity of the parental lines compared with ASC expressing variants in nude mice (Cifdaloz, 2010). The in vivo and in vitro data we have accumulated failed to provide a strong link between ASC expression, apoptotic activity and tumor growth potential of the tested melanoma cell lines.

Meanwhile, Okamoto et al. suggested that melanoma that express ASC are able to induce IL-1 beta secretion and inflammation signaling. This study suggested that IL-1 beta secretion facilitated melanoma development rather than provoking an antitumor response (Okamoto *et al.,* 2010)

In this part of the thesis, we take a neutral look at the functionality of ASC and NLRP3 inflammasome in primary human melanocytes and different melanoma cell lines. Confirming previously published observations, we show that primary human melanocytes have all the required components of Nlrp3 inflammasome including Asc at the mRNA level. However, out data suggest that, NLRP3 inflammasome in primary melanocytes is not functional as measured by IL-1 beta secretion in response to various NLRP3 activating signals (Figure 4.1b).

Although there are studies including ours which suggest the presence of Asc in protein level in melanocytes, we observed no expression of ASC in protein level in primary melanocytes in this particular study (Figure 4.3). Since there is no clear information about the number of passages and histology of the melanocytes involved in previous studies, we have room to speculate that melanocytes that were reported to be found to have ASC protein by western blot analysis were probably old cells. The reasoning for this trivial explanation comes from the observation that human primary melanocytes lose their histological characteristics, suggesting differentiation, when they are grown in culture for long. Moreover, cultured melanocytes have diminishing expression of melanin, which is the distinguishing fingerprint of a functioning melanocyte. Hence, a gradual change in inflammasomal machinery would not been surprising for cultured melanocytes with high number of passages.

In line with the explanation the primary melanocyte experiments provided, besides having lowered the expression of Asc in mRNA level, some of the melanoma cell lines, specifically number #9 and #10 (also #18) which were examined in detail, had functioning NLRP3 inflammasomes as demonstrated by their intact response to LPS+ATP treatment, the golden standard for NLRP3 activation. Confirming these observations, when the melanoma cells were infected with lentivirus carrying shRNA for Asc, ASC protein levels, as well as the functionality of NLRP3 inflammasome diminished dramatically as monitored by IL-1 beta secretion in response to NLRP3 inflammasome stimuli (Figure 4.4).

As a side observation, here we also report that, at mRNA level, Nlrp3 expression is upregulated in melanoma cells. This may be an example of a general increase in receptor proteins of inflammasomes since we also observed upregulation in expression of the receptors of two other inflammasomes examined: Nlrc4 and Aim2 (Figure 4.2).

Overall, melanoma cells examined here seem to turn the non-functional NLRP3 inflammasome machinery on by modifying the regulation of inflammatory proteins. It’s fair to speculate that resulting inflammatory activity and IL-1 beta secretion, which in turn would activate resident macrophages to secrete additional cytokines, may facilitate

melanoma growth and invasiveness by signaling for angiogenesis and providing necessary escape path by weakening blood vessel walls.

5.2. PART II : NLRP3 inflammasome activation in response to dsRNA

Nlrp3 was reported to be required for Caspase-1 cleavage and Il-1b secretion in response to a wide range of stimuli including ATP, nigericin, maitotoxin, S. aureus and L. monocytogenes, influenza virus, fungal pathogen Candida albican, Alzheimer's disease-assoicated amyloid deposits, monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD), crystalline asbestos, silica and aluminium adjuvants. Ability of NLRP3 inflammasome to be activated by such different stimuli made us and other scientists in the field curious on the mechanism of activation. Mainly three models were proposed to provide a general unifying explanation for NLRP3 activation mechanism. The first suggestion that NLRP3 senses changes in cytosolic potassium ion concentration (Franchi *et al.,* 2007) seems to be a good explanation; however this model fails to explain how each NLRP3 activating signal may cause K^+ efflux. Another model suggests that particulate matter such as silica crystals and aluminum salts damage lysosomes causing leakage of lysosomal molecules which are sensed by NLRP3 (Hornung *et al.,* 2008). The third model proposes that varying stimuli increase mitochondrial ROS production, which is, in turn, is sensed by NLRP3. Lysosomal damage and ROS models are limited to explain NLRP3 activation in response to certain stimuli, but not all.

Using macrophages differentiated from bone marrow of mice deficient in Tlr3 -/-, MyD88 -/-, Trif -/-, Mda-5 -/- , Ips-1, or both IPS-1 and Trif we show that Ips-1 is required for intracellular pIC recognition and extracellular dsRNA recognition pathways has no role in this mechanism (Figure 4.6). Following the unexpected finding that membrane damage and cell death in response to cytosolic pIC takes place independent of NLRP3 inflammasome activity, we stated investigating the effect of membrane damage on NLRP3 inflammasome activation (Figure 4.9).

Inflammasome activity analysis of Bax/Bak DKO macrophages and immunofluorescence assay for active Bax showed that, Ips-1 acts on apoptotic Bcl2 family members Bax/Bak triggering programmed cell death mechanism (Figure 4.10,

). Resulting membrane permeabilization causes loss of cytosolic K+ ion (Figure 4.12) which is sensed by NLRP3 leading to NLRP3 inflammasome activation (Figure 4.13).

Our data shows that it is the Bax/Bax mediated cell membrane permeabilization that causes K+ depletion and this ultimately causes NLRP3 activation and IL-1B secretion in response to recognition of pIC through IPS-1 signaling.

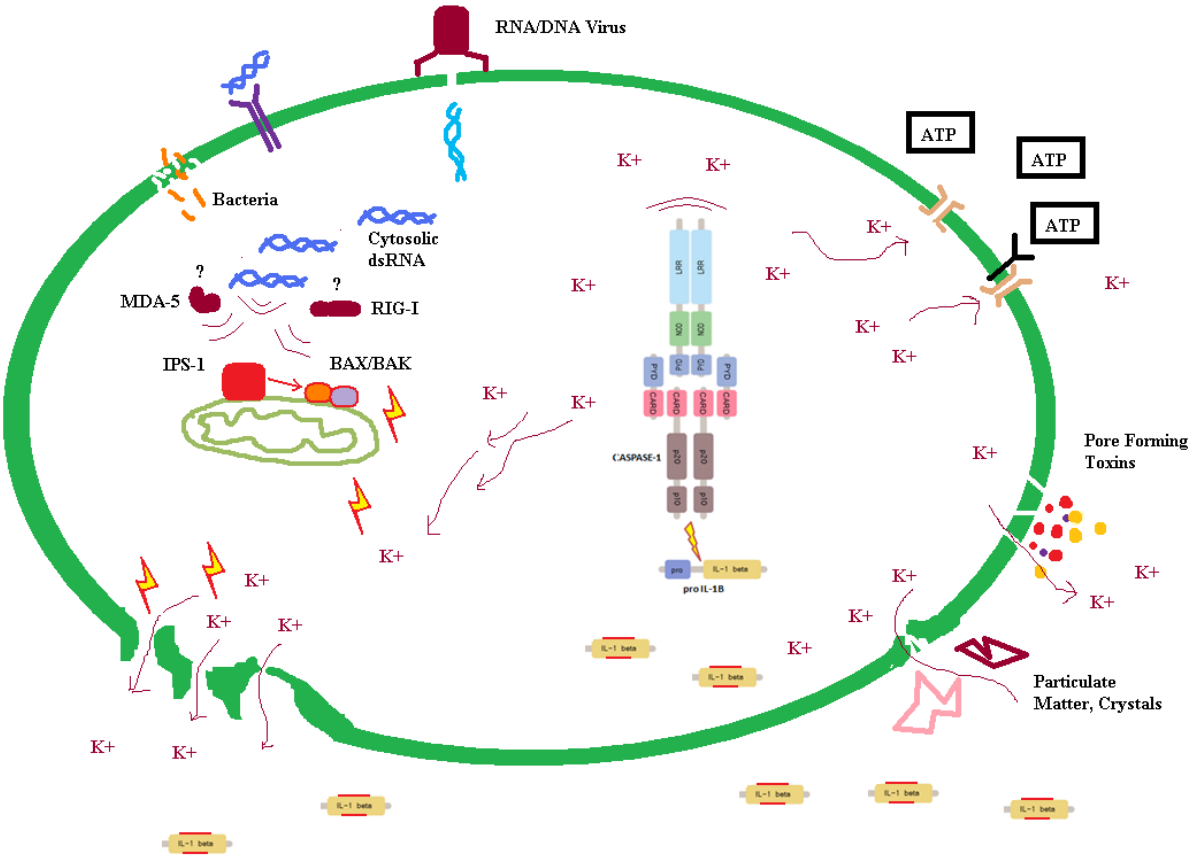

Figure 5.1. Proposed model for NLRP3 activation through various stimuli.ATP causes membrane permeability by keeping the P2X7 ion channels open. Pore forming toxins from pathogenic organisms, crystals, and particulate matter create pores on cell membrane or disrupt the membrane barrier, causing ion leakage. Cytosolic dsRNA activates cell death mechanisms through apoptotic Bcl-2 family members Bax/Bak.

More importantly, these observations provide an explanation to curious question on how NLRP3 can sense so many different kind of stimuli. By linking intracellular stimuli to membrane damage, and membrane permeabilization to K+ efflux, resulting in NLRP3 activation we provide a unifying understanding on how NLRP3 inflammasome works.

NLRP3 seems to work as a sensor of uncontrolled membrane permeabilization resulting either (i) from damaging signals from the organelles of the cell itself, (ii) through continuous activation of membrane channels, or (iii) through any extracellular organism or crystal that disturbs the plasma membrane (Figure 5.1). We propose that the ‘common target’ that was being searched for different stimuli activation NLRP3 is the plasma membrane integrity.

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