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TOLL-LIKE RECEPTORS AS MEDIATORS OF TUMOR CELL DEATH

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List of abbreviations

AnnV	Annexin V
BMDCs	Bone Marrow Dendritic Cells
HMGB1	High Mobility Group Box 1
i.t.	intratumoral
IFN	Interferon
IFNGR	Interferon-gamma receptor
IL	Interleukin
KC	Keratinocyte-derived chemokine
LPS	Lypopolysaccharide
LTA	Lipoteichoic acid
MHC	Major Histocompatibility Complex
MyD88	Myeloid Differentiation primary response gene (88)
nec-1	Necrostatin-1
NF-kB	Nuclear Factor Kappa B
PAMP	Pathogen Associated Molecular Pattern
PI	Propidium Iodide
Poly I:C	polyinosine-polycytidylic acid
PRRs	Pathogen Recognition Receptors
PS	Phosphatidylserine
RIP1	Receptor-Interacting Protein 1
tbp	TATA-binding protein
TLR	Toll-like receptor
TNF	Tumor Necrosis Factor

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Abstract

Recently, it has been demonstrated that invasive but nonpathogenic *Salmonella typhimurium* i.t. injected into melanoma B16-bearing mice led to complete regression of the tumor, although *Salmonella* was unable to directly kill B16 tumor cells *in vitro*. Since we found high levels of IFN-γ in infected tumor masses *in vivo*, we wondered whether this cytokine might be involved in tumor cell death. In this work, we have demonstrated that IFN-γ shows a cytotoxic effect only when administered in combination with *Salmonella*. In addition, this combined treatment is able to enhance TLR transcription, mainly TLR2 and TLR3, in B16 cells, suggesting a possible link between tumor cell death and TLRs, as already proposed in some studies. Our hypothesis is strengthened by the finding that, unlike IFN-γ, IFN-α does not induce neither tumor cell death nor TLR transcription. B16 tumor cell death induced by either TLR2 ligand Pam3SCK4 or TLR3 ligand poly I:C treatment in combination with IFN-γ indicates that both TLR2-TLR1 heterodimer and TLR3 are involved in tumor cell death, although they likely activate independent mechanisms. On the contrary, neither TLR2-TLR6 nor TLR4 seems to be able to efficiently kill tumor cells neither alone nor in combination with IFN-γ. Notably, several evidences suggest that *Salmonella* in combination with IFN-γ activates TLR2 rather than TLR3. However, since we cannot definitely rule out the existence of a TLR3 ligand in *Salmonella*, further experiments using anti-TLR2 and anti-TLR3 blocking antibodies will be necessary. Furthermore, in this study we demonstrate that B16 tumor cells undergo a first necrotic event in the first 24 hours of combined stimulations inducing IL-6 and KC release, probably through NF-kB activation. Moreover, caspase activation analysis has demonstrated that B16 cells undergo apoptosis as well, but a later time. In this regard, we detected mainly caspase-7 cleavage after 48 hours of combined treatments. However, incubation with the necrosis inhibitor necrostatin-1 (nec-1) strongly reduces caspase-7 activation, suggesting that a necrotic event might be responsible for the subsequent apoptosis, that may be independent of TLR engagement. B16 dying cells release HMGB1 as well, a marker of necrotic cell death, that is able to enhance tumor immunogenicity. However, since *Salmonella* alone triggers HMGB1 release without inducing cell death, HMGB1 may be actively secreted in response to *Salmonella*, but may also be passively released by necrotic dying cells. Because of the important role of HMGB1 in tumor immunogenicity, future studies will be performed with the aim of understanding how B16 cells release HMGB1. Our results suggest that this may be achieved through a TLR4 MyD88-independent pathway. Interestingly, we demonstrate for the first time that poly I:C induces TLR3 translocation to B16 cell surface in combination with IFN-γ. In this way, poly I:C can improve TLR3-mediated response thus probably inducing a stronger caspase-7 activation with respect to *Salmonella* and TLR2 ligand. Finally, different experiments have highlighted the importance of IFN-γ in this tumor cell death mechanism. In particular, IFN-γ seems to sensitize tumor cells before receiving a subsequent stimulation, up-regulating TLRs involved in tumor cell death (e.g. TLR3) or through other unknown mechanisms. These

results set the basis to improve the immunotherapy protocol developed in Maria Rescigno's laboratory, combining IFN-γ i.t. injection with *Salmonella* i.t. injection or replacing *Salmonella* with Pam3CSK4, in order to obtain a more prompt therapeutic effect avoiding possible side effects of *Salmonella*. In addition, these results provide a rationale for introducing poly I:C treatment into electrochemotherapy approach, a new method applied to cutaneous cancers treatment, melanoma included. Since it is based on the permeabilization of the cell membrane by means of short and intense electric pulses, it would allow the entry of poly I:C into the cytosol of both tumor and immune cells. Therefore, TLR3 ligands may be involved in both the killing of tumor cells and in activating the immune system. Importantly, this new system may overcome the use of a systemic treatment with IFN-γ and could be used to treat melanoma avoiding possible side effects.

1. Introduction

1.1. Cancer immunotherapy

Immunotherapy is a central component of many cancer treatments. In addition to microbial and mutated proteins, the immune system can recognize developmentally and tissue-restricted proteins, as well as proteins that are overexpressed by tumor cells. However, tumors frequently interfere with the development and function of immune mechanisms. Established therapies employ a variety of manipulations to enhance antitumor immune responses in a tumor-bearing host. Immunotherapy can be divided into two categories: active and passive. Active immunotherapy aims mainly to elicit the body's own response to attack the tumor cells, whereas passive immunotherapy relies on therapeutics that can directly mediate the killing of the tumor. Examples of active immune therapies include the introduction of adjuvants into the tumor microenvironment, the systemic delivery of cytokines, and cancer vaccines, whereas immunization with monoclonal antibodies, targeting regulatory cells and adoptive transfer of tumor-specific T lymphocytes are components of passive immunotherapy.[1]

1.1.1. Immune adjuvants

Cancer cells often express a variety of abnormal proteins that can serve as targets for an immune response (antigens). Although spontaneous immune responses to these antigens can occur, these reactions are rarely sufficient to cause tumor regression; however, the local administration of immune-activating agents (adjuvants) can induce tumor-associated inflammation and protective immunity. Immune adjuvants have already proven useful in the treatment of a range of early stage tumors. For superficial bladder cancer, immune therapy with live bacilli Calmette-Guérin (BCG), when combined with surgery, is more effective than conventional chemotherapy[2]. Moreover, other bacteria are promising for the development of microbial-based tumor therapies. An interestingly example is *Salmonella,* a Gram-negative bacterium, facultative anaerobe belonging to enterobacteria that colonizes preferentially the tumor if injected intravenously (i.v.) into tumor-bearing mice[3]. Due to this property and due to its pathogenicity, *Salmonella Thypi* (*S. Thypi*) has been considered as an interesting candidate for directed enzyme-prodrug-therapy as well as a possible biological tool for the diagnosis. However, the administration of wild type *S. Thypi* causes severe side effect in humans because of lipopolysaccharide (LPS) present on the bacterium. Attenuated strains of *Salmonella* were developed, but a phase I clinical trial demonstrated that treatments using these strains were unable on metastatic melanoma patients[4]. Therefore, the systemic route of administration is particular appealing to treat non-accessible metastases, but at the present time it is not particular efficient. However, many studies were conducted on the possible exploitations of *Salmonella* in mice. For example, an attenuated *Salmonella* expressing the *Escherichia Coli* cytosine deaminase, an enzyme capable of converting non toxic 5-fluorocytosine (5-FC) to the active antitumor agent 5-fluorouracil (5-FU) has been developed[5]. *Salmonella* was also used as a vector for oral cytokine-gene therapy in an experimental

model[6]. Moreover, it was described the antitumor properties of cytokine-expressing attenuated *Salmonella thyphimurium (*hereafter referred simply as *Salmonella)*, notably interleukin (IL)-2-expressing *Salmonella* [7] and CCL21-expressing *Salmonella*[8]. Finally, Avogadri and coworkers discovered that the *in vivo* injection of *Salmonella* into a tumor mass of a melanoma murine model has the capacity to (a) induce the complete regression of the treated tumor and (b) slow down the growth of another distal untreated tumor[9]. Complete tumor regression caused by the *in situ* injection of *Salmonella* is due initially, to an indirect (i.e. systemic) immune response against the *Salmonella*-infected cells, and subsequently, to a direct immune response against the tumor cells through cross-presentation of the tumor antigen by dendritic cells (DCs) to $CD8^+$ T cells in the draining lymph node.

Microbes often elicit immune responses by activating pattern-recognition receptors such as Toll-like receptors (TLRs). Purified TLR ligands have been evaluated as immune adjuvants and have shown considerable activity in preclinical models. The TLR7 agonist imiquimod was approved for the external warts caused by human papilloma virus (HPV); however, it has demonstrated efficacy also against low-grade epithelial tumors and precancerous lesions[10].

Unfortunately, any of immune adjuvants currently approved for cancer therapy is not suitable for systemic delivery. Current research has focused on identifying systemically active immune adjuvants which could be used to treat a wider range of tumors. Unlike imiquimod, TLR9 agonists can activate productive immune responses when delivered into the circulation[11]. Similarly, NKT cells activated by α-galactosylceramide (α-galcer) respond by producing of specific effector cytokines, including IFN-γ; this action triggers a potent antitumor immunity response[12].

1.1.2. Cytokines

Cytokines, secreted proteins with immune-modulating properties, can be delivered systemically to activate antitumor immunity. Although response rates are low, both the cytokines IL-2, a potent T cell growth factor, and interferon (IFN)-α, an important mediator in antiviral immunity, have been used to treat advanced melanoma and renal cell carcinoma (RCC), tumors that are generally resistant to standard chemotherapy[13-15]. Experiments in animal models suggest that IFN-α may play a role in antitumor immunity, and clinical responses to IFN-α are associated with therapy-induced autoimmunity, linking the effectiveness of IFN-α to an induction of an immune response[16]. The side effects of cytokine administration are severe and often dose limiting. Typically, cytokines induce symptoms that mirror those of systemic infection[13,14]. Despite the limitations of cytokine therapy, both IL-2 and IFN-α can induce durable responses in a subset of patients with melanoma[14]. In contrast to systemic cytokine therapy used primarily for immune modulation, local administration of the cytokine tumor necrosis factor (TNF)-α has been used to treat soft tissue sarcomas (STSs) of the limb and melanoma, making use of the toxic effects on both tumor cells and the tumor vasculature that are mediated by this cytokine[17].

1.1.3. Supportive therapy

Many forms of conventional chemotherapy have dose-limiting toxic effects on the bone marrow, including effects on the cells of the immune system. Immune toxicity, in particular neutropenia, can lead to substantial morbidity and mortality. Loss of neutrophils can predispose cancer patients to life-threatening bacterial sepsis. Supportive therapy aimed at rescuing immune cells is thus a critical component of many chemotherapy regimens for both solid and hematopoietic tumors, as well as many bone marrow transplantation protocols. To prevent neutropenia, many high-dose chemotherapeutic regimens are followed with an infusion of recombinant granulocyte (G) or granulocyte-macrophage (GM) colony stimulating factor (CSF)[18].

1.1.4. Prophylactic immune therapy

A number of cancers are caused by microbial infections, either directly or through the induction of chronic inflammation. As a result, therapies aimed at eradicating or preventing these infections act prophylactically against their associated tumors. The hepatitis B virus (HBV) infections of the liver can lead to chronic hepatitis, which can predispose people to the development of hepatocellular carcinoma (HCC). HBV vaccine was the first available vaccine that provided protection against an infection with known oncogenic potential[19]. More recently, a vaccine against HPV 16 and 18 has been developed specifically to prevent cervical carcinoma[20]. Like viruses, bacteria have also been associated with tumor development. The *Helicobacter pylori* bacteria is the primary cause of stomach cancer and is involved in mucosal-associated lymphoid tissue (MALT) lymphomas[21]. *H. pylori* colonization can be cleared with appropriate antibiotics, decreasing the risk of stomach cancer. A strong correlation also exists between chronic inflammation of the colon and colorectal cancer. This inflammation has been directly implicated in the development of colorectal cancer, as demonstrated by the efficacy of anti-inflammatory treatments in reducing cancer risk[22].

1.1.5. Monoclonal antibodies

The administration of tumor-targeting monoclonal antibodies has proven to be one of the most successful forms of immune therapy for cancer. The infusion of manufactured monoclonal antibodies can directly generate an immediate immune response. Monoclonal antibody therapies are typically not as toxic as conventional cytotoxic cancer chemotherapy, although binding to nonmalignant cells can, in some cases, induces significant adverse reactions. Nowadays, nine monoclonal antibodies, targeting six tumor-associated proteins, are clinically approved for the treatment of cancer. Five of these antibodies (alemtuzumab, gemtuzumab, rituximab, ibritumomab tiuxetan, and tositumomab) bind surface proteins that are highly expressed on hematologic tumors. Of these, rituximab is the most widely used as part of the standard treatment for non-Hodgkin's lymphoma (NHL)[23]. Trastuzumab, cetuximab, and panitumumab bind proteins of the

epidermal growth factor receptor (EGFR) family, either targeting EGFR itself (cetuximab and panitumumab) or targeting the related protein HER2/*neu* (trastuzumab). Both EGFR-targeting antibodies have been used for the treatment of metastatic colorectal cancer in patients who have previously failed standard chemotherapy[24]. Trastuzumab was the second monoclonal antibody approved for cancer therapy and is used for the treatment of invasive, HER2/*neu* positive breast cancer[25]. Bevacizumab is the only monoclonal antibody with anticancer activity that does not directly target malignant cells; instead, it binds vascular endothelial growth factor (VEGF), a critical mediator of tumor angiogenesis.Two monoclonal antibodies have been used to deliver cytotoxic therapy direcly to tumors by conjugating them to radioactive isotypes (ibritumomab tiuxetan and ^{131}I tositumomab) or to toxic chemicals (gemtuzomab).

By binding to their targets, antibodies exercise their functions through several effector mechanisms, including steric inhibition and neutralization, complement activation, and activation of cell-mediated cytotoxicity. Antibody binding alone is probably sufficient to provide some antitumor activity; however, therapeutic monoclonal antibodies may also function by recruiting other elements of the immune system to malignant cells. Some evidence indicates that complement-dependent cytotoxicity may contribute to the antitumor effects of rituximab[26]. In addition, ligation of activating fragment c receptors (FcRs) on neutrophils, monocytes, and natural killer (NK) cells can lead to antibody-dependent cellular cytotoxicity (ADCC)[27]. In order to overcome the limitations of current antibodies and thus enhancing the antitumor efficacy, second-generation monoclonal antibodies are under development. Diffusion of antibodies into tumors can be significantly improved by removing the antibody Fc region to generate $F(ab')_2$ fragments. However, many important antibody functions are mediated by the Fc region. As a result, such $F(ab')_2$ fragments are useful for antibody-conjugated cytotoxic therapy[28]. Monoclonal antibodies have been modified to alter serum half-life, either to extend their biology effects or to accelerate the clearance of toxin-conjugated antibodies. In addition, antibodies can assist DCs in the acquisition of tumor-associated antigen and in the presentation of these antigens to T cells[29]. Moreover, a large number of novel monoclonal antibodies with immune-modulating activity are under development for the treatment of cancer. Several of these antibodies directly antagonize negative regulatory circuits that are thought to be important in limiting antitumor responses; similarly, agonistic antibodies that activate T cell coreceptors are being developed to drive cytotoxic T cell responses.

The most clinically advanced immune-modulating antibodies block cytotoxic T lymphocyte antigen (CTLA)-4, an important negative immune-regulatory receptor expressed on a variety of immune cells, including activated T cells and regulatory T cells (Tregs). In the absence of CTLA-4, mice develop a lethal multiorgan inflammatory disease, underscoring the importance of CTLA-4 in immune homeostasis. Studies in animal models, as well as early clinical trials, have demonstrated enhanced antitumor activity following CTLA-4 blockade, particularly when used in conjunction with other tumor immune-modulating strategies[30]. Inhibition of the negative immune-regulatory receptor PD-1, expressed on activated T cells, B cells and

monocytes, can also improve antitumor immunity in animal models[31]. The anti-inflammatory cytokines IL-10 and TGF-β are among the most important immune-suppressive cytokines produced by tumors, and strategies to block this immune suppression represent an attractive target for immunotherapy[32,33].

Monoclonal antibodies that act as agonists of stimulatory receptors can directly augment antitumor immune responses. Several such antibodies have been developed to target TNF family costimulatory receptors, including glucocorticoid-induced tumor necrosis factor receptor (GITR), CD134 (OX40), CD137 (4-1BB), and CD40. GITR, CD134, and CD137 are expressed on T cells, and agonistic antibodies directed against each of these receptors enhance cytotoxic T cell function and increase the efficacy of antitumor immune therapy[1]. The receptor for the cytokine tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is also under investigation as a target for agonistic therapy either with TRAIL itself or with monoclonal antibodies. Work in experimental systems suggests that signals through the TRAIL receptor can have potent effects on tumors cells, directly inducing apoptosis, without inducing substantial systemic toxicities[34].

1.1.6. Targeting regulatory cells

The immune-suppressive tumor microenvironment is, in large part, maintained through the anti-inflammatory activity of both innate and adaptive regulatory cells. Tregs are a subset of T cells wich actively suppress T cell activation and prevent autoimmune disease resulting from pathological self-reactivity. These cells are overrepresented in a wide range of tumors, and increased numbers of Tregs correlates with poor prognosis[35]. Given the potential importance of Tregs in limiting antitumor immunity, methods for directly targeting them may be of clinical use. The drug denileukin diftitox is a conjugate of Diphtheria toxin and IL-2 and has shown some selective toxicity against Tregs[36].

1.1.7. Therapeutic cancer vaccines

Prophylactic cancer vaccines have been effective in a number of spontaneous tumor models in mice. These therapeutic vaccines must bypass immune-regulatory mechanisms that have already led to tumor tolerance. Many strategies for generating therapeutic immune responses to cancer have been attempted: antigen-specific vaccines, DC vaccines, and cytokine-based, whole tumor cell vaccines.

Antigen-specific vaccines

A wide range of vaccines based on single-tumor antigens has been tested in experimental systems, and several of them are currently in clinical trials. These vaccines, based on use of recombinant proteins or antigenic peptides mixed with immune adjuvants, can elicit coordinated T and B cell immune responses and have some efficacy in a variety of human tumors. Cancer testis antigens (e.g. MAGE-A3 and NY-ESO-1) are expressed by tumor cells as well as endogenously within the immune-privileged

environment of the testis, making immune response against them highly selective for tumor tissue. In addition, many tumors overexpress proteins with relatively low endogenous expression whereas other tumors express proteins with a narrow distribution in nonmalignant tissues; tissue-restricted proteins have been successfully targeted in melanoma, where a variety of melanocyte-specific proteins often continue to be expressed[37].

Dendritic cell vaccines

DCs are potent inducers of adaptive immunity, driving the activation of T cells in response to invading microorganisms. Immature monocyte-derived DCs can be readily loaded with antigenic peptides or proteins *in vitro*; these antigen-loaded DCs can then be used in an autologous transplant to induce antigen specific T cell responses[38]. Activated DCs have improved antigen-presenting abilities, as well as increased expression of T cell costimulatory proteins. DCs can be activated by a range of stimuli, including inflammatory cytokines and microbial products. Most clinical trials employ an inflammatory cytokine cocktail to mature DCs, although the use of microbial pattern-recognition receptor agonists has also been considered[39]. An alternative approach bypasses *in vitro* activation and instead injects immature, antigen-loaded DCs into an inflamed tissue. Once exposed to the inflammatory environment, antigen-loaded DCs can mature in a more physiological fashion and migrate to draining lymph nodes[40]. In addition, antigens can also be delivered directly to DCs *in vivo* using antibodies that bind DC surface receptors.

Cytokine-based tumor vaccines

When combined with a variety of cancer vaccines, cytokines can boost immune responses through the recruitment and maturation of a variety of immune effector cells. Several cytokines, including IL-2, IL-12, IFN-α, and GM-CSF, have been evaluated as vaccine adjuvants[41]. GM-CSF has been particularly potent when used to prime immune responses against whole tumor cell vaccines. GM-CSF primarily acts on myeloid cells and functions to recruit and mature DCs, enhancing the presentation of tumor antigens to the immune system. In cancer patients, injection of autologous, irradiated, whole tumor cells engineered to produce GM-CSF (GVAX) can induce coordinated B and T cell responses to a wide range of tumor antigens. In melanoma patients, these immune reactions have been associated with both partial and complete responses[42].

1.1.8. Adoptive T cell therapy

Adoptive T cell therapy relies on the *in vitro* expansion of endogenous, cancer-reactive T cells, which are harvested from cancer patients, manipulated, expanded and then reintroduced as a mechanism for generating productive tumor immunity. This technique enables the production of large numbers of autologous T cells which are specific for tumor-associated antigens (TAAs). It overcomes the problem of central tolerance and bypass the need for the host to activate the immune response against tumor antigens. $CD8^+$ cytotoxic T lymphocytes are the primary

effector cells in adoptive T cell therapy[43]. However, $CD4^+$ T cells may also play an important role in maintaining $CD8^+$ cytotoxic function, and transplantation of tumor-reactive $CD4^+$ T cells has been associated with some efficacy in metastatic melanoma[44]. Adoptive T cell therapy alone may be insufficient to induce clinically meaningful responses in most cancer patients; combination therapies using vaccines to increase the frequency of tumor-reactive T cells prior to, or immediately after, adoptive therapy may be another mechanism for increasing overall treatment efficacy.

1.2. Interferon-γ

1.2.1. The interferons (IFNs)

The interferons (IFNs) were originally discovered as agents that interfere with viral replication. They are now classified into type I and type II according to receptor specificity and sequence homology. The type I IFNs are IFN-α, IFN-β, IFN-ω, and IFN-τ, all of which are structurally related and bind to a common heterodimeric receptor (IFNAR, comprised of IFNAR1 and IFNAR2 chains). IFN-α and IFN-β are primarily induced in response to viral infection of cells. Although type I IFNs are secreted at low levels by almost all cell types, type II IFN, now known as IFN-γ, is produced predominantly by T lymphocytes, NKT cells and natural killer (NK) cells, but also by B cells and professional antigen-presenting cells (APCs) following activation with immune and inflammatory stimuli rather than viral infection. However, probably the two IFN systems may have evolved to complement each other to defend against a broad spectrum of pathogens[45]. IFN-γ is structurally unrelated to type I IFNs and binds to a different receptor (IFNGR). IFN-γ production by professional APCs (monocyte/macrophage, dendritic cells) acting locally may be important in cell self-activation and activation of nearby cells. IFN-γ secretion by NK cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFN-γ in the adaptive immune response[46]. IFN-γ production is controlled by cytokines secreted by APCs, in particular IL-12 and IL-18. Pathogens recognition induce macrophages to secrete IL-12 and chemokines that attract NK cells to the site of inflammation. IL-12 promotes IFN-γ synthesis in these cells. Moreover, the combined stimulation of IL-12 and IL-18 further increases IFN-γ production[47]. Negative regulators of IFN-γ are IL-4, IL-10, transforming growth factor-β (TGF-β), and glucocorticoids. In addition to functions in host defense, IFN-γ may also contribute to autoimmune pathology. Notably, in humans, IFN-γ is implicated in pathology of diseases such as systemic lupus erythematosus, multiple sclerosis, and insulin-dependent diabetes mellitus[48].

1.2.2. The IFN-γ receptor and its signaling mechanism

IFN-γ receptor (IFNGR) is composed of two ligand-binding IFNGR1 chains associated with two signal-transducing IFNGR2 chains. Biologically active IFN-γ is a noncovalent homodimer formed by self-association of two

mature polypeptides in an antiparallel orientation. IFN-γ signals through the Jak-Stat pathway, a pathway used by several cytokines, growth factors, and hormones to affect gene regulation. Ligand binding induces Jak2 autophosphorylation and activation, which allows Jak1 transphosphorylation by Jak2. Phosphorylation causes dissociation of a Stat1 homodimer from the receptor. Thus, Stat1 enters the nucleus and binds to promoter elements (GAS elements) to initiate or suppress transcription of IFN-γ-regulated genes[49] (Fig. 1-1). The first wave of IFN-γ-induced transcription occurs within 15–30 min of IFN-γ treatment. Many of the induced genes are in fact transcription factors (for example, IRF-1), which are activated by IFN-γ and are able to further drive regulation of the next wave of transcription. Stat1 presents two phosphorylation sites which are functionally important for efficient signaling (Y701 and S727). In particular, phosphorylation of Stat1 at S727 induced by different stimuli, including types I and II IFN, lipopolysaccharide (LPS), IL-2, IL-12, tumor necrosis factor α (TNF-α), and platelet-derived growth factor, is essential to activate transcription of target genes[50]. Moreover, it was found different mechanisms that control the extent of ligand stimulation of IFN-γ signaling. In fact, Stat1 activation is inhibited within 1 hour of IFN-γ treatment, despite the continued presence of extracellular IFN-γ[51]. The IRF gene family (IRF-1, IRF-2 and IRF-9) participate in IFN-γ signaling. Stat1 and NF-kB interaction with promoter elements dramatically increases IRF-1 transcription. Thus, IRF-1 expression is up-regulated in response to types I and II IFN, virus, or cytokines, whereas IRF-1 transcriptional regulatory activity is regulated independently in response to IFN-γ, virus, and dsRNA[52]. Interestingly, the IFN-γ and IFN-α/β signal pathways cross-talk at multiple levels to synergize or antagonize particular functions within the cell. In fact, the signal pathways and target genes used by types I and II IFN are partially overlapping. This cross-talk is biologically relevant, as cells *in vivo* are not stimulated with one cytokine in isolation, rather with a cytokine cocktail. For example, the Stat1:Stat2:IRF-9 complex (ISGF3) is activated by IFN-γ but is involved in type I IFNs production[53]. On the contrary, type I IFN can elicit type II IFN signaling molecule such as active Stat1 homodimers, which are able to activate transcription of target genes.

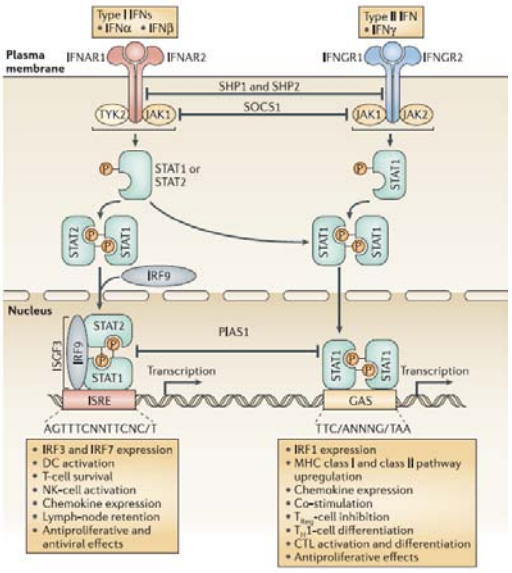


Figure 1-1. Interferon-induced signalling and transcription
(Dunn, G.P., et al. *Nature Reviews Immunology* 2006)

1.2.3. Cellular effects of IFN-γ

IFN-$\gamma^{-/-}$ and IFNGR1$^{-/-}$ mice show a normal development and their immune system appears to develop normally[54]. However, these mice show deficiencies in natural resistance to bacterial, parasitic, and viral infections. Interestingly, also human patients with inactivating mutations of the IFNGR1 or IFNGR2 chains show clinical presentation similar to the mouse models.

Class I and class II antigen presentation pathways

IFN-γ is able to up-regulate cell-surface class I MHC, an important feature for host response to intracellular pathogen, as it increases the potential for cytotoxic T cell recognition of foreign peptides and thus promotes the induction of cell-mediated immunity. Moreover, IFN-γ up-regulates LMP2, MECL-1, and LMP-7, the new subunits which replace the constitutive proteasome subunits to form the 'immunoproteasome'. In this way, the quantity of peptides for class I MHC loading is increased as overall expression levels of proteasome are increased. Also the quality and the repertoire of peptides is increased because of the cleavage specificity of the immunoproteasome that produce peptides better able to bind class I MHC and different from peptides cleaved by classic proteasome. Therefore, this mechanism improves $CD8^+$ T cell recognition of peptides:MHC class I complex and thus increases immune surveillance. Importantly, this mechanism may have evolved to ensure LMP2/LMP7/MECL-1-dependent epitopes are only produced in sites of inflammation and thus avoid autoimmunity without compromising appropriate T cell stimulation[55]. In addition, IFN-γ is also able to up-regulate other molecules involved in class I

antigen presentation pathway including TAP transporter, class I MHC heavy chain and light chain (β_2-microglobulin), and chaperones such as tapasin and GP96 implicated in aiding in the efficient assembly of peptide:MHCI complexes[48]. Furthermore, IFN-γ alone can efficiently up-regulate the class II antigen presenting pathway and thus promote peptide-specific activation of $CD4^+$ T cells[56]. Notably, it is able to induce class II MHC expression also in cells that do not constitutively express these genes (nonprofessional APCs).

Antiviral effect

IFN-γ is involved in antiviral mechanisms and in particular in up-regulation of key antiviral enzymes, most notably protein kinase dsRNA-regulated (PKR), which is a kinase greatly induced by types I and II IFN stimulation[57] and inactive in its constitutive form. dsRNA, a necessary intermediate in replication of RNA viruses, is the best characterized activator of PKR, causing its autophosphorylation. PKR is then activated for dsRNA-independent phosphorylation of specific cellular substrates. One of these substrates is the eIF-2α subunit, a rate-limiting factor in the normal cellular translational machinery. Phosphorylation by PKR inhibits viral and cellular protein synthesis. PKR is implicated in numerous other functions, including activation of NF-kB[58], TNF-α transcript splice regulation, induction of fas-mediated apoptosis[59], and regulation of Stat1 activity[60]. It is likely that IFNs induce many as-yet undiscovered antiviral proteins.

Antiproliferative effect

One of the most easily observed effects of IFN-γ is cell growth inhibition. The IFNs arrest the cell cycle at the G1/S checkpoint, although blockages of other cell cycle stages have been reported. Notably, IFN-γ blocks cells in G1/S inducing p21 and p27 ciclin-dependent kinases inhibitors (CKIs)[61], and down-regulating c-myc expression, responsible of activation of G1/S transition[62]. Additionally, IFN-γ is involved in apoptosis. Induction of apoptosis by signals such as DNA damage requires the IRF-1 tumor-suppressor gene. Levels of IRF-1 may be a deciding factor in whether IFN-γ induces or protects from apoptosis on treated cells. It is proposed that IFN-γ treatment of cells with high levels of functional IFNGR very rapidly activates Stat1, thereby producing high levels of IRF-1 that are able to induce apoptosis. In contrast, IFN-γ treatment of cells with low levels of functional IFNGR may activate Stat1 more slowly, thereby producing lower levels of IRF-1 that are not sufficient to induce apoptosis. For example, myeloid cells that express relatively high numbers of functional IFNGR on the cell surface are more sensitive to the proapoptotic actions of IFN-γ than other cells such as T cells. Experiments in which overexpression of functional IFNGR on normally low-level IFNGR-expressing cells changed the IFN-γ response of these cells from an antiapoptotic/proliferative phenotype to a proapoptotic phenotype are performed[63]. Many of the proapoptotic effects of IRF-1 are mediated by the IRF-1-induced caspase 1 (IL-1β-converting enzyme). Caspase 1 is a cysteine protease implicated in mediating macrophage apoptosis by various stimuli including LPS and is involved in generation of bioactive IL-1β and IL-18. Caspase 1 expression and resulting apoptosis can be IFN-γ-inducible[64]or can occur through IFN-γ-

independent IRF-1 activity. IFN-γ also induces a number of other proapoptotic molecules. These include PKR, death associated proteins (DAPs), cathepsin D, and surface expression of Fas and the TNF-α receptor.

Activation of microbial effector functions

The most important effect of IFN-γ on macrophages is the activation of microbial effector functions. Macrophages activated by IFN-γ kill pathogens and tumor cells primarily by production of ROS and reactive nitrogen intermediates (RNI) via induction of the NADPH oxidase system and iNOS, respectively. ROS are used to target extracellular pathogens during phagocytosis or that are too large for phagocytosis, whereas RNI target intracellular pathogens and upon appropriate stimulus, extracellular pathogens and tumor cells[48].

Immunomodulation and leukocyte trafficking

Unlike other IFNs, IFN-γ is able to coordinate the transition from innate immunity to adaptive immunity aiding in the development of a Th1-type response, directly promoting B cell isotype switching to IgG2a, and regulating local leukocyte endothelial interactions. IFN-γ up-regulates expression of adhesion molecules (e.g., ICAM-1, VCAM-1) and chemokines (e.g., IP-10, MCP-1, MIG, MIP-1α/β, RANTES) and thus, orchestrates the trafficking of specific immune cells to sites of inflammation. IFN-γ and NO produced at the site of inflammation cause local dilation of the blood vessels, thereby decreasing the local blood flow rate and causing gathering of blood in leaky vessels. Specific leukocyte subsets are instructed by the cytokine/chemokine milieu to extravasate into the tissue via interactions between adhesion molecules presented on leukocyte and endothelial surfaces ("diapedesis")[56].

IFN-γ priming of the macrophage LPS response

LPS, a cell wall constituent of Gram-negative bacteria, activates macrophage microbial effector functions and the production of proinflammatory cytokines (TNF-α, IL-1, IL-6). Macrophages recognition of LPS requires toll-like receptor 4. IFN-γ primes macrophages for more rapid and heightened responses to LPS[65] as well as other TLR agonists, such as unmethylated CpG motifs present in bacterial DNA (CpG DNA)[66]. Pretreatment with IFN-γ is necessary for the induction of some genes in response to LPS, not for all LPS-induced genes. For example, IFN-γ pretreatment promotes LPS-induced TNF-α but not procoagulant activity enzyme production[67]. IFN-γ receptor KO mice are highly resistant to LPS-induced toxicity[68]. This highlights the physiological significance of IFN-γ priming *in vivo*, as it demonstrates that IFN-γ is normally produced during the response to LPS and acts to amplify LPS-induced cellular responses. IFN-γ influences LPS-dependent signaling capabilities by promoting ligand-receptor interactions as well as downstream signaling machinery. In different experiments, IFN-γ was shown to promote transcription of TLR4, subsequent TLR4 surface expression, and LPS binding ability in macrophages[69-71]. Furthermore IFN-γ stimulation may promote the expression of the MD-2 accessory molecule, the MyD88 adaptor, and the IRAK signaling molecule.

In the macrophage-like cell line RAW264.7, it was found that IFN-γ pretreatment promoted NF-kB activation upon LPS exposure, as well as more rapid DNA-binding kinetics and faster degradation of the NF-kB inhibitor, IkBα[72]. In human monocytes also, IFN-γ pretreatment causes induction of active NF-kB upon LPS treatment[73]. When these cells were unstimulated, they exhibited high levels of the p50 subunit of NF-kB but only low levels of p65. IFN-γ priming caused an increase in p65 mRNA as a result of increased transcript stability. Many IFN-γ-inducible genes are also TNF-α-inducible, and these genes are often superinduced by the combination of these factors. TNF-α is a macrophage-derived cytokine secreted in response to LPS, which can act in an autocrine manner to mediate many LPS-induced effects via NF-kB.

1.2.4. IFN-γ and cancer immunoediting

Both mouse models and human clinical trials support the cancer immunosurveillance theory. This hypotesis holds that immune system protects the host against the development of cancers of non viral origin (tumor elimination). However, other studies demonstrated that tumor cells can escape or attenuate this immune pressure, either by sculpting tumor immunogenicity or by suppressing host-protective immune effector mechanisms. The dual opposing functions of immune system (host protection and tumor promotion) form the basis of the cancer immunoediting hypothesis. Cancer-immunoediting process consists of three phases: elimination (or protection), equilibrium (persistence) and escape (progression). It is thought that many different immune cells might interact with tumor cells from the earliest stages of transformation to the terminal phase of widespread metastasis. These interactions are controlled by endogenously produced interferons (IFNs) and recent work indicates that type I IFNs and type II IFN might have non-redundant functions in the cancer-immunoediting process by affecting distinct target-cell populations[48]. The IFN family has recently been shown to have obligate roles in the elimination phase of cancer immunoediting. In particular, endogenously produced IFN-γ is significant in this tumor surveillance. The antitumor activity of IFN-γ was observed in experiments performed to identify the cytokines required for the LPS-dependent rejection of transplanted Meth A cells (which are 3-metylcholanthrene (MCA)-induced fibrosarcoma cells of BALB/c mice). Cells treated with IFN-γ-neutralizing antibodies displayed compromised tumor rejection. Moreover, Meth A tumors grew significantly more rapidly in mice treated with the neutralizing IFN-γ-specific antibodies[74]. These studies revealed a critical role for IFN-γ in promoting rejection of transplantable tumors. The antitumor activity of IFN-γ was subsequently confirmed in primary tumorigenesis models. IFN-γ-insensitive 129/Sv mice lacking either the IFNGR1 subunit or Stat1, or wild type mice, were treated with different doses of carcinogen MCA and tumor development was monitored. At every dose of MCA, IFN-γ-insensitive mice developed tumors significantly more rapidly and with greater frequency compared to wild type mice[75]. The enhanced susceptibility to tumor formation was similar in mice that were unresponsive to IFN-γ only (i.e. IFN-γ $R^{-/-}$ mice) or to both IFN-γ as well as

IFNα/β (Stat1$^{-/-}$ mice), a result that suggested that IFN-γ played the major role in providing Stat1-mediated protection against development of primary carcinogen-induced tumors. These observations were recently confirmed by another group using C57BL/6 mice lacking the gene that encodes IFN-γ rather than the IFN-γ receptor[76]. Thus, the finding that endogenously produced IFN-γ prevents development of primary carcinogen-induced sarcomas is generalizable to mice on different genetic backgrounds. Another study showed that IFN-γ cooperates with other cytokines to prevent tumor formation, in particular with granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3)[77]. Additional studies revealed that IFN-γ and lymphocytes play important and interdependent roles in protecting the host against tumor development[78]. Studies of the contribution of IL-12 to antitumor immunity provide insight into the physiologically relevant stimuli for IFN-γ production during the cancer-elimination phase. In fact, IL-12 regulates the induction of IFN-γ expression in both the innate and the adaptive immune compartments. Injection of recombinant IL-12 into tumor-bearing mice reduces the rate of metastasis, slows tumor growth, and in some cases induces complete tumor regression. The critical requirement of IFN-γ in this process was demonstrated by the observation that the protective effects of IL-12 in tumor-bearing mice were ablated upon administration of neutralizing IFN-γ-specific antibodies[79]. These data show that endogenously produced IL-12 also promotes cancer elimination and mediates some of its antitumor effects through induction of IFN-γ expression.

In addition to their involvement in cancer immunosurveillance (elimination phase in cancer immunoediting), IFNs have been shown to be crucial components of the cancer-immunoediting process. Notably, IFN-γ has a key role in promoting tumor immunogenicity. In fact, unlike type I IFNs, IFN-γ acts on tumor cells directly during the antitumor response. Specifically, tumors that developed in IFNGR1-deficient mice were found to grow aggressively in immunocompetent recipients. However, when sensitivity to IFN-γ was conferred on the tumor cells by introducing the receptor subunit IFNGR1, the tumor cells became highly immunogenic and were rejected in a CD4^{+} and CD8^{+} T-cell-dependent manner. Therefore, tumors from IFNGR1-deficient mice represent sarcomas that cannot display their high antigenicity because of their inability to respond to IFN-γ[75]. The ability of IFN-γ to promote tumor rejection is mediated, at least in part, through its capacity to upregulate the MHC class I pathway of antigen processing and presentation in tumor cells. Actually, IFN-γ regulates various biological programmes that, *a priori*, could participate in abrogating tumor growth. They include the capacity of IFN-γ to inhibit cellular proliferation[61,80], to promote apoptosis through effects on the expression of caspases, CD95 (also known as FAS) and TRAIL[64], and to inhibit angiogenesis.

Concerning angiogenesis, the production of angiostatic molecules (angiostatin, endostatin) does not appear to be directly influenced by the presence or absence of IFN-γ. However, a three-member family of interferon-induced chemokines have been identified that can exert potent angiostatic actions: IP-10, Mig, and I-TAC. IP-10 was the first angiostatic chemokine family member to be identified as a protein that was rapidly induced and secreted in IFN-γ-treated cells[81]. This protein was found to have

potent chemotactic activity for T cells and promoted rejection of transplantable tumors when the tumor cells used were engineered to constitutively express high levels of IP-10[82]. Importantly, in this study, the protective effects of IP-10 required the presence of T lymphocytes in the host. Subsequent work revealed that exogenously administered IP-10 was capable of inhibiting angiogenesis *in vivo*[83]. Moreover, it was found that also IL-12 therapy inhibited angiogenesis and only in the IFN-γ–responsive tumors. Based on these results, the authors concluded that IL-12 therapy induced IFN-γ production in the host, which in turn induced IP-10 production within the tumor leading to blockade of neovascularization and inhibition of tumor growth. Subsequent studies have suggested that IFN-γ-induced IP-10 production also occurs in non-hematopoietic cells of the host and is critical for the angiostatic antitumor effect of IFN-γ[84].

IFN-γ increases tumor immunogenicity, but it is also able to regulate host immune cells to promote antitumor function. IFN-γ induces development of the Th1-cell lineage, rather than the Th2-cell lineage, that in turn promotes cell-mediated antitumor immune responses by facilitating cytotoxic T lymphocytes (CTL) maturation and macrophage activation[85]. Moreover, IFN-γ inhibits the generation and/or activation of naturally occurring ($CD4^+CD25^+$) regulatory T (Treg) cells[86].

Because it is probable that the IFN-γ-induced processes that are responsible for eliminating cancer cells might differ by tumor type, it will be important to use *in vivo* models of primary tumorigenesis to establish the physiological relevance of these IFN-γ-induced processes[87].

1.2.5. IFNs and cancer immunotherapy

Type I IFNs are involved in the cancer elimination phase, but in a different way than IFN-γ. Type I IFNs act on host cells rather than tumor cells. Several data indicate that endogenously produced or therapeutically administered type I IFNs mediate their antitumor effects mainly through acting on hematopoietic cells of the host. Furthermore, unlike IFN-γ, they do not control the immunogenicity of tumor cells. IFN-α and IFN-β have been extensively used for the treatment of several cancers. In fact, they are approved by the US Food and Drug Administration (FDA) for the management of hairy-cell leukaemia, AIDS-related Kaposi's sarcoma, follicular lymphoma, chronic myeloid leukaemia and melanoma[88]. For example, in the Eastern Cooperative Oncology Group Trial EST 1684, 287 patients with melanoma were treated by surgical excision, then randomized to groups of either follow-up observation or treatment with high doses of IFN-α for 48 weeks[14]. High-dose IFN-α therapy significantly increased both relapse-free time and overall survival time compared with untreated patients. ECOG Trial 1684 resulted in the FDA approval of high dose IFN-α for the adjuvant treatment of melanoma patients with thick lesions or node-positive disease. Moreover, a recent study supported the hypothesis that IFN-α augments the antitumor immune response[89]. In an ongoing clinical trial, individuals who developed autoantibodies or clinical manifestations of autoimmunity had significantly longer relapse-free survival compared with individuals who did not develop autoimmunity. Despite several sophisticated

clinical trials, much controversy still exists regarding appropriate patient selection, dosing regimens, and risks of significant toxicities associated with IFN-α. In North America IFN-α is used as a conventional treatment for high-risk melanoma patients. This is not the consensus worldwide, however, for European oncologists do not recommend interferon therapy for melanoma outside the scope of clinical trials[90]. The reasons for this discord are many, including inconsistent study results, severe IFN-related toxicities, as well as patient selection debates. Despite these controversies, IFN-α remains the only FDA-approved immunotherapeutic treatment for high-risk melanoma patients. Yet, researchers continue to search for the ideal IFN-α2b treatment regimen that will prove effective against melanoma without causing significant organ toxicities. It is apparent from clinical experience that despite FDA approval, high-dose IFN-α a is not considered to be the gold standard for adjuvant therapy of high risk, Stages I and II melanoma[91].

Unlike type I IFNs, IFN-γ is not approved by the FDA for the treatment of any cancers. Previous studies showed that treatment with IFN-γ had no benefit for patients with metastatic renal-cell carcinoma[92], advanced colon cancer[93] or small-cell lung cancer[94]. However, improved survival was observed when IFN-γ was used as an adjunct to therapy for individuals with stage-Ic–IIIc ovarian cancer[95], as well as when administered intravesically to individuals with transitional-cell bladder carcinoma[96] or when used in isolated-limb perfusion treatments of individuals with some nonmelanoma cancers of the extremities[97]. Despite the proven pivotal role of endogenously produced IFN-γ in animal models of antitumor immunity, the limited success of this cytokine in cancer-immunotherapy trials in humans might be explained by the following factors: tumor-cell insensitivity to IFN-γ, an inability to deliver IFN-γ locally (nearly all cells constitutively express functional IFN receptors) or an inability to therapeutically recapitulate the natural periodicity of IFN-γ production. Clinical trials of IFN-γ are ongoing[87].

1.3. Toll-like receptors (TLRs)

1.3.1. TLRs structure

Toll is a type I transmembrane receptor in *Drosophila*. The extracellular domain contains leucine-rich repeat (LRR) whereas the cytoplasmic domain shows striking similarity with that of IL-1R, and is referred to as Toll/IL-1R (TIR) domain. The Toll gene was identified as a gene essential for the dorsal-ventral development in the *Drosophila* embryo[98]. Subsequently, Toll signaling has been shown to play an essential role in the insect innate immune response against fungal[99] and Gram-positive bacterial infections[100]. Toll receptors are evolutionarily conserved and their homologs, namely Toll-like receptors (TLRs), are found in insects, plants and mammals. The first mammalian homologue of the *Drosophila* Toll receptor was identified in 1997 as hToll (now termed TLR4) by Medzhitov and colleagues[101]. To date, 10 members of the TLR family have been identified in humans (TLR 1-10) and 12 members in mice (TLR1-9, TLR11-13). TLRs are type I integral membrane glycoproteins composed of

extracellular, transmembrane and intracellular signaling domains[102]. The extracellular N-terminal domain consists of varying numbers of leucine-rich-repeat (LRR) motifs and it is responsible for binding so-called ''pathogen associated molecular patterns'' (PAMPs)[103,104]. The LRR domains are composed of 19–25 tandem LRR motifs, each of which is 20-30 amino acids in length, containing a conserved ''LxxLxLxxN'' motif and a variable part. LRRs are found in a diverse number of proteins and are involved in ligand recognition and signal transduction[105]. On the basis of their sequences and structural patterns, LRR-containing proteins can be divided into seven subfamilies. TLRs belong to the 'typical' subfamily. Each LRR region consists of 24 amino acids and they possess the conserved motif XLXXLXXLXLXXNXLXXLPXXXFX and display the unique horseshoe shape associated with LRR proteins. Interestingly, the LRR regions of TLR1, TLR2 and TLR4 have been shown to deviate in their conformation when compared with other 'typical' family members[106,107]. Their LRRs have been shown to be divided into an N-terminal region, a central region and a C-terminal region. Their central regions lack the conserved asparagine ladder normally associated with conferring stability on the horseshoe structure. This anomaly could allow these TLRs to vary their structural conformations and this could explain their ability to bind a variety of ligands as well as co-receptors which are essential for them to signal.

The intracellular C-terminal domain of TLRs is known as the Toll/IL-1 receptor (TIR) domain and shows high similarity to that of the IL-1 receptor (IL-1R)[108]. Intracellular TIR domains are composed of about 150 amino acid residues and after TLRs dimerization following ligand binding, they are involved in the interaction and recruitment of TIR domain-containing adaptor molecules to initiate downstream signaling pathway[109].

Structures of TLR3, TLR2 and TLR4 with their ligand was recently reported provide an understanding of ligand-induced activation of TLRs. In particular, it has been shown that these TLRs can form heterodimers such as TLR1-TLR2, TLR2-TLR6, TLR4-MD2 or a homodimer such TLR3-TLR3 after association with their respective agonist/antagonist ligands[106,110-114].

By comparing peptide sequences, TLRs can be classified into several subfamilies (Fig. 1-2)[115]. Although TLR1-9 are conserved between humans and mice, TLR10 is not functional in mice because of a retrovirus insertion, and TLR11, TLR12 and TLR13 are lost in human genomes. Moreover, the lack of responsiveness to TLR8 ligands in mouse models suggests that also murine TLR8 is not functional[116].

TLRs are expressed on various immune cells, including macrophages, dendritic cells, B cells, specific types of T cells, and even on nonimmune cells such as fibroblasts and epithelial cells. Expression of TLRs is not static but rather is modulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses. Furthermore, TLRs may be expressed extra- or intracellularly. While certain TLRs (TLRs 1, 2, 4, 5, and 6) are expressed on the cell surface, others (TLRs 3, 7, 8, and 9) are found almost exclusively in intracellular compartments such as the endosome, lysosome or the endoplasmic reticulum, and their ligands, mainly nucleic acids, require internalization to the endosome before signaling is possible.

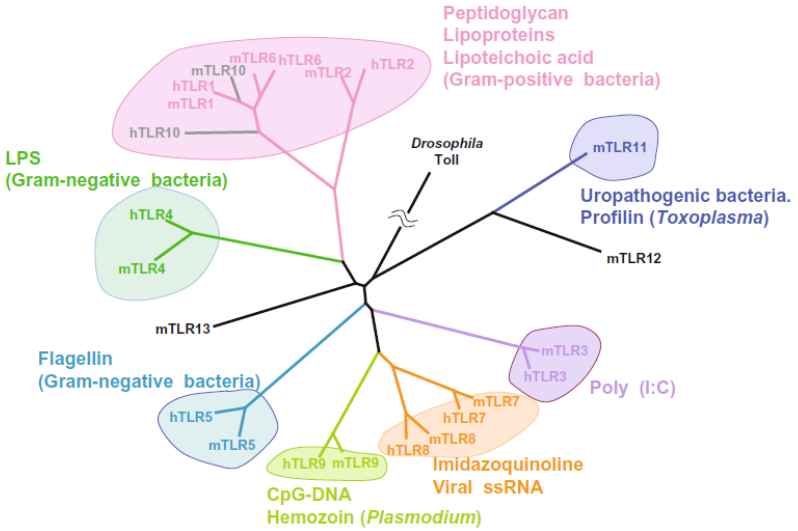


Figure 1-2. Phylogenic classifi cation of murine (*m*) and human (*h*) Toll-like receptors
(Kumagai, Y., et al. *J Infect Chemother* 2008)

1.3.2. TLRs and their ligands

Each TLR recognizes specific patterns of microbial components. TLR2 recognizes a wide range of PAMPs derived from various pathogens, ranging from bacteria, fungi, parasites and viruses[117]. These ligands include triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, porin from *Neisseria*, lipoarabinomannan from mycobacteria, zymosan (an insoluble preparation of yeast cell containing β-glucan, mannans, chitin, lipid and protein) from fungi, *Trypanosoma* GPI-mucin (tGPI-mucin) and hemagglutinin protein from measles virus. TLR2 forms a heterodimer with either TLR1 or TLR6, and these heterodimers appear to be involved in the differential recognition of lipoproteins with different lipid moieties[114,118]. TLR1-TLR2 heterodimer recognizes the bacterial triacylated lipopeptide, whereas the TLR2-TLR6 heterodimer recognizes the mycobacterial diacylated lipopeptide, LTA and zymosan. In addition, TLR2 forms a heterodimer with non-TLR molecules such as Dectin-1, CD14 and CD36. Dectin-1, a C-type lectin, was found to interact with TLR2 to recognize yeast pathogens and to elicit inflammatory responses. CD14 is involved in recognition of diacylated lipopeptide and lipoarabinomannan. CD36, a member of a class II scavenger receptor expressed on the surface of innate immune cells, is critical in sensing some but not all TLR2 ligands, including TLR2-TLR6 ligands. Moreover, TLR2 is able to bind different synthetic lipopeptides: the triacylated peptide Pam3CSK4 (TLR2/1) and the diacylated peptides, Pam2CSK4 (TLR2/6), SFSL1 (TLR2/6), R-FSL1 (TLR2/6/CD36). FSL1 is diacylated synthetic

lipoprotein derived from *Mycoplasma salivarium* and similar to active natural lipopeptide MALP-2.

TLR4 recognizes lipopolysaccharide LPS, a major constituent of the outer membrane of Gram-negative bacteria, which, through its lipid portion (lipid A), is a potent immunostimulatory molecule and causes septic shock. Recognition of LPS by TLR4 involves the accessory molecules MD-2[119], CD14[120], and LPS-binding protein (LBP)[121]. Dimerization of the TLR4-MD2 complex with another TLR4-MD2 complex on the cell surface only occurs following binding of LPS and this heterodimerization is required for signal transduction[113]. TLR4 is known to activate two signaling pathways - the myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and the TIR-containing adapter inducing IFN-β (TRIF)-dependent pathway (Fig. 1-3). The diversity of the structures of LPS among bacterial species may influence selective activation of these pathways. In addition to the detection of components of Gram-negative bacteria, TLR4 is implicated in the recognition of envelope proteins of viruses such as respiratory syncytial virus (RSV) and mouse mammary tumor virus[117].

TLR5, expressed by epithelial cells, monocytes, and immature DCs, recognizes flagellin, a protein component of bacterial flagella, the motility apparatus used by many microbial pathogens and is a potent activator of innate immune responses[122]. In fact, flagellin delivered to the cytosol can also activate the IPAF inflammasome and thus induce the secretion of the inflammatory cytokine interleukin 1β[123].

TLR3, 7, 8 and 9 are receptors for nucleic acid and its derivatives[124-126]. These intracellular TLRs appear to be sensors of foreign nucleic acids and trigger anti-viral innate immune responses by producing type I IFN and inflammatory cytokines. In particular, TLR3 is responsible for the recognition of double-stranded RNA (dsRNA). It was demonstrated that TLR3 binds polyinosine-polycytidylic acid (hereafter referred as poly I:C), a synthetic dsRNA that may mimic viral dsRNA generated during the replication of single-stranded RNA (ssRNA) viruses. TLR3 alone is not sufficient for anti-viral responses *in vivo*. TLR3 mRNA is detected in conventional dendritic cells (cDCs) and macrophages as well as by non-immune cells including fibroblasts and epithelial cells. Unlike immune cells, epithelial cells appear to express TLR3 on their cell surface. Strong expression of TLR3 is found in $CD8\alpha^+$ DCs with high phagocytic activity for apoptotic bodies of virus-infected or dsRNA-loaded cells. This allows dsRNA to gain access to TLR3 within cells and activate the signaling cascade to produce IL-12 p40 and IFN-β, suggesting a role of TLR3 in triggering cross-presentation, which processes exogenous antigens within the MHC class I pathway[127]. TLR3 is also implicated in the recognition of small interfering RNA (siRNA). TLR3 recognizes siRNA in a sequence independent manner and induces the production of IL-12 and IFN-γ, which efficiently suppress angiogenesis in a mouse model of choroidal neovascularization, indicating that siRNA-induced, TLR3-mediated innate immune responses, rather than suppression of gene expression, are important for the inhibition of angiogenesis[128].

TLR7 and TLR8 genes show high similarity to each other. Although both TLR7 and TLR8 are expressed in mice, murine TLR8 appears to be nonfunctional. Murine TLR7 and human TLR8 recognize synthetic

imidazoquinoline compounds such as imiquimod and resiquimod (R-848) and some guanine nucleotide analogs (e.g. loxoribine), all of which have anti-viral and antitumor poperties[125]. Subsequently, also viral ssRNA had been detected as ligands for TLR7 and TLR8. Both TLRs are expressed within the endosomal membrane. Many enveloped viruses get into the cytosol through the endosomal compartment. The phagolysosome is a highly acidified environment containing abundant degradation enzymes that may damage the viral particles, leading to ssRNA release and recognition by TLR7 or TLR8. Furthermore, when virus-infected apoptotic cells are taken up by phagocytes, viral RNAs may be released from these cells in the phagolysosome. Unlike virus particles whose genomes are sheltered in the capsid, self RNAs are subject to degradation by extracellular RNases when they are released from the cell, and rarely reach the endocytic compartment[117]. TLR7 is highly expressed on plasmacytoid dendritic cells (pDCs), a subset of DCs that rapidly secrete vast amounts of type I IFN in response to viral infection, whereas TLR8 was detected in various tissues, with the highest expression in monocytes.

TLR9 is a receptor for DNA with an unmethylated CpG-motif (CpG-DNA). Unmethylated CpG motifs are frequently present in bacterial DNA, but are rare in vertebrates[126]. Synthetic CpG oligodeoxynucleotides (ODNs) function as TLR9 ligands, and TLR9 recognition of DNA occurs independently of the base sequence. Viral DNA also stimulates the host immune system via TLR9 expressed by pDCs. In addition to DNA, hemozoin, a pigment from the malaria parasite *Plasmodium falciparum,* potently activates macrophages and DCs to produce inflammatory cytokines and chemokines through TLR9[129].

Mouse TLR11 is highly expressed in the kidney and bladder. Accordingly, TLR11 is likely to sense uropathogenic bacteria products although a ligand has not been identified yet[130]. TLR11 is also involved in the recognition of profilin, a protein from the parasite *Toxoplasma gondii tachyzoites*[131]. However, human TLR11 is nonfunctional because of the presence of a stop codon in the gene. Lastly, mouse TLR12 and TLR13 are not express in humans and their ligands are still unknown.

Toll-like receptors, in addition to exogenous pathogen-associated molecular pattern (PAMPs), are able to detect endogenous damage-associated molecular patterns (DAMPs), molecules released from injured or dying cells during inflammatory responses and tissue damages and, therefore, referred to as a danger signal. These endogenous ligands, recognized above all by cell-surface TLR2 and TLR4, include heat shock proteins (HSP60, HSP70, gp96 and HSP22), extracellular matrix degradation products (byglican, hyaluronan, fibronectin, fibrinogen, heparan sulfate, surfactant protein A), high-mobility group box 1 (HMGB1), β-defensin, and minimally modified low-density lipoprotein (LDL). These molecules trigger production of TNFα, IL-12 and nitric oxide by macrophages[132].

1.3.3. TLR signaling pathways

As above mentioned, dimerization of TLRs triggers activation of signaling pathways, which originate from a cytoplasmic Toll-like receptor (TIR) domain. The signaling cascades via the TIR domains are mediated by specific adaptor molecules including MyD88 (myeloid differentiation factor 88), MAL (MyD88 adaptor-like protein also known as TIRAP), TRIF (TIR domain containing adaptor inducing interferon-*β*), TRAM (TRIFrelated adaptor protein), and SARM (sterile-*α* and HEAT/Armadillo motifs-containing protein)[133]. These adaptor proteins also contain TIR domains and TIR-TIR interactions between receptor-receptor, receptor-adaptor, and adaptor-adaptor are critical for activating signaling. These adapters are selectively recruited to their respective TLRs, eliciting appropriate responses depending on the type of PAMP. TLRs signaling depend on two different pathways: the MyD88-dependent and the MyD88-independent pathway. The latter is also known as TRIF-dependent pathway.

MyD88-dependent pathway

MyD88 is utilized by all TLRs with the exception of TLR3 and drives NF-κB and mitogen-activated protein kinase (MAPK) activation to control inflammatory responses. TIRAP is recruited to TLR2 and TLR4 and functions as a sorting adapter that recruits MyD88 (Fig. 1-3B). Upon stimulation, MyD88 recruits IRAK-4 to TLRs through interaction of the death domains of both molecules, and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with TRAF6 which in turn activates transforming growth factor β-activated kinase 1 (TAK1). TAK1, in a complex with TAK1-binding protein (TAB1), TAB2 and TAB3, subsequently leads to the activation of two distinct signaling pathways involving IκB kinase (IKK) complex (Fig. 1-3C) or MAPK (Fig. 1-3D). MAP kinases phosphorylate and activate AP-1 transcription factors. In contrast, IKK complex induces phosphorylation and subsequent degradation of IκBα, which induces the nuclear translocation of the transcription factor NF-κB[134].

MyD88-independent pathway

The adaptor TRIF is used by TLR3 and TLR4 and initiates an alternative pathway leading to IRF3, NF-κB and MAPK to induce type I IFN and inflammatory cytokines. TRAM selectively serves to link TRIF to TLR4, but not TLR3. TRIF binds receptor-interacting protein 1 (RIP1) and TRAF6 leading to TRIF dependent NF-κB activation (Fig. 1-3E). TRIF also interacts with TRAF3 (Fig. 1-3F), which bridges to TBK1 and IKK*i*/IKKε, members of the noncanonical IκB kinases that directly activate IRF3. Phosphorylated IRF3 forms a dimer and translocates to the nucleus to induce the expression of target genes including IFN-β[129,134]. In pDCs, activation of TLR7 or TLR9 results in activation of a unique MyD88-dependent pathway leading to induction of type I IFNs through the transcription factor IRF7[117].

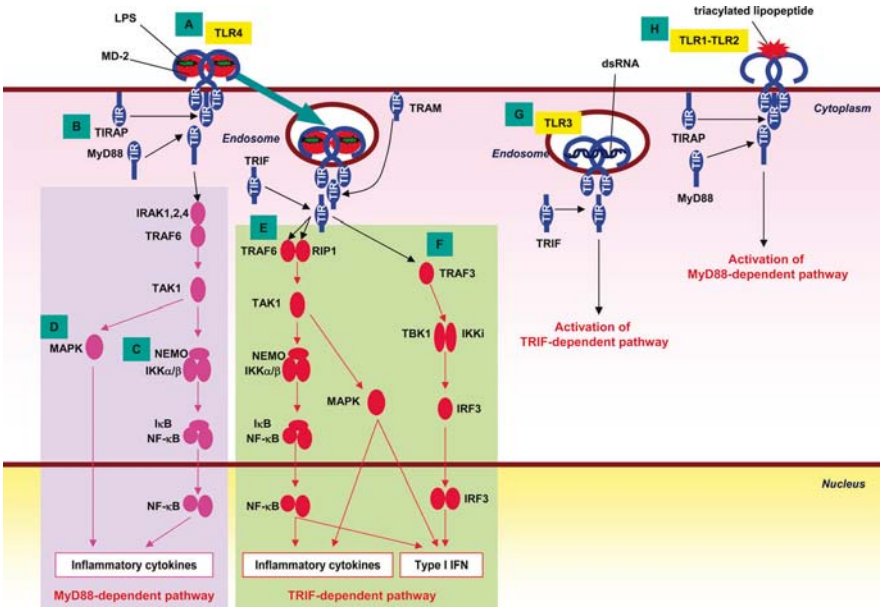


Figure 1-3. Signaling pathways triggered by TLR3, TLR4 and TLR1–TLR2
(Kawai, T., et al. *Int Immunol* 2009)

1.3.4. Function of TLRs

TLRs and host defence

Toll-like receptors are the best studied of a class of host receptors known as pattern recognition receptors (PRRs). They play a crucial role in the host defense against invading microorganisms by recognizing conserved structures named pathogen-associated molecular patterns (PAMPs). The expression of TLRs on mature cells has been extensively studied. TLRs are mainly expressed in human immune related cells, such as monocytes, neutrophils, macrophages, dendritic cells, T cells, B cells and NK cells. Differential expression patterns of the TLRs in immune cells were well reviewed[135]. In these cells, TLRs activation with their ligands derived from microbes triggers innate immune response to pathogens. However, more recently, the expression of TLRs on haematopoietic precursor cells has been detected, suggesting a possible role for TLRs in haematopoiesis. TLRs enhance the uptake of microorganisms by phagocytic cells and optimize microbial killing through the generation of reactive oxygen and nitrogen intermediates and stimulation of the neutrophil oxidative burst. TLRs also have a crucial role in mediating leukocyte recruitment to infected tissues. In addition, TLRs activate signals that are critically involved in the initiation of adaptive immune responses. In particular, activation of dendritic cells by TLRs induce T cell activation, the processing and presentation of microbial antigens, the upregulation of co-stimulatory molecules such as CD80 and CD86, which are necessary for the activation of naive $CD4^+$ T cells; and the

inhibition of regulatory T cell activity by the production of factors such as interleukin 6 (IL-6). Moreover, TLRs are also crucial for the activation and maturation of the B cell response during infection and vaccination. TLRs can also regulate the differentiation and maintenance of T and B cells by the production of IL-12, IL-23 and IL-27. These cytokines induce T helper type 1 (Th1) and Th17 cell development, and so help to promote the cell-mediated immune response.

TLRs, tissue repair and regeneration

In addition to their role in mammalian host defense from deleterious microbial infection, TLRs are also involved in various aspects of mammalian homeostasis such as development, the recognition of cellular and tissue injury, and tissue repair and regeneration. The repair and regeneration of tissue is a complex process. Firstly, TLRs may limit the extent of damage to initial injury in providing pro-survival signals and in preventing apoptosis. Subsequently, TLRs are involved in many events of the regeneration process. In tissue homeostasis, TLRs may be activated by microbial ligands during infectious injury or by endogenous ligands liberated from necrotic cells such as HMGB1 or extracellular matrix components as a consequence of non-infectious injury or repair[136].

1.3.5. TLRs and cancer

Since epithelia are continuously exposed to microbial challenges they are considered first line of defense against invading pathogens. Consequently, many TLRs are expressed by keratinocytes in skin and by epithelial cells in the gastrointestinal, respiratory and genitourinary tracts. Pathogen recognition by the TLRs expressed by these cells leads to the production of cytokines, chemokines and antimicrobial peptides[137]. Table 1-1 reported differential expression patterns of the TLRs in epithelia analyzed by reverse transcriptase PCR (PCR), by immunohistochemistry (IHC) or by Western blotting (WB). In some cases also the function was tested (FT).[138] Recently, several studies demonstrated that TLRs are also expressed by cancer cells (Table 1-2)[139], suggesting that TLR-signaling may play an important role in tumor development. In addition to various TLRs-expressing cancer types indicated in Table 1-2, many reports show that TLRs are expressed also on leukemia cell[140]. The most physiological role that TLRs play against cancer may be in preventing infection by microbial pathogens associated with the development of cancer. TLRs have been shown to be important in the recognition of microbial pathogens such as Epstein–Barr virus, hepatitis B and C virus, human papilloma virus, and *H. pylori,* all of which are important etiologic agents of human cancer. Functional TLR responses (in addition to those of other microbial PRRs) are likely to be important in whatever natural resistance humans have to these pathogens, and perhaps more importantly, in inducing protective immune response for cancer prevention by vaccines.

Surprisingly, different papers argue that TLRs on tumor cells may act as a double-edged sword, on one hand enhancing host immunity against the tumor by stimulating antigen presenting cells, and on the other hand

protecting the tumor from host surveillance. In fact, some studies suggest that enhancement of TLR function drives DC maturation and block $CD4^+CD25^+$T cell regulatory function, but in other cases the TLR function in tumors facilitates evasion of immune surveillance[141].

Table 1-1. Differential expression patterns of TLRs in epithelia
(modified from Yu, L., et al. *Cancer Immunology Immunotherapy* 2008)

Tissues	Toll-like receptors
Gastric epithelium	TLR4, TLR5, TLR9 (IHC)
Ileal mucosa	TLR3, TLR5 (IHC)
Active pouchitis	TLR2, TLR4 (IHC)
Colonic epithelium	TLR2, TLR3, TLR4, TLR5, TLR7, TLR9 (PCR); TLR2, TLR3, TLR4 (IHC); TLR4, TLR9 (WB)
Respiratory epithelium	TLR4, TLR9 (PCR); TLR4 (WB); TLR4, TLR9 (FT)
Fallopian tubes, endometrium, cervix and ectocervix	TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 (PCR)
Fallopian tubes, endometrium, cervix, ectocervix and vagina	TLR1, TLR2, TLR3, TLR5, TLR6 (IHC)
Endocervix, endometrium and fallopian tubes	TLR4 (IHC)
Cervical epithelium	TLR1, TLR2, TLR3, TLR5, TLR6 TLR7, TLR9, TLR10 (PCR); TLR3, TLR9 (FT)
Keratinocytes	TLR1, TLR2, TLR3, TLR4, TLR5, TLR9 (PCR); TLR2, TLR4 (FT)

Table 1-2. TLR expression in human cancer cells
(modified from Sato, Y., et al. *Cancer Microenviron* 2009)

Type of cancer	TLR
Gastric cancer	TLR2,TLR4,TLR5,TLR9
Colorectal cancer	TLR2,TLR3,TLR4,TLR5,TLR9
Ovarian cancer	TLR2,TLR3,TLR4,TLR5
Cervical cancer	TLR3, TLR4, TLR5,TLR9
Lung cancer	TLR2,TLR3,TLR4,TLR9
Prostate cancer	TLR4,TLR9
Melanoma	TLR2,TLR3,TLR4
Brain cancer	TLR2,TLR4
Breast cancer	TLR2,TLR3,TLR4,TLR9
Hepatocellular carcinoma	TLR2,TLR3,TLR4,TLR6,TLR9
Laryngeal cancer	TLR2,TLR3,TLR4

TLR as a positive regulator of cancer

Since TLRs appear to promote cell proliferation and tissue repair, their role in tumorigenesis and cancer is now being widely explored. In this

regard, MyD88 has been found to play a critical tumorigenic role in a mouse model of intestinal adenoma by upregulating the expression of tumor promoting genes, growth factors and various cytokines and chemokines[142]. Through its effects on IL-6 production, MyD88 has also been found to be a determining factor of gender disparity in the most common form of liver cancer, hepatocellular carcinoma. Chemically induced skin papillomas and sarcomas have also been associated with MyD88 activity. MyD88 has also recently been shown to be crucial to tumor promotion in the $Apc^{Min/+}$ and azoxymethane (AZO) model of spontaneous ($Apc^{Min/+}$) and carcinogen-induced (AZO) intestinal tumorigenesis[142]. It seems that MyD88 regulated the expression of many positive regulators of tumor promotion such as COX-2, matrix metalloproteinase MMP7, and cytosolic phospholipase A2 (cPLA2), which are important in many aspects of tumor growth. It remains to be determined whether these tumor promoting effects are mediated by specific TLRs. Interestingly, single nucleotide polymorphisms (SNPs) in several TLR loci seem to associate with higher cancer risk. For example, TLR4 polymorfism, as well as polymorphic variants of TLR1, 6, and 10 have been associated with increased prostate cancer risk. Other studies have linked TLR2 and TLR4 polymorphisms to increased cervical cancer and a TLR2-196 to 174del polymorphism to increased gastric cancer risk. Moreover, another TLR2 variant is associated with an increased risk of follicular lymphoma and a decreased risk of chronic lymphocytic leukaemia. Furthermore, the TLR4 Asp299 Gly variant was positively associated with the risk of mucosa-associated lymphoid tissue lymphoma and Hodgkin's lymphoma. Microsatelite GT polymorphisms of TLR2 gene and Asp299Gly polymorphism of the TLR4 gene is also associated with sporadic colorectal cancer among Croatians. It has been reported that sequences variants of TLR3 and TLR10 may be relevant to Nasopharyngeal carcinoma susceptibility in the Chinese population. Since the polymorfisms do not always affect TLRs expression levels, further investigations are necessary in order to elucidate the mechanism that links TLR polymorphisms with tumors[138].

There are a variety of mechanisms by which TLRs are thought to regulate tumor growth. First, TLRs can directly regulate tumor cell growth by modulating cell proliferation or survival signaling pathways. TLRs are expressed in various tumor cell types and are able to upregulate the NF-κB cascade and produce anti-apoptotic proteins that contribute to carcinogenesis and cancer cell proliferation. Regarding that, it was demonstrated that some TLRs ligands augment the growth of adoptively transferred tumors. Using a model of intravenous injection of a spontaneously metastasizing mammary adenocarcinoma cell line, it has been shown that systemic LPS administration increases both tumor migration and invasion to secondary sites from the bloodstream and angiogenesis at these sites[143]. In a similar model, but using a colonic adenocarcinoma cell line, intraperitoneal injection of LPS has been shown to increase proliferation and decrease apoptosis of metastatic tumors. Moreover, *in vivo* administration of TLR ligands may be protumorigenic due to action on both the tumor cells themselves and accessory cells in the tumor microenvironment. However, it was shown that several tumor cell lines

increased survival and proliferation also after *in vitro* stimulation. Notably, isolated plasma cells from patients with multiple myeloma were shown to express an increased repertoire of TLRs compared to plasma cells from healthy donors and stimulation of these cells with TLR ligands led to increased proliferation in part due to autocrine secretion of IL-6[140]. Such a direct effect of TLR ligation has been demonstrated by knocking down endogenous expression of TLRs in tumor cell lines before adoptive transfer. In these studies, the growth promoting effect of TLR4 on a TLR4-expressing colon cancer cell line occurred independently of exogenous administration of LPS[141], while the positive effect of TLR2 on *in vivo* hepatocellular carcinoma cell line growth was due to intratumoral administration of *Listeria monocytogenes*[144].

TLRs can also mediate cancer cell release of cytokines and chemokines that can recruit immune cells to enhance immunity in the tumor microenvironment. These immune cells release further proinflammatory cytokines, proangiogenic factors and growth factors, which impair the antitumor function of antigen-presenting cells (APCs) and effector T cells. Thereby, tumor cells are able to escape host surveillance. For example, versican, an extracellular matrix proteoglycan released by Lewis lung carcinoma (LLC), stimulates TLR2 on macrophages to produce tumor promoting cytokines such as TNFα, IL-1β, and IL-6[145]. Stimulation of the M26 mouse colon cancer cell line with LPS leads to the production of various soluble factors and proteins including IL-6, iNOS, IL-12, B7-H1, and B7-H2 and results in the inhibition of T cell proliferation and in the decreased NK and CTL cell cytotoxicity[141]. A similar immunosuppressive effect of nitric oxide and IL-6 was observed in the mouse hepatocarcinoma cell line H22 after TLR2-mediated stimulation with bacteria *Listeria monocytogenes*[144]. Stimulation of TLR2-4 expressed in human cutaneous melanoma with ligands specific for each TLR (zymosan for TLR2, poly I:C for TLR3, and LPS for TLR4), upregulated TLR expression and activated the adaptor protein MyD88 and NF-κB. After stimulation, TLRs induced several inflammatory cytokines and chemokines and melanoma cell migration increased[146]. TLR4-dependent signaling in the recipient was shown to be responsible for LPS-tumor growth by the increasing the levels of circulating TNF produced by host hematopoietic cells, which led to the up-regulation of NF-κB anti-apoptotic factors as Bcl-Xl, cIAP1, and cIAP2 in tumor cells[147]. In epithelial ovarian cancer cells, LPS can promote, directly from the tumor, the production of proinflammatory cytokines, tumor growth and paclitaxel chemoresistance. Notably, the activation of TLR4 in ovarian cancer cells results in a significant increase of X-linked inhibitor of apoptosis (XIAP) a major inhibitor of caspase-3 and -9 and phosphorylated Akt[148]. Thus, this suggests that TLRs also contribute to tumor-cell resistance to apoptosis and increased invasiveness. The inhibition of tumor-cell apoptosis by TLR-signaling is also observed in lymphoma cells and lung cancer cells[149]. The highly invasive MDA-MB-231 breast cancer cell line expresses TLR9, which when activated promotes MDA-MB-231 cell invasion by increasing the activity of matrix metalloproteinase 13 (MMP13), but not MMP8. Moreover, two earlier studies demonstrated that lipopolysaccharides may also promote tumor invasion through the lipopolysaccharides-activated NF-κB pathway

resulting in upregulation of iNOS and MMP2 and the β1 integrin subunit[143,150]. Thus, activation of TLR4 expressed by tumor cells results in adherence to extracellular matrix and endothelial cells promoting invasion and metastasis. In addition, a new *in vitro* study indicates that TLR9 agonists can stimulate prostate cancer invasion by increasing MMP13 activity[151].

However, it remains to be determined whether the TLR-mediated homeostatic response to tissue injury that is associated with tumorigenesis orchestrates processes such as angiogenesis that are ancillary to tumor promotion. TLR activation is known to stimulate angiogenesis *in vitro* through the expression of pro-angiogenic factors such as IL-8, vascular endothelial growth factor and metalloproteinases (MMPs), and there is *in vivo* evidence that TLRs might regulate the angiogenic switch.

Finally, recent studies report abnormally upregulated TLR signals in epithelial cells undergoing changes during chronic inflammation[152]. Chronic inflammation caused by autoimmune disease or microbial infections is an important risk factor for colorectal cancer (inflammatory bowel disease), gastric cancer (*Helicobacter pylori*), cervical cancer (human papilloma virus), liver cancer (hepatitis virus B and C), and hematologic malignancies (cytomegalovirus and Epstein-Barr virus). Notably, it was reported that more than 15% of cancers worldwide have a direct infectious origin[153].

TLRs as negative regulators of cancer

Microbial components have been used for many years to enhance anti-cancer immune responses. At the end of XIX century Coley found that repeated injections of a mixture of bacterial toxins from the Gram-positive bacteria *Streptococcus* and the Gram-negative bacteria *Serratia marcescens* served as efficient antitumor therapeutic agent demonstrating that microbial products, rather than infection per se, may have an effect against tumors. Shear and Turner later revealed that the antitumor effect of Coley's toxin was due to LPS[154], suggesting that the long appreciated antitumor effect of Coley's toxin can be attributed to stimulation of the host via TLRs. It is now clear that the anti-cancer effect of microbial components are mediated through TLR signaling. For example, the Bacillus Calmette-Guerin cell wall skeleton (BCGCWS) enhances the cytotoxicity of T cells and macrophages against cancer cells, and induces *in vivo* antitumor effect through TLR2 and TLR4 in the treatment of bladder cancer via intravesicular injection[155]. OK-432, a lyophilized preparation of group A *Streptococcus* is used in the treatment of cervical, gastric and oral squamous cell carcinoma and it was recently shown that the anti-cancer effect is mediated through TLR4[156].

Administration of purified ligands for TLRs has been demonstrated to have potent anticancer effects against established tumors in both mice and humans as a result of local (at the site of the tumor) and systemic delivery. Systemic administration of LPS has been used in Phase II clinical trials for the treatment of colorectal and lung cancer and leads to tumor regression when directly injected into adoptively transferred tumours[157]. For the latter, a similar result has also been shown on injection of flagellin[158]. Locally, application of synthetic ligands for TLR7 and TLR8, such as imiquimod, are under investigation for the treatment of skin cancer[159]. Imiquimod can inhibit tumor angiogenesis by inducing anti-angiogenic cytokines such as IFNs, IL-

10 and IL-12; down-regulating pro-angiogenic factors such as fibroblast growth factor β (FGFβ) and metalloproteinase-9 (MMP9); and promoting endothelial cell apoptosis. TLR agonists may be also effective when administered systemically for chronic lymphocytic leukaemia (CLL). They clear chronic lymphocytic leukaemia cells by enhancing the activity of natural killer and tumor-reactive T cells, or by altering the tumor microenvironment and inhibiting angiogenesis. However, TLR7 and TLR9 agonists can directly activate signaling pathways in CLL cells, leading to the production of cytokines and costimulatory molecules that render the tumor cells more sensitive to killing by cytotoxic T cells, immunotoxins and some chemotherapeutic drugs. The most studied TLR ligand used for its antitumor effect is the TLR9 ligand, CpG, which is under study for the treatment of brain, skin and renal cancer and lymphoma[160]. OM-174, a chemically defined TLR2/4 agonist, reduces tumor progression and prolongs survival in B16 melanoma mice treated with cyclophosphamide[161]. It appears that TLR2/4 agonists induce TNF-α secretion and inducible nitric oxide synthase (iNOS) expression. Nitric oxide is able to induce apoptosis of chemotherapy-resistant tumor cell clones. Moreover, TLR2/4-stimulation activates dendritic cell traffic and its associated tumor-specific, cytotoxic T cell responses. Therefore, TLR2/4 agonists seem promising molecules to prolong survival in cancer patients who relapse under chemotherapy[154]. Lastly, TLR4 agonists, including monophosphoryl lipid, have been used as adjuvant for vaccines against different pathogens

The administration of TLR agonists mediates antitumor activity by a multitude of mechanisms. To date, proapoptotic properties have been established or suggested for seven out of ten human TLRs. TLR2 was the first family member to be described as a death-inducing receptor, either after transfection, or directly in macrophages, neutrophils, trophoblasts, Schwann cells and microglia cells. TLR2 drives apoptosis through the Fas pathway. For example, the lipopeptide mediated apoptosis of HEK 293 cells is dependent on the TLR2–MyD88 pathway which appears to involve Fas associated death domain protein (FADD) and caspase-8[162,163]. Activated caspase-8 cleaves and activates caspase-3, which then executes the apoptotic program. In some conditions, the autocrine secretion of inflammatory factors such as TNF-α or the up-regulation of FasL may contribute to TLR2-triggered apoptosis. Moreover, TLR2 activation induces ROS generation and activation of apoptosis signal-regulating kinase 1 (ASK1). The ensuing sustained p38 MAPK phosphorylation leads to the activation of NF-kB and AP-1 as well as to the enhancement of cell death[164] (Fig. 1-4). Also TLR4, stimulated by LPS, is involved in apoptosis, but data regarding TLR4-triggered apoptotic pathways are more complex, since both the adaptors (MyD88 and TRIF) involved and the nature of the programmed cell death (extrinsic *versus* intrinsic) appear to vary with cell type (Fig. 1-4). In intestinal epithelial cells that highly express TLR5 on their basolateral membrane, purified flagellin simultaneously triggers TLR5-mediated proapoptotic and pro-inflammatory signaling. Consistently, when activation of either NF-kB or Akt was blocked, TLR5 ligands triggered the apoptosis of epithelial cells[165]. The TLR7 ligand Imiquimod induces the apoptosis of basal carcinoma cells, of transformed keratinocytes and of melanoma metastases

both *in vitro* and *in vivo*[166]. TLR8 was shown to trigger neuronal apoptosis[167]. CpGrich oligonucleotides (CpG ODN) not only trigger TLR9-mediated death of transfected cells, but also of glioma and colon cancer cell lines[168]. Lastly, also high doses of TLR3 agonist poly I:C can lead to apoptosis and have been shown to directly kill both tumor cells and ancillary cells of the tumor microenvironment. Unlike other TLRs, TLR3-triggered apoptosis exclusively depends on TRIF adaptor (Fig. 1-4). Notably, synthetic poly I:C induces apoptosis of human breast cancer cells in a TLR3-dependent manner involving the proapoptotic role of IRAK4 and NF-kB downstream of TLR3 as well as the activation of the extrinsic caspases[169]. Also melanoma cells, expressing TLR2, TLR3 and TLR4[146], can undergo apoptosis. Whereas stimulation of TLR4 on melanoma cells with LPS up-regulated the production of IL-8 and cell adhesion[170], the engagement of the receptor by TLR3 agonists can directly inhibit cell proliferation and induce tumor cell death when combined to treatment with either type I IFN or protein synthesis inhibitors[171]. TLR3-mediated cell death involves the activation of caspases and engages both extrinsic and intrinsic apoptotic pathways. These evidences suggest that TLR3 agonists represent very promising adjuvants for cancer vaccines, but also good cytotoxic agents in selected cancers.

Further investigations revealed that type I IFNs antagonize the proliferation promoting effect of TLR ligands. IFN-β prevents the degradation of p27, a cell cycle inhibitor, that conversely TLR ligands trigger in a MyD88 and Akt dependent manner to promote survival. These results are consistent with the observation that type I IFNs inhibit mesangial cell proliferation. Accordingly, it was shown that poly I:C and LPS, both of which induce type I IFNs, promote apoptosis[172]. Moreover, as above mentioned, IFN-α synergizes with poly I:C in triggering apoptosis in melanoma cell lines[171].

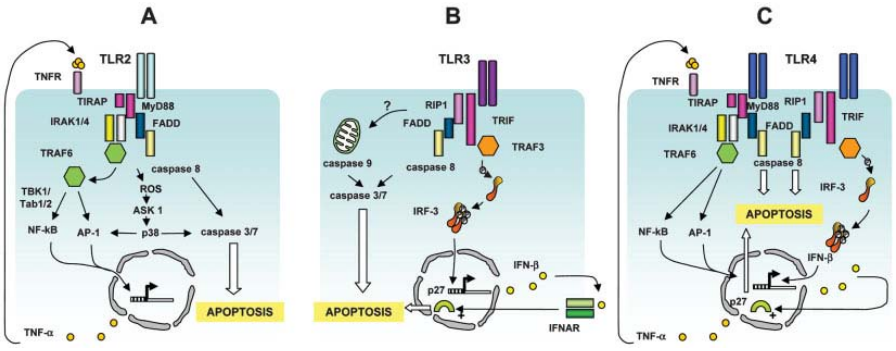


Figure 1-4. Different signaling pathways link TLR2, 3 and 4 to apoptosis
(Salaun, B., et al. *Eur J Immunol* 2007)

In addition, there are IFN-independent mechanisms of TLR-induced apoptosis. In fact, poly I:C triggers apoptosis in IFN-insensitive human prostate cancer cells by protein kinase C alpha (PKCα)-induced JNK and p38 activation[173]. Also retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated antigen 5 (MDA-5) helicases initiate a proapoptotic signaling pathway that is independentof type I IFNs. In human melanoma

cells, poly I:C triggers this signaling pathway that leads to efficient activation of mitochondrial apoptosis, requiring caspase-9 and Apaf-1. Surprisingly, this proapoptotic signaling pathway was also active in nonmalignant cells, but these cells were much less sensitive to apoptosis than melanoma cells[174]. In addition, a recent work report that poly I:C complexed with polyethyleneimine (PEI) to improve intracellular delivery, is able to induce an early but persistent autophagy and a late apoptotic program as well. This is in contrast to other systems where caspases are the initiating death signals and autophagy is induced to favor the clearance of partially degraded cytosolic components. Sustained lysosomal-dependent degradative process together with the activation of apoptotic caspases can ultimately converge in efficient tumor cell death[175].

TLR activation may also lead to tumor regression by directly or indirectly (TNF-mediated) increasing vascular permeability, recruiting of leukocytes (such as macrophages) involved in resolving tumor, direct and indirect activation of the tumor lytic activity of NK and cytotoxic T cells, and increasing the sensitivity of tumor cells to assisted killing such as via TRAIL, TNF, and granzyme B/perforin. The best appreciated role of TLR in cancer therapy has come from taking advantage of the function of TLRs in stimulating the adaptive immune response against microbial pathogens. These studies have sought to break tolerance to tumor self-antigens and induce antitumor effector immune responses by using TLR ligands as adjuvants (or even alone in TLR monotherapy) in cancer vaccines, as targets of gene therapy, and in raising antitumor antigen-specific T cells *in vitro* for adoptive transfer. The mechanisms by which TLRs induce effective antitumor adaptive immune responses include uptake, processing, and cross-presentation of tumor cells by dendritic cells, increased survival of dendritic cells, induction of co-stimulatory markers on professional antigen-presenting cells, induction of Th1 and CTL responses, and the inhibition of regulatory T cell activity[136]. Most studies have used exogenous TLR agonists to induce anti-cancer T cell responses that are very hard to induce under physiologic (endogenous) circumstances. However, two recent study has suggested a more physiologic role of TLRs in inducing antitumor T cell responses. In one study was reported that the antitumor efficacy of numerous chemotherapeutic agents to mice with established, adoptively transferred tumors was dependent on TLR4 and MyD88. The authors suggest that this phenomenon is due to the activation of TLR4 and induction of antitumor T cell immunity by HMGB1 released from dying tumor cells due to chemotherapy[176]. In a second report, C3H/HeJ mice with loss-of-function mutation in TLR4 that were treated with carcinogenic polyaromatic hydrocarbon 7,12-dimethylbenz(a)anthracene (DMBA) to induce skin tumors developed more tumors than wild-type mice, perhaps because of decreased activation of IFN-γ-dependent antitumor T cell responses[177].

1.4. Immunogenic cell death

Depending on the lethal stimulus, tumor cells can die by distinct cell death mechanisms including apoptosis and necrosis. Most chemotherapeutic agents kill tumor cells through an apoptotic pathway. Among these, only a few agents have the capacity to stimulate immunogenic cell death. Recent studies have revealed that treatment of tumor cells with anthracyclins, oxaliplatin or ionizing irradiation, but not with other apoptosis inducing drugs (such as mitomycin C, etoposide or staurosporin) induce a potent immune response *in vivo* when dying cells are injected in immunocompetent mice. Remarkably, it was demonstrated that the outcome of these treatments depends on the active contribution of the host immune system[178]. Whether tumor cell death is immunogenic or not depends to a large extent on the death-initiating stimulus. In fact some, but not all cell death inducers cause the exposure of immunogenic factors on the cell surface or the release of immunogenic signals into the extracellular space. In addition, the same anticancer agent can cause the exposure/release of immunogenic signals from some tumor but not from others, due to the fact that this exposure/release requires the intervention of specific signal transduction pathways[179]. Anticancer chemo- and radiotherapies induce cell death in rapidly proliferating tumor cells, as well as in cells of the hematopoietic system including the immune system. Since apoptosis, the main cell death modality, has a role during normal development and physiological cellular turnover, it has been thought to be intrinsically non-immunogenic or tolerogenic. On the contrary, pathological cell death, necrosis, is immunogenic and elicits inflammatory reactions. However, cells dying by apoptosis can be also highly immunogenic, whereas necrotic cells can be less immunogenic than cells undergoing an immunogenic form of apoptosis. Thus, it has been proposed that there are subtypes of cell death, such as immunogenic and non-immunogenic apoptosis, and that subtle differences in the composition of the cell surface and/or in the products that are secreted by the dying cells (which could include Damage-Associated Molecular Patterns, DAMPs) determine whether the death of the cell is immunogenic or not[180].

Cancer cells die through different mechanisms depending on the precise cause of death, such as hypoxia, shortage of nutrients, absence of essential growth factors or conventional anticancer treatments (radiotherapy and chemotherapy). Cell death can be classified, according to its morphological criteria, in type 1, 2 and 3 cell deaths that are apoptosis, autophagic cell death and necrosis, respectively[181].

1.4.1. Apotosis

Apoptotic cell death is morphologically defined by chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), shrinkage of the cytoplasm, few or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing and formation of apoptotic bodies, but cell integrity is maintained until the final stages of the process. There are several distinct subtypes of apoptosis that, although morphologically similar,

can be triggered by different biochemical routes (for example, through the intrinsic or the extrinsic pathway or with or without caspase activation). At later stages, apoptotic cells can acquire features of necrosis (termed secondary necrosis), namely swelling and membrane rupture[181].

Apoptosis is usually, but not exclusively, associated with caspase activation and mitochondrial membrane permeabilization. Caspases, an evolutionary conserved family of aspartate-specific cysteine proteases, are the major proteases responsible for the proteolytical cleavage of numerous substrates during this process[182]. To prevent undesired cell death as a consequence of unscheduled caspase activity, these proteases are produced as latent zymogens with an N-terminal prodomain of variable length preceding the catalytic domain. Caspases are classified in two groups: 'initiator caspases' (such as caspase-1, -8 and -9) and 'effector caspases' (such as caspase-3, -6 and -7). The former, with large prodomains, are first recruited into large protein complexes in which they undergo autoactivation. Activated initiator caspases subsequently free the latter ('effector caspases') of their short inhibitory prodomain, allowing them to cleave a large set of cellular substrates[183]. However, not all caspases participate in apoptosis. There are also the inflammatory caspases incuding caspase -1, -4, -5, -11, and -12 that seem to be involved in the maturation and secretion of proinflammatory cytokines such as interleukin IL-1β and IL-18 during infection and inflammation. Notably, caspase-7, besides its activation during apoptosis, has also been observed under inflammatory conditions. Interestingly, in macrophages stimulated with lipopolysaccharides (LPS) and ATP or infected with the Gram-negative pathogens *Salmonella typhymurium* and *Legionella pneumophila*, caspase-7 activation requires caspase-1 complexes named 'inflammasomes' (Fig. 1-5) rather than the caspase-8 and -9 protein complexes involved in apoptosis. Lamkanfi and coworkers found that activation of caspase-1 and -7 in response to LPS+ATP and *Salmonella* infection proceeded normally in macrophages deficient in caspase-3, demonstrating that caspase-3 is not required for activation of caspase-1 and -7. Similarly, caspase-1 and caspase-3 do not require caspase-7 for their activation[184,185]. However, caspase-7 is closely related to caspase-3 and both are involved in apoptosis. Caspase-3 and -7 can be activated in concert by the initiator proteases caspase-8 and -9 in response to classical apoptotic triggers such as death receptor engagement and UV irradiation. Importantly, caspase-3 and -7 have some overlapping, but also some distinct, roles in apoptosis. Caspase-3 controls DNA fragmentation and morphologic changes of apoptosis, whereas caspase-7 plays little role in these processes. In contrast, caspase-7 appears to be more important to the loss of cellular viability, although the combined role of both caspases is crucial in this area. Moreover, it should be noted that the importance of caspase-3 and -7 in apoptosis appears to be cell type- and stimulus-dependent[186]. A biochemical study demonstrated that caspase-3 and -7 exhibit differential activity toward multiple substrate proteins. Caspase-7 is more selective: of the 20 different substrates examined during this study, 12 were preferentially cleaved by caspase-3 whereas only one (cochaperone p23) was more susceptible to proteolytic processing by caspase-7[187].

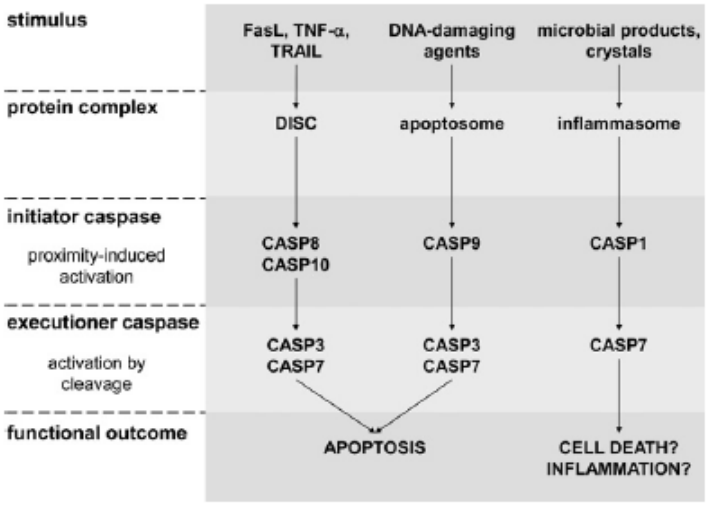


Figure 1-5. Overview of caspase activation mechanisms
(Lamkanfi, M., et al. *Int J Biochem Cell Biol* 2009)

Two major pathways can lead to the activation of the apoptotic program. The intrinsic pathway is initiated from within the cell and usually in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress. For example, staurosporine and ultraviolet irradiation (UV) are two inducers of mytochondrially mediated apoptosis. This pathway is under the strict control by members of the Bcl-2 protein family and involves the release of proapoptotic proteins that activate caspase enzymes from the mitochondria. The proteins of the Bcl-2 family contain signature domains of homology called Bcl-2 homology (BH) domains (termed BH1, BH2, BH3, and BH4) and can be subdivided into pro- and antiapoptotic members. Proapoptotic Bcl-2 proteins are divided into two subgroups based on the number of BH domains they contain. There are those with several BH domains (eg, Bax and Bak), and then there are those that only have the BH3 domain, such as Bid, Bad, Bim, Bmf, PUMA, and NOXA. BH3-only proteins activate the multi-BH domain proapoptotic proteins Bax and/or Bak, which then allow for permeabilization of the outer mitochondrial membrane (MOMP). The antiapoptotic Bcl-2 proteins Bcl-2 and Bcl-X_L act to prevent permeabilization of the outer mitochondrial membrane (MOMP). Upon membrane permeabilization, cytochrome *c* is then able to translocate into the cytosol and binds the adaptor apoptotic protease activating factor-1 (Apaf-1), forming a large multiprotein structure known as the apoptosome. Initiator caspase-9 is recruited into the apoptosome and activated. Thus, it can in turn activate the downstream effector caspases.

The extrinsic pathway depends on the binding of a series of specific ligands such as tumor necrosis factor (TNF), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) to their respective transmembrane

receptors, called death receptors[188]. The subsequent recruitment of adapter molecules like TRADD or FADD enables the binding and autoproteolytic activation of pro-caspase-8, which in turn leads either to a direct activation of effector caspases such as caspase-3 and -7 or rather stimulates an indirect pathway, namely by triggering MOMP with subsequent cytochrome *c* release, apoptosome activation and caspase-9-dependent caspase-3 and -7 activation[181]. Cells that undergo physiological apoptosis are rapidly and specifically recognized and engulfed by phagocytic cells. Phagocytosis by macrophages is associated with the release of anti-inflammatory mediators like transforming growth factor-β (TGF-β), prostaglandin E2 or platelet-activating factors, according with the hypothesis that apoptosis is immunologically silent due to the suppression of local inflammation. However, as above mentioned, the apoptosis is also immunogenic in some experimental situations[181].

1.4.2. Autophagy

Autophagy is an important eukaryotic response to cellular stress like protracted nutrient deprivation, hypoxia or infection. Macroautophagy (hereafter referred to as autophagy) involves the sequestration of cellular material within characteristic double- or multi-membraned autophagosomes and its subsequent degradation upon fusion of the autophagosomes with lysosomes. Autophagy serves as a major turnover mechanism to eliminate supernumerary or damaged organelles, intracellular pathogens, aggregate-prone proteins and superfluous portions of cytoplasm. Autophagy promotes survival by adapting cells to stress conditions. Nevertheless, persistent autophagy, which depletes the cell of organelles and critical proteins, can lead to a caspase independent form of cell death[189]. Autophagic cell death occurs in the absence of chromatin condensation but is accompanied by massive autophagic vacuolization of the cytoplasm[190]. In some experimental conditions the autophagy acts as a mechanism to execute cell death when apoptosis is inhibited. Moreover, autophagy deficiency can stimulate apoptosis and also necrotic cell death. However, inhibition of autophagy may also favor tumor growth[191]. Apart from its role as innate defense mechanism against invading pathogens, autophagy and digestion of endogenously synthesized cytosolic proteins enables their processing for MHC II presentation thus connecting autophagy with adaptive immunity[191].

1.4.3. Necrosis

Necrosis is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, mitochondrial dysfunction, rapid plasma membrane rupture and subsequent loss of intracellular contents. Necrosis lack of typical apoptotic features such as DNA cleavage and nuclear condensation[190]. Moreover, necrotic dying cells retain their ability to synthesize proteins, while translation is blocked in apoptotic dying cells. Necrosis has traditionally been considered merely as an accidental, uncontrolled form of cell death resulting from severe and acute injuries under conditions where apoptosis could not take place for some reason.

Nonetheless, programmed necrosis can occur as a result of the activation of specific signal transduction cascades, even during development and in adult tissue homeostasis. Support for a regulated necrosis mechanism came from studies of the death receptors. Interestingly, it was discovered that, in certain cell types, stimulation with FasL or TNF-α under apoptosis deficient conditions could induce cell death with morphological features of necrosis, instead of the extrinsic apoptotic pathway. This regulated cellular necrosis mechanism, discrete from apoptosis, was termed ''necroptosis'' [192]. Thus, the same upstream signal can produce different types of cell death as a function of the activation or inhibition of catabolic enzymes in the cell, underlining the close relation between cell death modalities. Necroptosis involves the obligatory activation of the RIP1, a death-domain-containing kinase associated with the death receptors and also with the adaptor protein TRIF. RIP1 is a serine/threonine kinase with a death domain (DD) and a caspase recruitment domain (CARD). RIP1-DD was shown to bind death receptors, such as tumor necrosis factor (TNF)-R1, TRAIL-R1 and TRAIL-R2, and to DD-containing adaptor proteins such as TNF-receptor-associated death domain (TRADD) and FADD. Besides, RIP1 interacting with TRIF is involved in TLR3 and TLR4 signaling pathways. In all cells and under different conditions, RIP1 is crucial for activating NF-kB, but it is also involved in activating MAPKs such as p38 MAPK, JNK and ERK. As already reported, apart from its role in apoptosis, recently RIP1 has been found to participate in necrotic cell death[192,193]. Notably, Kalai and colleagues demonstrated that RIP1 was required for initiation of both dsRNA-induced apoptosis and necrosis[194]. Moreover, the inhibition of HSP90 by geldanamycin led to lower expression levels of RIP1 and thus to the complete blockade of IL-6 release[195]. Finally, the kinase activity of RIP1, inhibited by the allosteric inhibitor Nec-1[192], seems to be essential only for cell death induction and ERK activation. By contrast, this property is dispensable for RIP1-induced activation of NF-kB and other MAPKs[193].

In contrast to apoptotic cells, whose remains are engulfed completely by phagocytes, necrotic cells are internalized by a macropinocytotic mechanism, meaning that only parts of the cell are taken up by phagocytes[196]. Unlike apoptosis, which only under certain circumstances exhibits an immunogenic response, necrosis is considered to be immunologically harmful at all times, because of the sudden release of proinflammatory mediators[181] (Fig. 1-6). Necrotic cell death often causes the release of proinflammatory cytokines, such as IL-8, IL-10, TNF-α[181,197] or of terminal mediators of inflammation like HMGB1[198]. Furthermore, in a recent study was demonstrated that also IL-6 is released in necrosis induced by TNF, anti-Fas and dsRNA after the activation of NF-kB and p38MAPK. However, the release of IL-6 was strongly reduced or even abolished in conditions of apoptotic cell death induced by the same stimuli[195].

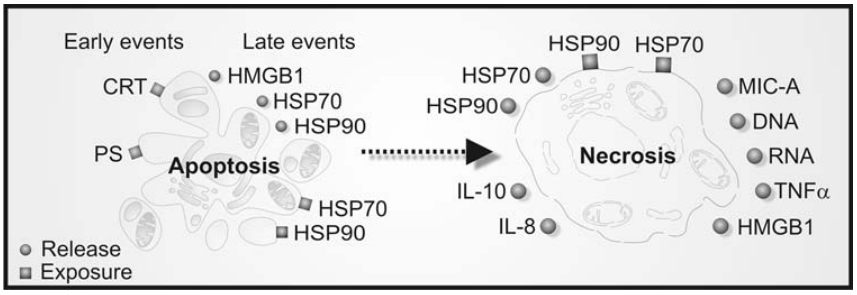


Figure 1-6. Immunogenic determinants of tumor cell death
(Kepp, O., et al. Apoptosis 2009)

1.4.4. Immunogenic effectors and their influence on the immune system

Calreticulin

Calreticulin (CRT) is a Ca^{2+}-binding chaperone that is usually located in the lumen of the endoplasmic reticulum (ER). Along with the ER-resident disulfide isomerase ERp57, CRT facilitates proper folding of most ER-chaperoned proteins. CT26 colon cancer cells and MCA205 fibrosarcoma cells treated with anthracyclins and ionizing irradiation have been found to translocate calreticulin to the cell surface at an early, pre-apoptotic stage, several hours before phosphatidylserine (PS) expose and before plasma membrane permeabilization. By contrast, in response to other cell death inducers, CRT exposure occurs concomitantly with PS exposure, at a late apoptotic stage. Notably, it was found that the interaction of CRT with ERp57, the caspases activation and the phosphorylation of the eukaryotic initiation factor 2α (eIF2α) are necessary for the pre-apoptotic CRT exposure and thus for the immunogenic outcome of anthracyclin treatment. Unlike, other cell death inducers targeting ER (like thapsigargin, tunicamiycin and brefeldin), mitochondria (arsenite, betulinic acid and C2 ceramide) or DNA (Hoechst 33342, camptothecin, etoposide and mitomycin C) fail to induce CRT exposure and immunogenic cell death. Thus, the translocation and exposure of CRT dictates the immunogenicity of cancer cell death, presumably because surface-exposed CRT facilitates the engulfment of dying tumor cells by DC[180].

Heat shock proteins

Heat shock proteins (HSPs) are a class of chaperones that can be induced by multiple different stressors. Under non-lethal stress conditions, HSPs function to protect cells by refolding damaged proteins or by redirecting them to proteasomal degradation. On the contrary, cellular stress can induce the exposure and the release of HSPs from necrotic dying cells. HSP70 and HSP90 can translocate from intracellular compartments to the cell surface of dying tumor cells and hence can participate in the activation of the immune system during necrosis[199,200]. However, HSPs overexpression is also able to inhibit apoptosis[201]. The recognition of HSPs exposed by tumor cells can be mediated by TLR4, which facilitates intracellular antigen

processing and presentation and by scavenger receptors that might stimulate DC maturation. Recently, it was reported that the proteasome inhibitor bortezomib induces the expression of HSP90 on the surface of dying human myeloma tumor cells and thus enhancing their immunogenicity[202]. In particular, in order to increase immunogenicity, the presence of HSPs at the surface of dying tumor cells facilitates their recognition by DC and/or stimulate the maturation of DC[190].

High-mobility group box 1

In healthy cells, the high mobility group box 1 protein (HMGB1) binds to chromatin and influences transcription and other nuclear functions. However, cells that undergo necrosis release HMGB1 which has proinflammatory properties[198]. HMGB1 can either be actively secreted from inflammatory cells or passively released from necrotic cells. Although it was initially thought that HMGB1 is released from the nucleus only during primary necrosis, recent data indicate that HMGB1 can also be released during secondary necrosis that occurs following apoptosis and after autophagic cell death. Recently, the redox status of HMGB1 has been discovered to be important for its immunological potential during apoptotic release[203]. HMGB1 is strongly up-regulated in breast cancer, colon cancer, melanoma, pancreatic cancer and prostate cancer. The release of HMGB1 from the nucleus of dying tumor cells to their cytoplasm and subsequently to the extracellular space during later stages of apoptosis constitutes a crucial step in the activation of antigen presenting cells[204]. HMGB1 has been shown to bind to at least three different surface receptors expressed on DC, namely the receptor for advanced glycosylation (RAGE), TLR2 and TLR4[178]. The binding of HMGB1 to TLR4 in DCs can facilitate the processing and presentation of tumor derived antigens by inhibiting fusion of phagosomes with lysosomes, thereby preventing the precocious degradation of tumor antigens and enabling their traffic towards the antigen-presenting compartment. Neutralization or knockdown of HMGB1 or knockout of TLR4 abolishes the capacity of dying tumor cells to elicit anticancer immune responses both *in vitro* and *in vivo*. Syngenic tumor implantend in tlr4$^{-/-}$ mice responded poorly to local radiotherapy or systemic chemotherapy as compared to similar tumors implanted in WT mice[204]. Also the adapter molecule MyD88 (but not TRIF) is important for the perception of immunogenic cell death. Thus, a TLR4/MyD88 dependent pathway participates in the chemotherapy-induced anti-cancer immune response. In fact, breast cancer patients bearing a loss-of-function allele of TLR4 that reduces the affinity of TLR4 for HMGB1 developed metastases more rapidly than patients bearing the normal allele of TLR4 after local radiotherapy and systemic anthracyclin therapy[176].

Nucleotide release

During apoptotic and necrotic cell death, degrading cellular corpses release nucleotides, RNA and DNA, which may exert immunostimulatory effects. RNA which is released during cell death can interact with TLR3 on the surface of DC[205], double-stranded DNA can stimulate macrophages and DC. Nucleotides may stimulate the maturation of DC accompanied by an

activation of the NF-kB signaling. Studies have shown that RNA can be released by tumor or normal cells and detected in blood[206]. In a recent study, TLR3 expression was upregulated in melanoma cells incubated with purified total RNA from normal peripheral blood lymphocytes (PBL) or allogeneic melanoma cells, and TLR activation promoted melanoma cell migration. Thus, RNA derived from melanoma cells can act as a TLR3 ligand and facilitate migration of melanoma cells, without support from immune cells[146]. According to this previous work, Sato and coworkers have shown how interactions between TLR3 and RNA could create and maintain a tumor microenvironment. In this microenvironment, cancer cell death might stimulate cancer progression if nucleic acid fragments released by the dead tumor cells are transfected into normal cells, thereby changing the normal cell's properties. The authors argue that normal cells in the tumor microenvironment might also be transfected by microRNA released from tumor cells, because these small RNA molecules (20–22 base pairs) are easily taken up by cells. This phenomenon could explain the expression of tumor-related proteins by normal cells in the tumor microenvironment., but it has yet to be demonstrated *in vivo*[139].

Other DAMPs

A recent study has identified SIN3A-associated protein 130 (SAP130), which is a component of small nuclear ribonucleoprotein, as another DAMP that is released by necrotic and late apoptotic cells. SAP130 specifically binds to macrophage-inducible C-type lectin (MINCLE). Neutralization of MINCLE with a specific antibody inhibited the recruitment of neutrophils and the production of pro-inflammatory cytokines following intraperitoneal injection of dead tumor cells, indicating a role for the SAP130‑MINCLE interaction in the immunogenicity of dead cells *in vivo* [190]. However, the expression and function of MINCLE by DCs has yet to be directly examined. Dying cells also expose or release another as yet unidentified DAMP that is recognized by the receptor C-type lectin domain family 9, member A (CLEC9A), which is expressed by $CD8\alpha^+$ DCs[207]. This activates spleen tyrosine kinase (SyK) and results in cross-presentation of associated antigens on MHC class I molecules important for the induction of immune responses by dying cells. Finally, in response to oncogenes or DNA damaging agents, cells can express MHC class I polypeptide-related sequence A (MICA) or the retinoic acid early transcript 1 (RAE1) that are ligands for the stimulatory immune receptor expressed by natural killer (NK) cells and T cells, NKG2D[181].

Inflammatory cytokines

Dying tumor cells can release proinflammatory cytokines that can induce an immune response. Necrotic but not apoptotic cells are able to act on fibroblasts, macrophages and DCs, activating NF-kB and inducing the expression of genes that are involved in inflammatory responses and tissue repair[199] including the cytokine induced neutrophil chemoattractant (KC) and macrophage inflammatory protein-2, metalloproteinase 3 and vascular endothelial growth factor, TNF-α, IL-8, IL-10, and IL-6[195,197]. Unlike, these proinflammatory features seem to be absent from apoptotic cells[196].

Therefore, the induction of an immune response by a general and unspecific release of multiple immunogenic factors seems to be a unique feature of necrosis.

2. Aim of the Thesis

Previous results obtained in melanoma B16 tumor-bearing mice showed that intratumoral (i.t.) injection of invasive but nonpathogenic *Salmonella enterica* serovar *typhimurium* (*Salmonella*) led to the regression of even bulky tumors in 50-100% of mice[9]. Moreover it was found that the complete tumor regression induced by i.t. injection of *Salmonella* was initially due to the activation of the immune system against the infected cells and subsequently to a direct immune response against the tumor cells through cross-presentation of the tumor antigen by dendritic cells to $CD8^+$ T cells in the draining lymph node[9]. Interestingly, this immunotherapeutic approach was able to break ignorance or tolerance of the immune system against the tumor thus leading to the development of a systemic anti-cancer immune response. *Salmonella*, however, was unable to directly induce B16 cell death.

It is well-known in literature that TLRs play a key role in host immunity through detection of pathogens and that they are expressed not only on immune cells, but also on several tumor cells[138]. Moreover, many studies reported that TLRs might mediate apoptotic cell death, in tumor cells as well[162-164,169,171]. This mechanism could in turn enhance the immunogenicity of tumor cells and thus allows the immune system to recognize and eradicate tumors. Since *Salmonella* presented various PAMPs that might bind TLRs present on tumor cells we were not able to rule out the possibility that tumor regression could be directly induced by *Salmonella* through a TLR-mediated mechanism. However, since *Salmonella* was unable *in vitro* to directly kill B16 cells, other mechanisms should contribute to tumor cell death *in vivo*. Since we detected high levels of IFN-γ *in vivo* after *Salmonella* infection and IFN-γ is a cytokine that has also an antiproliferative effect[48], it could be involved in tumor cell death.

Therefore, in this study, we wanted to understand the role of IFN-γ and *Salmonella* in B16 cell death.

Specific aims:

1. To test *in vitro* whether *Salmonella* in combination with IFN-γ or IFN-α is able to induce B16 cell death.
2. To analyze TLR expression on B16 cells both untreated and subjected to combined stimulation with *Salmonella* and IFN-γ or IFN-α.
3. To examine the role of TLR2 and TLR3, highly up-regulated in B16 cells by *Salmonella* in combination with IFN-γ, very likely involved in tumor cell death, through *in vitro* stimulations with TLR2 and TLR3 synthetic ligands.
4. To discriminate between necrosis and apoptosis as the possible causes of dependent cell death of B16 cells *in vitro*.

3. Materials and Methods

3.1. Mice, cells and bacterial strain

Five-week-old female C57BL/6J mice were purchased from Charles River and maintained in Specific Pathogen Free stabulary at IFOM-IEO Campus, Milan, Italy. All mouse studies were carried out in accordance with the guidelines established in the Principles of Laboratory Animal Care (directive 86/609/EEC).

The murine melanoma B16F10 cell line (called throughout the paper B16 a kind gift from Dr. P. Dellabona) was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol (complete RPMI). The melanoma B16BL6 (BL6) and the breast carcinoma 4T1 murine cell lines were cultured in complete RPMI, whereas the murine lung carcinoma LLC1 cell line was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin.

The murine dendritic cell line DC1 was generated in Maria Rescigno's laboratory following an established methodology.

Bone marrow-derived dendritic cells (BM-DCs) were generated from bone marrows of C57/BL6J mice. The bone marrow was flushed from femurs and tibias with sterile DPBS (Dulbecco's Phosphate-Buffered Saline) and resuspended by pipetting until a single cell suspension was obtained. The suspension was filtered through a 40 µm-cell strainer to remove debris. Viable cells, excluding red blood cells, were then counted using Trypan Blue and 1.5×10^6 cells were seeded in non tissue-treated dishes (Bibby Sterilin Ltd) with 10 ml of RPMI 1640 medium supplemented with 10% North American Fetal Bovine Serum, 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM 2-mercaptoethanol, 0.1 mM Non-Essential Aminoacids and 30% supernatant from GM-CSF (Granulocyte-Macrophage colony-stimulating factor) – producing fibroblasts (NIH-3T3 cell line). Cultures were maintained at 37°C, 5% CO_2. After 3 days, 10 ml of fresh medium were added. At day 6, 10 ml of medium were removed from each plate and replaced with 10 ml of fresh medium. At day 9, non adherent-cells were collected and used for subsequent experiments.

Salmonella typhimurium SL3261AT on SL1344 background was a $aroA^-$ metabolically defective strain and was grown at 37°C in Lurian broth.

3.2. Reagents and antibodies

LPS, LTA, Necrostatin-1 and poly I:C were purchased from Sigma-Aldrich; IFN-γ and flagellin were purchased from Alexis, whereas IFN-α was Roferon®-A (Roche). Pam3CSK4 and Pam2CSK4 were obtained from Invivogen. In flow cytometry were used FITC-Annexin V (BD Pharmigen) and different antibodies: Alexa Fluor® 647 anti-mouse TLR2 (e-Bioscience, 51-9021), FITC anti-mouse TLR3 (Imgenex, IMG-516C), phycoerythrin (PE) anti-mouse IFNGR1 (e-Bioscience, 12-1191), PE anti-mouse MHCI (BD Pharmigen). For Western blot analysis were used the following primary antibodies: rabbit anti-cleaved caspase-3 (Cell Signaling, #9664), rabbit anti-cleaved caspase-7 (Cell Signaling, #9491), mouse anti-vinculin (Sigma),

rabbit anti-NF-kB p65 (Santa Cruz, sc-372) or rabbit anti-histone H3 (Abcam, ab1791). Anti-mouse horseradish peroxidase-conjugated IgG (Calbiochem) or anti-rabbit horseradish peroxidase-conjugated IgG (BioRad) were the secondary antibodies used.

3.3. Analysis of IFN-γ in the tumor mass

B16 was induced in C57BL/6J mice through the injection of 10^5 B16 cells in the flank of these mice. 10 days later, when the tumor was visible and palpable, mice were injected with 10^8 CFU of *Salmonella* or PBS as a control. 1, 3 and 7 days post-injection the tumors were removed and smashed in 500 µl PBS containing 0.5% triton, incubated on ice for 1 hour and centrifuged at 13000 rpm at 4°C for 15 minutes. Supernatants were analyzed for the presence of IFN-γ by ELISA (R&D System), according to manufacturer's instructions.

3.4. *In vitro* infection with bacteria and treatment with different stimuli

Single bacterial colonies were grown overnight and restarted the next day at 1:10 of the original volume up to an $A_{600\ nm}$ = 0.6 corresponding to 0.6 x 10^9 CFU/ml. Murine cell lines (seeded at 2 x 10^5 cells/well in 6-well plates and grown for 18 h) were incubated with bacteria for 2 hours, in a ratio of 1:50 (cells/bacteria), in the appropriate medium without antibiotics. Subsequently, cells were washed with PBS and incubated in medium supplemented with 50 µg/ml gentamicin in order to kill extracellular bacteria. After infection, IFN-γ (100 U/ml) or IFN-α (2000 U/ml) was added where stated.

Murine cell lines were plated in 6-well plates (2 x 10^5 cells/well) and grown for 18 hours. Cells were incubated with stimuli in complete medium with and without IFN-γ (100 U/ml) or IFN-α (2000 U/ml) for 3, 6, 8, 24 or 48 hours depending on the experiment. The single bacterial component used were LPS (1 µg/ml, Sigma), flagellin (0.1 µg/ml, Alexis) and LTA (10 µg/ml, Sigma). Different synthetic TLR ligands were also used, such as Pam2CSK4 (0.05, 0.1 or 1 µg/ml, Invivogen), Pam3CSK4 (0.5 or 1 µg/ml, Invivogen), poly I:C (50 µg/ml, Sigma). Necrostatin-1 inhibitor (nec-1, Sigma) were added simultaneously to different stimuli at 50 µM.

3.5. Real-time PCR (Q-PCR)

Total RNA was isolated by B16 cells using RNeasy Mini Kits (Qiagen) according to manufacturer's instructions, adding a DNase digestion step to eliminate eventual genomic DNA contamination. Subsequently, 1 µg RNA from each sample was reverse-transcribed using 200 U SuperScript® II RT (Invitrogen) and random primers (Invitrogen) in a total volume of 20 µl, according to the manufacturers' protocol. One µl (50 ng) of cDNA was amplified by PCR using the SYBR Green PCR Master Mix (Applied Biosystem) and specific primers of interest listed in Table 3. Real-time PCR

was carried out using a 7500 Real-Time PCR System (Applied Biosystem) and the amplification was performed with 40 cycles of 15 s at 95°C and 60 s at 59°C. Our samples were analyzed for the expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR11, TLR12, TLR13, MyD88, IkBα. To normalize each sample for total RNA content, control housekeeping gene (TATA-binding protein, tbp) was used under similar PCR conditions. The relative expression level was calculated with the $2^{[-\Delta\Delta C(T)]}$ method and was expressed as a fold change. All PCR experiments were performed in triplicate and the standard deviations were calculated and displayed as error bars.

Table 3-1. List of murine primers used for real time PCR

Gene	Forward Primer	Reverse Primer
TLR1	5' - TGAGGGTCCTGATAATGTCCTAC - 3'	5' - AGAGGTCCAAATGCTTGAGGC - 3'
TLR2	5' - AAGAGGAAGCCCAAGAAAGC - 3'	5' - CGATGGAATCGATGATGTTG - 3'
TLR3	5' - GTGAGATACAACGTAGCTGACTG - 3'	5' - TCCTGCATCCAAGATAGCAAGT - 3'
TLR4	5' - GGACTCTGATCATGGCACTG - 3'	5' - CTGATCCATGCATTGGTAGG - 3'
TLR5	5' - CAGACGTGTATTGCATGTACCCTAA - 3'	5' - GACCGCATGGCTTCCTCTT - 3'
TLR6	5' - GACTCTCCCACAACAGGATACG - 3'	5' - TCAGGTTGCCAAATTCCTTACAC - 3'
TLR7	5' - GAAGTTGGCTTTTGTCCTAATGCT - 3'	5' - TATCGGAAATAGTGTAAGGCCTCAA - 3'
TLR8	5' - CAAACAACAGCACCCAAATGAA - 3'	5' - AGGCAACCCAGCAGGTATAGT - 3'
TLR9	5' - ATGGTTCTCCGTCGAAGGACT - 3'	5' - GAGGCTTCAGCTCACAGGG - 3'
TLR11	5' - GCTTCTGTTGATTATCCTTCCTCTGA - 3'	5' - GTCCACATAATTTCCACCAACAAG - 3'
TLR12	5' - GGTCTCCCGCTATTTCACATTC - 3'	5' - CCTCCACAGTCCGAGGTACAAC - 3'
TLR13	5' - CAGAGGCCATTAGTGACATACCTAGA - 3'	5' - AAGTCCACCAGAGCAGACAGATT - 3'
MyD88	5' - CATGGTGGTGGTTGTTTCTGAC - 3'	5' - TGGAGACAGGCTGAGTGCAA - 3'
IkBα	5' - GAGGACGGAGACTCGTTCCTG - 3'	5' - TGTTCTGGAAGTTGAGGAAGGC - 3'
tbp	5' - CTGGAATTGTACCGCAGCTT - 3'	5' - TCCTGTGCACACCATTTTTC - 3'

3.6. Flow cytometry

B16 cells untreated or treated with various stimuli for different times, depending on the experiment, were analyzed for TLR2, TLR3, MHCI and IFNGR1 protein levels. For extracellular staining, the cells were incubated with Alexa Fluor® 647 anti-mouse TLR2 (1:100, e-Bioscience) or FITC anti-mouse TLR3 (1:50, Imgenex) or phycoerythrin (PE) anti-mouse IFNGR1 (0.5 µg/10^6 cells in 100 µl, e-Bioscience) or PE anti-mouse MHCI (1:100, BD Pharmigen) in 1% normal mouse serum for 1 hour at 4°C, protected from light. For intracellular staining of TLR3, 1 µg/10^6 cells cells were fixed, permeabilized with Cytofix/Cytoperm (BD Phamigen), incubated with FITC anti-mouse TLR3 (Imgenex), according to the manufacturers' protocol. All stained cells were acquired on FACScalibur (BD) and data were analyzed using FlowJo software.

3.7. Cellular apoptosis analysis

Cellular apoptosis was analyzed by cytofluorimetry after double staining procedure with FITC-Annexin V (BD Pharmigen) and propidium iodide (PI). Briefly, cells untreated or subjected to different stimulations were

harvested and washed twice in PBS, resuspended in 50 µl FITC-Annexin V diluited 1:50 in Annexin Buffer (Hepes 10 mM, NaCl 150 mM, $MgCl_2$ 1 mM, $CaCl_2$ 3.6 mM, KCl 5 mM) and incubated 1 hour at room temperature, protected from light. Then cells were washed in Annexin Buffer and resuspended in PBS. After adding 1 µl of PI solution 50 µg/ml in PBS, cells were analyzed immediately by flow cytometry using FACScalibur (BD). As positive controls to assess cell death, BMDCs cells were either cultured with 3 µM staurosporine for 6 hours or UV irradiated (50 J/m^2), whereas B16 cells were UV irradiated with different doses (30, 40 or 50 J/m^2) 24 hours before analysis. Viable cells were defined as Ann-/PI- ; early apoptotic cells as Ann+/PI- ; late apoptotic or necrotic cells as Ann+/PI+, Ann-/PI+.

3.8. Protein extraction

Nuclear and cytosolic fractions:

In order to analyze NF-kB nuclear translocation, B16 cells were plated in 6-well plates (2 x 10^5 cells/well) and grown for 18 hours. 24 hours after treatment with *Salmonella* and IFN-γ (100 U/ml) singularly or simultaneously administered, cells were washed in PBS and detached with a cell lifter in 1 ml PBS. After two washes in PBS, cells were resuspended in 250 µl L1 buffer (Tris pH 8.0 50 mM, EDTA pH 8.0 2 mM, NP40 0.1%, Glycerol 10%) containing protease inhibitors and incubated for 5 minutes on ice. Subsequently, cells were centrifuged at 3000 rpm for 5 minutes at 4°C; the supernatant contained the cytosolic fraction, whereas the pellet contained the nuclear fraction. The pellet was washed in L1 buffer and then resuspended in 100 µl RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), containing protease inhibitors, and incubated on ice. Lastly, nuclear fractions were subjected to several cycles of sonication in a sonicating water bath and then quantified.

Total extract:

In order to study caspase activation, B16 cells were plated in 6-well plates (2 x 10^5 cells/well) and grown for 18 hours. 6, 24, or 48 hours after different treatments, tumor cells were washed with PBS and then lysed by adding 100 µl SDS sample buffer (62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red) per well. Immediately, cells were scraped off the plate and then the extracts were transferred to a microcentrifuge tube and incubated on ice. Subsequently, total extracts were sonicated 10–15 seconds to shear DNA and reduce sample viscosity. 20 µl sample were boiled to 95–100°C for 5 minutes, cooled on ice, centrifuged for 5 minutes and finally loaded onto SDS-PAGE gel (10 cm x 10 cm).

3.9. Western blot analysis

SDS-PAGE and Western blotting were carried out by standard procedures. Nitrocellulose membranes were blocked in 5% (w/v) bovine serum albumin (BSA) in TBS, 0.05% (v/v) Tween20 (for anti-NF-kB p65 and anti-histone H3 antibodies) or in 5% (w/v) dried milk in TBS, 0.1% (v/v)

Tween20 (for anti-cleaved caspase-3, anti-cleaved caspase-7 and anti-vinculin antibodies) for 1 hour at room temperature. Membranes were subsequently probed with rabbit anti-NF-kB p65 (1:200, Santa Cruz) or rabbit anti-histone H3 (1:5000, Abcam) or mouse anti-vinculin (1:10000, Sigma) antibodies for 2 hours at room temperature; whereas membranes probed with anti-cleaved caspase-3 or anti-cleaved caspase-7 (1:1000, Cell Signaling) rabbit antibodies were incubated overnight at 4°C. After extensive washes in TBS, 0.1% (v/v) Tween20, membranes were incubated with anti-mouse horseradish peroxidase-conjugated IgG (Calbiochem) or with anti-rabbit horseradish peroxidase-conjugated IgG (BioRad) antibodies for 1 hour. After a final wash in TBS, 0.1% (v/v) Tween20, visualization was carried out with ECLPlus (GE Healthcare). Bands were quantified by densitometry using NIH Image-based software Scion Image (Scion Corporation).

3.10. Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6, IL-10, IL-12p70, IL-1β, TNF-α, KC (BD) and HMGB1 (BIOTREND) protein levels in cultured supernatants were quantified using ELISA according to manufacturer's instructions.

3.11. Statistical analysis

Values are presented as means ± S.D. or S.E. Statistical analyses were usually performed using Student's t-test comparing untreated cells data with treated cells data. Sometimes comparisons among different stimulations were performed. Significance was defined as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

4. Results

4.1. *Salmonella* in combination with IFN-γ is able to induce tumor cell death and to up-regulate different TLRs on B16 cells

4.1.1. IFN-γ is highly expressed in *Salmonella* infected tumors and in combination with bacteria is involved in in vitro tumor cell death

In a recent work carried out in Maria Rescigno's laboratory, it was demonstrated that intratumoral (i.t.) injection of invasive but nonpathogenic *Salmonella enterica* serovar *typhimurium* (*Salmonella*) in established murine B16F10 (hereafter referred simply as B16) melanomas led to complete regression of the tumors, which did not recur in up to 60% of cases. Moreover, mice that completely rejected the tumor following i.t. *Salmonella* treatments developed signs of vitiligo and were protected from a subsequent challenge of the same melanoma in a different location[9]. These observations strongly suggested that bacterial treatments can lead to the development of a systemic anti-cancer immune response, even when administered locally. On the basis of these previous findings, we performed a first *in vivo* experiment using the same animal model. We intratumorally injected B16 bearing mice with *Salmonella* or PBS as a control and 1, 3, and 7 days later tumors were removed and smashed to analyze the presence of IFN-γ. Interestingly, the ELISA assay revealed that infected tumor masses expressed high level of IFN-γ (Fig. 4-1). Therefore, since Avogadri and coworkers observed that *Salmonella* is not directly toxic for tumor cells, we wondered if IFN-γ could be involved in tumor cell death. In this regard, it was demonstrated that IFN-γ treatment is able to increase cell surface MHC class I. Notably, B16 melanoma cells stimulated with IFN-γ both *in vitro* and *in vivo*, up-regulated MHC class I, thus enhancing the anti-tumor effect of tumor immunotherapy[208]. Moreover, it was found that IFN-γ itself had an antiproliferative effect by both blocking the cell cycle and inducing apoptosis[48]. However, a lot of clinical protocols for cancer treatment are based on adjuvant immunotherapy with IFN-α, whereas IFN-γ has limited exploitation in cancer-immunotherapy trials in humans. In the light of these data, we performed an *in vitro* experiment to assess tumor cell death after treatment with IFN-α (2000 U/ml) or IFN-γ (100 U/ml) in combination with *Salmonella* infection. To this purpose, murine B16 melanoma cells were either treated with single stimuli (such as, *Salmonella*, IFN-α, IFN-γ) or infected with *Salmonella* in simultaneous combination with IFN-α or IFN-γ. As a positive control to asses cell death, B16 cells were UV irradiated (50 J/m^2). 24 hours later, cell death was evaluated by double staining procedure with FITC-Annexin V (AnnV) and propidium iodide (PI) and subsequent cytofluorimetric analysis. As shown in Figure 4-2, IFN-γ was able to significantly increase the percentage of dead cells compared to untreated cells, but only if associated to bacterial infection. On the contrary, IFN-α did not result in enhanced tumor cell death either alone or in combination with *Salmonella*. Moreover, in accordance with previous results[9], *Salmonella* did not directly kill tumor cells. Surprisingly, a very low percentage of AnnV positive and PI negative B16 cells was detected, indicating that all dead cells

were undergoing late apoptosis or necrosis (data not shown). Interestingly, ELISA analysis performed on the supernatants revealed that IFN-γ, a cytokine generally expressed in immune cells, was produced also in untreated tumor cells (about 100 pg/ml). No difference in IFN-γ content was observed in every condition except for samples in which recombinant IFN-γ was added (data not shown). In order to verify whether a correct IFN-α concentration was used, a titration experiment was carried out. Subsequently, B16 cells were stimulated with IFN-α at different concentrations (100, 500, 1000, 2000, 3750 U/ml) and cell surface MHCI protein expression was assessed by cytofluorimetric analysis. Tumor cells were also triggered with IFN-γ (100 U/ml) as a control. We found that IFN-α up-regulated MHCI only at highest concentrations (2000 U/ml and 3750 U/ml) to the same extent of IFN-γ (data not shown), confirming that an appropriate IFN-α concentration had been chosen.

On the whole, these data suggest that most likely IFN-γ, highly present in infected tumor masses and *in vitro* expressed by B16 cells is able to induce tumor cell death in association with *Salmonella.*

4.1.2. TLRs mRNA basal levels in B16 cells

Since *Salmonella* express a number of pathogen-associated molecular patterns (PAMPs) we wondered whether some of these could directly interact with tumor cells. Toll-like receptors (TLRs) are able to bind different PAMPs and recently, there has been evidence that they are expressed not only on immune cells, but also on some types of murine[14143] and human[146,148,170,171] tumor cells. Among these, different melanoma cell lines can greatly express them. In order to test whether B16 melanoma cells presented TLRs, we analyzed mRNA levels of all TLRs (1-13, except for the pseudogene TLR10) by real-time PCR (Q-PCR). The expression of each gene was normalized to the housekeeping TATA binding protein (tbp) mRNA level. As a positive control, we tested the different primers couples on cDNA obtained from DC1 dendritic cells treated with different stimuli, such as LPS, lipoteichoic acid (LTA) or flagellin in order to induce TLRs upregulation (Fig. 4-3A) Both murine cell lines expressed all TLR mRNAs, except for TLR12. In particular, B16 cells strongly expressed TLR3 and TLR4, but also TLR1 and TLR2 were present even if at lower amounts (Fig. 4-3B). Very low levels of TLR5 and TLR6 (Ct=30) were detected, whereas TLR7, TLR9 and TLR13 were substantially absent (Ct>32). Lastly, apart from TLR12, neither TLR8 (not functional in mice[116]) nor TLR11 were expressed (Fig. 4-3B).

4.1.3. mRNA levels of TLRs 1-6 in B16 cells after bacterial infection associated to IFN-α or IFN-γ treatment

Having assessed the presence of different TLRs in B16 cells, we wondered whether *Salmonella* might up-regulate TLRs mRNA levels in combination with IFN-α or IFN-γ. In particular, we focused our subsequent studies only on TLRs that we found at detectable levels (such as, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6). Thus, B16 cells were infected with *Salmonella* and simultaneously treated with IFN-α (2000 U/ml) or IFN-γ (100

U/ml). As controls, B16 cells were left untreated or were triggered with *Salmonella*, IFN-α or IFN-γ alone. 3, 6 and 24 hours after stimulation total RNA was extracted and reverse-transcribed to cDNA. Then, real-time PCR was performed to analyze the transcript level of the six TLRs. Interestingly, we found that *Salmonella* and IFN-γ synergize to up-regulate different TLRs, such as TLR1, TLR2, TLR3 and TLR6. Figure 4-4A shows that TLR1 transcript level increases 24 hours after simultaneous administration of *Salmonella* and IFN-γ, whereas TLR2, TLR3 and TLR6 are significantly up-regulated already 3 hours after treatment (Fig. 4-4B,C,E). Among all these TLRs, TLR2 showed the highest increase in mRNA level after *Salmonella* infection associated to IFN-γ stimulation (Fig. 4-4B). TLR3 transcription was also significantly induced by the synergistic effect of *Salmonella* and IFN-γ, even if to a lesser extent compared to TLR2 transcription. Importantly, IFN-γ was able to strongly up-regulate TLR3 mRNA level by itself (Fig. 4-4C). Lastly, although TLR6 transcript was not readily detected in the steady state, it was increased by *Salmonella* infection coupled to IFN-γ. However, also *Salmonella* and IFN-γ singularly administered were able to slightly induce TLR6 mRNA level (Fig. 4-4E). On the contrary, TLR4 and TLR5 transcription remained almost unchanged upon every stimulation at any time (Fig. 4-4D and data not shown, respectively). Surprisingly, unlike IFN-γ, IFN-α was not able to increase TLR transcription either alone or in combination with *Salmonella*. In fact, the increase in TLR1, TLR2, TLR3 and TLR6 transcription observed in B16 cells after simultaneously administration of *Salmonella* and IFN-α was primarily due to *Salmonella* effect.

Taken together, these results indicate that B16 cells do not up-regulate any TLR transcripts upon treatment with single stimuli (*Salmonella*, IFN-γ or IFN-α), with the exceptions of TLR3 that is significantly induced by IFN-γ and TLR6 that is weakly up-regulated by both IFN-γ and *Salmonella*. Remarkably, *Salmonella* and IFN-γ act in synergy to increase mainly TLR2 transcription, but also TLR1, TLR3 and TLR6 mRNA levels. In contrast, IFN-α does not trigger any variation, not even in combination with bacteria. Therefore, since we have demonstrated that IFN-γ treatment associated to *Salmonella* infection is involved in tumor cell death as well as in induction of TLR transcription, we might easily hypothesize that TLR are able to trigger cell death of melanoma cells. Importantly, most likely TLR4 is not involved in tumor cell death, since its mRNA level does not change after any stimulations.

4.1.4. Protein levels of TLR2 and IFN-γ receptor (IFNGR1) on B16 cells

Since we found that *Salmonella* and IFN-γ increased mainly TLR2 transcription in B16 cells, we decided to test also TLR2 protein level. To this purpose, B16 cells untreated or subjected to different stimulations were processed and stained for fluorescence-activated cell sorting (FACS) analysis, using an anti-TLR2 antibody, after 24 hours of treatment. Consistent with our previous real-time PCR results, FACS analysis revealed that B16 cells were able to express a basal level of TLR2 protein on their surface that strongly increased mainly when *Salmonella* infection was coupled to IFN-γ treatment (Fig. 4-5). In such conditions, we observed an

approximately 4-fold increase in TLR2 protein level. On the contrary, as expected, *Salmonella* and IFN-α did not synergize to up-regulate TLR2 protein. Moreover, also IFN-γ and IFN-α singularly administered did not induce any significant variations, whereas *Salmonella* caused a 2-fold increase of TLR2 protein. Finally, we analyzed the TLR2 protein expression in a time course experiment 3, 6 and 24 hours after treatments. The highest protein level was reached at 24 hours whereas, as previously mentioned, the increase in TLR2 transcription was observed approximately already after 3 hours of stimulation (data not shown). Subsequently, in order to assess the expression of IFN-γ receptor (IFNGR1) on B16 cells, *Salmonella* and IFN-γ were simultaneously or singularly administered to tumor cells. As a control, B16 cells were left untreated. After 24 hours of stimulation tumor cells were processed and stained with an anti-IFNGR1 antibody in order to perform a FACS analysis. Figure 4-6 shows that B16 cells express high basal levels of IFN-γ receptor on their surface. Notably, *Salmonella* infection slightly reduced the number of IFNGR1-positive cells. On the contrary, although IFN-γ treatment alone or in combination with *Salmonella* did not increase the number of IFNGR1-positive cells, it was able to enhanced the expression of its receptor in some of them.

Altogether these data suggest that B16 cells express both TLR2 and IFNGR1 on their surface. Moreover, TLR2 protein expression is up-regulated by the synergistic effect of *Salmonella* and IFN-γ, whereas IFNGR1 protein basal level is very high and increases after IFN-γ stimulation.

4.1.5. Single bacterial components synergize with IFN-γ to increase TLR transcription

Once the synergistic effect of *Salmonella* and IFN-γ in inducing TLR transcription confirmed, we wondered whether even single bacterial components were able to up-regulate TLRs mRNA level in B16 cells. In this regard, we tested three bacterial components, such as lypopolysaccharide (LPS), lipoteichoic acid (LTA) and flagellin, capable of binding different TLRs. In particular, LPS, a component of the outer membrane of Gram-negative bacteria, is a ligand of TLR4; LTA, a major constituent of the cell wall of Gram-positive bacteria, binds TLR2; finally, flagellin, the principal component of bacterial flagella, is the agonist of TLR5. Hence, B16 cells were cultured with LPS, LTA or flagellin in combination or not with IFN-γ. As controls, tumor cells were either left untreated or triggered with *Salmonella* and IFN-γ singularly or simultaneously administered. After 3, 6 and 8 hours we analyzed the mRNA level of TLRs 1-6 by real-time PCR. Consistent with our previous Q-PCR data, this analysis showed that TLR2 transcription was induced at the highest level when *Salmonella* was administered in combination with IFN-γ. Notably, although already after 3 hours of treatment there was a clear increase in TLR2 transcription, the maximum peak was reached after 8 hours when TLR2 mRNA level was 200 folds higher than in untreated cells. Interestingly, the same or even stronger up-regulation was achieved upon stimulation with LPS plus IFN-γ, whereas LPS singularly administered induced only a 9-fold increase. A similar result was obtained when B16 cells were infected with bacteria or treated with IFN-γ alone.

Conversely, LTA and flagellin significantly induced TLR2 transcription, but only when added with IFN-γ, whereas in the absence of IFN-γ these bacterial components were unable to up-regulate TLR2 transcript. (Fig. 4-7A). Unlike TLR2, TLR3 transcription was improved essentially by IFN-γ, even if, as previously demonstrated, a synergistic effect between *Salmonella* and IFN-γ was observed. In contrast, no synergy was detected between IFN-γ and other stimuli. Moreover, all single bacterial components, as well as whole bacteria, did not influence TLR3 expression (Fig. 4-7B). In accordance with our first experiment, we found that TLR4 transcript was highly expressed in B16 cells. Nevertheless, TLR4 mRNA level did not change significantly after any stimulations (Fig. 4-7C). Finally, B16 cells displayed low mRNA levels of TLR1, TLR5 and TLR6. In particular, whereas TLR5 and TLR1 transcripts did not change significantly within 8 hours of any treatment (data not shown), TLR6 mRNA was up-regulated after simultaneous administration of IFN-γ with bacteria or with bacterial components LPS or LTA, at the same time point (Fig. 4-7D).

Subsequently, we also analyzed mRNA levels of TLRs 1-6 after 24 hours of stimulation. No single bacterial components was able to increase mRNA expression of any TLRs at this time point. Interestingly, we detected that TLR1 and TLR4 transcript levels increased also in untreated tumor cells in a time dependent manner, indicating that mRNA expression seemed to be affected even by the percentage of cell confluence (data not shown).

In summary, these results suggest that LPS, flagellin and LTA are able to up-regulate differently only some of the TLRs and only when associated to IFN-γ treatment. In particular, TLR1, TLR4 and TLR5 remain unchanged upon any stimulation at all times. LPS is the *Salmonella* component responsible for increasing TLR2 transcription. In fact, we found that LPS synergizes with IFN-γ to consistently up-regulate TLR2 more than whole bacteria administered with IFN-γ. Moreover, LPS in combination with IFN-γ induces also TLR6 transcription in a similar manner to whole *Salmonella*. Importantly, LPS does not up-regulate neither TLR3, nor its receptor TLR4. Flagellin, another *Salmonella* component, in synergy with IFN-γ induces only TLR2 transcription, but to a lesser extent than LPS. On the contrary, LTA from Gram-positive bacteria, significantly increases TLR2 and TLR6 mRNA levels. In addition, we observed that the effect of bacterial components appears within 8 hours of treatment, whereas the action of *Salmonella* in combination with IFN-γ is more lasting. Lastly, whereas LTA in synergy with IFN-γ up-regulates its receptor TLR2, and also TLR6, a co-receptor of TLR2, unexpectedly, LPS and flagellin do not up-regulate their own receptors TLR4 and TLR5,respectively, but only TLR2, indicating that a possible cross-talk between different TLR pathways might exist.

4.1.6. The role of IFN-γ on TLRs mRNA levels

In order to better understand the role of IFN-γ in up-regulating TLRs mRNA level, we decided to culture B16 cells with IFN-γ for 24 hours and subsequently to infect or incubate tumor cells with single stimuli (LPS, flagellin, LTA) for further 8 hours (IFN 24h + stimulus 8h). On the other hand, vice versa we treated B16 cells with *Salmonella* or single stimuli without IFN-

γ and 24 hours later, we added IFN-γ into the medium for 8 hours (stimulus 24h + IFN 8h). As controls, we administered bacteria (or stimuli) and IFN-γ either simultaneously (stimulus + IFN) or singularly (stimulus 8h) for 8 hours. 32 hours after the first treatment, we extracted mRNA and we performed a real-time PCR analysis to detect mRNA level of TLRs 1-6. As expected, Figure 4-8A shows that the simultaneous administration of *Salmonella* or LPS with IFN-γ induces a considerable up-regulation of TLR2 mRNA (blue bars), as already detected in the previous experiment. Surprisingly, the same effect was obtained in B16 cells pretreated 24 hours with IFN-γ and subsequently infected or treated with LPS (pink bars), but not in tumor cells infected or incubated with LPS for 24 hours and then treated with IFN-γ (black bars). Unexpectedly, LPS added in IFN-γ pretreated cells induced a minor TLR2 transcript up-regulation than *Salmonella*. Besides, consistent with our previous analysis, *Salmonella* infection, LPS and IFN-γ singularly administered triggered only small variations in TLR2 mRNA level (green bars). Conversely, flagellin and LTA induced a slight but significant TLR2 mRNA increase only when administrated simultaneously with IFN-γ. TLR3 mRNA expression also seemed to be influenced by IFN-γ pretreatment and subsequent *Salmonella* infection (Fig. 4-8B, pink bar) similarly to simultaneously administration (blue bar). Consistent with our previous experiments, IFN-γ was the major responsible of TLR3 mRNA expression, whereas every single stimulus did not affect TLR3 mRNA level (green bars). Surprisingly, unlike *Salmonella*, there was a synergistic effect between LPS and IFN-γ only in B16 cell pretreated 24 hours with IFN-γ and afterwards with LPS (pink bar). On the contrary, TLR6 transcription enhanced only upon simultaneous administration of IFN-γ with *Salmonella*, LPS or LTA (data not shown), as already reported in previous experiments. Lastly, TLR1 transcription increased only upon simultaneous treatment with bacteria and IFN-γ, whereas TLR4 and TLR5 transcript levels did not change significantly in response to various treatments (data not shown).

In this way, we further demonstrated that IFN-γ and *Salmonella* (probably through LPS) in simultaneous combination act in synergy in order to strongly increase TLR2 mRNA level. In particular, since a similar effect was obtained only when B16 cells were pretreated with IFN-γ and subsequently infected, it seems that IFN-γ is important in triggering a pathway (e.g. TLR3-mediated pathway or other) that facilitate the subsequent *Salmonella* action, resulting in an amplified induction of TLR2 mRNA transcription. Interestingly, a similar effect, but to a lesser extent, was observed on TLR3 mRNA expression. However, unlike TLR2, there was no synergy between LPS and IFN-γ simultaneously administered, but only when the cells were pretreated with IFN-γ and then stimulated with LPS. On the contrary, IFN-γ pretreatment does not affect the levels of TLR1, TLR4, TLR5 and TLR6 mRNAs.

4.1.7. *Salmonella* in combination with IFN-γ up-regulates TLRs primarily on murine melanoma cells

In order to ascertain if the fact that *Salmonella* synergizes with IFN-γ in up-regulation of the different TLRs, was confined to this tumor model, we

carried out similar studies in another mouse melanoma cell line (B16BL6) and in two metastatic breast and lung mouse carcinomas, 4T1 and LLC1, respectively. To this purpose, we analyzed TLR2, TLR3 and TLR4 transcription in melanoma B16BL6 (BL6) cell line 24 hours after the combined stimulation with *Salmonella* and IFN-γ. As controls, BL6 cells were either untreated or triggered with bacteria or IFN-γ singularly administered. Q-PCR results obtained in BL6 tumor cells untreated or subjected to different stimuli, closely mirrored those observed in B16 cells. In particular, the basal level of TLRs mRNA in BL6 untreated cells was similar to the one found in B16 cells (data not shown). Besides, as shown in Figure 4-9A, triggering of BL6 tumor cells with bacteria in combination with IFN-γ induced a significant TLR2 mRNA increase. IFN-γ alone was able to significantly up-regulate TLR3 mRNA, but a synergic effect with *Salmonella* was shown also in this case (Fig. 4-9B). Moreover, TLR4 mRNA expression remained unchanged (Fig. 4-9C).

On the contrary, 4T1 and LLC1 cells showed a different behavior in TLRs expression compared to melanoma cell lines. In particular, compared to B16 cells, 4T1 and LLC1 cells expressed lower basal levels of TLR1 mRNA that remained unchanged after stimulation (Fig. 4-10A). Regarding TLR2 mRNA, 4T1 and LLC1 tumor cells expressed higher or lower basal levels, respectively, with respect to B16 cells. Moreover, in 4T1 carcinoma cell line, the combined stimulation with *Salmonella* and IFN-γ elicited a weak increase in TLR2 mRNA levels (Fig. 4-10B). In order to confirm these Q-PCR results, TLR2 protein levels analysis was performed. In particular, we found that 4T1 cells expressed higher TLR2 basal level compared to B16 or LLC1 cells. Moreover, unlike B16 cells, 4T1 and LLC1 tumor cells did not significantly up-regulate TLR2 protein after 24 hours of combined stimulation with *Salmonella* and IFN-γ (data not shown). In addition, Q-PCR analysis revealed that TLR3 was the most expressed TLR in 4T1 tumor cells and its basal level was similar to the one found in B16 cells. Conversely, in LLC1 a low basal level of TLR3 mRNA was detected (Fig. 4-10C). IFN-γ treatment was able to enhance TLR3 transcription in B16 and 4T1 cell lines, but only in B16 cells a further increase of TLR3 was observed after the synergistic effect between bacteria and IFN-γ. On the contrary, TLR4 was expressed at very high levels in both LLC1 and B16 tumor cells, whereas it was detected in 4T1 cells to a lesser extent. However, TLR4 mRNA level remained unchanged after all stimulations in every cell line analyzed (Fig. 4-10C). Finally, we observed that 4T1 showed TLR6 mRNA basal levels higher than B16 and LLC1 cells. Moreover, unlike B16 cells, IFN-γ alone is sufficient to induce TLR6 mRNA expression in both carcinoma cell lines (Fig. 4-10D).

On the whole, these data suggest that both murine melanoma cell lines analyzed (B16 and BL6) show a similar TLR transcription pattern. This is likely due to their common origin. In fact, BL6 was derived from B16F10 (B16) but they display different metastasis behavior; BL6 cells can generate spontaneous lung metastases from a primary subcutaneous tumor, whereas the B16 cells do not[209]. Accordingly, we found that simultaneous treatment with *Salmonella* and IFN-γ is able to enhance TLR2 and TLR3 in both cell lines, suggesting that B16 and BL6 cells might undergo a similar fate through the same TLRs-mediated mechanism. Conversely, lung carcinoma

LLC1 cell line shows TLR2, TLR4 and TLR6 basal levels comparable to those observed in B16 cells. However, these tumor cells express very low levels of TLR1 and TLR3. Importantly, TLR transcription does not change after *Salmonella* infection coupled to IFN-γ stimulation. On the other hand, breast carcinoma 4T1 cell line shows a different TLR transcription pattern compared to the other cell lines analyzed. Notably, 4T1 cells present higher basal levels of TLR2, TLR3 and TLR6 compared to B16 cells, whereas TLR1 and TLR4 are less expressed. Like in LLC1 cells, no synergy between *Salmonella* and IFN-γ in enhancing TLR transcription was observed. Surprisingly, only TLR2 transcription seems to be induced by *Salmonella* and IFN-γ simultaneously administered in 4T1 cells, although this result was not confirmed by TLR2 protein levels analysis, perhaps because of its very high basal levels. Since, unlike B16 cells, 4T1 and LLC1 cell lines express different basal levels of TLRs that are not up-regulated by *Salmonella* in combination with IFN-γ, it would be interesting to also analyze tumor cell death of these cell lines in order to better understand the mechanism leading to cell death. In this way, we might verify whether *Salmonella* infection associated to IFN-γ treatment was able to kill these tumor cells as well and whether TLRs levels were really necessary to induce cell death.

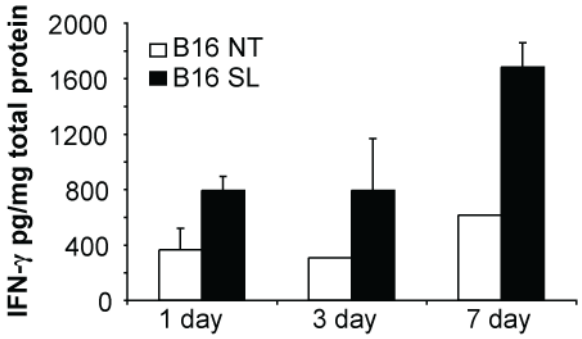


Figure 4-1. IFN-γ in infected tumor masses. B16 established tumors were treated (B16 SL, black bars) or not (B16 NT, white bars) with *Salmonella*. 1, 3, and 7 days later, mice were sacrificed and tumors were smashed and analyzed for IFN-γ production. Error bars: S.E.

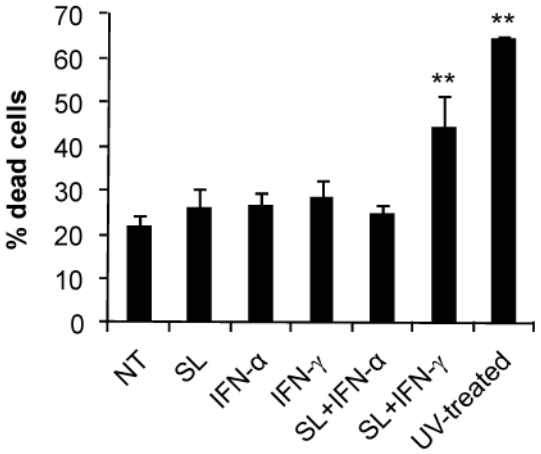


Figure 4-2. *Salmonella* in combination with IFN-γ induces tumor cell death *in vitro*. B16 cells were singularly or simultaneously treated with *Salmonella* (SL), IFN-α, IFN-γ for 24 hours and the percentage of stimulated dead cells compared to untreated dead cells (NT) was evaluated by double staining procedure with FITC-Annexin V and propidium iodide and subsequent cytofluorimetric analysis. As a positive control, B16 cells were UV irradiated (50 J/m^2). Data represent the mean ± S.E. of three independent experiments. Significance according to Student's t-test: **p<0.01.

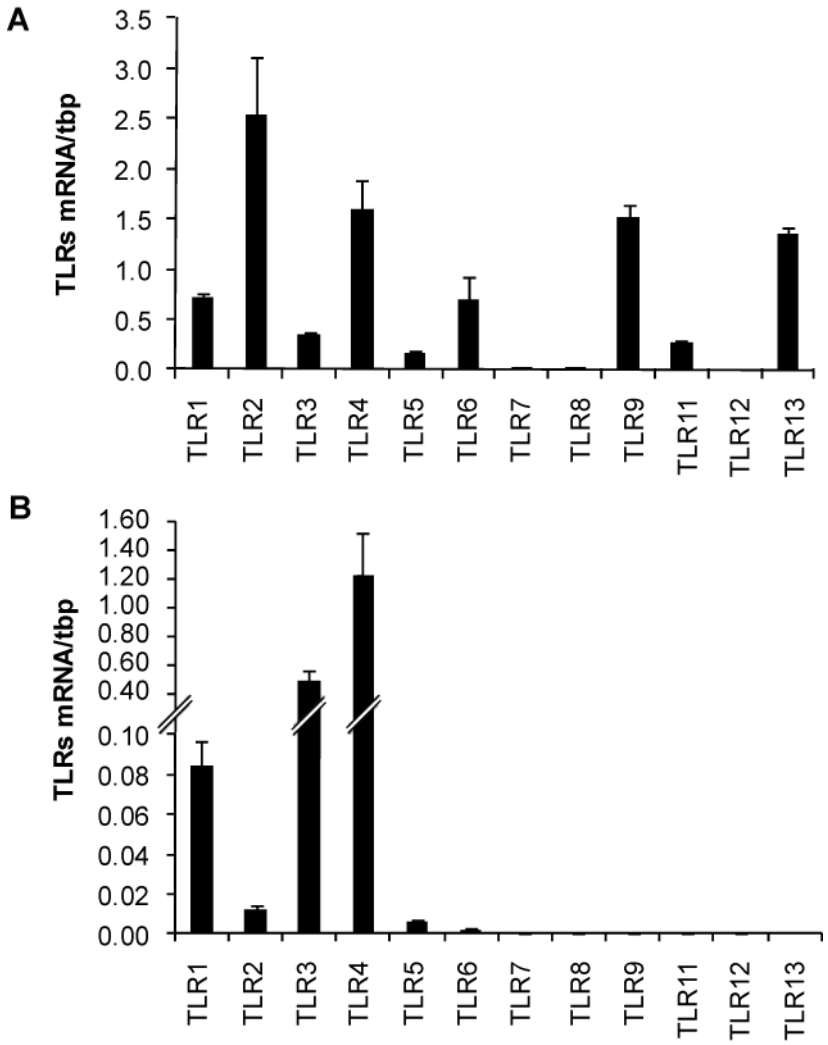


Figure 4-3. B16 and DC1 cells express several TLRs. Real-time PCR analysis of TLR1-9,11,12,13 mRNA contents normalized to the housekeeping TATA-binding protein (tbp) mRNA, in DC1 (**A**) and B16 (**B**) cells. Data represent the means ± S.D. and are representative of three independent experiments.

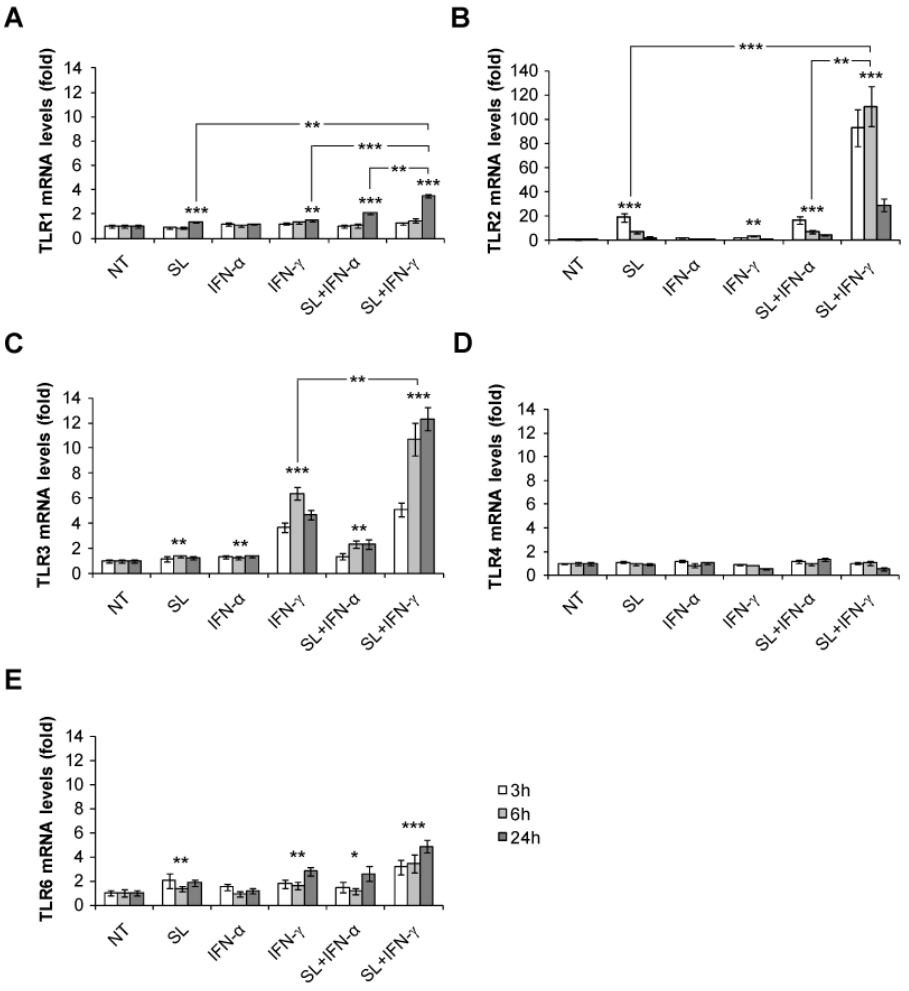


Figure 4-4. *Salmonella* in combination with IFN-γ up-regulates different TLRs in B16 cells. Real-time PCR analysis of TLR1 (**A**), TLR2 (**B**), TLR3 (**C**), TLR4 (**D**), and TLR6 (**E**) mRNA levels in treated cells, in comparison with untreated (NT) cells. B16 cells were analyzed 3, 6, and 24 hours after *Salmonella* infection (SL), IFN-α or IFN-γ treatment, and combined stimulations. Data represent the means ± S.D. and are representative of three independent experiments. Significance according to Student's t-test: *p<0.05, **p<0.01 and ***p<0.001.

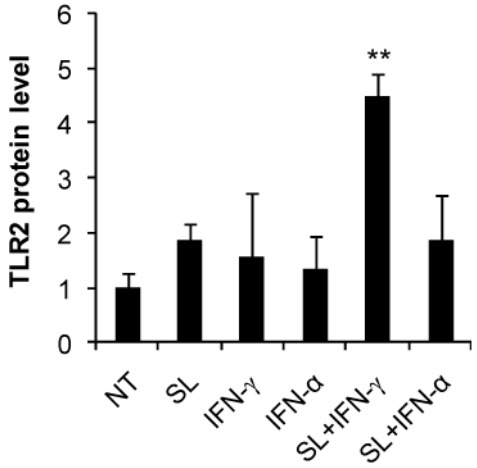


Figure 4-5. *Salmonella* in combination with IFN-γ up-regulates TLR2 protein level in B16 cells. B16 cells were both left untreated (NT) and singularly or simultaneously treated with *Salmonella* (SL), IFN-α, or IFN-γ. After 24 hours stimulation, extracellular TLR2 protein level on B16 cells was determined by FACS analysis, using an Alexa Fluor® 647 anti-mouse TLR2 antibody. The percentage of treated TLR2 positive cells was normalized to the percentage of untreated TLR2 positive cells. Data represent the mean ± S.D. of three independent experiments. Significance according to Student's t-test: **p<0.01.

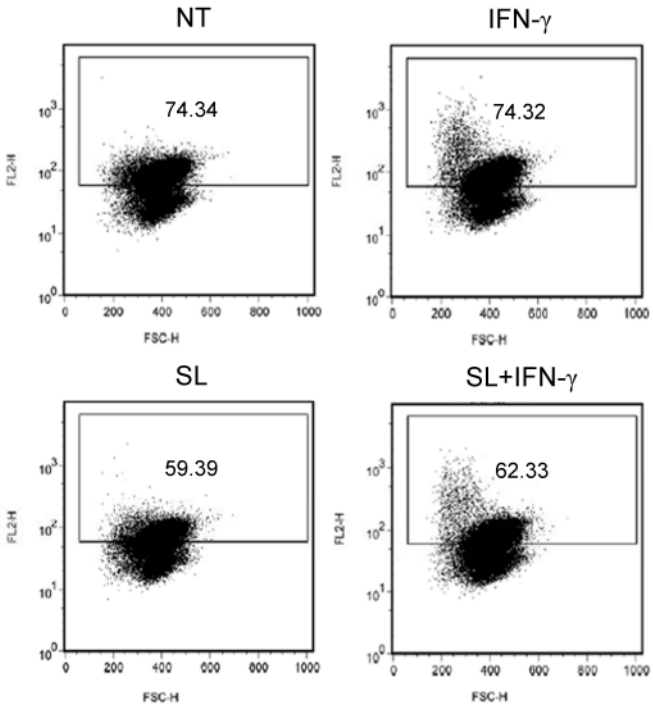


Figure 4-6. B16 cells express IFNGR1 on their surface. B16 cells were both left untreated (NT) and singularly or simultaneously treated with *Salmonella* (SL) and IFN-γ. 24 hours after treatment, extracellular IFNGR1 protein level on B16 cells was determined by FACS analysis, using a PE anti-mouse IFNGR1 antibody. The percentages of IFNGR1 positive cells are indicated. One of three independent experiments is shown.

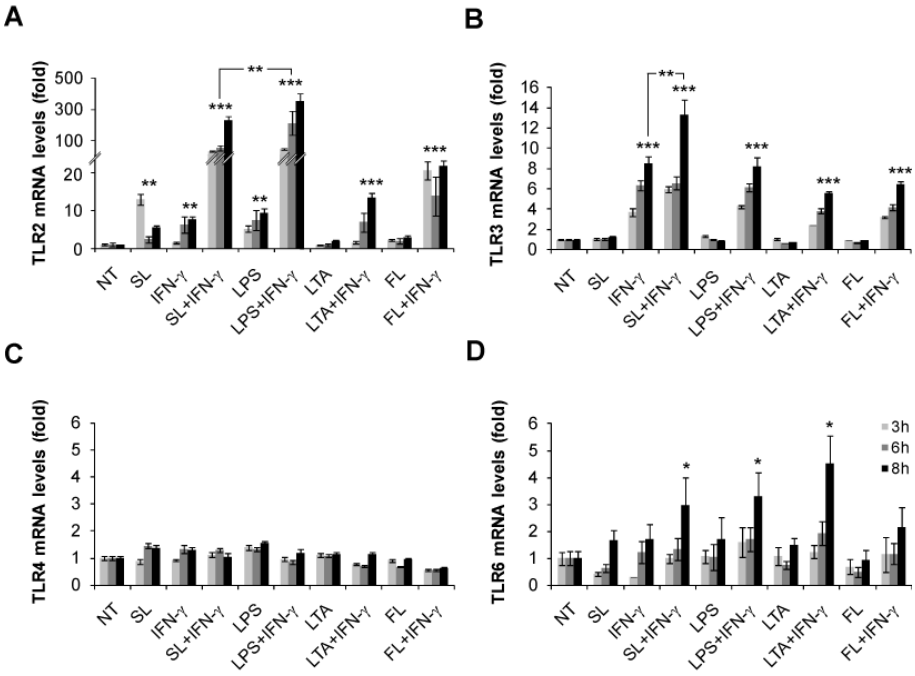


Figure 4-7. Single bacterial components synergize with IFN-γ to increase TLR transcription. Real-time PCR analysis of TLR2 (**A**), TLR3 (**B**), TLR4 (**C**), and TLR6 (**D**) mRNA levels in treated B16 cells, in comparison with untreated (NT) cells. B16 cells were analyzed 3, 6, and 8 hours after *Salmonella* infection (SL), lypopolysaccharide (LPS), acid lipoteichoic (LTA), flagellin (FL), or IFN-γ treatment, and combined stimulations with IFN-γ. Data represent the means ± S.D. and are representative of three independent experiments. Significance according to Student's t-test: *p<0.05, **p<0.01 and ***p<0.001.

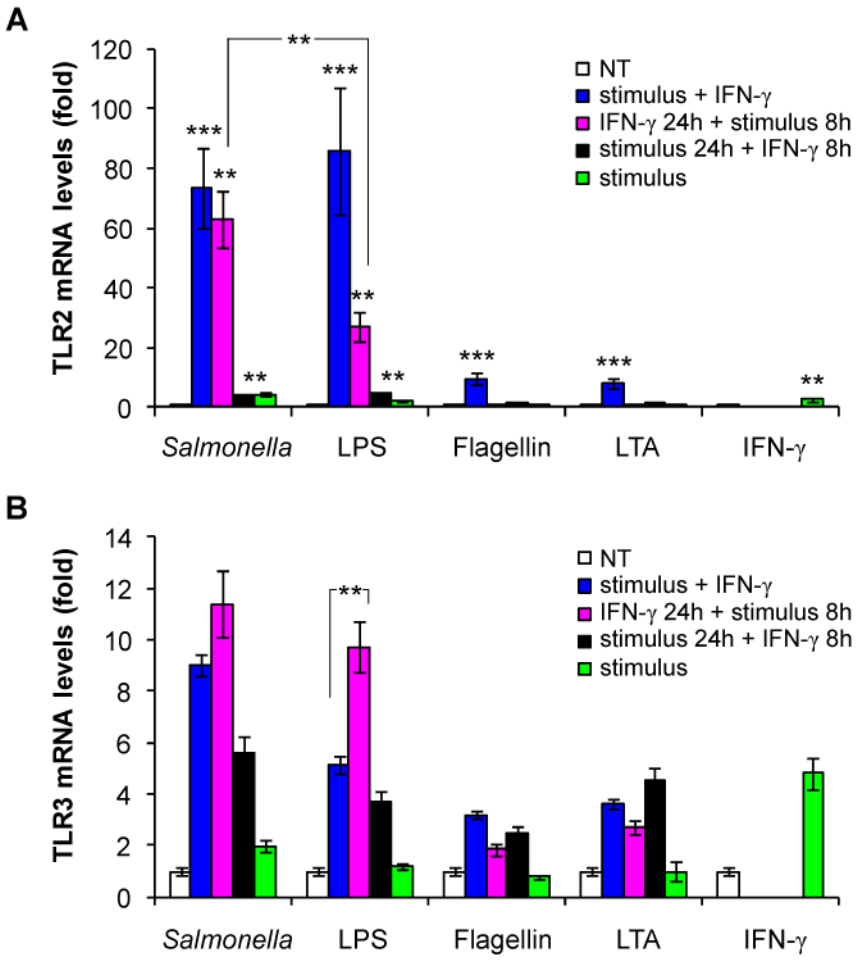


Figure 4-8. The role of IFN-γ on TLR2 and TLR3 mRNA levels in B16 cells. Real-time PCR analysis of TLR2 (**A**) and TLR3 (**B**) mRNA levels in treated B16 cells, in comparison with untreated cells (white bars). B16 cells were pretreated with IFN-γ for 24 hours and then stimulated with *Salmonella*, LPS, flagellin, LTA or IFN-γ for 8 hours (pink bars), or vice versa (black bars). As controls, B16 cells were treated with stimuli alone (green bars) or in combination with IFN-γ (blue bars) for 8 hours. Data represent the means ± S.D. and are representative of three independent experiments. Significance according to Student's t-test: **p<0.01 and ***p<0.001.

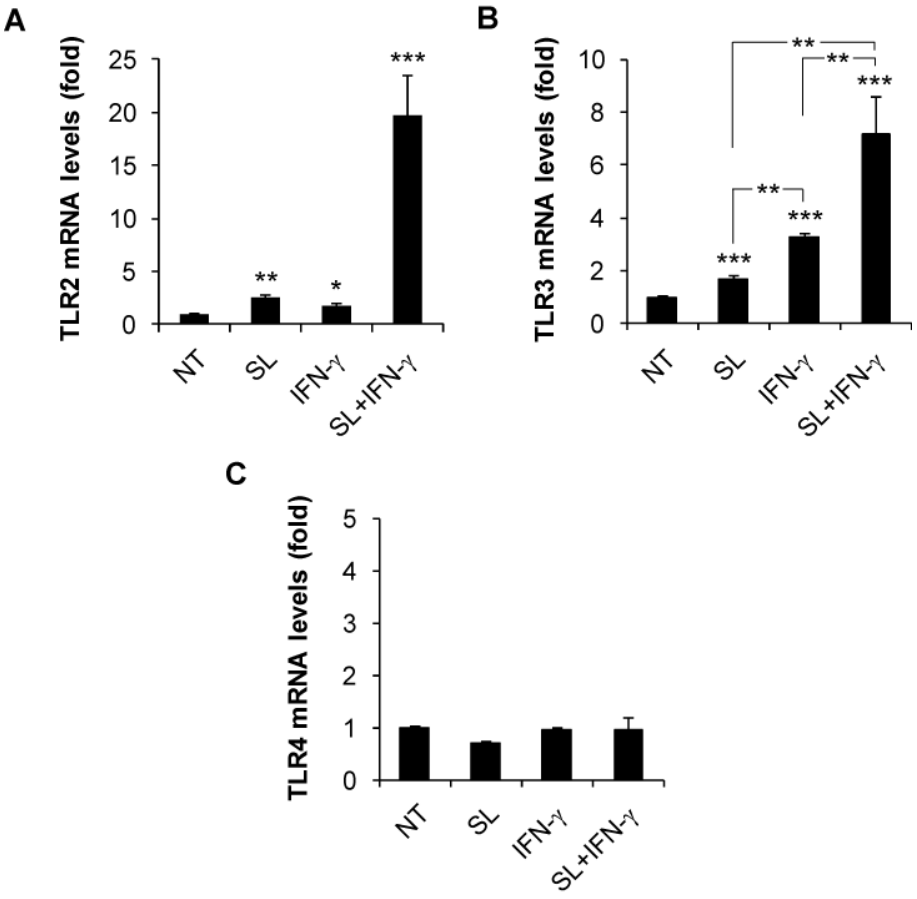


Figure 4-9. TLRs expression levels in BL6 melanoma cells. Real-time PCR analysis of TLR2 (**A**), TLR3 (**B**), and TLR4 (**C**) mRNA levels in treated cells, in comparison with untreated (NT) cells. BL6 cells were analyzed 24 hours after *Salmonella* infection (SL), IFN-γ treatment, and combined stimulations. Data represent the means ± S.D. and are representative of three independent experiments. Significance according to Student's t-test: *p<0.05, **p<0.01 and ***p<0.001.

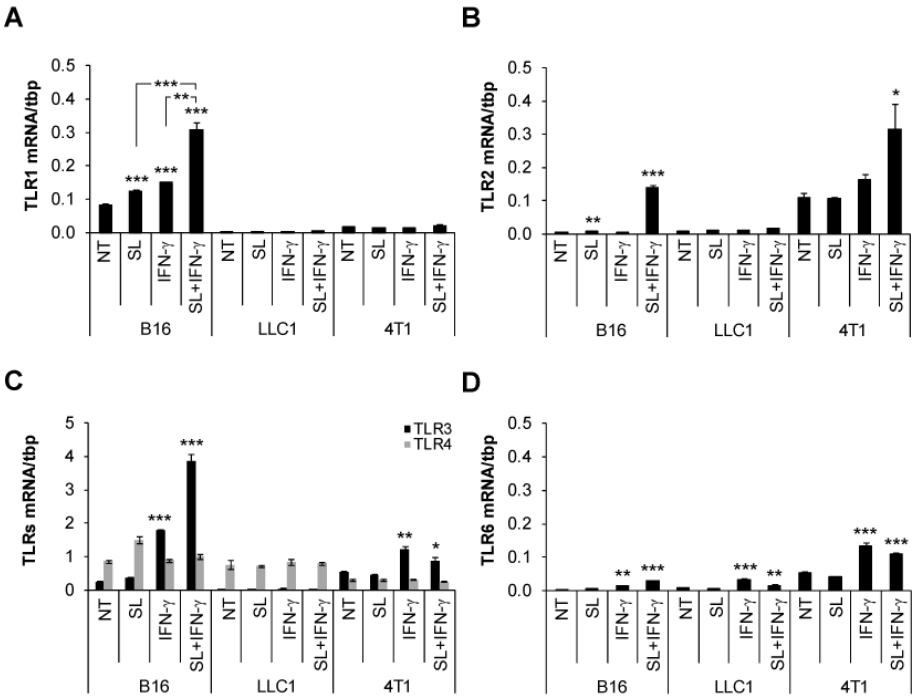


Figure 4-10. TLRs expression levels in B16, LLC1, and 4T1 tumor cells. Real-time PCR analysis of TLR1 (**A**), TLR2 (**B**), TLR3, TLR4 (**C**), and TLR6 (**D**) mRNA levels in B16, LLC1, and 4T1 tumor cells. Cells were analyzed 24 hours after *Salmonella* infection (SL), IFN-γ treatment, and combined stimulations. The Y axis of panel **C** represents TLR3 and TLR4 mRNA quantities normalized to the housekeeping TATA-binding protein (tbp) mRNA. Other panels display mRNA levels of treated cells in comparison with untreated (NT) cells. Data represent the means ± S.D. and are representative of three independent experiments. Significance according to Student's t-test: *p<0.05, **p<0.01 and ***p<0.001.

4.2. TLR2-TLR1 heterodimer and TLR3 seem to mediate B16 tumor cell death

4.2.1. Pam3CSK4 and poly I:C in combination with IFN-γ induce B16 tumor cell death

In this study, we found that *Salmonella* in combination with IFN-γ was able to induce B16 tumor cell death. Moreover, this combined stimulation strongly up-regulated TLR2 protein level. However, Q-PCR analysis demonstrated that also the transcription of TLR3 and TLR2 co-receptors, such as TLR1 and TLR6, increased upon this treatment. These findings allowed us to hypothesize that TLRs expressed on B16 cells might mediate tumor cell death induced by *Salmonella* in combination with IFN-γ. Consistent with our results, in literature was reported that a link between TLRs and apoptosis really exists, as already extensively discussed in Paragraph 1.3.5. In particular, it was demonstrated that both TLR2 and TLR3 mediate apoptotic pathways, even in tumor cells[162-164,169,171]. Furthermore, a recent study reported that TLR3 engagement by TLR3 agonists in human melanoma cells led to caspase activation and tumor cell death when combined to pretreatment with either IFN-α or cycloheximide[171]. However, since in our previous experiments we obtained the best results when tumor cells were stimulated in combination with IFN-γ, we decided to treat B16 cells with TLR3 ligand, poly I:C, a synthetic analog of dsRNA, in association with either IFN-α or IFN-γ. Thus, in a preliminary experiment we cultured B16 cells with poly I:C at 50 µg/ml either alone or in combination with IFN-α or IFN-γ and tumor cell death was evaluated by double staining procedure with FITC-Annexin V (AnnV) and propidium iodide (PI) and subsequent cytofluorimetric analysis after 24 hours of stimulation. Plots reported in Figure 4-11 show that poly I:C is able to induce tumor cell death only when administered with IFN-γ. Notably, about 84% of untreated cells was viable (double negative AnnV-/PI-) whereas only 57% of cells simultaneously treated with poly I:C and IFN-γ represented viable cells. Conversely, in our cell line we did not detect the cytotoxic effect of IFN-α associated to poly I:C observed in human melanoma cells. In fact, in this condition we found a percentage of viable cells comparable to untreated cells (87% of AnnV-/PI-) or to cells incubated with poly I:C alone, as well.

Furthermore, up to now TLR3 ligands are little known, whereas several ligands of TLR2 have been discovered. Notably, TLR3 recognizes double-stranded RNA (dsRNA), a molecular pattern associated with viral infection[124], whereas TLR2 is involved in the recognition of a wide array of microbial molecules representing broad groups of species such as Gram-positive and Gram-negative bacteria, as well as mycoplasma and yeast[117]. Interestingly, TLR2 forms a heterodimer with either TLR1 or TLR6, and these heterodimers appear to be involved in the differential recognition of lipoproteins with different lipid moieties[114,118]. Therefore, since *Salmonella* does not express a well-known TLR3 ligand, although it presents various PAMPs that are able to bind TLR2, we might hypothesize that *Salmonella* interacts with TLR2 and its co-receptors, instead of TLR3. Thereby, in order to evaluate whether TLR2 activation could really lead to B16 tumor cell

death, we tested two different TLR2 ligands, such as Pam3CSK4 and Pam2CSK4, in combination with IFN-γ. Pam3CSK4 is a synthetic tripalmitoylated lipopeptide (LP) that mimicks the acylated amino terminus of bacterial LPs and it signals through TLR2-TLR1 heterodimer. In contrast, Pam2CSK4 is a synthetic diacylated lipopeptide that binds TLR2-TLR6 heterodimer. Thus, in a subsequent experiment, B16 cells were cultured with Pam2CSK4 or Pam3CSK4 either alone or in the presence of IFN-γ. Importantly, the proper concentration of TLR2 ligands was determined in preliminary experiments. We initially tested Pam2CSK4 at two different concentrations 50 or 100 ng/ml, and Pam3CSK4 at 0.5 or 1 µg/ml. After 24 hours of stimulation in the presence or in the absence of IFN-γ, cell death was evaluated. We observed that Pam3CSK4 at 1 µg/ml in combination with IFN-γ was able to induce cell death, whereas Pam2CSK4 did not show any effect (data not shown). Thereby, in order to rule out the possibility that this was not due to an exceedingly low Pam2CSK4 concentration, we subsequently cultured B16 cells with this TLR2 ligand at a 10-fold higher concentration. 24 hours later, cell death was measured. Figure 4-12 shows that the percentage of viable cells does not significantly change in all tested conditions compared to the untreated cells sample. In this way, we further confirmed that Pam2CSK4 was not able to trigger tumor cell death, not even at a very high concentration. On the basis of these preliminary data, we decided to use Pam2CSK4 at 100 ng/ml and Pam3CSK4 at 1 µg/ml in all the following experiments.

Importantly, it was reported in literature that TLR4 also is able to mediate apoptosis in cells stimulated with LPS. Since LPS is a component of *Salmonella* cell wall and our previous Q-PCR data indicated that TLR4 mRNA was highly present in B16 cells, we could not exclude a possible TLR4 engagement in B16 tumor cell death. To this purpose, we cultured tumor cells with LPS alone or in combination with IFN-γ. As positive controls, B16 cells were either UV irradiated (50 J/m^2) or incubated with poly I:C in combination with IFN-γ. 24 hours later, B16 cells both untreated (negative control) and subjected to the different treatments were double stained with FITC-Annexin V (AnnV) and propidium iodide (PI) and cell death was evaluated by FACS analysis. Figure 4-13 shows that Pam3CSK4 at 1 µg/ml induces a significant tumor cell death only when administered in combination with IFN-γ, in accordance with the previous experiment. The percentage of dead cells was comparable to the one detected in B16 cells incubated with IFN-γ in combination with poly I:C treatment or *Salmonella* infection. Consistent with our previous results, Pam2CSK4 at 100 ng/ml both alone and in the presence of IFN-γ was not able to kill B16 cells. Finally, LPS only if simultaneously incubated with IFN-γ induces a slight tumor cell death. However, since we used a standard LPS containing contamination of bacterial components such as lipopeptides (TLR2 ligands) we cannot exclude that this effect may be due to TLR2 rather than TLR4 activation.

Altogether these data suggest that *Salmonella* might interact with TLR2-TLR1 heterodimer that in turn would activate a tumor cell death mechanism. In addition, also TLR3 stimulation with poly I:C is able to induce a similar effect. However, TLR2 and TLR3 activation does not occur in the absence of IFN-γ. Thus, this cytokine seems to sensitize B16 cells to a

subsequent stimulation, up-regulating TLRs involved in tumor cell death or maybe activating another unknown mechanism. Interestingly, these results show that neither TLR2-TLR6 homodimer nor probably TLR4 mediate tumor cell death. In order to rule out a TLR4 engagement, further experiments using ultra-pure LPS, that activates only TLR4 pathway, will be necessary.

4.2.2. Both Pam3CSK4 and poly I:C, in combination with IFN-γ, are able to increase TLR2 and TLR3 transcription

In previous experiments we demonstrated that B16 cells underwent cell death upon combined stimulation with *Salmonella* and IFN-γ and that this treatment was able to up-regulate mainly TLR2 and TLR3, but also TLR1 and TLR6. Subsequently, we observed that B16 tumor cell death was also induced by IFN-γ in combination with either Pam3CSK4 or poly I:C. Thereby, we wondered whether these stimulations could lead to the TLRs up-regulation previously observed in infected B16 cells. To this purpose, in a first experiment, B16 cells were cultured with IFN-γ in combination with either Pam3CSK4 at 1 µg/ml or Pam2CSK4 at 100 ng/ml. As controls, B16 cells were either not stimulated or triggered with single stimuli. After 7 hours and 24 hours of treatment, total RNA was extracted and a Q-PCR analysis was performed. The results obtained revealed that Pam3CSK4 was able to strongly enhance TLR2 mRNA level when incubated in combination with IFN-γ. However, Pam3CSK4 alone also induced a lower but significant increase in TLR2 transcription. On the contrary, this TLR2 ligand up-regulated TLR3 mRNA level only when administered with IFN-γ (Fig. 4-14A,B). The other TLR2 ligand, Pam2CSK4, was able to slightly increase TLR2 and TLR3 transcription only when added simultaneously with IFN-γ. Actually, TLR3 up-regulation was primarily due to IFN-γ effect (Fig. 4-14A,B). In addition, neither TLR2 ligands up-regulated TLR1, TLR4 and TLR6 (data not shown). Finally, the combined treatment with Pam3CSK4 and IFN-γ induced enhanced MyD88 mRNA level, the adaptor protein involved in TLR2 pathway, as well (Fig. 4-14C).

A subsequent experiment was performed in order to assess whether TLR transcription was also influenced by the TLR3 ligand poly I:C simultaneously administered with IFN-γ. To this purpose, B16 cells were simultaneously incubated with poly I:C at 50 µg/ml and IFN-γ. In addition, we infected tumor cells with *Salmonella* and IFN-γ as a positive control, whereas as negative control B16 cells were left untreated. Finally, tumor cells were also stimulated with single stimuli. 24 hours later, total RNA was extracted and a Q-PCR was carried out. Figure 4-15A shows that poly I:C in combination with IFN-γ induces a stronger increase in TLR2 transcription compared to the combined treatment with *Salmonella* and IFN-γ. However, this increase was previously observed at an earlier time point (after 6-8 hours), indicating that this latter condition was actually able to induce higher level of TLR2 mRNA, similar to the former stimulation (Fig. 4-4B). On the contrary, the simultaneous incubation of B16 cells with poly I:C and IFN-γ enhanced TLR3 transcription more than positive control treatment (Fig. 4-15B). In fact, TLR3 mRNA did not exceed a 15-fold increase, not even at earlier time points, as reported in our previous experiments. Moreover, poly

I:C alone was also able to enhance its receptor at mRNA level. In contrast to combined stimulation with *Salmonella* and IFN-γ, simultaneous administration of poly I:C and IFN-γ did not up-regulate TLR1, TLR4 and TLR6 (data not shown). Surprisingly, this treatment was able to induce an increase in MyD88 mRNA level to a higher extent as *Salmonella* infection coupled to IFN-γ treatment (Fig. 4-15C).

Since Q-PCR data demonstrated that Pam3CSK4 and poly I:C in combination with IFN-γ increased mainly TLR2 transcription in B16 cells and that in turn this receptor was very likely involved in tumor cell death, we decided to assess also TLR2 protein levels. To this purpose, we performed again the previous experiment on B16 cells, administering Pam2CSK4, Pam3CSK4, poly I:C and *Salmonella*, in combination or not with IFN-γ. In this case, B16 cells both untreated and subjected to different treatments were processed and stained for FACS analysis, using an anti-TLR2 antibody, after 48 hours of stimulation. Consistent with our Q-PCR results, FACS analysis revealed that TLR2 protein is up-regulated on B16 cell surface after IFN-γ incubation associated to either Pam3CSK4 or poly I:C treatment. Interestingly, in B16 cells treated with IFN-γ, TLR3 ligand is able to increase TLR2 protein level more than *Salmonella* infection (Fig. 4-16).

On the whole, these results suggest that stimulations that induce tumor cell death, that is Pam3CSK4, poly I:C or *Salmonella* in the presence of IFN-γ, are also able to up-regulate TLR2 and TLR3. However, conversely to *Salmonella*, TLR2 and TLR3 ligands do not increase TLR1 and TLR6 mRNA levels. Therefore, we may hypothesize that the increased expression of TLR2 and TLR3 is necessary to induce tumor cell death. On the contrary, this mechanism seems not to be affected by the expression of TLR1 and TLR6. In fact, we observed that in the presence of high levels of TLR2 and TLR3, basal levels of TLR1 and TLR6 are sufficient to obtain the same cytotoxic effect. In addition, since Pam2CSK4 does not kill tumor cells, we may further suppose that TLR6 is not involved in this cell death mechanism. Moreover, we found that Pam3CSK4 and poly I:C are able to up-regulate mainly the transcription of their own receptors, namely TLR2 and TLR3, respectively. However, Pam3CSK4 induces also an increase in TLR3 mRNA level, whereas poly I:C up-regulates both TLR2 transcript and protein level, suggesting a possible cross-talk between both TLR2 and TLR3 pathways. Consistent with our hypothesis, we found that poly I:C is also able to up-regulate MyD88, the principal adaptor protein of TLR2 pathway. Furthermore, TLR3 ligand up-regulates also TLR2 protein levels, to a higher extent than *Salmonella*.

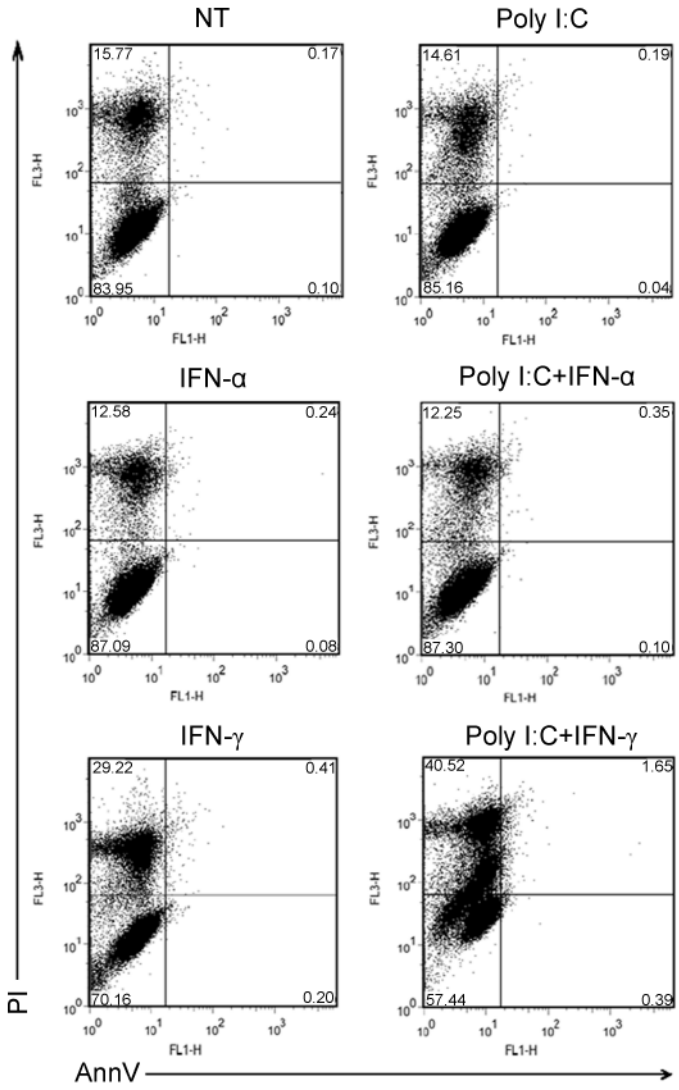


Figure 4-11. Poly I:C in combination with IFN-γ induces tumor cell death. B16 cells were treated with poly I:C (50 µg/ml) in combination with IFN-α (2000 U/ml) or IFN-γ (100 U/ml). As controls, B16 cells were either left untreated (NT) or stimulated with single stimuli. 24 hours after treatment B16 cells were stained with FITC-Annexin V and propidium iodide and subjected to FACS analysis. One of three independent experiments is shown.

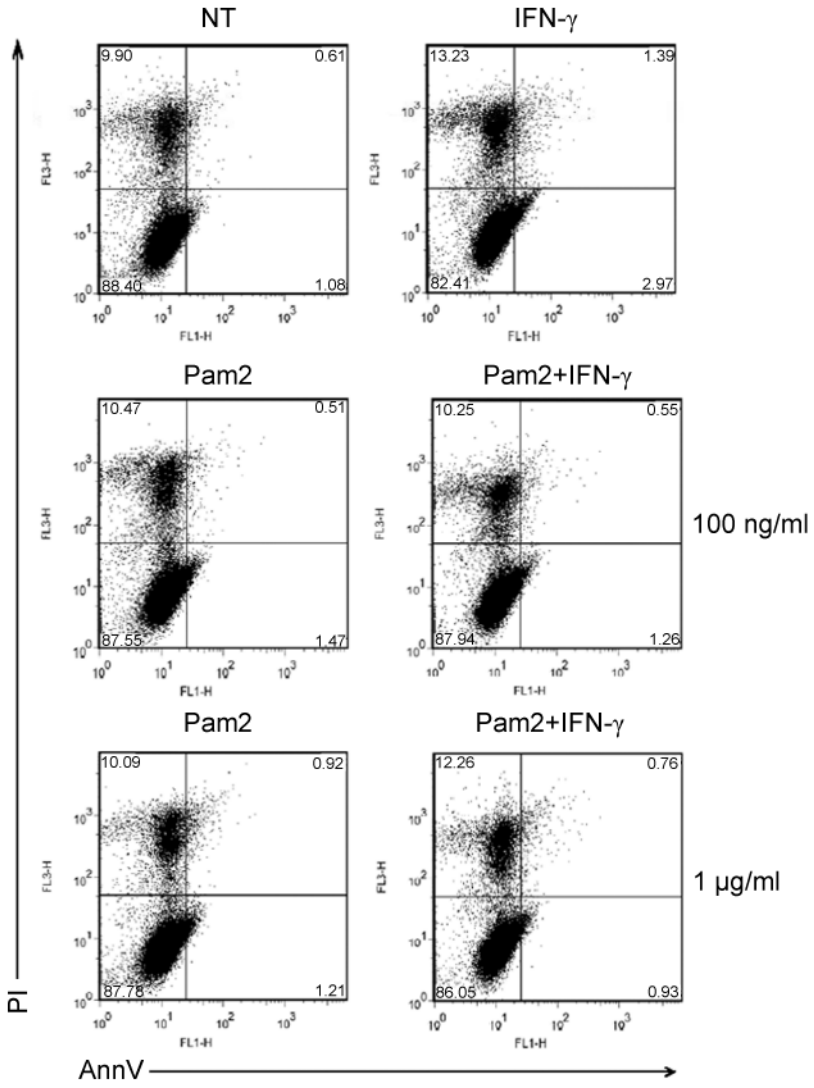


Figure 4-12. Pam2CSK4 does not induce tumor cell death. B16 cells were treated with Pam2CSK4 (Pam2) at 100 ng/ml or at 1 µg/ml alone or in combination with IFN-γ (100 U/ml). As a control, B16 cells were left untreated (NT). 24 hours after treatment B16 cells were stained with FITC-Annexin V and propidium iodide and subjected to FACS analysis. One of two independent experiments is shown.

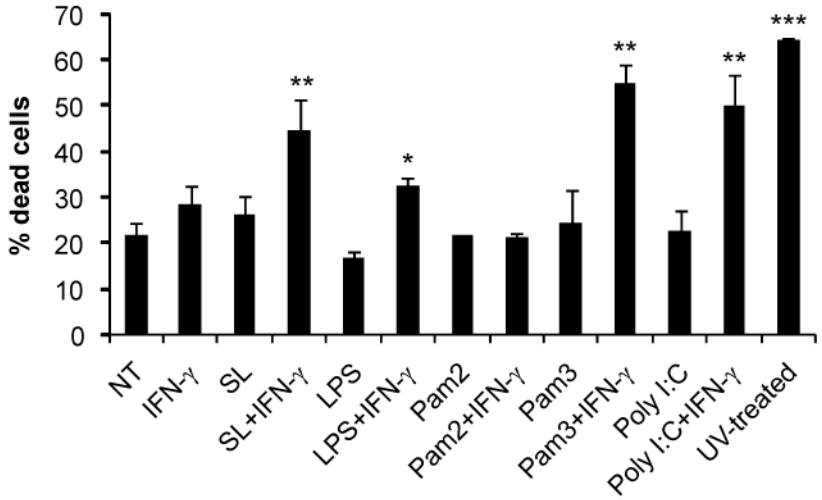


Figure 4-13. Combined stimulations induce tumor cell death *in vitro*. B16 cells were treated with *Salmonella* (SL), lypopolysaccharide (LPS), Pam2CSK4 (Pam2), Pam3CSK4 (Pam3), or poly I:C alone or in combination with IFN-γ for 24 hours. As controls, B16 cells were either left untreated (NT) or UV irradiated (50 J/m^2). The percentage of treated dead cells compared to untreated dead cells was evaluated by double staining procedure with FITC-Annexin V and propidium iodide and subsequent cytofluorimetric analysis. Data represent the mean ± S.E. of three independent experiments. Significance according to Student's t-test: *p<0.05, **p<0.01 and ***p<0.001.

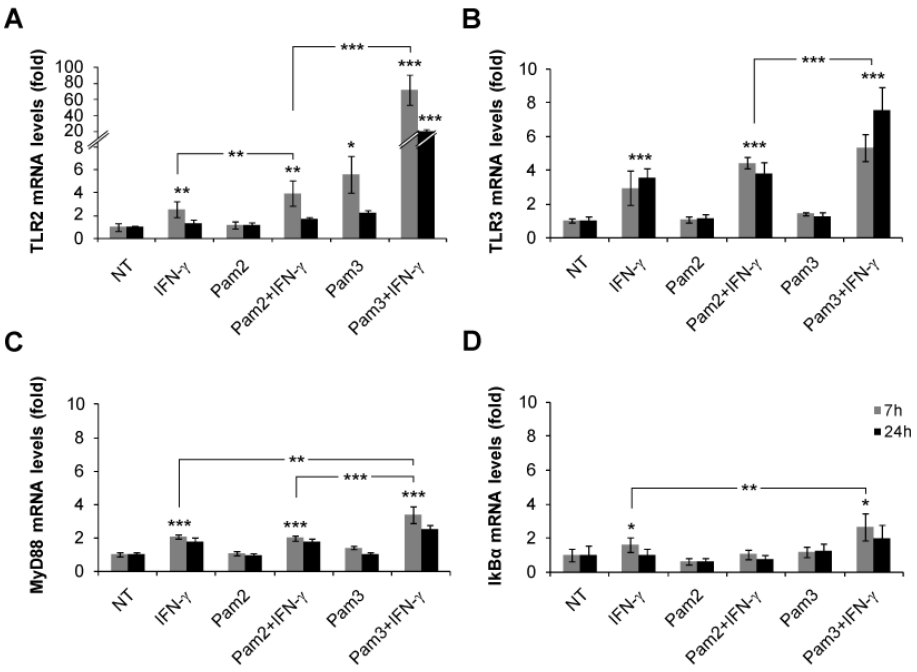


Figure 4-14. Pam3CSK4 in combination with IFN-γ increases TLR2, TLR3, MyD88 and IkBα mRNA levels. Real-time PCR analysis of TLR2 (**A**), TLR3 (**B**), MyD88 (**C**), and IkBα (**D**) mRNA levels in treated B16 cells, in comparison with untreated (NT) cells. B16 cells were analyzed 7 and 24 hours after stimulation with Pam2CSK4 (Pam2), Pam3CSK4 (Pam3), singularly or simultaneously administered with IFN-γ. Data represent the means ± S.D. and are representative of three independent experiments. Significance according to Student's t-test: *p<0.05, **p<0.01 and ***p<0.001.

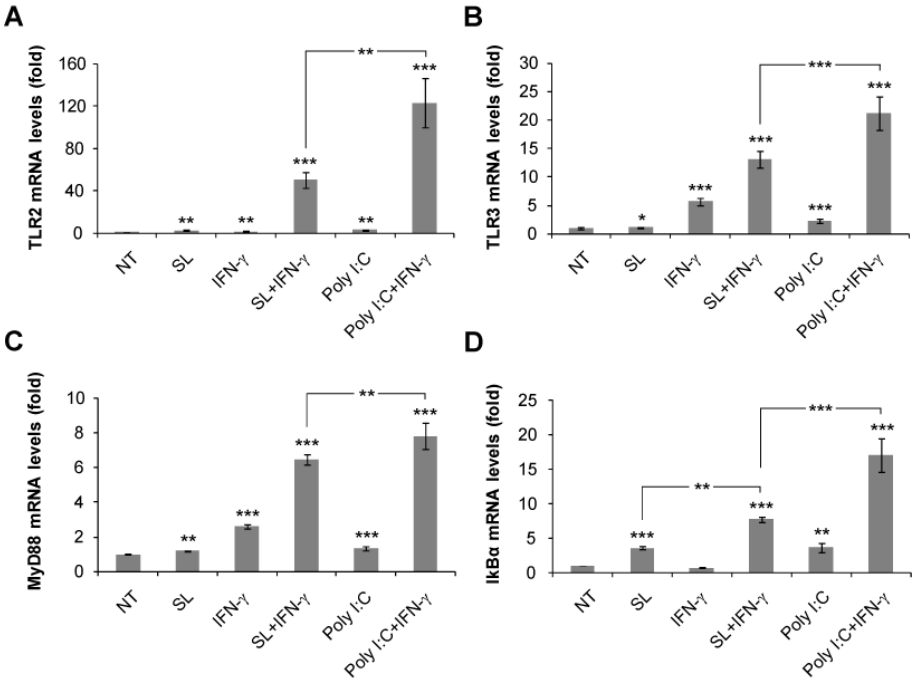


Figure 4-15. Poly I:C in combination with IFN-γ increases TLR2, TLR3, MyD88 and IkBα mRNA levels. Real-time PCR analysis of TLR2 (**A**), TLR3 (**B**), MyD88 (**C**), and IkBα (**D**) mRNA levels in treated B16 cells, in comparison with untreated (NT) cells. B16 cells were analyzed 24 hours after *Salmonella* infection (SL) or poly I:C treatment, singularly or simultaneously administered with IFN-γ. Data represent the means ± S.D. and are representative of three independent experiments. Significance according to Student's t-test: *p<0.05, **p<0.01 and ***p<0.001.

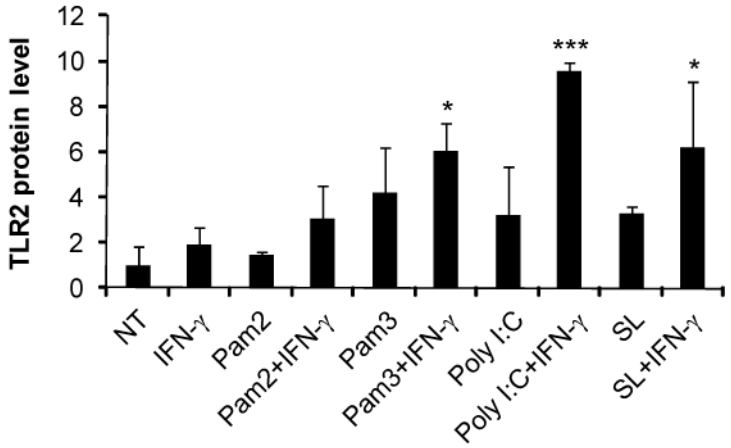


Figure 4-16. *Salmonella*, Pam3CSK4 and poly I:C in combination with IFN-γ up-regulate TLR2 protein level on B16 cells. B16 cells were treated with *Salmonella* (SL), Pam2CSK4 (Pam2), Pam3CSK4 (Pam3), or poly I:C alone or in combination with IFN-γ. After 48 hours stimulation, extracellular TLR2 protein level on B16 cells was determined by FACS analysis, using an Alexa Fluor® 647 anti-mouse TLR2 antibody. The percentage of treated TLR2 positive cells was normalized to the percentage of untreated (NT) TLR2 positive cells. Data represent the mean ± S.D. of three independent experiments. Significance according to Student's t-test: *p<0.05, ***p<0.001.

4.3. B16 cells undergo TLR-mediated early necrosis and caspase-mediated late apoptosis, after cytotoxic stimulations

4.3.1. Treated B16 cells do not undergo apoptosis within 24 hours

Our experiments demonstrated that different stimuli, such as *Salmonella*, Pam3CSK4 and poly I:C, in combination with IFN-γ were able to induce B16 tumor cell death after 24 hours treatment. Moreover, this mechanism seemed to be mediate by some of the TLRs present on tumor cells, mainly TLR2 and TLR3. Since, as already discussed, several studies suggested the involvement of TLRs in apoptosis, we wondered whether also B16 cells would undergo apoptotic cell death after cytotoxic stimulations. To this purpose, we decided to investigate B16 tumor cell death mechanism analyzing both phosphatidylserine (PS) exposure and caspase activation. B16 cells were either infected with *Salmonella* or incubated with Pam3CSK4 or poly I:C, in the presence or in the absence of IFN-γ. As controls, tumor cells were either left untreated or triggered with UV irradiation (30, 40 or 50 J/m^2), a classical apoptotic stimulus. In order to analyze PS exposure, a marker of early apoptosis, B16 cells were double stained with FITC-Annexin V and PI after 24 hours stimulation. Staining cells simultaneously with FITC-Annexin V and the non-vital dye propidium iodide allowed us to discriminate intact cells (Ann-/PI-), early apoptotic (Ann+/PI-) and late apoptotic or necrotic cells (Ann+/PI+, Ann-/PI+). In this regard, FACS analysis revealed that at this time point no significant percentage of double positive Ann+/PI+ or single positive Ann+/PI- cells were present in any sample. On the contrary, high levels of Ann-/PI+ cells were detected in all samples treated with combined stimulations, that is *Salmonella*+IFN-γ (Fig. 4-17A), Pam3CSK4+IFN-γ (Fig. 4-17B), and poly I:C+IFN-γ (Fig. 4-11). However, not even all positive controls showed an apoptotic behavior, but only high percentages of Ann-/PI+ cells (Fig. 4-17C). In addition, in the same experiment, we performed a Western blot analysis in order to investigate caspase activation. Therefore, after 24 hours treatment, B16 cells both untreated and subjected to different stimulations were lysed and total protein extracts were analyzed by Western blotting using anti-cleaved caspase-3 and anti-cleaved caspase-7 antibodies. Consistent with FACS results, we detected no bands in any sample, except for UV-treated positive controls. In contrast to FACS data, UV irradiations at any dose were able to activate both caspase-3 and caspase-7 cleavage already 24 hours after treatment (data not shown). This suggests that the translocation of PS from the inner cytoplasmic leaflet of the plasma membrane to the outer (cell surface) leaflet could take place at an earlier time point. To this purpose, a time course experiment was carried out. The experiment was similar to the one previously described, although in this case B16 cells were processed and double stained with FITC-Annexin V and PI for FACS analysis 3, 6, and 24 hours after stimulation. Moreover, as a control, we decided to analyze a longer time as well, that is 48 hours. Interestingly, neither Ann+/PI- nor Ann+/PI+ cells were detected at any times, suggesting that in B16 cells PS

exposure is not a reliable marker of apoptosis. Hence, we focused our analysis on caspase activation to assess apoptotic cell death. Western blot analysis showed that neither caspase-3 nor caspase-7 cleavage fragments were detected by specific antibodies 3, 6 and 24 hours after treatments. Surprisingly, the samples treated with IFN-γ in combination with *Salmonella*, Pam3CSK4 or poly I:C for 48 hours showed high levels of cleaved caspase-7, as shown in Figure 4-18. Conversely, only in samples treated with either *Salmonella* or Pam3CSK4, in combination with IFN-γ, a weak cleaved caspase-3 signal was detected (Fig. 4-18).

On the basis of these results we hypothesized that B16 cells after combined stimulations might undergo a first necrotic event followed by a later apoptotic one. 24 hours after combined treatments a significant percentage of tumor cell death was observed, even without caspase activation, indicating that B16 cells might initially die through a necrotic mechanism. However, even 48 hours after treatments FACS analysis did not show any Ann+/PI- or Ann+/PI+ cells, although at this time point B16 cells activate apoptotic pathways leading to caspase-7 and, to a lesser extent, caspase-3 cleavage. These data suggest that B16 cells might undergo a late apoptotic pathways activation. Moreover, since we have not found a complete correlation between FACS data and Western blotting results, we cannot exclude that B16 cells are actually not able to translocate PS on their cell surface. Lastly, in order to rule out a possible experimental mistake, we double stained bone-marrow dendritic cells (BMDCs) with AnnV and PI after stimulation with two classical apoptotic inducers, staurosporine and UV irradiations. In particular, BMDCs were incubated with 3 μM staurosporine for 6 hours or were irradiated with UV (40 J/m^2). As shown in Figure 4-19, in both triggered samples, we detected significant percentages of both Ann+/PI- and Ann+/PI+ cells, indicating that BMDCs were undergoing apotosis.

4.3.2. B16 cell death is associated to release of HMGB1, IL-6, and KC

Cells dying by necrosis readily lose membrane integrity and release intracellular contents, which may cause an inflammatory response. Therefore, unlike apoptotic cells that retain membrane integrity and can be rapidly phagocytosed by macrophages or by surrounding cells before lysis, necrotic dying cells are considered immunogenic at all times[181]. Many studies report that necrotic cells are potent inducers of NF-kB. NF-kB activation by necrotic cells resulted in dramatic induction of expression of genes involved in the inflammatory response, such as proinflammatory cytokines. Notably, cells dying by necrosis often produce and release IL-6, IL-8 (or KC, its functional counterpart in mouse), IL-10, TNF-α[181,195,197]. Interestingly, necrotic cell-induced NF-kB was found dependent on the TLR signaling pathways, mainly TLR2 and TLR3[197,210]. Furthermore, it has been recently discovered that cells that undergo necrosis release HMGB1 which has proinflammatory properties[198]. Importantly, HMGB1, a nuclear factor that enhances transcription, can either be actively secreted from inflammatory cells or passively released from necrotic cells. In order to assess whether B16 cells treated with *Salmonella*, Pam3CSK4 or poly I:C in combination

with IFN-γ really undergo a first necrotic event, cell culture supernatants were collected 24 and 48 hours after stimulations and analyzed by ELISA for HMGB1 release. Consistent with our cell death results, we found that B16 cells triggered with the combined stimulations released high levels of HMGB1 already after 24 hours of treatment. Surprisingly, *Salmonella* alone induced high release of HMGB1 as well (Fig. 4-20), although we demonstrated that bacteria alone did not kill tumor cells. Thus, we cannot rule out the possibility that HMGB1 is actively secreted instead of passively released from necrotic cells. In addition, no significant differences between HMGB1 levels detected after 24 or 48 hours of treatment were observed (data not shown).

Similarly, we performed a time course experiment in order to analyze the release of different cytokines, such as IL-6, IL-10, IL-12p70, KC, and TNF-α, most of which had been found associated to necrotic cell death. B16 cells were stimulated with *Salmonella*, Pam3CSK4, or poly I:C alone or in combination with IFN-γ. In addition, as controls, we tested the cytokine release also in B16 cells either untreated, or stimulated with IFN-α alone or coupled to *Salmonella* infection. After 3, 6, and 24 hours the supernatants were collected and ELISA assays were performed. Importantly, TNF-α, IL-10, and IL-12p70 were not released by B16 cells upon every stimulation at any time (data not shown). On the contrary, significant detectable levels of IL-6 and KC, a neutrophil-specific chemokine, were observed, but only starting from 24 hours stimulation. In particular, B16 cells triggered with all single stimuli, except for IFN-γ and IFN-α, were able to release significantly high levels of both IL-6 and KC compared to untreated cells. Moreover, *Salmonella* in combination with IFN-γ strongly enhanced IL-6 and KC release. The simultaneous administration of the other stimuli with IFN-γ increased IL-6 and KC levels, although high variability among different experiments was detected (Fig. 4-21A,B). In addition, B16 cells triggered with IFN-α coupled to *Salmonella* infection were not able to induce either IL-6 or KC release. As above mentioned, NF-kB is involved in transcription of proinflammatory cytokines and it can be activated by TLR pathways. In this regard, we wondered whether the observed cytokine production was a consequence of previous TLR pathways activation. To this purpose, we performed Q-PCR analysis to measure mRNA level of IkBα, a marker of NF-kB activation. IkBα is an inhibitor protein which masks the nuclear localization signal (NLS) of NF-kB. Upon cell stimulation, degradation of IkBα leads to nuclear translocation of NF-kB. NF-kB can rapidly induce the transcription of IkBα gene and the newly synthesized IkBα protein accumulates into the nucleus where it is responsible for inhibition of kB-dependent transcription[211]. Q-PCR experiments revealed that all combined stimulations, except for Pam2CSK4, were able to strongly enhance IkBα transcription (Fig. 4-14D and 4-15D). Notably, poly I:C in combination with IFN-γ induced the highest level of IkBα transcription (about 15-fold increase). Interestingly, *Salmonella* and poly I:C alone were able to up-regulate IkBa transcription to a similar extent as Pam3CSK4 in combination with IFN-γ (Fig. 4-14D and 4-15D). However, in order to further confirm NF-kB activation, we analyzed NF-kB nuclear translocation by Western blot analysis, using an anti-p65 antibody. This experiment was carried out only

on B16 simultaneously infected with *Salmonella* and incubated with IFN-γ. As controls, tumor cells were either left untreated or triggered with single stimuli. 24 hours after treatment, B16 cells were lysed and cytosolic fraction was separated from nuclear fraction. Subsequently, cytosolic and nuclear fractions were subjected to Western blot analysis and probed with anti-p65 antibody in order to detect NF-kB protein. Consistent with our Q-PCR results, Figure 4-22 shows that NF-kB translocates into the nucleus after combined stimulation with *Salmonella* and IFN-γ, but also after *Salmonella* infection alone, even if to a lesser extent.

Finally, we also analyzed IL-1β secretion by ELISA assay in order to clarify the pathway leading to caspase-7 activation. In fact, caspase-7, besides its activation during apoptosis, has also been observed under inflammatory conditions. Accordingly, it has been shown that caspase-7 is cleaved by caspase-1, an inflammatory caspase involved in the maturation and secretion of IL-1β[184]. Since B16 cells activated caspase-7 after 48 hours of combined stimulations, we protracted stimulations until 48 and 72 hours. Nonetheless, IL-1β was totally absent in all samples at any time (data not shown).

Altogether these results indicate that B16 cells release HMGB1, a marker of necrotic cell death, already 24 hours after IFN-γ treatment in combination with *Salmonella* or Pam3CSK4 or poly I:C, suggesting that tumor cells may undergo necrosis. However, *Salmonella* alone stimulates HMGB1 release as well, although it does not kill tumor cells. Thus, we cannot rule out the possibility that HMGB1 is actively secreted rather than passively released by necrotic cells. In addition, already 24 hours after combined stimulations, B16 cells are able to release high levels of IL-6 and KC, proinflammatory cytokines likely induced by NF-kB activation and often released by necrotic cells. On the whole, these findings support our hypothesis; tumor cells undergo a necrotic event within 24 hours of stimulation, releasing large amounts of HMGB1, IL-6 and KC. Subsequently, they undergo apoptotic pathways activation leading to caspase-7 cleavage. In this regard, the lack in IL-1β secretion rules out the possibility that caspase-7 activation is a consequence of an inflammatory event that is caspase-1 mediated.

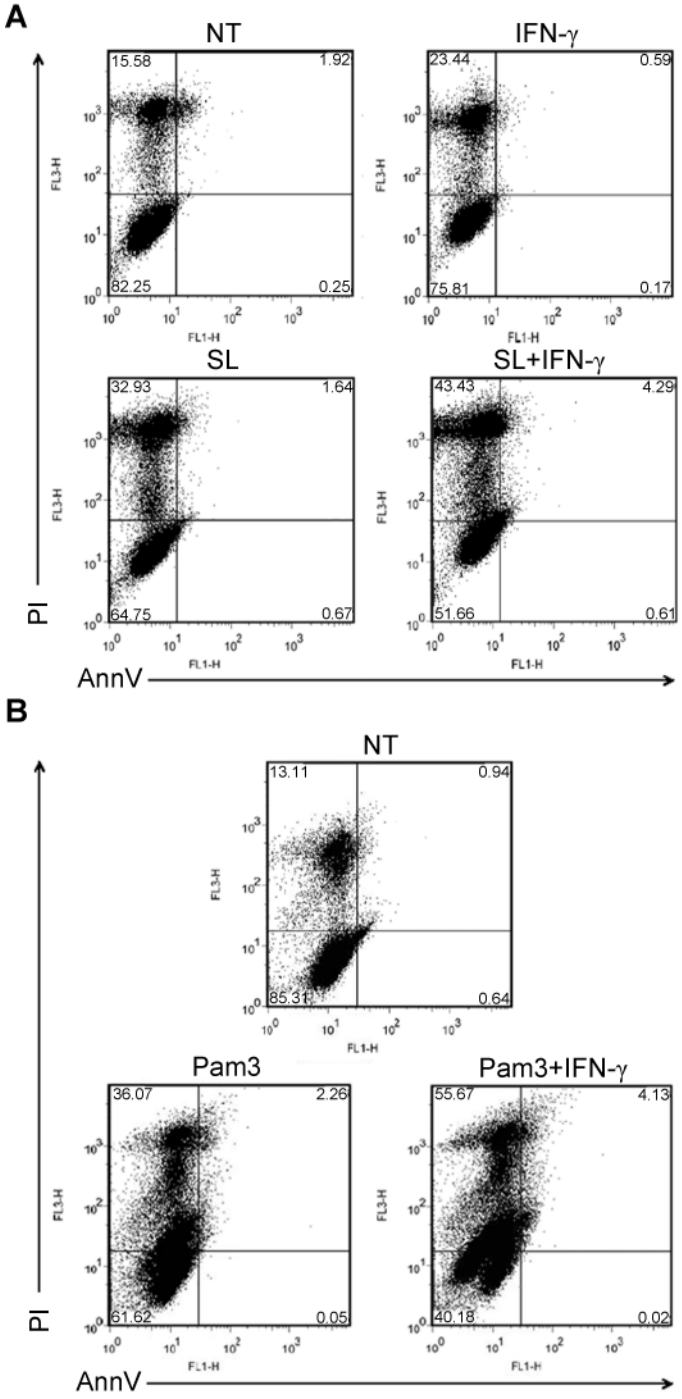
4.3.3. Caspase-7 cleavage is reduced by necrosis inhibitor nec-1

A small-molecule inhibitor of necrotic cell death was recently identified and named necrostatin-1 (nec-1). An analysis of its molecular target identified necrostatin-1 as an inhibitor of RIP1, a death-domain-containing kinase that has been found to participate in necrotic cell death[192,193]. In order to better understand B16 cell death mechanism, we tested whether nec-1 could inhibit TLR-mediated cell death. Firstly, we performed a Western blot analysis in order to investigate caspase-7 activation. Therefore, B16 cells were either left untreated, as controls, or triggered with *Salmonella*, Pam3CSK4 or poly I:C alone or in combination with IFN-γ in the presence or in the absence of nec-1 (50 μM). After 24 and 48 hours treatment, B16 cells both untreated and subjected to different stimulations were lysed and total protein extracts were analyzed by Western blotting using anti-cleaved

caspase-7 antibody. In accordance with our previous experiment, no bands were detected in samples stimulated for 24 hours (data not shown). On the contrary, we found that 48 hours after treatment *Salmonella*, Pam3CSK4 and, to a higher extent, poly I:C synergized with IFN-γ to activate caspase-7, as shown in Figure 4-23. Surprisingly, densitometric analysis revealed that caspase-7 cleavage was reduced by inhibitor nec-1 of about 60%, demonstrating that necrosis inhibition reduced the subsequent caspase-7 mediated apoptotic event. In addition, in the same experiment, cell culture supernatants were collected and analyzed for HMGB1, IL-6 and KC release by ELISA. Interestingly, nec-1 was able to significantly reduce cytokine release in all samples showing high levels of HMGB1, IL-6 and KC (Fig. 4-24). On the whole, this result strongly supports our hypothesis on TLR-mediated tumor cell death mechanism, even if a further experiment to evaluate cell death in the presence of nec-1 will be necessary.

4.3.4. Poly I:C induces TLR3 translocation to the cell surface in combination with IFN-γ

It is well-known that TLR3 is found almost exclusively in intracellular compartments such as the endosomes and that its ligands require internalization before signaling is possible. TLR3 mRNA has been detected in immune cells as well as in fibroblasts and epithelial cells[129]. Since we did not transfect poly I:C into B16 cells, but we simply added it into the medium, we wondered whether TLR3 might be exposed on tumor cell surface. To this purpose, TLR3 protein localization on B16 cells was analyzed. B16 cells, either untreated or triggered with different stimuli, were processed and subjected to both intracellular and extracellular staining using an anti-TLR3 antibody, after 24 hours treatment. Subsequent FACS analysis showed that, as expected, TLR3 was intracellularly expressed at very high levels in untreated cells as well and this percentage of positive cells reached the maximum increase after IFN-γ stimulation (data not shown). On the contrary, TLR3 was present on cell surface at very low levels, but poly I:C treatment in combination with IFN-γ was able to strongly induce TLR3 translocation to the B16 cell surface (Fig. 4-25). This result might explain the strong caspase-7 activation induced by poly I:C in association with IFN-γ observed in Western blot analysis.


A
NT
IFN-γ
SL
SL+IFN-γ
15.58
1.92
82.25
0.25
23.44
0.59
75.81
0.17
32.93
1.64
64.75
0.67
43.43
4.29
51.66
0.61
PI
AnnV
FL3-H
FL1-H
B
NT
Pam3
Pam3+IFN-γ
13.11
0.94
85.31
0.64
36.07
2.26
61.62
0.05
55.67
4.13
40.18
0.02

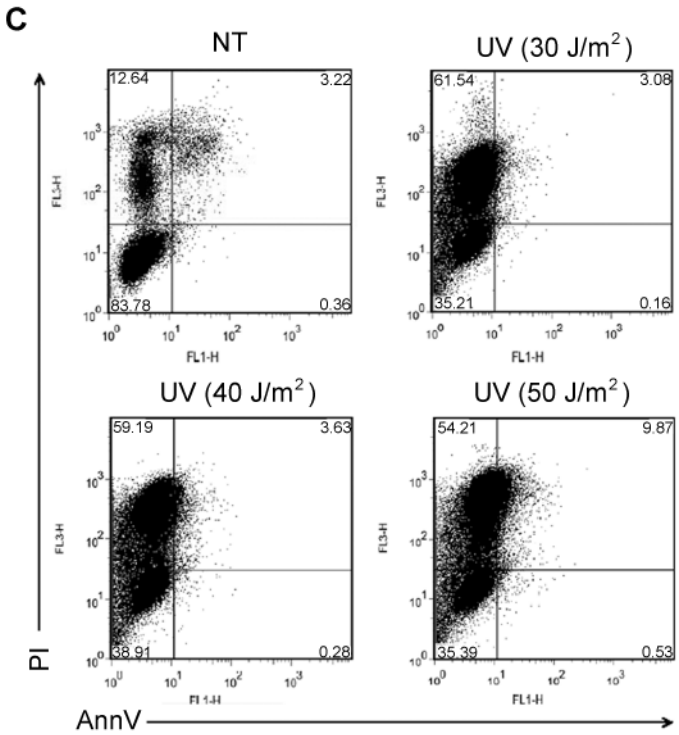


Figure 4-17. Treated B16 cells do not undergo apoptosis within 24 hours. B16 cells were singularly or simultaneously treated with *Salmonella* (SL) and IFN-γ (**A**) or with Pam3CSK4 (Pam3), alone or in combination with IFN-γ (**B**). As positive controls, B16 cells were triggered with UV irradiation (30, 40 or 50 J/m^2) (**C**). As control of each experiment, B16 cells were left untreated (NT). 24 hours after treatment, B16 cells were stained with FITC-Annexin V and propidium iodide and subjected to FACS analysis. One of three independent experiments are shown.

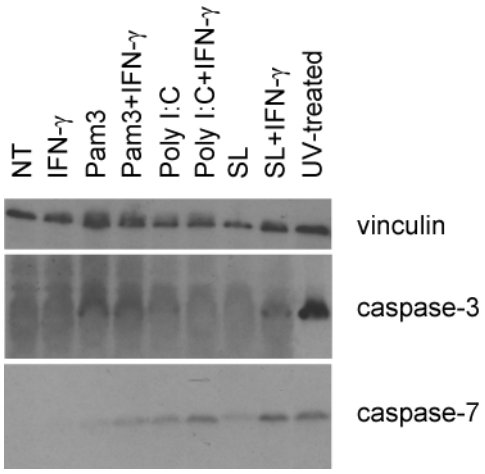


Figure 4-18. Late caspase activation in treated B16 cells. Caspase-3 and caspase-7 activation analysis in B16 cells treated with *Salmonella* (SL), poly I:C, or Pam3CSK4 (Pam3) either alone or in combination with IFN-γ. As controls, B16 cells were left untreated (NT) or UV irradiated (50 J/m^2). After 48h treatment, caspase activation was assessed through the evaluation of the corresponding cleavage fragments by Western blot analysis using anti-cleaved caspase-3 and anti-cleaved caspase-7 antibodies. Anti-vinculin antibody was used as loading control. Densitometric analysis was carried out using NIH Image-based software Scion Image (Scion Corporation). One of two independent experiments is shown.

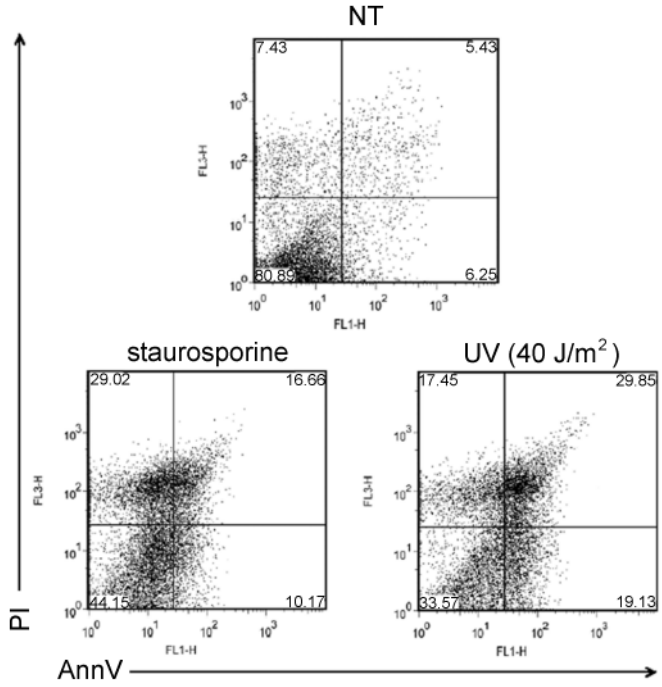


Figure 4-19. Apoptotic BMDCs translocate phosphatidylserine to the cell surface. Bone-marrow dendritic cells (BMDCs) were left untreated (NT), treated with staurosporine (3 µM) for 6 hours, or UV irradiated (40 J/m^2). BMDCs were stained with FITC-Annexin V and propidium iodide and subjected to FACS analysis. One of two independent experiments is shown.

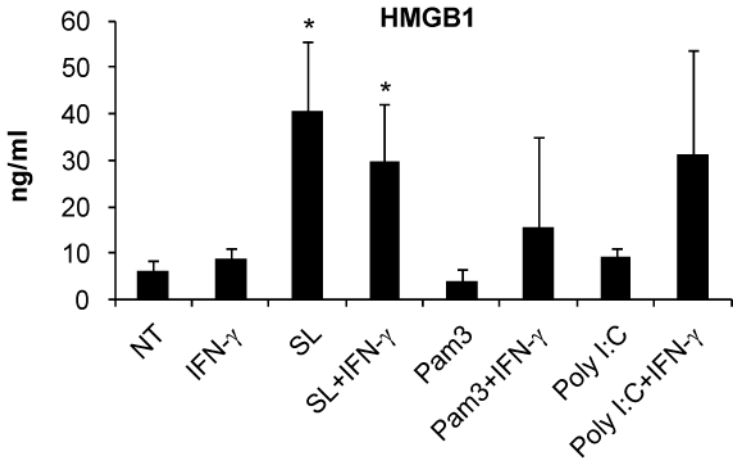


Figure 4-20. HMGB1 release. B16 cells were treated with *Salmonella* (SL), Pam3CSK4 (Pam3), or poly I:C, alone or in combination with IFN-γ. As a control, B16 cells were left untreated (NT). After 24 hours stimulation, cell culture supernatants were collected and cytokine levels determined by ELISA. Data represent the mean ± S.D. of three independent experiments. Significance according to Student's t-test: *p<0.05.

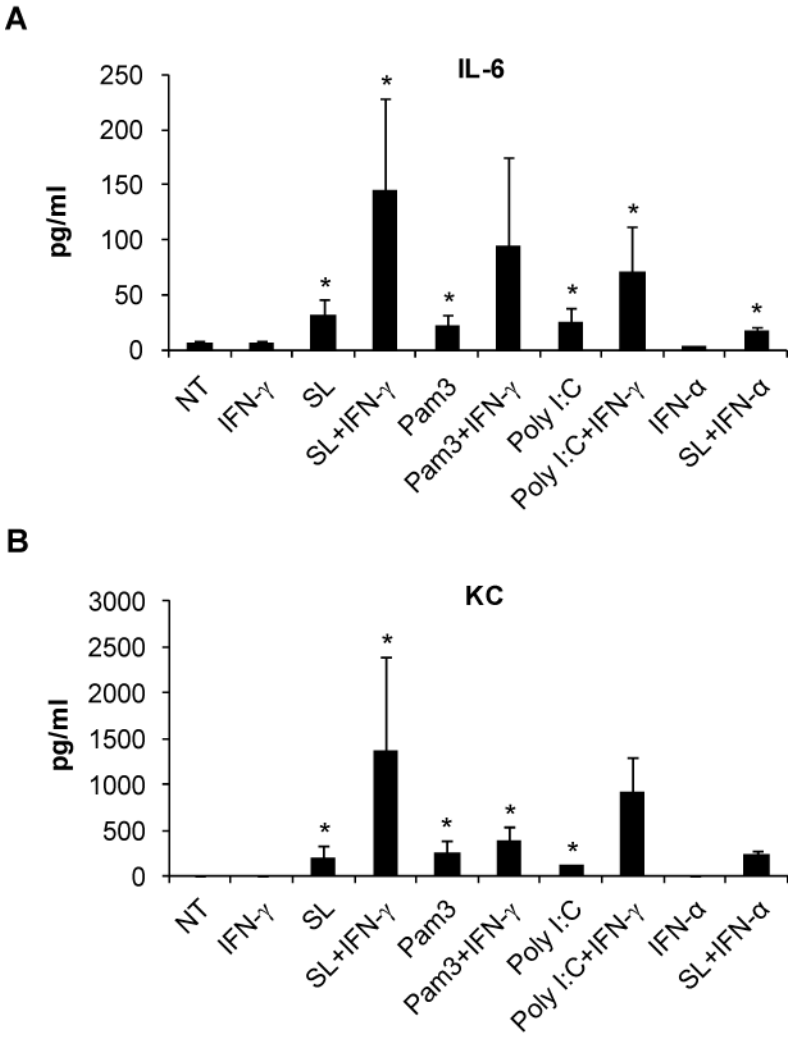


Figure 4-21. IL-6 and KC release. B16 cells were infected with *Salmonella* (SL), Pam3CSK4 (Pam3), or poly I:C, alone or in combination with IFN-γ. As controls, B16 cells were left untreated (NT) or infected with *Salmonella* in combination with IFN-α. After 24 hours stimulation, cell culture supernatants were collected and IL-6 (**A**) or KC (**B**) levels determined by ELISA. Data represent the mean ± S.D. of three independent experiments. Significance according to Student's t-test: *p<0.05.

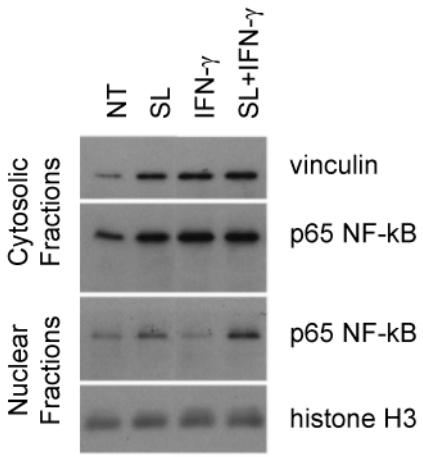


Figure 4-22. *Salmonella* in combination with IFN-γ activates NF-kB. B16 cells were singularly or simultaneously treated with *Salmonella* (SL) and IFN-γ for 24 hours. Western blots of cytosolic and nuclear fractions were probed with anti-p65 antibody. Cytosolic and nuclear fractions controls were performed with anti-vinculin and anti-histone H3 antibodies, respectively. Densitometric analysis was carried out using NIH Image-based software Scion Image (Scion Corporation). One of two independent experiments is shown.

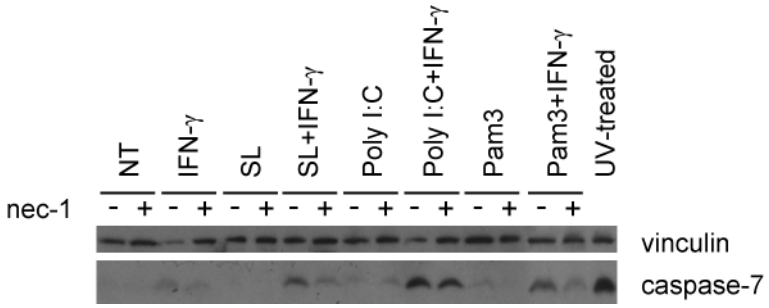


Figure 4.23. Necrostatin-1 reduces caspase-7 activation. Caspase-7 activation analysis in B16 cells treated with *Salmonella* (SL), poly I:C, or Pam3CSK4 (Pam3) either alone or in combination with IFN-γ. As controls, B16 cells were left untreated (NT) or UV irradiated (50 J/m^2). B16 cells both untreated (NT) and treated were either incubated (+) or not (-) with necrostatin-1 (nec-1) (50 μM). After 48h treatment, caspase-7 activation was assessed through the evaluation of the corresponding cleavage fragment by Western blot analysis using an anti-cleaved caspase-7 antibody. Anti-vinculin antibody was used as loading control. Bands were quantified by densitometry using NIH Image-based software Scion Image (Scion Corporation). One of two independent experiments is shown.

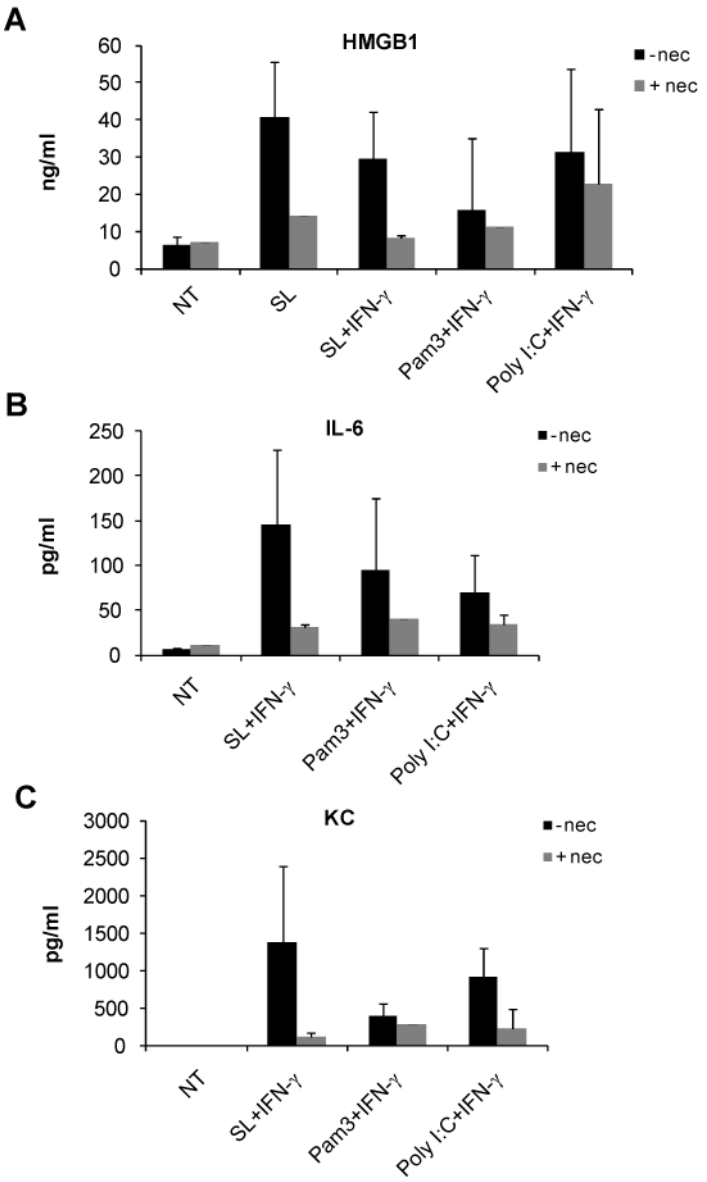


Figure 4-24. Necrostatin-1 reduces HMGB1, IL-6 and KC release. B16 cells were either left untreated (NT) or infected with *Salmonella* (SL), Pam3CSK4 (Pam3), or poly I:C in combination with IFN-γ. In addition, B16 cells were either incubated (+) or not (-) with necrostatin-1 (nec-1) (50 µM). After 48 hours stimulation, cell culture supernatants were collected and HMGB1 (**A**), IL6 (**B**), or KC (**C**) levels determined by ELISA. Data represent the mean ± S.D. of two independent experiments.

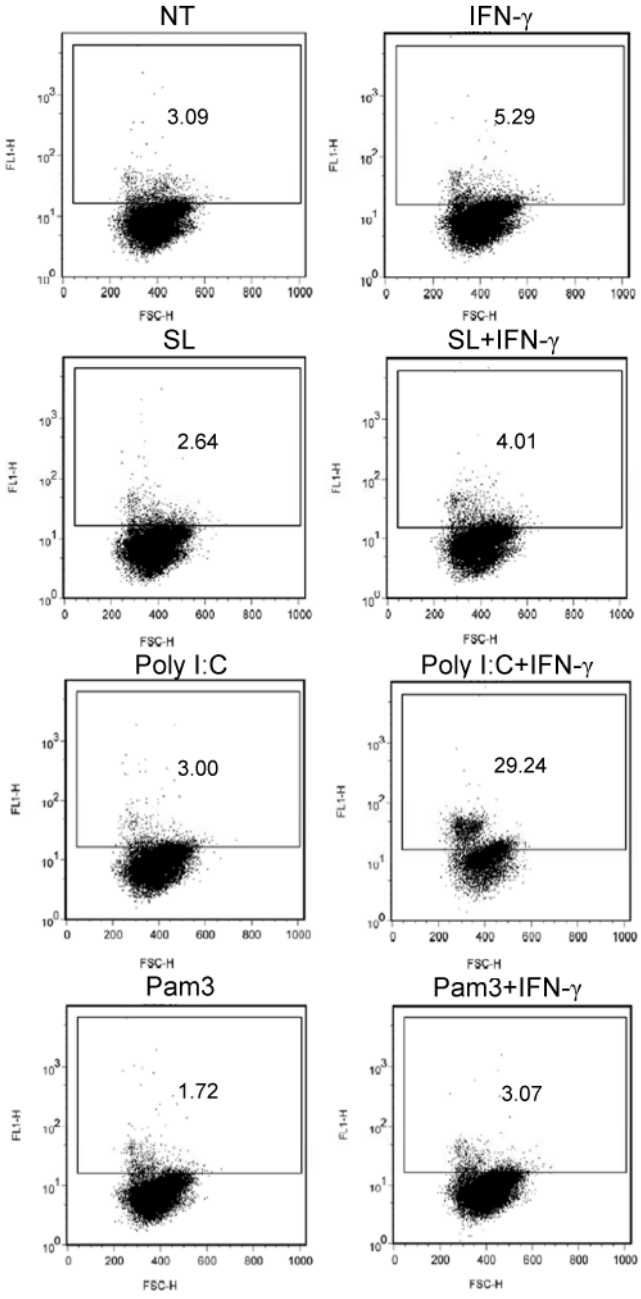


Figure 4-25. TLR3 translocation to B16 cell surface. B16 cells were treated with *Salmonella* (SL), Pam3CSK4 (Pam3), or poly I:C, alone or in combination with IFN-γ. After 24 hours stimulation, extracellular TLR3 protein level on B16 cells was determined by flow cytometry using a FITC anti-mouse TLR3 antibody. The percentages of TLR3 positive cells are indicated. One of three independent experiments is shown.

5. Discussion

In this study, we have shown that *Salmonella* infection is able to induce a significant tumor cell death *in vitro* when administered in combination with IFN-γ, a cytokine that we have found at high levels in infected tumor masses *in vivo*. Surprisingly, unlike IFN-γ, IFN-α has not shown a cytotoxic effect on B16 cells when administered in combination with *Salmonella*, although it is used in therapy for the treatment of several cancers, advanced melanoma included.

Recent studies have shown that TLRs, belonging to pathogen recognition receptors (PRRs), are expressed not only on immune cells, but also on different human and murine cancer cells. Since it is well-known that *Salmonella* displays several PAMPs, we have hypothesized that B16 tumor cells could sense microbial infection through their own TLRs. In this regard, we have found that B16 cells express detectable levels of TLRs 1-6 in the steady state, and, after combined stimulation with IFN-γ and *Salmonella*, tumor cells increase mainly TLR2 and TLR3 levels, but also TLR1 and TLR6 mRNAs. On the contrary, TLR4 and TLR5 remain unchanged upon any treatment. Q-PCR analyses upon stimulation with single bacterial components, such as LPS, flagellin and LTA, have revealed that LPS is likely the *Salmonella* component responsible for enhancing TLR2 transcription. Conversely, TLR3 mRNA level is primarily induced by IFN-γ treatment. Tumor cell death associated to increase in TLRs levels after simultaneous stimulation with *Salmonella* and IFN-γ has suggested that a link between cell death and TLRs could really exist. Consistently, IFN-α does not show any effect on TLR transcription, which may explain its inability to induce B16 cell death.

In accordance with our hypothesis, several studies reported in literature demonstrate that TLRs can be able to activate apoptotic mechanisms, even in tumor cells[162-164,169,171]. In particular, it has been shown that TLR2, TLR3 and TLR4 are involved[164]. Although all these TLRs are highly expressed on B16 cells, we have primarily focused on TLR2 and TLR4 for four important reasons: 1) TLR2 is strongly up-regulated after bacterial infection in combination with IFN-γ treatment at both mRNA and protein level; 2) mRNA levels of TLR1 and TLR6, both TLR2 co-receptors, increase after simultaneous administration of *Salmonella* and IFN-γ; 3) So far a TLR3 ligand associated to *Salmonella* has not been identified, whereas *Salmonella* presents both LPS and several lipoproteins that are able to bind TLR4 and TLR2, respectively; 4) TLR4 mRNA is present at very high basal levels, although it does not change after any stimulation. Accordingly, cell death experiments have shown that Pam3CSK4, a synthetic ligand of TLR2-TLR1 heterodimer, induces a strong tumor cell death, only if in combination with IFN-γ. Unexpectedly, LPS caused only a slight cell death when associated to IFN-γ, that we cannot exclude may be due to TLR2 activation via TLR2 ligand contamination of the LPS preparation. On the contrary, the other TLR2 ligand Pam2CSK4, that binds TLR2-TLR6 heterodimer, is not cytotoxic. In addition, since a recent work has suggested that human melanoma cells undergo apoptotic cell death after TLR3 engagement by its ligand poly I:C in combination with IFN-α pretreatment, we have also tested B16 tumor cell death after poly I:C treatment in combination with either IFN-α or IFN-γ. Interestingly, we have observed that poly I:C in combination with

IFN-γ causes a significant cell death, whereas it does not show any effect in association with IFN-α. We don't know whether this discrepancy may be due to a different capacity of B16 cells to sense type I versus type II IFNs. On the whole, these results allowed us to demonstrate that both TLR2-TLR1 heterodimer and TLR3 mediate B16 tumor cell death. By contrast, TLR4 may not act directly, although further experiments with ultra-pure LPS will be necessary to confirm these data. This characteristic of TLR-IFN-induced cell death may not be unique to B16 cells as metastatic breast and lung carcinoma, 4T1 and LLC1, as well as another melanoma cell line (BL6) also show a response to *Salmonella* in terms of TLR modulation. It would be interesting to assess the role of TLRs in cell death in these model systems.

Transcriptional analyses have demonstrated that a possible cross-talk between different TLR pathways exists in B16 cells. Notably, LPS alone but above all when coupled to IFN-γ, strongly enhances TLR2 transcription, although it does not affect mRNA level of its receptor TLR4. Flagellin also increases transcription of TLR2 instead of TLR5. Pam3CSK4, apart from its receptor, significantly increases TLR3 mRNA level, in combination with IFN-γ. Lastly, the combined stimulation with TLR3 ligand poly I:C and IFN-γ is able to induce very high levels of TLR2, more than *Salmonella* in association with IFN-γ, and to increase MyD88 transcription as well. However, we do not know whether all these cross-talks are functionally important in the cell death mechanism observed.

We have also observed that all cytotoxic stimulations, that is Pam3CSK4, *Salmonella*, and poly I:C, in combination with IFN-γ, are able to kill tumor cells to a similar extent. This suggests that both TLR2 and TLR3 induce cell death, although they likely activate independent mechanisms. In particular, since *Salmonella* does not express a well-known TLR3 ligand, although it presents several PAMPs that are able to bind TLR2, we hypothesize that *Salmonella* induces B16 cells death through a TLR2-mediated mechanism. On the contrary, as already discussed, LPS may contribute to cell death by strongly increasing TLR2 transcription. However, since we cannot completely rule out the existence of a TLR3 ligand in *Salmonella*, further experiments with anti-TLR2 and anti-TLR3 blocking antibodies will be necessary.

When we analyzed the nature of cell death induced by TLR/IFN-γ, we found that B16 tumor cells undergo first a necrotic event occurring within 24 hours of treatments. In fact, neither translocation of PS nor caspase activation was observed in any conditions after 24 hours stimulation, not even at early times. As we have not detected PS exposure at any time points, not even in UV-treated positive controls that, on the contrary, activate both caspase-7 and caspase-3 already at 24 hours, we may argue that PS exposure is not a reliable marker of apoptosis in B16 cells. In accordance with a necrotic event, we have found high levels of IL-6 and KC in cell culture supernatants already starting at 24 hours from combined treatments. In this regard, it has been reported in literature that these cytokines are often produced and released by dying necrotic cells [181,195,197]. In addition, it has been demonstrated that necrotic cells, sometimes even through TLR pathways[197,210], are able to strongly activate NF-kB that in turn fosters the expression of genes involved in inflammatory response. Notably, our

analysis has revealed that *Salmonella*, Pam3CSK4 or poly I:C in combination with IFN-γ activate NF-kB. On the whole, these findings suggest that TLR pathways stimulation induces IL-6 and KC release, through a NF-kB-mediated mechanism, in B16 cells, confirming our hypothesis of necrotic cell death. However, to further assess that tumor cells undergo necrosis, it would be necessary to perform lactate dehydrogenase (LDH) assay that measures LDH activity released from the cytosol upon damage of the plasma membrane of necrotic dying cells into the supernatant.

On the other hand, caspase activation analysis has demonstrated that after a first necrotic event observed within 24 hours of combined treatments, B16 cells undergo the activation of an apoptotic pathway leading to caspase-7 cleavage after 48 hours. The necrosis inhibitor, nec-1, is able to significantly reduce caspase-7 cleavage (about 60%), indicating that blocking necrosis leads to a decrease in apoptosis. This correlates with a significant inhibition in IL-6 and KC release. However, we cannot rule out an independent TLR engagement. This suggests that the initial necrotic events leads to a late apoptotic event. Further experiments will be necessary to understand which apoptotic mechanism (i.e. intrinsic or extrinsic pathway) is activated by the combined stimulations in B16 cells. In this regard, the lack of IL-1β secretion has allowed us to rule out a possible engagement of inflammatory caspases, such as caspase-1. In fact, it is reported in literature that caspase-7 is involved not only in apoptosis, but also in an inflammatory event leading to caspase-7 activation that is caspase-1 mediated[184].

In addition, we have also detected high levels of HMGB1 in supernatants of B16 cells after 24 hours treatment with all combined stimulations. Accordingly, since HMGB1 is a marker of necrotic cell death, this result further suggests that B16 may undergo necrosis within 24 hours. However, since *Salmonella* alone induces a strong HMGB1 release, although it does not kill tumor cells, we cannot exclude that HMGB1 could be actively secreted rather than passively released by necrotic cells. Since it is well-known that HMGB1 release constitutes a crucial step in the activation of antigen presenting cells[204], thus improving the tumor immunogenicity, future studies will be performed with the aim of understanding how B16 cells can induce HMGB1 release. It is likely that TLR4 could be involved, in fact *Salmonella* alone, probably through LPS, strongly enhances HMGB1 release more than poly I:C or Pam3CSK4 either alone or in combination with IFN-γ. Moreover, we have observed that RIP1 inhibition not only blocks necrosis but also reduces HMGB1 release, probably inhibiting TRIF-mediated TLR4 MyD88-independent pathway.

In this study, we have also demonstrated that TLR3 is able to mediate B16 tumor cell death when stimulated with poly I:C in combination with IFN-γ. Interestingly, increased TLR3 transcription and intracellular TLR3 protein level depend essentially on IFN-γ treatment. Moreover, we provide a new evidence demonstrating that poly I:C induces TLR3 translocation to B16 cell surface in combination with IFN-γ. In this way, poly I:C, simply added into the cell culture medium, can improve the TLR3-mediated response thus likely inducing a stronger caspase-7 activation compared to *Salmonella* or Pam3CSK4.

Our results have highlighted the importance of IFN-γ in activating this TLR-mediated cell death mechanism. IFN-γ is a cytokine involved not only in cancer immunosurveillance in promoting tumor rejection, but also in cancer-immunoediting process. Notably, since it acts on tumor cells directly during the antitumor response, IFN-γ increases tumor immunogenicity up-regulating the MHC class I pathway of antigen processing and presentation in tumor cells; promoting apoptosis; inhibiting cellular proliferation and angiogenesis[16]. Importantly, we have observed that IFNGR1 is expressed at high levels on B16 cell surface, although it increases after IFN-γ treatment. Moreover, we have found that B16 cells pretreated with IFN-γ and then stimulated with *Salmonella* or other stimuli (but not vice versa) up-regulates TLR2 and TLR3 to a similar extent as simultaneous administration. These results suggest that IFN-γ sensitizes tumor cells to a subsequent stimulation, up-regulating TLRs involved in tumor cell death (e.g. TLR3) or through other unknown mechanisms. However, we have not provided evidence for a clear mechanism of action of IFN-γ, and further in-depth studies will be necessary. In addition, following the work by Salaun and coworkers who demonstrated that human melanoma cells pretreated with IFN-α and stimulated with poly I:C undergo apoptosis[171], it would be interesting to pretreat B16 cells for 24h with IFN-γ and then add the different stimuli in combination with IFN-γ, to analyze the transcriptional pattern and tumor cell death.

In light of these new findings, we will improve the immunotherapy protocol developed in Maria Rescigno's laboratory, and now into the clinic, by combining *Salmonella* with IFN-γ i.t. injection in order to obtain a more prompt therapeutic effect. Importantly, since IFN-γ induces a lot of side effects when systemically administered, a direct injection into the tumor site could be less toxic and thus more easily applicable to clinical trials. Moreover, in order to avoid the possible side effects due to *Salmonella* infection in this immunotherapy protocol, we will investigate whether Pam3CSK4 i.t injected is able to completely replace bacteria. Finally, on the basis of the previous results obtained in this study, we will also focus on poly I:C treatment. In this regard, we have provided evidence that poly I:C is able to trigger tumor cell death after simultaneous treatment with IFN-γ. Moreover, this combined stimulation is necessary to translocate TLR3 to B16 cell surface and thus to amplify caspase-7 activation, more than *Salmonella* and TLR2 ligand. For these reasons it would be interesting to introduce poly I:C treatment into electrochemotherapy approach. This new method is an efficient, local, inexpensive treatment of cutaneous cancers, including melanoma. It is based on the application of cell-membrane-permeabilizing electric pulses on tumor cells in order to allow the entry of highly cytotoxic non-permeant drug, like bleomycin or cisplatin, into the cytosol[212]. Interestingly, electric pulses would facilitate the entry of injected poly I:C into both immune cells and tumor cells thus avoiding the additional administration of IFN-γ. Notably, poly I:C interacting with TLR3 present in immune cells could function as adjuvant of immune system, whereas its entry into tumor cells likely fosters mechanisms of cell death that could be mediated not only by TLR3, but also by MDA5 dsRNA sensor. In this regard, it has been recently reported that poly I:C, complexed with a polyethyleneimine (PEI) carrier to improve its cytosolic delivery, triggers

cytosolic MDA-5 to kill melanoma cells through the induction of proapoptotic factor NOXA[175]. Therefore, this system could be used to treat melanoma avoiding possible side effects induced by IFN-γ, allowing the entry of poly I:C, and thus the induction of its antitumor effects, in both tumor and immune cells.

In conclusion, the results obtained in this work set the basis for several new immunotherapeutic approaches aimed to use TLR engagement to activate on one side immune cells and on the other to induce the immunogenic cell death of tumor cells so to activate a tumor-specific response.

6. References

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