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# BACTERIAL GENOTOXINS: AN INTERFACE BETWEEN INFECTION AND CANCER BIOLOGY

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# Bacterial genotoxins: an interface between infection and cancer biology
# THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my Italian and Portuguese families*

# ABSTRACT

Infections contribute to the development of more than 20% of all malignancies in humans. While strong evidence exists for a number of virus-associated cancers, the role of bacterial infections is still poorly appreciated although there are evidences that link chronic bacterial infections to increased risk of certain tumors. In the context of bacteria-induced carcinogenesis, bacterial genotoxins might contribute to increase the occurrence of genomic instability during chronic infection. These toxins are produced by several Gram-negative bacteria and are unique among other bacterial toxins since they are able to reach the nucleus and cause DNA damage in intoxicated cells.

In this thesis we have investigated the contribution of two bacterial genotoxins to tumor development in acute and chronic intoxication, and their role in the context of chronic infections, using *in vivo* and *in vitro* models. We have used as a model the Cytolethal Distending Toxin (CDT) produced by several Gram-negative bacteria and the Typhoid Toxin (TT) produced by *Salmonella enterica* serovar Typhi.

In the context of acute intoxication, CDT-induced DNA damage triggers the activation of the integrin β1, increase of focal adhesions formation and cell spreading in epithelial cells during short-term intoxication experiments **(Paper I)**. This phenotype is mediated by an inside-out integrin signaling and is drastically reduced when the actin cytoskeleton remodeler NET1 is knocked down. We have also demonstrated that two components of the endosomal sorting complex required for transport (ESCRT) known to regulate integrin intracellular trafficking, ALIX and TSG101, are important for the anchorage-independent survival induced by sustain integrin activation.

Chronic intoxication with CDT leads to increased mutation frequency, accumulation of chromosomal aberrations and enhanced anchorage-independent growth *in vitro* **(PaperII)**. Cells treated with sub-lethal doses of CDT during a period of 220 days showed an altered DNA damage response and a higher activation of the MAP kinase p38 signalling, which is known to play an important role in cell survival during acute CDT intoxication.

In order to investigate the role of genotoxins *in vivo* and their carcinogenic potential we designed a new *Salmonella* infection model in mice where we used a *S. enterica* serovar Typhimurium (*S.* Typhimurium) strain able to produce the Typhoid Toxin and, as a control, an isogenic strain that expressed an inactive Typhoid Toxin **(PaperIII)**. We have infected immunocompetent sv129 mice for up to 6 months and the presence of an active version of the toxin led to a reduced mortality rate and higher persistence of the bacteria in some organs suggesting its role in the establishment of a chronic infection. Mice infected with the toxigenic *S.* Typhimurium strain also showed an altered inflammatory response in intestine and liver and a more pronounced intestinal microbiota alteration compare to the mice infected with the non-toxigenic strain.

To better understand how the TT is delivered to the host cells we further investigate its secretion and internalization mechanisms *in vitro* **(Paper IV)**. We have demonstrated that the Typhoid Toxin is loaded into outer membrane vesicles (OMVs) and is secreted by *S.* Typhimurium bacteria in the *Salmonella* containing vacuole (SCV). Vesicles are released by exocytosis-like process in the extracellular space, where they can deliver the genotoxic component to bystander cells.

Our data give new important contributions in the studying of bacterial genotoxins and their role in chronic bacterial infection and cancer development.

# AIMS OF THE THESIS

Bacteria can be associated with cancer development but the mechanisms by which this occurs are still unknown. We have investigated whether bacteria-producing DNA damage agents can contribute to carcinogenesis by looking at different aspects:

1. Characterization of the CDT-induced survival responses **(Paper I)**
2. Assessment of the carcinogenic potential of CDT *in vitro* **(PaperII)**
3. Characterization of the role of the Typhoid Toxin in the context of *Salmonella* chronic infection *in vivo* **(PaperIII)**
4. Characterization of the Typhoid Toxin secretion and delivery pathways *in vitro* **(PaperIV)**

# LIST OF SCIENTIFIC PAPERS

I. **Levi L,** Toyooka T, Patarroyo M, Frisan T. (2015)
Bacterial Genotoxins Promote Inside-Out Integrin β1 Activation, Formation of Focal Adhesion Complexes and Cell Spreading.
PLoS One. 2015 Apr 13;10(4):e0124119

II. Guidi R, Guerra L, **Levi L**, Stenerlow B, Fox JG, Josenhans C, Masucci MG, Frisan T (2013)
Chronic exposure to the cytolethal distending toxins of Gram-negative bacteria promotes genomic instability and altered DNA damage response.
Cell Microbiol 15: 98-113

III. Guidi R, Krejsgaard TF, **Levi L**, Del Bel Belluz L, Rouf S, Candela M, Turroni S, Tebaldi T, Viero G, Rhen M and Frisan T
The Typhoid toxin promotes chronic infection and alters the host inflammatory response

IV. Guidi R, **Levi L**, Rouf SF, Puiac S, Rhen M, Frisan T (2013)
Salmonella enterica delivers its genotoxin through outer membrane vesicles secreted from infected cells.
Cell Microbiol 15:2034-2050

# CONTENTS

# 1 BACTERIA AND CANCER

Historically several bacteria have been putatively associated to cancer development. Already in the 1772, *Mycobacterium tuberculosis* was thought to be the cause of bronchogenic carcinomas [1] even though it is still not clear today if there is or not an effective link between tuberculosis and cancer. A wide variety of studies on bacteria associated with carcinogenesis are under way and this is an up-to-date research area with many points to be clarified.

*Helicobacteri pylori, Salmonella* Typhi*, Streptococcus bovis* and *Chlamydia pneumoniae* are associated with different types of cancer such as gastric cancer, mucosa associated lymphoid tissue (MALT) lymphoma, gallbladder cancer, colorectal cancer and lung cancer respectively [2-6]. Although there are many evidences on the connection between these bacteria and cancer development, their possible role in carcinogenesis is still not completely clarified.

### *H. pylori* and cancer

The major bacterial cause of human cancer today is *H. pylori*, which was classified as being carcinogenic for humans in 1994 by the WHO (World Health Organization) and linked to two different types of gastric cancer: MALT lymphoma and gastric adenocarcinoma [6]. *H. pylori* is present in the stomachs of at least half of the world's population and, if left untreated, generally persists for the host life time [7,8]. In developed countries, *H. pylori* probably increases the risk of developing gastric cancer by sixfold but its presence is sometimes difficult to detect because *H. pylori* colonization diminishes in the presence of premalignant lesions [9]. One of the most important factors that characterizes *H. pylori* strains is the presence of the *cag* island, a horizontal acquired locus that contains 31 genes [10,11]. Most of the genes belonging to the *cag* island encode for type IV secretion system (T4SS) proteins,

which export proteins from bacterial cells. The cytotoxin-associated gene A (CagA) is one of the factors transported through the T4SS into the epithelial cells, where it undergoes tyrosine phosphorylation [12]. Once phosphorylated, CagA affects cell shape, increases cell motility, abrogates junctional activity, and promotes an epithelial to mesenchymal transition-like phenotype [13].

Several studies demonstrated that compared with *cagA*$^{-}$ strains, *H. pylori cagA*$^{+}$ strains significantly increase the risk of developing severe gastritis, atrophic gastritis, peptic ulcer disease and distal gastric cancer (reviewed in [7]).

### *S.* Typhi and cancer

Besides *H. pylori*, there are other known examples of bacterial-associated cancer development. One of the most important is *Salmonella* Typhi infection and gallbladder cancer in individuals who become chronic asymptomatic carriers after typhoid fever [14].

*S.* Typhi is still a worldwide threat for human health and it is estimated that at least 22 million people worldwide suffer typhoid fever every year [15]. This bacterium is endemic in many regions of South America, Africa, and Asia [16]. Typical symptoms include fever, malaise, headache, nausea, and abdominal pain, which are caused by a systemic infection that can lead to high temperature and severe diarrhea if left untreated [17]. A considerable percentage of infected people (2-5%) [18] becomes chronic carrier and it is still unknown why this happens only in some of the patients. Such individuals are considered as a *reservoir* of the infection that persists in the gallbladder and can then spread out again through the shedding of *S.* Typhi in the stools for a long period of time after the infection has taken place [19].

Chronic carriers show an increased risk (8.47 times) of developing carcinoma of the gallbladder compared with people who have had acute typhoid and have cleared the infection

[3]. In a recent study by Scanu and colleagues it has been shown that *S.* Typhi DNA can be detected in gall bladder carcinoma (GBC) samples from Indian patients while no trace of the bacterium is found in samples coming from the Netherlands. Although *S.* Typhi DNA has been found in most of the Indian samples, the bacterium itself is only rarely detected in the tumor cells suggesting that *S.* Typhi may induce transformation, but viable bacteria are not required to keep the transformation state [20].

**Chronic infection, inflammation and cancer**

Rather than link cancer to specific organisms, most of the research effort has been put in understanding the mechanisms behind the carcinogenesis induced by bacteria and this represents the main question in this field.

Chronicity of infection is a crucial point in cancer-associated bacterial infection. In literature many studies demonstrated that there is a correlation between bacteria and cancer but an indisputable direct link has not been proven to date. In most of these studies it seems clear that bacteria are not sufficient to lead to cancer development on their own. A chronic inflammation is needed to start the process and an independent accumulation of mutations in genes involved in oncogenesis signaling pathways might be quite relevant. One such example is represented by *Helicobacter hepaticus*, which is the cause of chronic active hepatitis that then leads to hepatocellular carcinoma formation in A/JCr mice [21].

More recently another interesting finding has proven the association of the gut microbiota and colorectal cancer. Inflammatory bowel disease (IBD), one of the three highest risk factors for colorectal cancer (CRC), can occur when the homeostasis between the intestinal microflora and the host immune system is disrupted [22]. Whether individual pathogenic species or entire microbial communities trigger inflammation is still unknown [23]. A good *in vivo* model to study the commensal-dependent ulcerative colitis is the T-bet$^{-/-}$ RAG2$^{-/-}$

ulcerative colitis (TRUC) mice that present a T-bet deficiency in the innate immune system [24]. TRUC mice develop a severe and highly penetrant colitis and spontaneously progress to colonic dysplasia and rectal adenocarcinoma, resembling human IBD-associated colorectal cancer [25]. The authors have shown that tumorigenic and pre-tumorigenic lesions can be reduced by treatment with antibiotics, suggesting a significative role of bacterial-induced carcinogenesis [25].

An enrichment of *Fusobacterium* species has been found in colorectal cancers adjacent to normal tissues even though it is not clear if these bacteria directly contribute to carcinogenesis [26-28]. In 2013 Kostic and colleagues analyzed the effects of *F. nucleatum* in $Apc^{Min/+}$ mice. They showed an increase in tumor multiplicity and a recruitment of myeloid cells, which can promote tumor progression [29].

In another study Wu and colleagues showed that the enterotoxigenic *Bacteroides fragilis* (ETBF) triggers colitis and colonic tumors in multiple intestinal neoplasia (Min) mice ($Apc^{Min/+}$) [30].

In all the examples cited above, inflammation seems to be one of the central and most important connections to cancer development.

The main question at this point is: what are the molecular players in the pro-tumourigenic inflammation process?

The nuclear factor κB (NF-κB) family and the Signal Transducer and Activator of Transcription 3 (STAT3) are long known as important mediators in the inflammatory process, and also demonstrated to play a key role in inflammation-associated carcinogenesis [31,32].

NF-κB and STAT3 proteins are transcription factors ubiquitously expressed in the cytoplasm of all cells. Upon extracellular stimuli these proteins translocate to the nucleus and activate

transcription of important inflammatory-response genes, which encode for anti-apoptotic proteins, chemokines, cytokines and growth factor receptors [33].

In absence of signals, NF-κB transcription factors are present in their inactive form bound to inhibitory proteins called I-κBs. In response to external stimuli such as cytokines, viral and bacterial products, different cellular receptors can activate NF-κB through the IκBs kinases (IKKs). These enzymes are composed by three subunits: catalytic subunits IKKα and IKKβ and the regulatory subunit IKKγ/NEMO. IKKs are able to phosphorylate IκBs leading to ubiquitin-dependent degradation and the subsequent nuclear entry of the released NF-κB dimers [34]. Once in the nucleus, NF-κB can exert its activity and activate the transcription of different genes whose products are involved in the inhibition of apoptosis and in the stimulation of cell growth [35].

Similarly to NF-κB, STAT3 is kept in an inactive form in the cytoplasm of non-stimulated cells but it does not require the degradation of an inhibitor [36,37]. STAT3 activation depends instead on the phosphorylation of the tyrosine 705 (Tyr705) by the Janus Kinase (JAK) JAK1 that leads to dimerization through phosphotyrosine-SH2 domain interaction [38,39]. STAT3 activity in the nucleus might be increased by the phosphorylation of the Ser727 by members of the Mitogen-Activated Protein Kinases (MAPK) and c-Jun *N*-terminal kinase families [40]. Development and homeostasis are the two main mechanisms in which STAT3 is involved and ablation of its gene leads to developmental abnormalities and death [41]. Prolonged activation of STAT3 is associated with different types of malignancies and its activated nuclear form has been found in different kind of cancers such as breast, lung, skin, colon, etc … [31,37,42,43].

One of the hallmarks for cancer is represented by the ability of malignant cells to enter to an anti-apoptotic and prosurvival state that prevents cell death [44]. Anti-apoptotic genes, such as Bcl-xL, Bcl-2 and c-IAP-2, are often targeted by NF-κB and STAT3 [45,46]. The inhibition of

STAT3 or NF-κB leads to an increase of p53-induced apoptosis demonstrating that these two factors have an important effect also on the most important tumor suppressor [47,48].

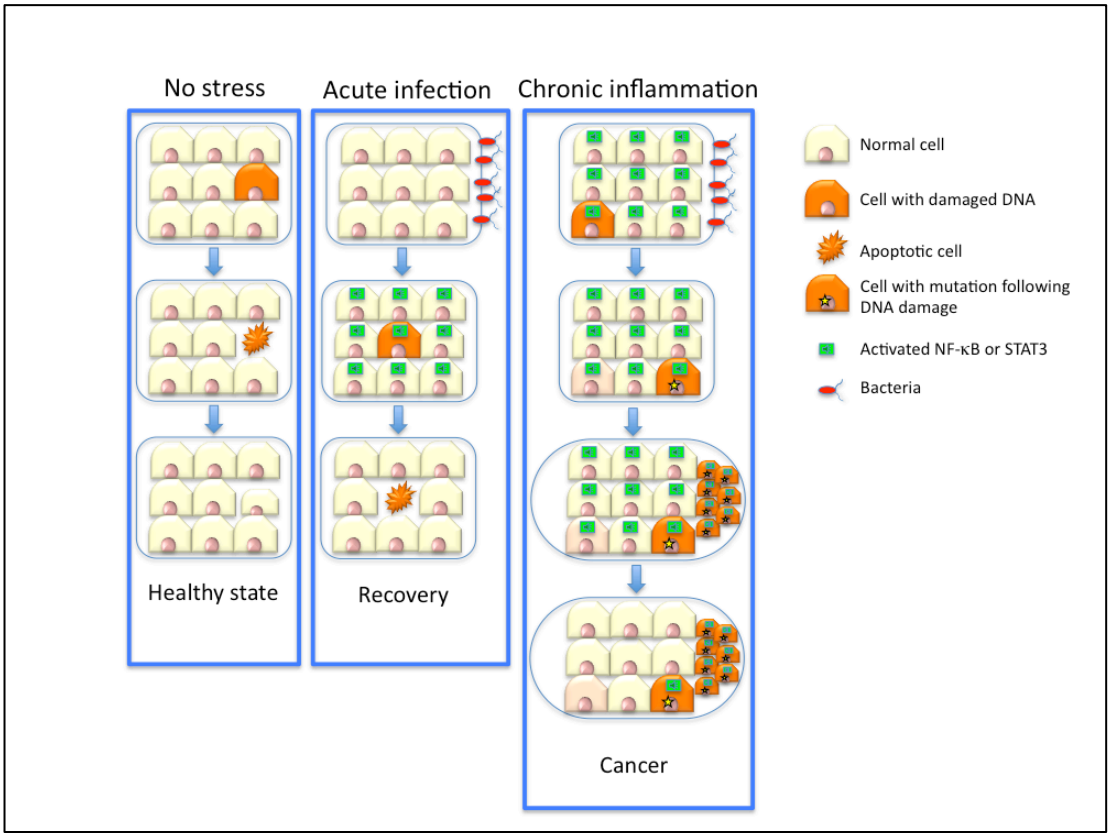


**Fig. 1:** Scheme of the effects of acute or chronic inflammation on tissues. In normal conditions cells with damaged DNA undergo apoptosis and are substitute by normal cells (healthy state). During acute infection NF-κB and/or STAT3 are activated for a short time and DNA-damaged cells normally undergo to apoptosis (recovery). During chronic infection the persistent activation of NF-κB and/or STAT3 can lead to uncontrolled cell growth due to inhibition of apoptosis (Cancer). Adapted from [49].

The molecular mechanisms by which this occurs are still under debate but what is known is that in acute infections the negative regulation of p53 is not carcinogenic and only a long-term effect, as for chronic inflammation, can be associated with cancer development [50]. NF-κB or STAT3-activated/p53-suppressed state has to last for a sufficient time in order to over come the p53-dependent apoptosis, accumulate genetic and epigenetic alterations and lead to a transformed phenotype. This assumption is in line with the fact that a significant fraction of

cancers express a wild-type p53, which may be inactivated by other mechanisms such as a constant activation of STAT 3 and NF-κB. Thus inhibition of NF-κB and/or STAT3 in cancer cells harboring a wild-type p53 might represent a new therapeutic advantage in cancer treatment [48,49]. On the other hand, the accumulation of mutations can be also increased by the production of Reactive Oxigen and Nitrogen Species (ROS and RNS), which are produced in high amount by inflammatory cells during chronic infections. As a result the overproduction of these molecules can cause oxidative and nitrative stress, which in turn can contribute to the damage of different biomolecules such as DNA, RNA, lipids and proteins [51]. Figure 1 summarizes the effects of acute and chronic infection induced by activation of NF-κB or STAT3.

**Bacterial genotoxins**

Besides the indirect effects of bacterial infections on the carcinogenic process via the induction of chronic inflammation and constitutive activation of the non-classical oncogenes NFκB and STAT3, and production of endogenous DNA damaging agents, several bacteria effectors may contribute to the tumor initiation and/or progression. These bacterial toxins are collectively known as cyclomodulins [52] and can either block apoptosis and promote cell proliferation (e.g. Cytotoxic Necrotizing Factor, CNF and *Pasteurella multocida* Toxin, PMT, produced by *Esherichia coli* and *Pasteurella multocida* respectively ) or can induce DNA damage, thus directly contributing to acquisition of genomic instability. The latter are known as bacterial genotoxins and so far only three of them have been identified. Two are protein toxins: the Cytolethal Distending Toxin (CDT) produced by several Gram-negative bacteria and the Typhoid Toxin produced by *Salmonella* Typhi [53,54]. The third bacterial genotoxin described is called Colibactin and is a peptide-polyketide genotoxin, produced by strains belonging to the phylogenetic group B2 of *Escherichia coli* [55].

**Cytolethal distending toxin**

The first bacterial genotoxin described is the Cytolethal Distending Toxin (CDT) family. Gram-negative bacteria such as *Escherichia coli*, *Shigella dysenteriae*, *Aggregatibacter actinomycetemcomitans*, *Campylobacter* spp., *Helicobacter* spp. and *Haemophilus ducreyi*, produce CDT, which causes DNA damage. Such damage induces cell cycle arrest and cellular distention in epithelial cells as well as apoptosis in cells of lymphoid origin. CDT is an $AB_2$ toxin, composed by the CdtB active subunit ("A"), and two accessory subunits ("B"), CdtA and CdtC as shown in Figure 2 left panel. The genes encoding for CDT (*cdtA, cdtB, cdtC*) are transcribed from a single operon [53]. The active subunit (CdtB) presents a canonical four layered-fold structure of the DNase I family, and it has been shown to cleave naked DNA in vitro and promote single and double strand breaks in intoxicated cells [56-58]. Mutations of the CdtB catalytic domain and the $Mg^{2+}$ binding site impair CDT intoxication activity [56,59].

The two "B" subunits are lectin-type molecules, sharing structural homology with the B-chain repeats of the plant toxin ricin [60]. The crystal structure of the holotoxin shows the presence of a groove between the CdtA and CdtC subunits and a patch of aromatic residues on CdtA, adjacent to the groove. These structures may play a relevant role in modulating toxin binding to the cognate receptor, still unknown, on the cell surface [60]. This step is essential to mediate internalization of the holotoxin into the target cells [60].

Upon binding to an unknown receptor, CDT is internalized via endocytosis and transits through the early and late endosomal compartments [61]. Similarly to other cytosolic acting toxins, such as ricin, shiga and cholera toxins [62], the CdtB active subunit requires translocation via the endosomal compartment, transit through the Golgi complex and transport to the endoplasmic reticulum (ER) in order to reach their targets [63]. In Figure 4 CDT internalization is summarized in right side of the picture.

Unlike other bacterial toxins, the target of CDT is nuclear, and the toxin has not been detected in the cytosol by biochemical assays. Furthermore, intoxication occurs in cells displaying a non-functional ERAD (Endoplasmic-reticulum-associated protein degradation) pathway suggesting that the toxin may be directly translocated from the ER to the nucleus [61,63]. The mechanism by which CDT is translocated from the ER to the nucleus and the cellular partners involved are still unknown.

Eshraghi and colleagues have recently demonstrated that three important components of the ERAD machinery (Derlin-2 (Derl2), the E3 ubiquitin-protein ligase Hrd1, and the AAA ATPase p97) are necessary for CDT intoxication. Nevertheless two motifs required for Derlin-dependent retrotranslocation of ERAD substrates have been found to be dispensable for CDT intoxication. These data suggest that an atypical ERAD pathway might be involved in CDT trafficking in intoxicated cells [64].

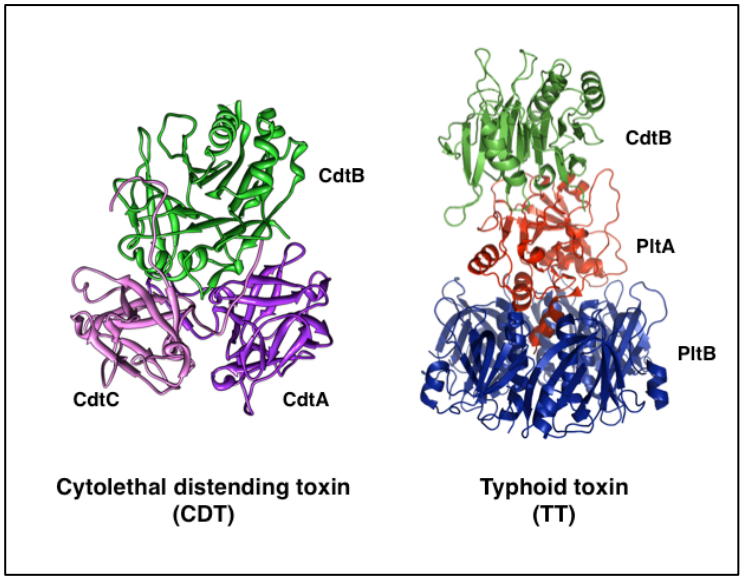


**Fig. 2:** Crystal structures of the Cytolethal Distending Toxin (left) and the Typhoid Toxin (right)

**Typhoid toxin**

TT is an $A_2B_5$ toxin where the A subunits, PltA and the *S.* Typhi CdtB (StCdtB), are homologues of the A subunits of the pertussis toxins and the cytolethal distending toxins,

respectively [54]. The PltA has an ADP-ribosyl transferase activity but its cellular targets have not yet been identified [54] while StCdtB induced DNA damage into the target cells [65]. PltB represents the "B" subunit and it is organized into a pentameric structure [54,66]. In Figure 2 (right panel) is shown the crystal structure of the Typhoid Toxin.

Although the role of TT in *S.* Typhi infection is still unknown, Song and colleagues showed that intraperitoneal injection of purified and active TT in C57BL/6 mice, leads to symptoms similar to the ones of acute phase of typhoid fever (loss of weight, malaise, etc.)[54]. The authors have further shown that the symptoms are exclusively induced by a functional CdtB, since only mutation of this subunit, but not of the other "A" subunit, PltA, promotes typhoid-like symptoms [54].

Interestingly the presence of the toxin seemed to affect also the immune system by strongly reducing the circulating neutrophils [54].

Further studies by the same group showed that intraperitoneal injection of a mutant strain of *S.* Typhi deleted of the PltB subunit, exhibits increased replication in the liver and spleen of immunodeficient $Rag2^{-/-}$ $\gamma c^{-/-}$ humanized mice [67]. These data suggest that a functional TT may be important for the establishment of a persistent infection by slowing down the replication of *S.* Typhi in the infected tissues [67].

**Colibactin**

Colibactin is produced by *E. coli* strains belonging to the phylogenetic group B2 [55]. The effects of colibactin on target cells are enlargement of the cell body and the nucleus and arrest of cell proliferation. Interestingly these effects are contact-dependent and are not observed when bacteria are separated from the cells with a membrane or if cells are treated only with the supernatant of bacterial culture [55]. Genes responsible for the production of an active colibactin are located on a genomic region called *pks* island. Nougayrede and colleagues have

shown that infection with *pks* positive bacteria induced DNA double strand breaks and a consequent activation of the DNA damage response in eukaryotic cells [55].

Cuevas-Ramos and colleagues studied the effects of colibactin on DNA damage in a mouse intestinal loop model and in the colon of antibiotic treated BALB/cJ mice. Colon loops and mice were infected with an *E. coli* strain harboring the *pks* island for 6 hours and 5 days, respectively and an isogenic strain (*clbA* mutant) unable to produce a functional toxin was used as negative control [68]. The central role of the *clbA* gene in colibactin biosynthesis was confirmed in both models and the expression of *clbA* was also associated with induction of DNA damage and consequent phosphorylation of the histone H2AX (a marker for DNA damage) in mouse enterocytes [68].

So far I have been describing different factors that have a direct or indirect effect in damaging the DNA. It is important at this point to discuss more in details about the DNA damage response and whether genotoxins, and specifically CDT and TT, can contribute to carcinogenesis.

# 2 DNA DAMAGE RESPONSE

The DNA is one of the most stable molecules found in nature but despite this, its integrity is threatened everyday by multiple factors. It has been estimated that the DNA of a single cell of the human body can experience between $10^4$-$10^5$ damages per day [69]. The causes of these damages can be exogenous (e.g. ionizing radiation (IR), radiomimetic chemicals, UV radiation) or endogenous (e.g. ROS, RNS, stalled replication forks, etc…) DNA damaging agents, which can lead to DNA double strand breaks (DSBs) or single strand breaks (SSBs), thymidine dimers, base oxidation, etc…

Catastrophic consequences are avoided due to a number of mechanisms that maintain the genomic integrity, generation after generation. These mechanisms are part of the DNA Damage Response (DDR) where a set of different proteins are involved in cell cycle checkpoints and DNA repair.

Bacterial genotoxins induce mainly DNA DSBs [58], thus I will review in this chapter the repair mechanisms that are activated in response to this type of DNA damage

**NHEJ or HR?**

DSBs can be repaired through at least two different pathways: homologous recombination (HR) or non-homologous end-joining (NHEJ) [70-72]. The choice of which repair pathway will be used depends essentially on the phase of the cell cycle in which the damage occurs. HR requires a homologous DNA template, thus it can be activated exclusively in S- and G2-phase. Conversely, cells in G1 or G0-phase relay on NHEJ as repair process [69]. The main difference between the two pathways is that while in the HR the DNA is repaired in an error-free process using the sister chromatid as template, during NHEJ part of the damaged DNA is

resected and/or processed with consequently loss of information. HR and NHEJ pathways are summarized in Figure 3.

In general DNA repair is carried out by an intricate group of pathways that involve several enzymes including nucleases, helicases, kinases, phosphatases, etc. The regulation of all these factors is crucial in order to maintain the integrity of the genome upon DNA damage and cells have developed different mechanisms to increase the reliability of the system [69].

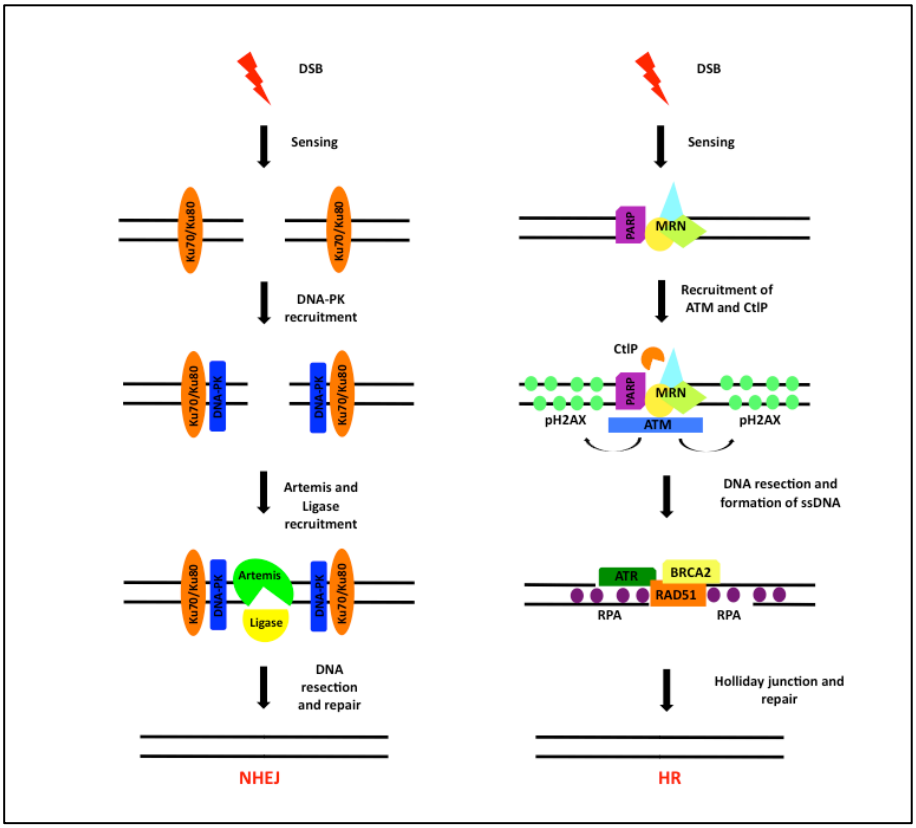


**Fig. 3:** Schematic representation of NHEJ (left panel) and HR (right panel) DNA repair systems in response to DSBs.

### Sensing the damage

The first step of the DDR is the sensing of the damage, which is initially mediated by members of the poly(ADP-ribose) polymerase (PARP) family for the HR and by the Ku heterodimer (Ku70/Ku80) for the NHEJ.

Among the 16 members of the PARP family, only PARP 1 and PARP2 are involved in the DDR and, as for the Ku70/Ku80, they localize to the site of the damage within seconds [73,74].

The role of PARP1/2 and Ku70/Ku80 is crucial for the decision of which repair pathway will be used (NHEJ or HR). While Ku heterodimer leads to NHEJ, PARP1/2 can activate either HR or an alternative version of NHEJ (alt-NHEJ). The majority of the higher eukaryotes, and especially mammals, generally prefer to repair DSBs through the classical NHEJ (c-NHEJ) and this pathway has been extensively described in different articles [75,76] where it is clear the intervention of at least seven different proteins: Ku70/Ku80, DNA-PK, Artemis, X-rays cross complementing 4 (XRCC4), XRCC4-like factor (XLF) and DNA ligase IV (LIGIV) [77].

On the other hand the alt-NHEJ mechanism has been not yet well elucidated but it has been demonstrated that it is much less used compare to c-NHEJ and somehow repressed by the presence of Ku70/Ku80, which repress also the HR pathway [78].

In summary even though the HR system is safer in terms of preserving the exact sequence of DNA, cells are more prone to use NHEJ mechanism to repair damaged DNA and this might be due to the fact that this alternative is much faster and efficient [79].

**Propagation of the signal and DNA repair**

The propagation of the signal is the second step of the DDR and starts with the recruitment and the activation of the DNA-dependent protein kinase (DNA-PK) by Ku70/Ku80 or the MRE11/RAD50/NBS1 (MRN) protein complex by PARP1/2 [74].

During NHEJ, the Artemis nuclease is recruited upon DNA-PK loading leading to resection of the DNA in order to generate the right 3'-hydroxyl and 5'-phosphate ends essential for ligase activity and DNA repair [69,70].

The Ataxia Telangiectasia Mutated protein (ATM) is recruited by the NBS1 subunit of the MRN complex during the HR leading to the propagation of the signal and DNA repair [69,70]. Alternatively to ATM, the ATM Rad3-related (ATR) protein kinase is recruited in case of SSBs or at later stages of DSBs repair leading to a switch from ATM to ATR at the site of the damage [80].

ATM and ATR are serine/threonine protein kinases able to recruit other DDR factors to the site of damage through a phosphorylation-dependent process. One of the most important features of this process is the phosphorylation of the histone H2AX (γ-H2AX) that leads to the propagation of the signal over several megabases around DSBs and starts a cascade of factors assembly [81,82].

During HR, the MRN complex is important for the stabilization of the DNA ends through the binding of the MRE11 subunit to DNA. This subunit has also exo- and endonuclease activities, which are fundamental for the DNA resection during the repair step [83]. The second subunit of the complex is RAD50 that belongs to the SMC (Structure Maintenance of Chromosomes) family and contains ATPase domains that interact with both MRE11 and the DNA ends at the site of the damage [83]. NBS1 does not possess a known enzymatic activity itself but participates in the DDR by mediating protein-protein interactions at the site of the DSBs and in particular associates with ATM via its C-terminal region [83,84].

In case of HR activation, the MRN complex promotes the activation of the ATM kinase leading to DNA resection, through CtlP and BRCA1 and formation of single strand DNA (ssDNA). Formation of ssDNA is crucial for the switch from ATM to ATR, which recruits RAD51 and BRCA2 that are important for the correct alignment of the sister chromatids. At this point the Replication Protein A (RPA 1) can sense the ssDNA and start the formation of the Holliday Junction (HJ) together with BRCA2 [70].

During NHEJ repairing pathway the Artemis nuclease processes the breaks and the XRCC4/LIG4 carries on the ligation of the two broken filaments [70].

Other important substrates activated by ATM and ATR are represented by the downstream effectors kinases CHK2 and CHK1 respectively, which can act on different substrates involved in cell cycle progression, apoptosis, and gene transcription [85]. Like other DDR factors, CHK2 and CHK1 do not stay at the site of damage but are released and dispersed in the nucleus where can target the CDC25 family phosphatases, the tumor suppressors p53 and BRCA1 and the kinase Wee1 [86-88]. Modulation of these factors leads to cell cycle arrest either in G1 phase, through the activation of p53 and p21, or in G2 phase, via inactivation of the CDC25 phosphatases and activation of Wee1 kinase leading to inactivation of the cyclin-dependent kinase CDC2 [89].

**DDR upon CDT and Typhoid Toxin intoxication**

CDT-intoxicated cells show many similarities to the DNA DSBs response described above and resemble the effects of the DDR induced by IR, a well-known genotoxic stress, which can act as carcinogen [90,91]as summarized in Figure 4.

CDT-induced DNA damage leads to activation of the ATM kinase, phosphorylation of histone H2AX, re-localization of the DNA repair proteins, such as MRE11 and RAD50, to the site of the damage activation of the tumor suppressor p53 and its transcriptional target, the cyclin-dependent kinase inhibitor p21, inactivation of CDC25 phosphatases and accumulation of the inactive hyper-phosphorylated form of CDC2 [91-97].

Our group has further demonstrated that the DDR signal coordinated by the sensor kinase ATM is transduced from the nucleus to the cytoplasm via the translocation and dephosphorylation of the guanine exchange factor (GEF) NET1 that in turns activates the

small GTPase RHOA [98,99]. RHOA activation can then lead to the promotion of two different pathways. The RHOA kinases ROCKI and ROCKII control the formation of the actin stress fibers, while the mitogen-activated protein kinase (MAPK) p38 promotes cell survival [99]. To further characterize the effectors of the CDT-induced survival response, Guerra et al. screened a *Saccharomyces cerevisiae* deletion library and selected 78 deletion mutants with reduced growth rate following inducible expression of CdtB. Among the human horthologs identified, the flap-endonuclease 1 (FEN1) and the tumor susceptibility gene 101 (TSG101) were shown to regulate DNA repair and endocytosis, respectively, and also share common interacting partners with RHOA. Further studies demonstrated that FEN1 but not TSG101 is involved in cell survival, MAPK p38 phosphorylation, RHOA activation and actin stress fibers formation in response to DNA damage [100].

**Integrin activation in response to CDT-induced DNA damage**

In **Paper I**, we have further characterized the integration of the DDR response with the re-organization of the actin cytoskeleton and activation of survival signals. Indeed, actin stress fibers are usually anchored at focal adhesion complexes that form upon engagement of integrins with components of the extracellular matrix (ECM) [101,102].

Integrins are heterodimeric transmembrane receptors formed by the non-covalent association of α and β subunits, but unlike classical signaling receptors (e.g., receptor tyrosine kinases), they do not possess enzymatic activity. Integrins activation is rather related to their ability to assume different affinity states that can be regulated bidirectionally: "outside-in" signaling induced by the components of the ECM or "inside-out" signaling induced by intracellular events that lead to the binding of integrins activator molecules such as talin and kindlin to the integrins cytoplasmic domain of β subunits [103].

Generally integrins are involved in different dynamic cellular processes such as cell proliferation, differentiation, survival and prevention of anoikis [104]. Many of these processes are de-regulated in tumor cells [105]. Thus, based on the strong re-organization of the cellular cytoskeleton and the formation of RHOA-dependent actin stress fibers in cells exposed to CDT, we assessed whether exposure to bacterial genotoxins promotes integrin activation, and which are the biological consequences of this activation.

Adhesion assays on intoxicated cells showed that time-dependent intoxication was associated with increased cell spreading and promotion of paxillin-positive FA (focal adhesion) after only 20 minutes of seeding on fibronectin-coated coverslips. Interestingly the same phenotype was observed also in cells infected with *Salmonella* Typhimurium strain MC71, expressing the typhoid toxin (MC71TT). This result suggests that the response observed is a general reaction to the bacterial genotoxins-induced DNA damage. FACS analysis and antibody function blocking experiments demonstrated that the enhanced adhesion properties induced by intoxication were mediated by activation of integrin β1 and α5. Moreover, the use of a specific inhibitor of the DNA damage sensor kinase ATM, demonstrated that the integrin-mediated cells adhesion was coordinated by this DNA damage sensor kinase , and it is part of the classical response to agents that induced DNA DSBs, since it was also observed in irradiated cells (**Paper I**).

As mentioned above one of the key factors that leads to stress fibers formation and rearrangement of the actin cytoskeleton, upon CDT-induced DNA damage, is the GEF protein NET1 [99]. In **Paper** I, we demonstrated that knocking down expression of the endogenous NET1 protein drastically decreased the spreading capacity of intoxicated cells upon seeding on fribronectin-coated coverslips. Nevertheless, the activation level of integrin β1 did not change, suggesting that that this effector regulates events downstream of the interaction of the integrin β1 with the ECM.

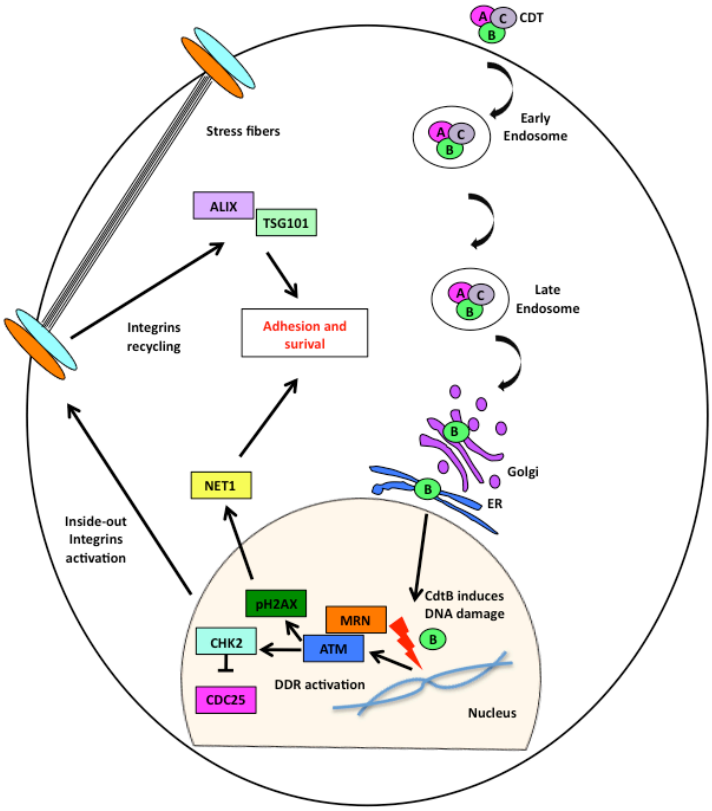


**Fig. 4:** Summary of CDT internalization pathway, DNA damagae response upon intoxication and inside-out integrins activation.

As of today there are many examples of inside-out integrin signaling pathways and most of them are related to the cross-talk with membrane bound growth factor receptors or cytokine receptors. Members of the epidermal growth factor (EGF) receptor family, such as EGFR and ERBB2, may contribute to tumor and metastasis progression in different types of tumors, including breast and pancreatic cancers. High levels of ERBB2 in breast cancer patients can induce spontaneous mammary tumour formation and tumour cell invasion in cooperation with integrin α6β4 [106].

In pancreatic cancer, EGF stimulates metastasis *in vivo* through the activation of the integrin αvβ5. The EGF receptor (EGFR) activation induces a Src-dependent phosphorylation of p130 CAS leading to activation of Rap1, a small GTPase involved in αvβ5 integrin activation [107].

The stimulation of the chemokine receptor CXCR4 by its ligand, the cell-derived factor 1 (SDF-1), increases the expression of integrins, such as α5β1 and αvβ3 and metastasis progression [108,109].

In our study we have demonstrated that the inside-out activation of the integrin β1 can also start in response to nuclear events such as DNA damage.

A very important aspect in integrins regulation is their recycling and trafficking from and to the plasma membrane. Integrins can either be endocytosed and recycled back to the cell membrane or transported via the ESCRTs (Endosomal Sorting Complex Required for Transport) to the multivesicluar body (MVBs), for further trafficking to the lysosome [110]. In our work we show that knock down of the ESCTR components ALIX and TSG101, leads to an inhibition of cell spreading on fibronectin coated coverslips.

To understand the biological significance of this novel DDR-dependent inside-out mode of integrin activation, we assessed whether it was associated with cell survival. The rational of this choice was the previous data obtained in our laboratory and demonstrating an important role of the RHOA-NET1 axis in promoting survival of intoxicated cells [100].

Inhibition of integrin activation-dependent signaling by siRNA-mediated knock down of TSG101 and ALIX expression increased the mortality rate of CDT-intoxicated cells when adhesion was prevented by seeding the cells onto wells pre-coated with 1% agarose. Since TSG101 does not influence the adhesion-dependent cell survival [100], integrin activation may play a role specifically on the regulation of cell survival in absence of adhesion.

Our work is in line with previous observation demonstrating that different doses of radiation induce enhanced cell adhesion in a variety of normal and transformed human cell lines resulting in a dose-dependent upregulation of β1, β3 and α5 integrins [111-114]. However, we did not detect a general up-regulation of the surface expression of integrin β1, but rather an increased affinity for the ECM (**Paper I**), suggesting that there may be multiple mechanisms

that regulate integrin signaling, which may differ depending on the cell type or stages of cell differentiation.

**Integrins, metastasis and drug resistance**

In the context of the exposure to a potentially carcinogenic agents such as DNA damaging CDTs, activation of the integrin signaling may have important implications for metastatic disease and drug resistance.

Metastasis is a multistep process that requires a regulation of several cellular processes such as cell attachment/detachment, epithelial to mesenchymal transformation, cell survival, cell migration and invasion, etc.. These processes are regulated by several adhesion molecules and growth factors, and integrins represent a crucial player in the initiation and progression of metastasis [115]. In order to colonize distant organs, cancer cells must be able to detach from the primary tumor, get access to the blood vessels and survive within the blood stream. An example is the activation of the αvβ3 integrins in human breast cancer cells, which induce tumor cell arrest during blood flow through interaction with platelets and leukocytes leading to cell survival [116]. Besides their role in the metastatic process, integrins can promote higher resistance to ionizing radiation and cytotoxic drugs. These phenomena are known as Cell Adhesion Mediated Drug Resistance (CAM-DR) and Cell Adhesion Mediated Radioresistance (CAM-RR) [112,113]. It has been shown that interaction between cells and the ECM promoted radiation-induced G1 or G2 cell cycle arrest giving to the cells time to repair the DNA and not undergoing apoptosis [117,118].

Integrins promoting cell survival effects have been observed also in response to chemotherapies drugs through the activation of antiapoptotic pathways mediated by p42/p44 MAPK and p38 MAPK [119].

Our data contribute not only to understand better the effects of infections with genotoxin producing bacteria, but also to find new insights in the contest of integrins-induced cell survival upon genotoxic injuries.

So far I have been describing what are the pathways and the most important factors activated in response to acute intoxication with CDTs. In the next chapters I will illustrate what are the consequences of chronic toxin exposure in an *in vitro* and *in vivo* models.

# 3 GENOMIC INSTABILITY

Chronic activation of DNA damage response, promotion of survival signals and inhibition of apoptosis are only the starting points for cancer development. In order to acquire a malignant phenotype, cells have to accumulate several mutations, which give them the capacity to get growth, survival, or metastasis advantage. This process is called genomic instability (GI) and represents one of the enabling characteristic of cancer [44].

GI has been found to be common to the majority of human cancers but it is still unknown when it starts and what is the molecular basis controlling it. Different forms of GI have been observed in cancers but the most common is the chromosomal instability (CIN), in which there is a change in the number or conformation of chromosomes [120]. Other types of GI have been described such as the microsatellite instability (MSI), which is characterized by the expansion or contraction of repeating units present in microsatellite sequences and forms of genomic instability that are characterized by increased frequencies of base-pair mutations [121-123].

**Mutation of DNA repair genes**

All these types of GI have been observed in hereditary cancers and linked to mutations in DNA repair genes. One example is represented by the hereditary non-polyposis colon cancer (HNPCC or Lynch syndrome) caused by a mutation in the DNA mismatch repair protein MSH2 gene leading to MSI [121]. Germline mutations in the MYH gene, which is implicated in the DNA base excision repair (BER), are also associated with colorectal tumors [123]. Other hereditary cancers such as breast and ovarian cancer, leukemia and lymphomas are associated with the presence of CIN and mutations within DNA repair genes such as BRCA1, BRCA2,

RAD50, NBS1, Werner syndrome and Bloom syndrome helicases (WRN and BLM), RecQ protein-like 4 (RECQL4) and Fanconi anemia genes [124-126].

**GI and oncogenes activation**

Deregulation of oncogenes and tumor suppressors is also strongly related to GI.

It has been observed that in precancerous lesions there is a strong activation of the DNA damage response to DSBs evidenced an ATM-dependent phosphorylation of the histone H2AX, CHK2 and p53, and the presence of p53 binding protein 1 (53BP1) foci [127,128]. Evidences of the presence of DSBs has been observed also in cancer lesions but in this case the DNA damage checkpoint pathway was compromised mostly due to mutations in the TP53 gene, which encodes the tumor suppressor p53 [127,128]. These data suggest that before the establishment of TP53 mutations, genomic instability is already present in precancerous lesions caused by induction of DNA DSBs. Interestingly one of the most evident differences between precancerous lesions and normal tissues is the presence of constitutive activated oncogenes, which are able to induce DNA DSBs, DDR and genomic instability by increasing DNA replication stress. [128-131].

Replication stress is defined as slowing or stalling in replication fork progression and it can be caused but different sources: depletion of nucleotides, DNA lesions, collisions between replication and transcription complexes, etc… [132].

Overexpression or constitutive activation of oncogenes such as c-MYC and HRAS can lead to increasing of replication initiation or origin firing, which in turns can result in depletion of nucleotides and/or increasing of collisions between replication and transcription complexes [133,134]. Oncogene-induced stalling in replication fork might lead to the collapse of the stalled fork, which can cause DNA DSBs and genomic instability [135,136].

**Activation of tumorigenic barriers**

To protect themselves from de-regulated proliferation due to the occurrence of GI or other pro-carcinogenic events, cells have evolved different defense mechanisms. Permanent cell-cycle arrest (senescence) and apoptosis are defined as tumorigenic barriers and represent the most powerful defense against uncontrolled cell proliferation. Cells that undergo senescence are in a state of irreversible cell-cycle arrest and show activation of typical senescence markers like β-galactosidase both *in vitro* and *in vivo* [137]. On the other hand apoptosis leads to a programmed cell death, which is characterized by cytochrome-c release and caspases activation [138]. In the context of activation of tumorigenic barriers, the transcription factor p53 represents a master regulator. This protein coordinates the activation of senescence and apoptosis in response to several cellular stress such as DNA damage, hypoxia and nutrient deprivation [139,140]. Although p53 is able to mediate both senescence and apoptosis in normal cells, it is still unknown which are the mechanisms that lead to one pathway or another [141].

**ROS as a trigger for genomic instability**

As mentioned in the first chapter of this thesis ROS production might represent another source of GI. Oxidative stress can cause DNA damage detected by the presence of 8-oxo-2'-deoxyguanosine (8-oxodG), which represents the main product of DNA oxidation [142]. This oxidative DNA lesion can lead to site-specific mutagenesis and G to T or A to C transversions, which have been found in different types of tumors carrying mutated oncogenes or tumor suppressor genes [143,144].

Malignant transformation due to ROS has been observed in different *in vitro* and *in vivo* models [145-147]. It has been shown that C3H/10T1/2 mouse fibroblast exposed to human neutrophils or hydrogen peroxide undergo malignant transformation. Cells injected right after treatment in nude mice gave rise to malignant and benign tumors formation [145].

ROS play also an important role in inflammatory conditions that precede cancer like in patients with chronic hepatitis or *H. pylori*-induced gastritis [148-150].

Drake and colleagues have shown that patients with *H. pylori*-induced gastritis have a high level of ROS in the stomach, which decreased with the eradication of the bacterium [149]. Moreover *H. pylori*-positive patients show a higher level of oxidative DNA damage in gastric mucosal samples compare to healthy volunteers [151].

Further studies performed in BigBlue® mice have shown that after 6 months of *H. pylori* infection, the gastric mutation frequency was 1.7-fold higher than in uninfected mice [152]. Mutations were mainly transversions and so associated to oxidative DNA damage, which is in line with increase in the iNOS (Inducible nitric oxide synthase) messenger RNA expression [152].

**CDT and genomic instability**

These data bring us to the following question: Is the role of bacteria infection in promotion of genomic instability limited only to chronic production of ROS, or could bacterial genotoxin contribute directly this process? To address this issue, we investigated on the effects of chronic exposure to sublethal doses of CDT in eukaryotic cells (**Paper II**).

Rat fibroblasts and human colon epithelial cells (HCT116) have been exposed to sub-lethal doses of CDT for a long period of time and followed to monitor the occurrence of genomic instability and tumor progression. The sub-lethal dose of CDT is referred to the amount of toxin that does not induce an acute cell cycle arrest but it is still able to cause DNA damage. As a control, cells were treated with a mutated version of CDT unable to induce DNA damage. A total of 14 sublines were selected after 220 days in presence of CDT in two

independent experiments. Seven sublines, exposed to the mutant toxin for the same period of time, were used as negative control.

After the treatment most of the selected sublines showed an increased mutation frequency, accumulation of chromosomal aberrations and enhanced anchorage-independent growth. A similar effect was described by Cuevas-Ramos and colleagues in 2010 when they performed a short-term infection of mammalian cells with *E. coli* strains producing colibactin [68]. The authors showed that short-term exposure of the hamster CHO cell line to a low infection dose of *pks* positive *E. coli,* induced DNA damage response followed by cell division with signs of incomplete DNA repair. Consequently anaphase bridges, chromosome aberrations, ring chromosomes, translocations and aneuploidy were observed in infected cells 72h post-infection. These aberrations persisted in dividing cells up to 21 days after infection and treated cells showed an increased rate of mutation frequency and capacity of growing in an anchorage-independent manner [68].

These data suggest that *pks* positive *E. coli* could play an important role in the promotion of colon cancer formation by continuously provoking DNA damage at the enterocytes level.

However our study showed that a substantial increase in the frequency of mutations and significant changes in chromosomal aberrations was observed only after 130 days and 60 days of continuous exposure to CDT of the Big Blue fibroblasts and HCT116 cells, respectively. These data highlighted the fact that increasing of mutation frequency and the acquisition of a malignant phenotype require a long period of time and short-term experiments or co-cultivation with live bacteria might lead to under- or overestimation of the results.

In order to verify whether the chronic intoxication with CDT affects the DNA damage response (DDR), these cells were left untreated, to be sure that the toxin effect could not interfere with the result, and then exposed to IR to test their response to DNA damage. All the

sublines, selected in the presence of the active toxin, showed a lower level of phosphorylated histone H2AX compare to the control, which were cells treated for the same period of time with a non-functional CDT. A quite high percentage of cells compare to the control were also able to continue cycling through the G1 phase after treatment with etoposide instead of arresting in G2 phase. Based on the findings published by Guerra et. al in 2008, we tested whether activation of MAPK p38 had a role also in the survival of chronic-intoxicated cells [99]. We demonstrated that the activation of the MAP kinase p38 signalling was much higher in the cells intoxicated with the functional CDT compare to the control, and selective inhibition of this kinase promoted a rapid cell death of the sublines selected in the presence of the active toxin.

Interestingly we also observed that long-term exposure to CDT caused a twofold increase in the level of intracellular ROS compare to short-term intoxication or control cells.

Taken together these results tell us that long-term exposure to CDT gives to the cells the ability to over come the tumorigenesis barrier, enhances the acquisition of a malignant phenotype and that production of ROS might have a synergistic effect on tumor progression.

As mentioned before in this chapter, impairment of the DDR might represent a crucial point in the starting of the genomic instability process and accumulation of mutations. In **Paper II** we have demonstrated that chronic exposure to CDT resulted in impaired DNA damage response and DNA repair and this might play a very important role in the case of chronic infections with genotoxin-producing bacteria.

Several *in vivo* models have studied the carcinogenic effect of bacterial genotoxins *in vivo*. The most compelling evidences are those reported by Arthur and colleagues. The authors have used an animal model of Inflammatory Bowel Disease (IBD) and colitis-associated CRC, and demonstrated the carcinogenic potential of colibactin. Treatment with the colon-specific carcinogen azoxymethane (AOM) induced a higher incidence of invasive

adenocarcinoma in germ free IL 10 knock-out mice mono-colonized with a *pks* positive *E. coli* [153]. Deletion of the *pks* genotoxic island reduced tumor multiplicity and invasion in Il10$^{-/-}$ mice treated with AOM, without altering intestinal inflammation [153].

These observations were strengthen by the detection of *pks* positive *E. coli* in 66.7% and 40% of CRC and IBD patients respectively compared to 20% found in non-IBD/non-CRC controls, providing a stronger evidence of the association between the presence of *pks*$^{+}$ bacteria, chronic intestinal inflammation and CRC [153].

*In vivo* study on *H. hepaticus* long-term infection in A/JCr mice showed that both the isogenic mutant lacking CDT activity and the wild-type *H. hepaticus* induce chronic hepatitis at 4 and 6 months post-infection [154]. However the mice infected with the mutant strain during a period of 10 months did not develop any hepatic dysplasic nodules while the animals infected with the toxigenic strain did [154]. These data confirm that CDT has an important role in the induction of preneoplastic lesions *in vivo.* Nevertheless these results do not give information on the molecular changes that CDT promotes in order to lead to the acquisition of malignant phenotype such as type of genetic alterations and the factors involved in promotion of cell survival, which is what we have described in **Paper II**.

**A new *in vivo* model**

These evidences suggest that the presence of an active genotoxins in the context of a chronic infection may be carcinogenic. These data are extremely relevant in the context of *S.* Typhi: the only genotoxin producing bacterium that can establish a chronic asymptomatic infection and is associated with increased risk of cancer development in human subjects [3,155,156].

Studying the role of the Typhoid Toxin in immunocompetent animal models represents a challenge, since *S.* Typhi is exclusively a human pathogen. To overcome this obstacle, we

have designed a model of *S.* Typhimurium expressing the Typhoid Toxin genes, which can promote a chronic infection in immune competent mice. Having developed this model, we can now ask two important questions:

1. Is the chronic infection with the genotoxin producing S. Typhimurium pro-carcinogenic?
2. What is the role of this toxin in the context of *Salmonella* infection?

# 4 DEVELOPMENT OF A NEW MODEL TO STUDY THE CARCINOGENIC POTENTIAL OF GENOTOXINS *IN VIVO*

***Salmonella enterica***

The genus *Salmonella* consists of two species: *Salmonella bongori*, a commensal of cold-blooded animals, and *Salmonella enterica* (divided into six subspecies) [157]. *Salmonella enterica* subspecies can be classified on the basis of the O (lipopolysaccharide [LPS]) surface antigen into more than 50 serogroups and into over 2400 serotypes or serovars when strains are differentiated by both their O and H (flagellar) antigens [158]. The majority of the serovars associated to human infections belong to *S. enterica* ssp. I, which are responsible for enteric or typhoid fever and colitis/diarrheal disease through the ingestion of contaminated food or water [159]. Typhoid fever, a severe systemic infection, is caused by the exclusively human pathogens *S. enterica* serovar Typhi and *S. enterica* serovar Paratyphi A and B. Colitis instead is a localized infection caused mainly by *S. enterica* serovar Typhimurium and serovar Enteritidis, which are able to infect a wide range of animal hosts besides humans [160,161].

In this chapter, I will discuss mainly about *S.* Typhi and *S.* Typhimurium infection strategies since these are the bacteria relevant for our studies.

Upon ingestion of contaminated food or water the bacterium is able to survive the low pH of the stomach via the activation of the acid tolerance response, which helps to maintain the intracellular pH higher than the one of the extracellular environment [162]. After reaching the ileum, a second barrier against *Salmonella* is represented by the mucus layer that protects the intestinal epithelium. However the bacterium can penetrate this layer using flagella and chemotactic movements [163]. In mice, *S. enterica* seems to preferentially adhere to and invade the microfold (M) cells of the Peyer's patches (PPs) in the intestinal epithelium via

pinocytosis, although active endocytosis in enterocytes can also occur [164]. Right after adhesion, the invasion process starts with the induction of a significant rearrangement of the host cells cytoskeleton and consequent formation of membrane ruffles that engulf adherent bacteria into large vesicles called *Salmonella*-containing vacuoles (SCVs) [165-169]. In the SCVs, *Salmonella* survives and replicates protected against eventual lysosomal degradation and exploiting cellular trafficking to access to nutrients [170-172]. A fraction of SCVs transcytoses to the basolateral membrane and releases the internalized bacteria to the submucosa where they are pinocytosed by macrophages [159]. Migration of infected macrophages can facilitate a systemic dissemination of bacteria through the bloodstream to other tissues such as spleen and liver [160,173].

In order to induce a bacterial-mediated endocytosis and hijack cellular pathways, *Salmonella* needs to produce and deliver different effectors. The majority of the virulence factors genes are located within highly conserved *Salmonella* pathogenicity islands (SPIs) and the effectors proteins are delivered to the host mostly through the Type III Secretion System (T3SS). This organelle is a multi-subunit protein with a characteristic needle-like structure capable of injecting effectors directly from bacterial cytoplasm into the host cell cytosol [174]. T3SS are found in several Gram-negative pathogens and have a key role in the contact-dependent translocation of substrate proteins into host cells [175]. *Salmonella* encodes for two T3SS, called T3SS-1 and T3SS-2, located in two different SPIs: SPI-1 and SPI-2 respectively [176].

Effectors proteins translocated via the T3SS-1 are involved in cytoskeleton reorganization, triggering invasion into non phagocytic cells, such as fibroblasts and epithelial cells, establishment of permissive intracellular niche and induction of expression of proinflammatory cytokines and chemokines [177-179].

The T3SS-2 is expressed upon *Salmonella* internalization into the SCVs and SPI-2 effectors are involved in manipulating the vesicular trafficking in order to enhance intracellular

survival and establish systemic infection [180]. Very important for the survival of *Salmonella* in the host cell is the production of *Salmonella*-induced filaments (SIFs). These tubular structures originate from the SCVs and aggregate along a scaffold of microtubules extending throughout the cell [181]. Among the key factors in SIFs production, only SifA seems to be absolutely required and it has been shown that *S.* Typhimurium ΔsifA mutants are less virulent in mice and have a lower intracellular replication in cultured macrophages but not in epithelial cells as reported by Stein and colleagues [182,183]. In order to start the SIFs production, the N terminus of SifA binds to a host protein called SifA kinesin interacting protein (SKIP), which connects the SCVs to microtubule motor kinesin [184,185]. On the other hand the C terminus of SifA binds to members of the Rho small GTPase family and the two events together trigger the formation of SIFs [185].

These findings indicate that the production of SIFs is important for the pathogen replication into the host cell.

Since *S.* Typhi is restricted to humans, there are no suitable animal models to study the effect of typhoid fever pathogenesis. For many years S. Typhimurium has been used in a systemic infection model using susceptible mouse strains in which it reproduces many of the characteristics of the human typhoid fever [186] [187]. Mice susceptibility is due to a non-functional natural-associated macrophage protein 1 (Nramp1) gene ($Nramp^{-/-}$), which is important for the destabilization of the metabolic activity in the SCVs [188].

On the other hand, the use of mouse strains carrying a functional Nramp gene ($Nramp^{+/+}$) represents a good model to study the chronic infection observed in S. Typhi carriers [189]. While BALB/c and C57BL/6 are $Nramp^{-/-}$ mice strains, the sv129 mice carry the wild type version of the Nramp1 gene and have been used by Monack and colleagues in order to study the mechanisms of persistent infection [189]. In this study, mice were infected with $10^8$ *S.* Typhimurium bacteria and tissue colonization, fecal shedding, and tissue pathology were

monitored along a period of twelve months. Although at the end of the experiment bacteria were still detectable in spleen, liver and gall bladder of only few mice, almost in all the animals the *Salmonella* persisted within macrophages in the mesenteric lymphnodes (MLNs) [189].

**A novel *Salmonella* infection model**

Following the analysis of these data, we decided to use sv129 mice and chronically infect them with the *S.* Typhimurium fully virulent strain MC1 or the attenuated MC71 strain, which carries a single point mutation within the *polynucleotide phosphorylase* (*PNPase*) gene [190]. *S.* Typhimurium does not express the Typhoid Toxin, therefore the *pltB–pltA* and *cdtB* genes of *S.* Typhi were cloned into the chromosome of *S.* Typhimurium under the control of their endogenous promoters. The new toxigenic strains (MC1/MC71-TT) and the isogenic strains lacking for the *cdtB* gene (no toxigenic MC1/MC71- Δ*cdtB*) were used to infect sv129 mice orally, the natural route of Salmonella infection (**Paper III**).

The animals were randomized into three groups: one group was left uninfected, a second group was infected with MC1- or MC71-TT strain and the third group was infected with the control strain MC1- or MC71- Δ*cdtB* at the infectious dose of $10^8$ bacteria per mouse, as reported by Monack and colleagues[189] and summarized in Figure 5. As expected, approximately 40% of the mice infected with the control MC1- Δ*cdtB* strain died within the first 15 days post-infection (p.i.) [189].

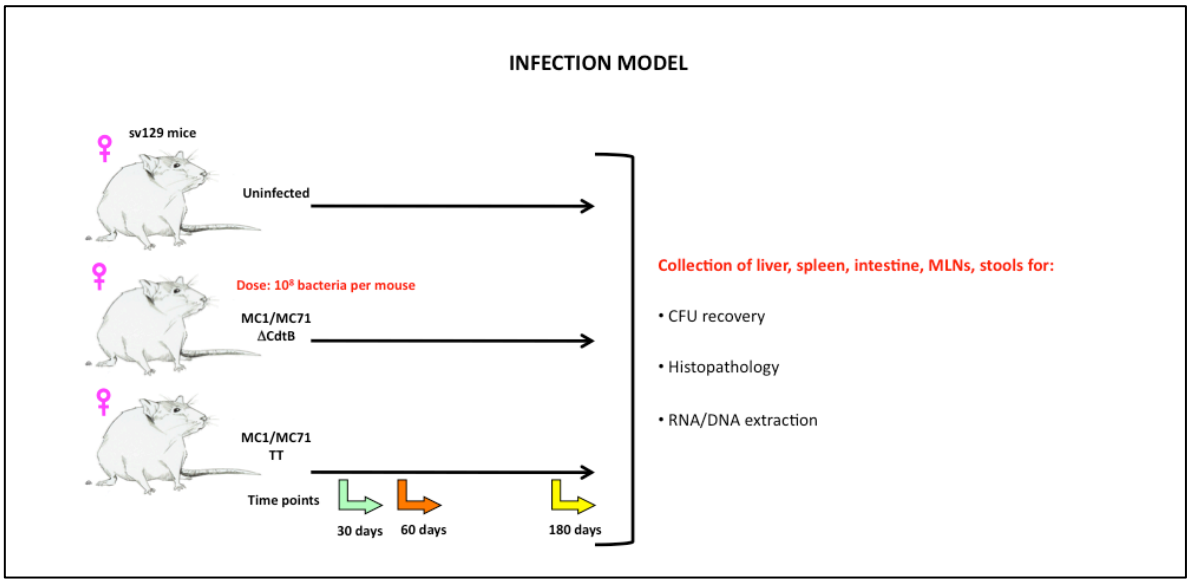


**Fig. 5:** Infection model for the short- and long-term infection with MC1/M71 ΔCdtB and MC1/MC71 TT strains

Interestingly, we observed a reduced mortality rate in mice infected with the MC1-TT strain compare to the ones infected with the MC1- Δ*cdtB* strain. MC71-TT and MC71- Δ*cdtB* infected mice followed a similar trend although with a less pronounced effect. Mice that overcome this critical period of 15 days survived and did not present any obvious symptoms, independently of the strain used. The enhanced survival of mice infected with the toxigenic strain is not due to an impair ability of MC1-TT or MC71-TT to invade the host, since equal amount of bacteria was recovered in the MLNs, liver and spleen of all the mice 30 and 60 p.i.. However, the presence of a functional toxin was associated with higher frequency of mice carrying *S. enterica* 180 days p.i. in liver, MLNs and cecum, indicating that it may contribute to the establishment of a chronic infection.

To further investigate the effects of the TT on the host response, we performed histological and transcriptomic analysis at 60 days p.i. in mice infected with the MC1 and MC71 strains. Data showed that the presence of the Typhoid Toxin reduced tissue damage and the host inflammatory response in the intestine. On the other hand infection with the toxigenic strain enhanced signs of severe pathology and the levels of inflammation in the liver. The reduced

intestinal inflammation is reminiscent of the capacity of *S.* Typhi to exert a silent intestinal invasion, followed by systemic symptomatic spread [191]. The presence of a bacterial genotoxin may suppress the host immune response like in the case of CDT, which is able to block the proliferation and induce apoptosis of T- and B-lymphocytes[54,91].

The anti- and pro-inflammatory effect observed in the intestine and in the liver, respectively, might be due to a different type of immune response compare to the intestine: activation and recruitment of suppressive regulatory T lymphocytes in the intestine versus a Th1/Th17 pro-inflammatory response in the liver [192].

**Effects of the Typhoid Toxin on the microbiota**

Interesting data came also from the intestinal microbiota analysis performed on bacterial DNA extracted from stool samples collected from mice infected with MC71 strains. Toxin-specific effects on the host response seemed to be associated with a pronounced and long-lasting alteration of the intestinal microbiota. Phylogenetic analysis showed a significant increase of Proteobacteria, which is associated with non-alcoholic fatty acid liver disease, and may contribute to the hepatic inflammation observed in mice infected with the toxigenic strain [193]. We also performed an analysis more focused on the family level that revealed a reduction of the butyrate producers *Lachnospiraceae* in particular in mice infected with the MC71-TT strain. Previous studies have demonstrated that butyrate can negatively influence both viability and virulence gene expression during *Salmonella* invasion [194]. This suggests that the reduction of the *Lachnospiraceae* that we observed might represent an advantage for *Salmonella* infection and invasion, and may also explain why we detected the presence of the MC1 toxigenic strain in the cecum of infected mice at 30 and 180 days post-infection.

Streptomycin pre-treated mice have been used as model in many studies because these animals have a much more efficient intestinal colonization with *S.* Typhimurium [195], due to

alteration of the microbiota composition and possibly the reduction of butyrate levels, allowing *Salmonella* invasion in the cecum [196,197]. Although this suggests that intestinal colonization increased because of a lack of competition with the microbiota components, on the other hand does not give any prove regarding the mechanisms of interaction, the causes or the effects of the *Salmonella* interplay with the intestinal microbiota. In this context, gnotobiotic mice might represent a good model to study the complicated relationship between *Salmonella* and the normal microflora since the composition of their microbial community can be controlled. Winter and colleagues have used this model to demonstrate that ROS produced during *Salmonella* Typhimurium infection react with luminal thiosulfate to produce tetrathionate, which can then be used as respiratory electron acceptor by *Salmonella*. This mechanism provides *Salmonella* with a big advantage compare to the other bacterial components of the intestinal microbiota, which have problems to survive the changes resulted from the *Salmonella*-induced inflammatory response [198].

In our study, we have used wild type immunocompetent mice, not pre-treated with any antibiotics and infected through the natural route of *Salmonella* infection (oral infection). These conditions gave us the possibility to look at the effects of *Salmonella* from a different prospective without altering the system, and may offer new tools to assess how the typhoid toxin alters the host-microbiota environment thus favoring *Salmonella* colonization of the cecum.

In conclusion, we have developed a unique model in which we have tried to be as close as possible to a real *Salmonella* infection to study the effects of bacterial genotoxins in the context of acute and long-term infections.

# 5 TYPHOID TOXIN SECRETION AND DELIVERY

*In vivo* experiments with a *S.* Typhimurium expressing the TT represent a very important tool to better understand the role of this toxin during *Salmonella* infection. In order to exert its effect on the target cells, the Typhoid Toxin has to be secreted from the producing bacterium and access the nuclear compartment.

Haghjoo and colleagues demonstrated that the Typhoid Toxin activity and the expression of the active subunit CdtB require the internalization of the bacteria in the host cell [65]. This data suggests that the toxin, in order to reach its target in the nucleus, has to cross the SCV membrane. Interestingly, none of the T3SSs are required for the delivery of CdtB [65]. In 2008 Spanó and colleagues proposed a mechanism by which the toxin is produced in the SCVs and secreted in a soluble form in the extracellular environment where they can target bystander cells [66]. However, the mechanism(s) of secretion from the producing bacterium as well as the mode of exit from the SCV have not been characterized. We have addressed this issue in **Paper IV**.

**OMVs**

Several lines of evidence have demonstrated that bacterial toxins, including the genotoxic CDT are secreted via outer membrane vesicles (OMVs), vesicles produced and shed by Gram-negative bacteria. OMVs appear at the electron microscope as spherical objects with a diameter of 50-250 nm and a bilayer membrane [199].

Electrophoresis analysis and activity assays have demonstrated that OMVs contain a high abundance of LPS and periplasmic proteins, while components of inner membrane and cytoplasmic components are lacking or highly depleted. These data suggest that OMVs are not the result of random budding or a bacterial lysis [200-202] [201,202]. The details of the OMVs

production are still unknown but in general these vesicles originate when the outer membrane (OM) bulges and encapsulates periplasmic components [203]. Thus proteins loaded into OMVs have to cross the inner membrane and reach the periplasmic space. Periplasmic translocation occurs mainly through two mechanisms: Sec- or Tat-mediated translocation across the cytoplasmic membrane [204,205]. The main difference between these two pathways is that the Sec system is responsible for the transport of newly synthesized proteins, while Tat system transports already folded proteins [206].

It has been shown that the CdtB subunit of the Typhoid Toxin contains a *sec*-dependent secretion signal that delivers the protein in the periplasmic space [65]. In **Paper IV**, bioinformatics analysis demonstrated the presence of a N-terminal signal peptide that would mediate a sec-dependent translocation into the periplasmic space, where the assembled Typhoid Toxin can be packed and released via OMVs. To prove this hypothesis, we took advantage of the MC71-TT strain, generated in our laboratory for the *in vivo* analysis. Since the toxin is produced only in the SCV, upon internalization in the host cells, we mimic this environment by growing the bacteria in minimal medium pH 5.8 [207]. OMVs produced in these conditions were purified by differential ultracentrifugation. Immuno-gold labelling followed by electron microscopy analysis showed that the secreted LPS positive vesicles contained the active subunit, which could be detected only when the vesicles were disrupted by sonication, indicating that the toxin is contained within the vesicles.

**TT-loaded OMVs deliver cytotoxicity to bystander cells**

The purified vesicles were able to transfer the genotoxic activity to bystander cells. Paracrine internalization of typhoid toxin-loaded OMVs by bystander cells was dependent on dynamin-1, indicating that the vesicles were internalized by an active endocytosis process. Internalization has been previously demonstrated for OMVs carrying other bacterial protein

toxins such as the Heat Labile enterotoxin (LT) of the Enterotoxigenic *E. coli* (ETEC). The VacA-loaded OMVs of *H. pylori* also require endocytosis to deliver their content to cells [208-210]. Conversely, CDT-loaded OMVs from *A. actinomycetemcomitans* fuse with the cholesterol rich domains of the target cells and release the toxin cargo directly in the cytosol [211]. Thus it may be possible that there are several pathways for OMVs internalization within the host cells, and it will be interesting to evaluate what regulate the choice between endocytosis and fusion with the plasma membrane. These data indicate that the field of OMVs internalization is still at its infancy and more research in needed to clarify all these relevant issues.

The subsequent induction of DNA damage was blocked by treatment with brefeldin A (BFA), indicating that retrograde transport of the Typhoid Toxin through the Golgi complex is required, similarly to the soluble CDT [63]. We could not detect co-localization of the labeled OMVs with fluorescent Golgi tracker, indicating that the toxin has been released from these vesicles at the level of the endosomal compartment(s) (unpublished observation).

### *Salmonella* Typhimurium infected cells secrete TT-loaded vesicles

Our data show that the typhoid toxin is secreted in vesicles. However, *Salmonella* produces this effector only within the SCV once internalized into the host cell, indicating that the OMVs have to cross several membranes in order to access the nuclear compartment of the infected and bystander cells as summarized in Figure 6. To assess whether OMVs are released from the infected cells into the extracellular medium, we purified the extracellular vesicles by differential ultracentrifugation, followed by sucrose gradient fractionation.

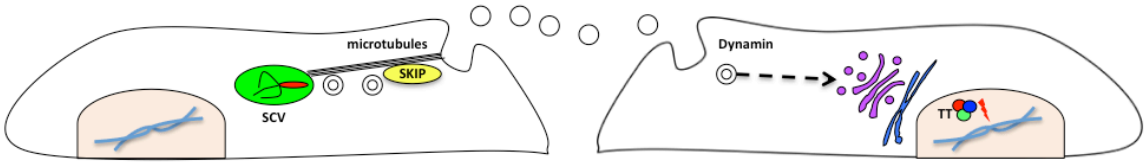


**Fig. 6:** Proposed model for Typhoid Toxin secretion and delivery.

Immuno-gold labeling using a specific antibody directed against the *Salmonella* LPS followed by electron microscopy analysis revealed that the infected cells released approximately 30% of LPS positive vesicles with an average diameter of 120 nm, comparable to that of a OMVs subset released from bacteria grown in MM5.8 medium. Bacteria defective for the *sifA* gene, which are not able to form an intact SCVs, could not induce the release of extracellular vesicles (EVs) carrying the genotoxic activity. Treatment with nocodazole or cytochalasin D, which interfere with microtubule and actin polymerization, reduced the release of toxin-loaded vesicles of approximately 50%. Taken together these data suggest that the release of EVs containing the Typhoid Toxin required an intact SCVs and relied on anterograde transport toward the cellular cortex on microtubule and actin tracks.

Very little is known about OMVs released from infected cells but there are evidences of the presence of OMVs in the cytoplasm of *Salmonella*-infected cells [212,213]. Our study contributes to better understand this mechanism but further investigation is needed and could give new and interesting insights in the context of intracellular bacteria infection strategies.

Generally OMVs represent an excellent tool that bacteria can use to deliver different types of cargoes (membrane-bounds proteins, lipids, quorum-signaling molecules, toxins, etc…), which can reach distant targets protected by the vesicles and, if it is necessary, in high concentrations [214].

An interesting question at this point is: are OMV produced also *in vivo*?

Fiocca and colleagues have found VacA-loaded OMVs in biopsy specimens of *H. pylori* infected tissues similar in appearance and composition to *H. pylori* vesicles made *in vitro* [215]. These data suggest that OMVs are produced also *in vivo.*

In conclusion our work provide new insights on the mode of secretion and delivery of exotoxins by cells infected with intracellular bacteria.

# 6 CONCLUSIONS

This thesis aimed to study different aspects of the bacterial genotoxins in order to better characterize the cellular responses induced upon intoxication and the role that they have during infection *in vitro* and *in vivo*. Our data cover a broad range of questions about CDT and Typhoid Toxin biology from acute and chronic intoxication, and from *in vitro* and *in vivo.*

1. Acute CDT intoxication *in vitro* to study the effects on integrin activation and survival signals
2. Chronic CDT intoxication *in vitro* to better characterize long-term consequences on molecular and phenotypic changes
3. Chronic and acute infection with *S.* Typhimurium *in vivo* to assess the role of the Typhoid Toxin in a new murine model
4. *In vitro* characterization of secretion and delivery pathways for the Typhoid Toxin

We described a novel inside-out integrin signaling pathway, which contributes to increase cells spreading and survival after short-term exposure to CDT. Our long-term intoxication experiments highlighted the carcinogenic potential of CDT *in vitro* and led us to set up a new murine model to investigate if this was true also *in vivo*. We decided to infect normal immunocompetent mice through the natural route of *Salmonella* to be as close as possible to the reality and this set up gave us the possibility to find something completely unexpected. We did not observe signs of cancer development but we may have revealed the physiological role of this toxin in the context of bacterial infection. Now we can use different models such as knock out mice for p53 and ATM to assess the carcinogenic properties of the Typhoid Toxin.

The characterization of the Typhoid Toxin trafficking from the infected cells to the extracellular space and its internalization in bystander cells contributed to better understand how intracellular bacteria can deliver their effectors. It would be very interesting to study these pathways also *in vivo* and see if TT-loaded OMVs can be found also in organs not directly infected with *Salmonella*.

Genotoxins-producing bacteria represent still a very open field in cancer research and many questions are still without an answer.

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