ANTI-TUMORAL IMMUNE RESPONSE AND BISPHOSPHONATES

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Master's Thesis
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January 2012

UNIVERSITY OF EASTERN FINLAND, Faculty of Health Sciences
School of Pharmacy: Biopharmacy
HIRVASNIEMI, PETRA JOHANNA: Anti-Tumoral Immune Response and Bisphosphonates
Master's Thesis, 83 pages
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January 2012

Keywords: Bisphosphonates, Cancer, Immunotherapy, γδ T cells, IPP, ApppI

Abstract

Nitrogen-containing bisphosphonates (N-BPs) are widely used to block bone destruction in cancer patients with bone metastasis because they are found to be effective inhibitors of osteoclast-mediated bone resorption. In addition to their antiresorptive effects, preclinical evidence strongly suggests that N-BPs possess also anticancer activity. Some of the activities associated with N-BPs are related to the activation of human γδ T cells that straddle the interface of innate and adaptive immunity. Vγ9Vδ2 T cells recognize *in vitro* a wide array of transformed cells and are activated *in vivo* in various tumors. Vγ9Vδ2 T cells are able to induce protective immunity against different cancers by directly killing tumor cells and by producing inflammatory cytokines that provoke antitumor properties of other immune effector cells. Human γδ T cells can be activated in a non-MHC dependent fashion either by low molecular mass phosphoantigens, or by agents that provoke the accumulation of endogenous pyrophosphates such as isopentenyldiphosphate (IPP).

The literary part of this Master's Thesis summarizes the functions of immune system and γδ T cells as well as the properties of bisphosphonates on the perspective of cancer immunotherapy. The experimental part concentrated on the intentional activation of Vγ9Vδ2 T cells by N-BPs as well as IPP/ApppI accumulation and secretion in human breast cancer cells. Co-culture with purified Vγ9Vδ2 T cells led to IPP/ApppI-dependent killing of N-BP (ZOL or RIS) treated breast cancer cells. Without Vγ9Vδ2 T cell expansion, N-BPs were not able to induce cancer cell death. A clear correlation was seen between the anticancer activities of Vγ9Vδ2 T cells and the intracellular IPP/ApppI levels in both ZOL and RIS-treated breast cancer cells *in vitro*. However, further studies are required to confirm that IPP/ApppI is secreted in extracellular media, and that this would promote Vγ9Vδ2 T cell migration to tumors.

ITÄ-SUOMEN YLIOPISTO, Terveystieteiden tiedekunta
Farmasian laitos: Biofarmasia
HIRVASNIEMI, PETRA JOHANNA: Anti-Tumoral Immune Response and Bisphosphonates
Pro Gradu –tutkielma, 83 sivua
Ohjaajat: FT Ismahène Benzaïd, FaT Johanna Räikkönen ja FaT Hannu Mönkkönen
Tammikuu 2012

Avainsanat: bisfosfonaatit, syöpä, immunoterapia, γδ T-solut, IPP, ApppI

Tiivistelmä

Bisfosfonaatteja, jotka sisältävät sivuketjussaan typpiryhmän (N-BP:t), on pitkään käytetty estämään luukudoksen tuhoutumista syöpäpotilailla, sillä niiden on todettu ehkäisevän tehokkaasti osteoklastivälitteistä luun hajoamista. Luuhun kohdistuvien suojelevien vaikutuksen lisäksi näillä N-BP:illa näyttäisi prekliinisten tulosten perusteella olevan myös suoria vaikutuksia syöpäkudokseen. Osa N-BP:en vaikutuksista voidaan yhdistää elimistön γδ T-soluihin. γδ T-solut ovat vähemmän tunnettuja T-lymfosyyttejä, joita ei voida puhtaasti luokitella synnynnäisen eikä adaptiivisen immuunijärjestelmän soluihin. Vγ9Vδ2 T-solut pystyvät tunnistamaan laajan valikoiman muuntautuneita soluja *in vitro*, ja aktivoituvat kohdatessaan useita kasvaintyyppejä *in vivo*. Vγ9Vδ2-T solut pystyvät puolustamaan elimistöä useita syöpätyyppejä vastaan joko tappamalla kasvainsolut suoraan, tai tuottamalla tulehdusvälitteisiä sytokiineja, jotka vuorostaan aktivoivat useita muita immuunijärjestelmän tuhoajasoluja. γδ T-soluja voidaan aktivoida MHC-riippumattomilla tavoilla joko pienikokoisilla luonnollisilla fosfoantigeeneilla tai synteettisillä molekyyleillä, jotka saavat aikaan isopentyylidifosfaatin (IPP) kertymisen soluun.

Tämän Pro Gradu –tutkielman kirjallisessa osassa kerrotaan lyhyesti immuunijärjestelmän ja γδ T-solujen toiminnan periaatteista sekä tiivistetään bisfosfonaattien historia syövän immunoterapian näkökulmasta. Kokeellisessa osassa keskityttiin Vγ9Vδ2 T-solujen aktivointiin N-BP:en avulla ja tutkittiin IPP/ApppI:n kerääntymistä ihmisen rintasyöpäsoluihin sekä niiden mahdollista eritystä solujen ulkopuolelle. Kun N-BP:illa (ZOL tai RIS) käsiteltyjä syöpäsoluja inkuboitiin puhdistettujen Vγ9Vδ2 T-solujen kanssa, voitiin havaita IPP/ApppI-tasoista riippuvainen syöpäsolujen kuolema. Vastaavasti, ilman Vγ9Vδ2 T-soluja N-BP:t eivät pystyneet aiheuttamaan syöpäsolujen kuolemaa. Vγ9Vδ2 T-solujen kyky aiheuttaa ZOL- tai RIS-käsiteltyjen syöpäsolujen kuolema näyttäisi lisäksi olevan suoraan verrannollinen syöpäsoluista mitattujen IPP/ApppI -pitoisuuksien kanssa *in vitro*. Lisää tutkimuksia tarvitaan kuitenkin todistamaan, että IPP tai ApppI erittyisivät myös syöpäsolujen ulkopuolelle ja sieltä käsin houkuttelisivat Vγ9Vδ2 T-soluja syöpäkudokseen.

Acknowledgements

I made the experimental part of this Master's Thesis in Lyon, France, at INSERM research unit UMR 1033 in the spring 2011. I would mostly like to thank my supervisor, Ph.D. Ismahène Benzaïd, for her valuable help and guidance throughout the research project. Also I would like to thank my supervisors Ph.D. Hannu Mönkkönen, who introduced me this subject and Ph.D. Johanna Räikkönen, who guided me with the literature part of this Master's Thesis.

In addition, I would like to thank all the INSERM employees, who helped me with the research, made me feel like home there, and persistently tried to teach me speak French instead of English.

Jyväskylä, January 2012

Petra Hirvasniemi

Table of Contents

List of Abbreviations

AMP	Adenosine monophospate
APC	Antigen-presenting cell
ApppI	Triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester
ATP	Adenosine triphosphate
BP	Bisphosphonate
BSA	Bovine serum albumin
DC	Dendritic cell
DMAPP	Dimethylallyl diphosphate
FBS	Fetal bovine serum
FPP	Farnesyl diphosphate
FPPS	Farnesyl diphosphate synthase
GGPP	Geranylgeranyl diphosphate
HMBPP	(*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HMG-CoA	Hydroxymethyl-glytaryl-coenzyme-A
HPLC-ESI-MS	High performance liquid chromatography - negative ion electrospray ionization mass spectrometry
HPV	Human papilloma virus
HSP	Heat-shock protein
IL	Interleukin
INF	Interferon
IPP	Isopentenyl diphosphate
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
N-BP	Nitrogen-containing bisphosphonate
NK cell	Natural killer cell
NKT cell	Natural killer T cell

Non-N-BP	Non-nitrogen-containing bisphosphonate
NPP	Nucleotide pyrophosphatase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PP_i	Pyrophosphate
RIS	Risedronate
TCR	T cell receptor
TIL	Tumor infiltrating lymphocyte
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	T-regulatory cell
ZOL	Zoledronic acid

I REVIEW OF THE LITERATURE

Anti-tumoral immune response and bisphosphonates

1 INTRODUCTION

Cancer is a one of the major health problems and causes of morbidity and mortality worldwide. In Finland only, almost 30 000 new cancers emerge every year (Syöpärekisteri 2011). Breast cancer is by far the most common cancer among women, whereas men generally suffer from prostate cancer. Although 5-year cancer specific survival rates in these cancer types are high, up to one third of patients with advanced breast cancer develop bone metastases, which significantly decrease the long-term survival (Suva et al. 2011). Therefore the fight against these and many other cancer forms remains one of the fixed stars of our time in the field of science research. Within past decades scientists have taken massive steps forwards in developing new treatments, and the conventional cancer therapies, surgery, radiation therapy and chemotherapy, will in the foreseeable future yield to immune based treatments.

Cancers arise from the uncontrolled and progressive growth of progeny of single transformed cell (Janeway et al. 2001). Therefore, curing cancer means that all malignant cells must be removed or destroyed, without killing the patient. The growth of malignant tumors is determined mostly by the proliferative and invasive capacity of the transformed cells. The basic idea of using immune system to combat cancer dates back to 1890, when immunization against microbial diseases was found to be a success (Urban et al. 2010). In principle, targeting the immune system against tumors has been, and still is, a promising therapeutic strategy. Truth is, however, that the process has been slow and barely victorious because of the difficulties in distinguishing malignant cells from normal ones (Ge et al. 2011, Urban et al. 2010).

The recent clinical and preclinical experiments have established that although tumor cells are derived from human cells, they express certain antigens that are recognized as foreign agents by the immune system (Abbas et al. 2007). These tumor associated antigens are expressed either uniquely by tumors or at significantly higher levels by tumor cells than normal cells and their discovery has provided new tools for targeting immune responses to specific malignancies.

2 CANCER AND IMMUNOTHERAPY

The first attempts to fight cancer using basic immunology were seen in the early 1920s, when a surgeon, William Coley, developed a mixture of bacterial toxins known as Coley's toxin (D'Elios et al. 2009). He deliberately infected cancer patients with Coley's toxin and was able to induce even complete tumor regression in some patients. Next remarkable steps were taken in 1976, when T cell growth factor interleukin-2 (IL-2) was identified and cloned, and it became possible to study immune cells *ex vivo*. Current evidence strongly suggests a role for the immune system in the treatment of cancer for a few reasons: tumors are 100 times more likely detected from patients undergoing immunosuppressive medications than people with normal immune functions. Then again, spontaneous cancer regressions have been observed amongst patients with heightened activity of immune system. As scientists discover more and more about the functions of immune system every day, the expectations to create immune-based strategies against cancer keep increasing.

2.1 Properties and overview of immune responses

To understand the mechanisms of potential immune based cancer therapies, one must first appreciate the fundamental principles of the human immune system. Briefly, the immune system is usually defined as a number of mechanisms that work together to protect individuals from infections (Abbas et al. 2007). These mechanisms can, however, be elicited also by noninfectious substances like macromolecules, such as proteins and polysaccharides. The mechanisms of the immune system are highly complex, but can be roughly divided between innate and adaptive immune system. Innate immunity is responsible for the early reactions, and adaptive immune responses develop later, usually days after the infection, and are in charge of increased antigenic specificity and memory (Abbas et al. 2007, Janeway et al. 2001). These two, innate and adaptive immune system together, provide us an extremely effective defense system, which allows us to spend our lives surrounded by potentially dangerous microbes, and become ill relatively rarely.

2.1.1 Innate immune system

Innate immunity, also sometimes referred as natural or native immunity, consists of four components that are in place already before infection (Abbas et al. 2007). The earliest line of defense is epithelia, and the antimicrobial substances on its surface. If microbes pass the physical and chemical barriers, they face the cells of innate immunity, such as leukocytes (neutrophils, macrophages, dendritic and natural killer cells), circulating effector proteins (collectins, pentraxins and members of complement system) and cytokines, responsible for coordinating and regulating other cells of the innate immunity (Fig. 1A).

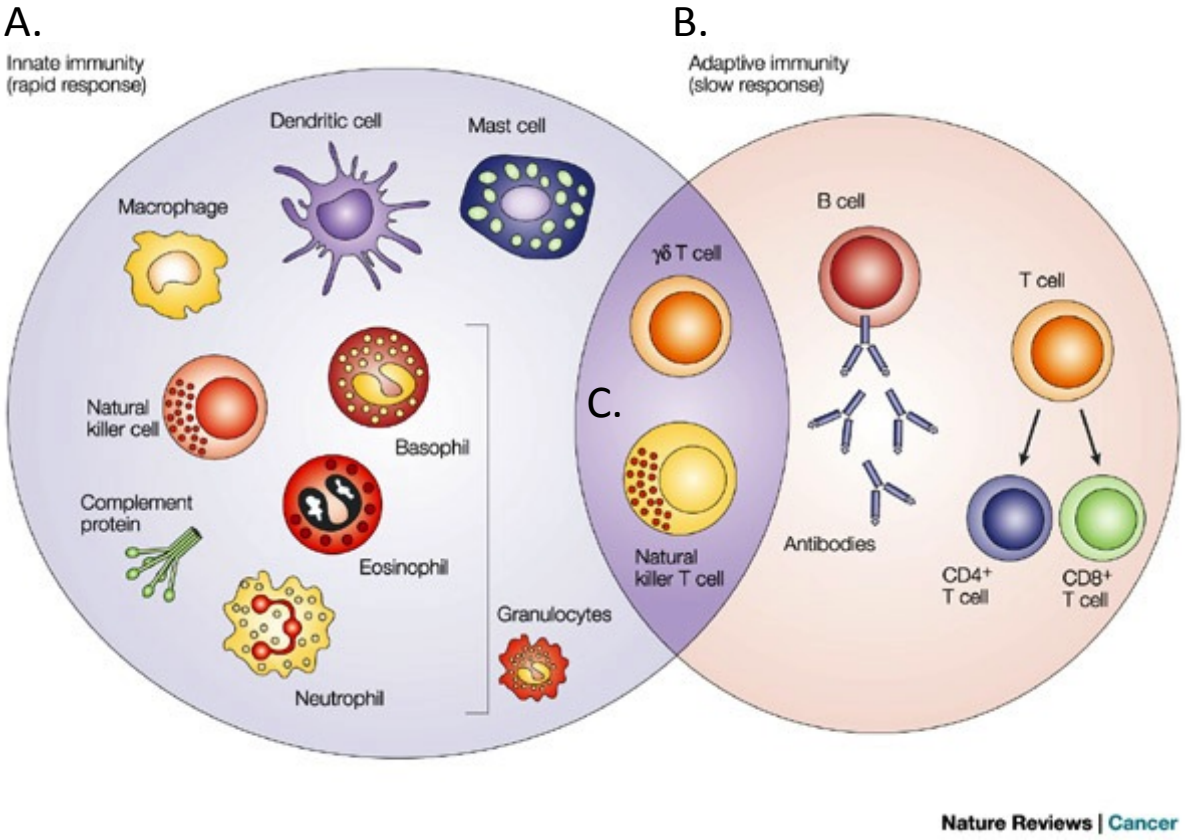

Fig. 1. A) The innate immune system consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. B) The adaptive immune system consists of antibodies, B cells, and CD4+ and CD8+ T lymphocytes. C) Natural killer T cells and γδ T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity (Dranoff 2004).

The innate immune system can recognize only limited number of structures that are characteristic to microbial pathogens (Abbas et al. 2007). These structures are not presented on mammalian cells, but are found for example on viruses, bacteria and fungi. Due to this specificity for microbial products the innate immunity, unlike adaptive immunity, is not known to react against self-structures in healthy tissues. On the other hand, innate immune system is able to recognize stressed, infected or injured host cells based on certain altered membrane phospholipids produced by these cells.

When microbes have breached epithelial barriers and entered into tissues or circulation, each cell type of innate immune system plays its own role (Abbas et al. 2007, Janeway et al 2001). Macrophages and natural killer (NK) cells use their surface receptors to recognize micro-organisms (like bacteria) and secrete cytokines and chemokines, which attract phagocytes from the bloodstream. The released cytokines and chemokines together with the complement system initiate a cellular reaction called inflammation. Inflammation can be compactly defined by words heat, pain, redness and swelling, all of which represent the effects of circulating effector proteins on the local blood vessels (Janeway et al. 2001). Quite similarly, also mast cells, a population of cells presented under many epithelia and in serosal cavities, respond to microbial products by secreting cytokines and lipid mediators to promote inflammation. Later, cytokines induce the expression of adhesion molecules, causing eventually the circulating leucocytes to migrate and bind to the vessel walls on the site of inflammation. Cytokines can also enhance the microbiological activities of phagocytes, and stimulate natural killer cell (NK cell) and T cell responses.

As more phagocytes (neutrophils and macrophages) migrate from blood into inflammation site, they begin to identify microbes, ingest them into vesicles by phagocytosis and destroy the ingested microbes in phagolysosomes (Abbas et al. 2007). Neutrophils are white blood cells, which mediate the earliest phases of inflammatory responses, and they can migrate to sites of infection within a few hours after the entry of microbes. Macrophages respond to microbial invasion equally fast as neutrophils, but they survive much longer at the site of inflammation, which makes them the dominant effector cells present few days after infection. NK cells are lymphocytes, which are able to recognize and kill infected cells by a combination of activating and inhibitory receptors. NK cells also provide a source of cytokine interferon-γ (INF-γ), which activates macrophages to destroy phagocytosed microbes. Dendritic cells (DCs) have phagocytic capabilities, but they also express pattern recognition receptors and respond to microbes by secreting cytokines. DCs can also capture and display

microbial antigens to T lymphocytes, which makes them as important linking connection between innate and adaptive immune responses.

2.1.2 Adaptive immune system

The adaptive immunity has a unique ability to specifically recognize all pathogens and to provide protection also against reinfections (Abbas et al. 2007, Janeway et al. 2001). In outline, there are two types of adaptive immune responses, called humoral immunity and cell-mediated immunity. Humoral immunity is mediated by antibodies produced by B lymphocytes, and cell-mediated immunity is conducted by T lymphocytes (Fig. 1B). Diversity and specificity of adaptive immune system is based on clonal selection of B and T lymphocytes that bear antigen-specific receptors (Janeway et al. 2001). The random recombination of variable receptor gene segments and the pairing of different variable chains generate these specific lymphocytes, which each bear a distinct receptor.

Adaptive immune system is needed when the innate immune responses fail to eliminate infections. Activation of the innate dendritic cells is a first step for induction of adaptive immune response. After uptake of a pathogen in infected tissue, DCs travel to a nearby lymph node, where they mature into antigen-presenting cells (APCs). APCs are able to activate pathogen-specific lymphocytes and secrete cytokines that influence both innate and adaptive immune responses. When activated lymphocyte encounters its specific foreign antigen, it can proliferate to daughter cells, which differentiate into effector cells and eliminate the infectious antigen. A part of these effector cells differentiates further into memory cells, which remain in the system for years after the infection and are ready to activate quickly if same pathogen is encountered again.

Different pathogens have distinct lifestyles, what for our immune system requires different response mechanisms to their detection, recognition and destruction (Janeway et al. 2001). For this purpose, lymphocytes have two different kinds of antigen receptors. The surface immunoglobulin receptors of B cells are adapted to recognize antigens that are presented outside the cells of the body, which is the most common site of bacteria. T cells, by contrast, have smaller antigen receptors, and they can detect peptide fragments generated inside infected cells, like those due to viruses. Peptide antigens must be attached to host proteins that are encoded by genes in major histocompatibility complex (MHC) and expressed on cell surface (Abbas et al. 2007).

T lymphocytes develop in thymus (hence the name) and can be divided into two functionally distinct populations called helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) (Abbas et al. 2007). $CD4^+$ T cells recognize peptides presented by MHC II antigens and they produce cytokines, whereas $CD8^+$ T cells recognize MHC I presented antigens and eliminate the reservoirs of infection by directly killing the infected cells. Another population of T lymphocytes is T-regulatory cells (Tregs), which are able to suppress the function of other T cells. Tregs are probably induced in an effort to regulate immune responses, and to maintain immune homeostasis and peripheral tolerance towards self antigens (Ge et al. 2010, Abbas et al. 2007).

In humoral immunity, activated B lymphocytes secrete antibodies that recognize and bind to microbial antigens and toxins, and promote their elimination by numerous effector mechanisms like activating phagocytosis and the complement system (Abbas et al. 2007). However, intracellular microbes such as viruses and some bacteria can survive and proliferate inside the host cells, where they are inaccessible to circulating antibodies. In cell-mediated immunity $CD4^+$ T lymphocytes secrete cytokines that enhance B cell antibody production and cytolytic T cell functions, as well as promote macrophages to kill phagocytosed microbes (Dranoff 2004). At the same time, $CD8^+$ T lymphocytes kill infected host cells primarily through the perforin-granzyme pathway and death ligands, like tumor necrosis factors (TNF) and transmembrane protein FAS.

In the interface of innate and adaptive immune system there are yet two populations of immune cells called as γδ T cells and natural killer T (NKT) cells (Fig. 1C) (D'Elios et al. 2009, Abbas et al. 2007). NKT cells form a numerically small and unique lymphocyte subpopulation, where the cells express both NK receptor and T cell receptor. T lymphocytes, expressing the γδ T cell receptor (TCR), represent a lineage, which clearly distinguishes from the more numerous MHC-restricted $CD4^+$ and $CD8^+$ T cells (Abbas et al. 2007, Morita et al. 1995). γδ T cells and NKT cells may represent an important bridge between innate and adaptive immunity, functioning as lymphocytes in the first line of defense against many pathogens. γδ T cells and their importance in cancer immunotherapy are further discussed in Chapter 3.

2.2 Immunotherapy for tumors

All cancers are said to be different, yet they all arise from the uncontrolled proliferation of transformed cells and spread clones to multiply (Abbas et al. 2007). The malignancy of tumor cells is largely determined by their proliferative capacity and their ability to invade host tissues and to metastasize to distant sites. In addition, another important factor in the growth of malignant tumors is their ability to evade or overcome the host immune surveillance.

Immunologic approaches in the treatment of cancer patients have held great promises because of their specificity for tumor cells (Abbas et al. 2007). These immunologic therapies can work essentially in three different ways (D'Elios et al. 2009). Firstly, the number of effector cells can be increased or cytokines can be added in the system, which both stimulate cytotoxicity of immune system. Secondly, tumor cells can be altered so that they become more immunogenic and also more vulnerable to immune cells. And finally, there are treatments that enhance patient's susceptibility to traditional cancer treatments, such as stimulation of the bone marrow function with growth factors during chemotherapy or radiation.

2.2.1 Immune recognition of tumors

Complex interactions with the host immune system shape tumors throughout their development and form the basis for cancer immunotherapies (Dougan and Dranoff 2009). Some of these host responses may inhibit tumor growth, but some can also provoke cancer by maintaining chronic inflammation and producing factors that drive tumor growth and angiogenesis. Sometimes, when transformed cells begin to proliferate, immune responses fail to prevent their development to malignant tumors because they still resemble normal cells in many respects (Abbas et al. 2007). Most tumors tend to be very poorly immunogenic because they express only few antigens, which can be recognized as foreign. The minority of tumors that elicit strong immune responses includes those induced by oncogenic viruses, or by carcinogens that often cause mutations in normal cellular genes.

Tumors grow and spread rapidly, and they can therefore overwhelm the capacity of immune system (Abbas et al. 2007). In order to prevent cancer from spreading, immune system would have to find and eliminate every single malignant cell. Naturally, tumors have also developed their own mechanisms to escape immune surveillance and improve their tolerance against

latest therapies (D'Elios et al. 2009). For example loss of tumor antigen expression, MHC downregulation, secretion of immunosuppressive cytokines or generation of Tregs illustrate well the capacity of tumor to transform in different tumor cells.

Anti-tumor immune responses are mediated through both antigen-specific and nonspecific mechanisms (D'Elios et al. 2009). Components of innate immune system detect cancer cells non-specifically, based on the cell surface molecules such as MICA and MICB, which are frequently expressed in the cancer cells (Fig. 2) (Dranoff 2004). These stress-related genes function as ligands for NKG2D receptors expressed on the surface of NK cells and other cytotoxic lymphocytes and macrophages. Dendritic cell in turn, can use CD36 and the α_V β_5 integrin to phagocytose apoptotic tumor cells. Macrophages and DCs also use scavenger receptors and CD91 to ingest heat-shock proteins (HSPs), complexed with tumor-derived peptides that are released from necrotic cancer cells.

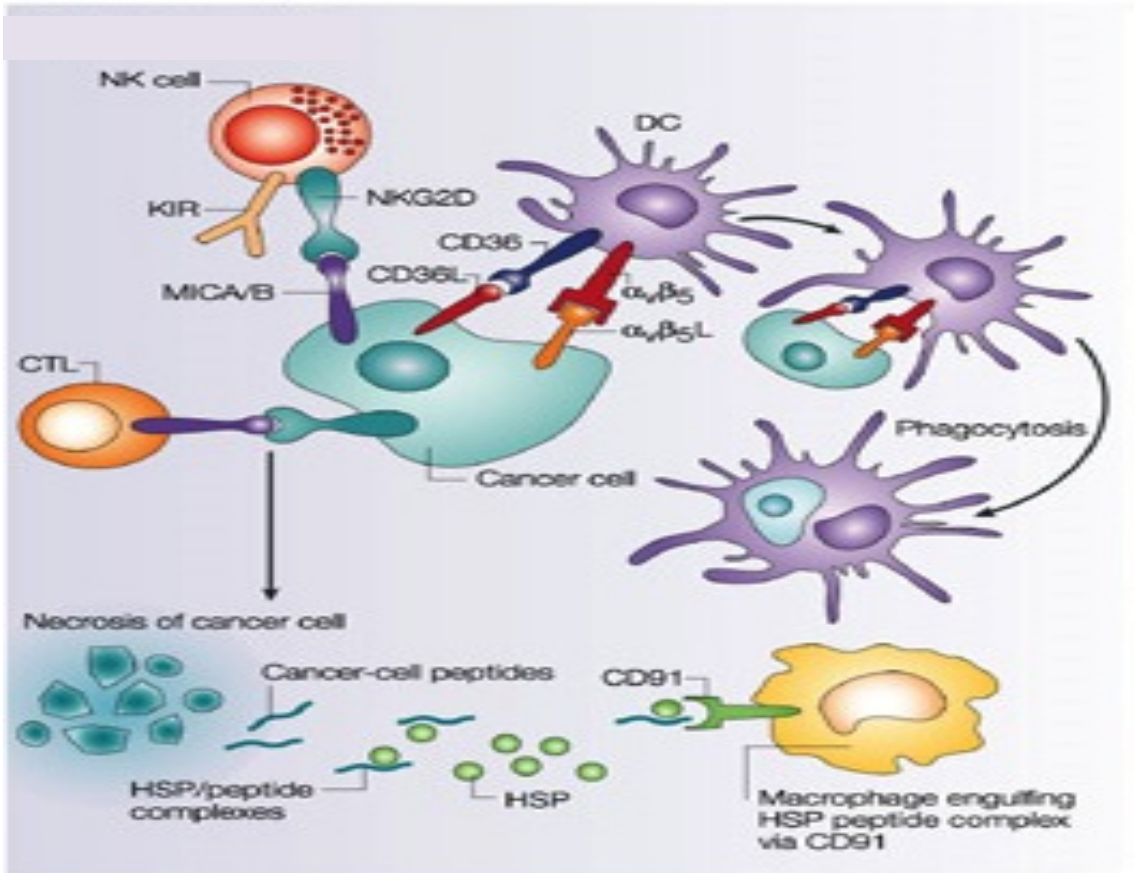

Fig. 2. Innate immune cells recognize cancers through germ-line encoded pattern-recognition receptors and other cell surface molecules (Dranoff 2004)

The adaptive immune system usually exploits an indirect pathway to recognize cancer cells (Abbas et al. 2007, Dranoff 2004). This mechanism is called cross-priming by DCs, because the dendritic cells can capture dying tumor cells or debris for MHC presentation (Fig. 3). DCs migrate to regional lymph nodes and stimulate tumor-specific lymphocytes. More specifically, these mature DCs express co-stimulatory molecules such as B7-1 and B7-2 which activate CD4+ and CD8+ T cells to react with the MCH-restricted tumor peptides produced by the cancer cells. Activated CD4+ T cells and NKT cells express CD40 ligand, which further stimulates DC maturation through CD40 signaling. Furthermore, CD4+ T cells and DCs can also trigger B cells to produce tumor protein-reactive antibodies.

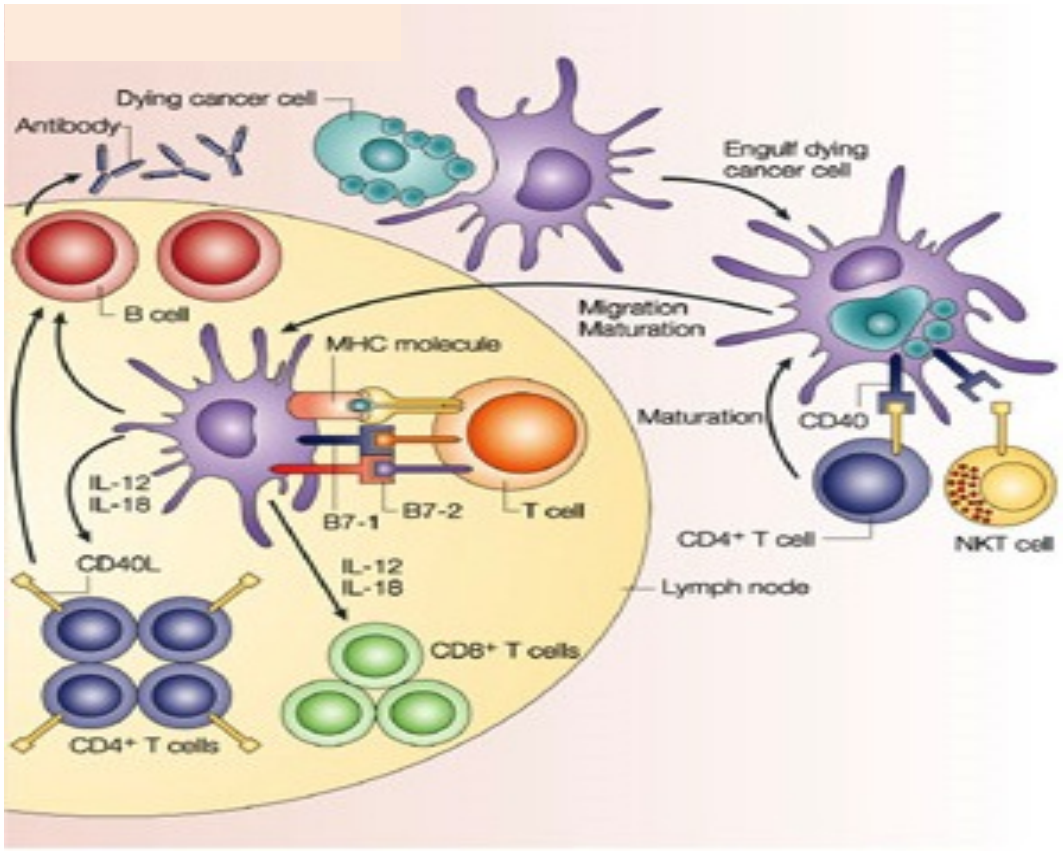

Fig. 3. Adaptive immune cells are initially stimulated to recognize cancer cells through cross-priming by DCs, because tumor cells do not express co-stimulatory molecules that are important for T cell activation (Dranoff 2004)

2.2.2 Established immunotherapies

Immune based cancer therapies have shown great challenges but also great opportunities in the past ten years (Dougan and Dranoff 2009, Abbas et al. 2007). As scientists have discovered more about the functions of immune system, new expectations have emerged for developing more effective immunotherapeutic expedients for prevention and treatment of cancer. Because of the substantial number of cancer types proven resistant to traditional treatments (surgery, chemotherapy and radiation), numerous approved immunotherapies are used to activate patients' own immunity (Dougan and Dranoff 2009). For example monoclonal antibodies, immune adjuvants, and vaccines against oncogenic viruses have already become standard treatments for a variety of cancers, and immune modulation is an integral part of supportive care for many high-dose chemotherapy treatments.

Monoclonal antibodies

The administration of tumor-targeting monoclonal antibodies has emerged since the middle of 1990s and proven to be one of the most successful forms of specific immunotherapy for cancer (Dougan and Dranoff 2009, Abbas et al. 2007). When manufactured monoclonal antibodies are infused to cancer patients, they generate immediate immune responses that bypass many of the limitations of endogenous immune response. Eight monoclonal antibodies (mAbs) are currently in clinical use in Finland, and four of them (rituximab, alemtuzumab, ofatumumab and ibritumomab tiuxetan) bind to surface proteins that are highly expressed in hematologic tumors (Duodecim Lääketietokanta 2011). Four others (trastuzumab, cetuximab, panitumumab and bevacizumab) are used in the treatment of solid tumors, although these have not shown similar efficacy than e.g. rituximab in Non-Hodgkin's lymphoma. For example epidermal growth factor receptor (EGFR) –targeting cetuximumab and panitumumab are used for the treatment of metastatic colorectal cancer in patients who are not responding to chemotherapy (Dougan and Dranoff 2009). Current mAb therapies are typically less toxic than conventional chemotherapy agents, although binding to non-malignant cells can sometimes cause significant adverse reactions. In addition, animal-origin of these antibodies can lead to hypersensitivity reactions.

Computational design methods have allowed also the modulation of mAbs and creation of dimeric bispecific antibodies from two different mAbs (Ge et al. 2010, D'Elios et al. 2009). This is achieved by combining two antigen binding sites, one directed against a tumor associated antigen of the cancer cell, while the other is directed against a triggering molecule

such as CD16 for NK cell. Also many other variations like coupling tumor-specific antibodies with toxic molecules, radioisotopes and anti-tumor drugs have been examined in order to improve their effectiveness (Abbas et al. 2007).

Cytokines

As discussed earlier, tumor cells induce only weak immune responses because they lack the cell-surface antigens recognized as foreign by immune cells (Abbas et al. 2007). One potential approach to boost host responses to tumors is to provide cytokines, which have potential both to induce nonspecific inflammatory responses and to stimulate T cells. Cytokines are small cell-signaling protein molecules, which posses immune-modulating properties, and can be delivered systematically to activate both innate and adaptive immune responses (Dougan and Dranoff 2009). Two most frequently used cytokines, IL-2 and INF-α, are used to treat advanced melanoma and renal cell carcinoma, both of which are usually resistant to chemotherapy. Another cytokine, TNF-α, is administrated locally to treat melanoma and soft tissue sarcomas of the limb. The side effects of cytokine administration can be severe. They resemble strong systemic infection, and are in most cases the reason for dose limitations. However, regardless of the limitations of this therapy, all these three and several other cytokines are able to induce durable responses as mediators of anti-tumor immunity.

Immune adjuvants

Non-specific approach to cancer immunotherapy is the use of immune adjuvants, locally administered immune-activating agents, which provoke local inflammation reaction and induce protective immunity (Ge et al. 2010, Dougan and Dranoff 2009). Adjuvants work by stimulating local antigen presenting cells, such as DCs, macrophages and B cells via Toll-like receptors (TLRs) and pathogen-associated recognition molecules. Clinical studies have shown that immune adjuvants are effective only against early stage tumors, but when combined with surgery they can be more effective than chemotherapy with very low risk for adverse reactions. These therapies include, for example, injection of live bacilli Calmette-Guérin (BCG) in superficial bladder cancer. The injection creates initial nonspecific inflammation, which leads to a strong innate immune response and tumor cell death (Ge et al. 2010). Besides the tumor cell death, patient usually maintains lasting cellular immunity against the bladder cancer. Another immune adjuvant is imiquimod, used in treatment of

external wards caused by human papilloma virus (HPV) and vulval intraepithelial neoplasia typically associated with HPV types 16 and 18 (Dougan and Dranoff 2009).

Vaccines

Classic vaccination strategies are also used against number of cancers that are caused by microbial infections directly or through the induction of chronic inflammation (Dougan and Dranoff 2009). The first available vaccine, that provided protection against an infection with known oncogenic potential, was the vaccine for the hepatitis B virus. More recently, a vaccine against HPV types 16 and 18 has been developed specifically to prevent cervical carcinoma and other genital epithelial tumors.

Supportive therapies

Supportive therapies, aimed at rescuing immune cells during chemotherapeutic regimens, have become a routine component of cancer treatments (Dougan and Dranoff 2009). These include, for example, infusions of granulocyte colony stimulating factors to prevent neutropenia and infection-related mortality after a high-dose chemotherapy. Folic acid derivate (leucovorin) is used to prevent bone marrow and gut epithelial cell loss after high-dose methotrexate treatment.

2.2.3 Novel approaches

Disappointingly, established nonspecific immunotherapies have only been effective for a small minority of fortunate people (Ge et al. 2011). Thus, the focus is now been directed towards specific humoral methods with more clinical success. Current knowledge has thus far lead to the generation of second generation immune-modulating antibodies, cytokine-based tumor vaccines and few specific *in vitro* T cell clones with auspicious anti-tumor activity (D'Elios et al. 2009, Dougan and Dranoff 2009). Yet, these specific immunotherapies are far from becoming commercially available, mainly due to many difficulties in conducting sufficient amount of randomized clinical trials. Despite the extensive range of approaches, it appears that all the latest strategies attempt either to extend patients' natural anti-tumoral immunity, or to interfere the immune-regulatory circuits that maintain tumor tolerance (Dougan and Dranoff 2009). These specific approaches, such as novel immune adjuvants, use of exogenous immune-activating stimuli, expansion of tumor-reactive T cells, and

antagonization of regulatory pathways of immune tolerance are likely used in future to create tailored treatment combinations for each patient.

Dendritic Cells

Dendritic cells (DCs) are found to possess valid significance as the most powerful APCs between innate and adaptive immunity (D'Elios et al. 2009). Because of their ability to activate several effector cells (NK, NKT, T and B cells), researchers have shown great interest in DC-based vaccinations. Monocytoid DCs are readily available from the peripheral blood monocytes and can be quickly activated *ex vivo* using cytokines and growth factors in order to induce cell differentiation and to increase immunogenicity (Ge et al. 2010, D'Elios et al. 2009). After the activation, DCs are pulsed with antigens and either re-injected into patient or used to expand anti-tumor lymphocytes, which will then be dosed to patient. An alternative approach bypasses *in vitro* activation, and injects immature antigen-loaded DCs directly into an inflamed tissue (Dougan and Dranoff 2009). Here, the maturation of DCs and migration to lymph nodes occurs more naturally. Already more than 150 clinical trials (by 2009) have shown that DC-based vaccines can be overall administered safely and with minor side effects (D'Elios et al. 2009). Although this therapy clearly induces antigen-specific T cell immunity, sufficient clinical responses are still slight, at least in late-stage tumors. More trials, where DCs are applied earlier during the course of the disease are needed in order to see whether the smaller tumor masses are more easily accessible to immune cells.

Natural killer and natural killer T cells

Ample strategies are also explored with a view to modulate either natural killer (NK) or natural killer T (NKT) cell functions (D'Elios et al. 2009). Both cell types play an important role in host defense against tumors, where the NK cell functions seem to be more poorly understood. Several clinical trials have corroborated the effects of systemic administration of cytokines, interferons and monoclonal antibodies in NK cell differentiation and activation, but still the therapeutic breakthroughs remain rather distant. For example difficulties with selection of NK cell type, crucial criteria of donor genotype, size of alloreactive subset, and the different immune status of the patients need to be exceeded before the development of potential adoptive NK transfer therapies.

More promising results have been obtained from the clinical trials with NKT cells (D'Elios et al. 2009). Although distinct subsets of NKT cells appear to hold different anti-tumor

capacities, they all are, when activated, usually able to induce cancer cell death through expression of various effector molecules (perforins, FasL, TRAIL, INF-γ and IL-4). NKT cell activation is tipped to occur either via direct recognition of tumor-derived antigens or glycolipid components, or it can also be associated with cytokine-delivered inflammation. However, because of human NKT cells are rare, clinical trials (phase I and II) have been carried out using DCs, pulse-treated with α-GalGer (a glycolipid antigen of CD1d in TCR), in order to expand NKT cells in *in vitro* cultures. Intravenous injections of these DCs have thus far shown only mild side effects, and the auspicious efficacy suggest their use as a potent new tool for immunomodulation.

T cells

One progression of cellular immunotherapy has been the use of tumor infiltrating lymphocytes (TILs) and the effector T cells found in the lymph nodes nearby tumors (Ge et al. 2010, D'Elios et al. 2009, Dougan and Dranoff 2009). In these therapies, T cells are harvested from the cancer tissue of the patients, manipulated and then reinjected to patient in order to generate productive tumor immunity. At least two different strategies can be applied herein. One is to manipulate T cells to express selected TCRs (with or without the antigen-binding domain of B cell receptor) for tumor recognition. Another way is to expand T cells either through exposure to specific tumor antigens or through stimulation with activating antibodies. Problems of these therapies are often related to insufficient amounts of T cells in cancer biopsies, slow manipulation process, and poor antigenicity leading to insufficient potency of TILs as a single treatment.

Also, other types of T cells are examined intensively. Allogeneic mixed lymphocyte reactive T cells and genetically modified T cells with suicide switches are few promising trends (Ge et al. 2010, D'Elios et al. 2009). Equally, methods to eliminate functions of T-regulatory cells would likely improve clinical results of cancer immunotherapy, and earnest investigation of Tregs in cancer has blossomed in the last five years. Last but not least, γδ T cells have also demonstrated impressive cytotoxic effector activity, and they are comprehensively discussed in the next chapter.

3 γδ T CELLS

The γδ T cell population was first described 25 years ago, and yet today these cells present challenges to our understanding (Urban et al. 2010). In fact, key questions about T cell selection, repertoire maintenance, activation, and the control of effector functions of γδ T cells remain still unanswered. The defensive role of γδ T cells in infections is certainly interesting, as these cells cannot be directly classified either as a part of innate or adaptive immunity. As a matter of fact, they seem to form an independent population of innate-like circulating lymphocytes, with very particular functions and distribution (Beetz et al. 2008, Kabelitz et al. 2007). In peripheral blood of most healthy adults, γδ T cells account for 1-10 % of circulating T cells. By contrast, in other anatomic locations, such as intestine or skin, they can constitute even the majority of all T cells.

3.1 Differentiation and development

Immune system relies on several types of functionally different T cell subsets, but essentially only two genetically distinct lineages exist: αβ and γδ T cell lineages (Beetz et al. 2008). The major subunit, which expresses αβ TCR, recognizes peptides presented on the surface of antigen presenting cells (APCs) by major histocompatibility complex. The cells of the minor subunit, composed of γ and δ chains, do not absolutely require this presentation, although the presence of APCs improves some of their direct responses. Obviously, this begs the question why immune system has maintained two different types of TCR throughout evolution, as there are no major differences between γδ and αβ T cells effector functions. Key for this question may reside in selective recognition, because γδ TCR seems to recognize a number of novel ligands that are not seen by conventional αβ T cells (Kabelitz et al. 2007). This provides an additional pathway for local immunosurveillance and also immediate significance for tumor defense. Most of the γδ T cells lack also the expression of CD4 and CD8, which supports the recognition of unconventional antigens by MHC nonrestricted means.

3.1.1 T cell development

Peripheral blood T lymphocytes develop normally from pluripotent precursor cells in thymus after complex series of signaling events (Urban et al. 2010). These thymocytes progress through at least 4 DN (double negative) stages (Fig.4). Somatic rearrangement of genes encoding the TCR chains β, γ and δ begins in DN2 stage, and γδ T cells emerge mostly from the DN2 and DN3 stages. Successful recombination of γ and δ chains leads to expression of γδ TCR on the cell surface. Then again, successfully recombinated β chains pair with a surrogate preTCRα chains and differentiate further into DN4 and DP stage, where they are selected to recognize either MCH class I or MCH class II molecules.

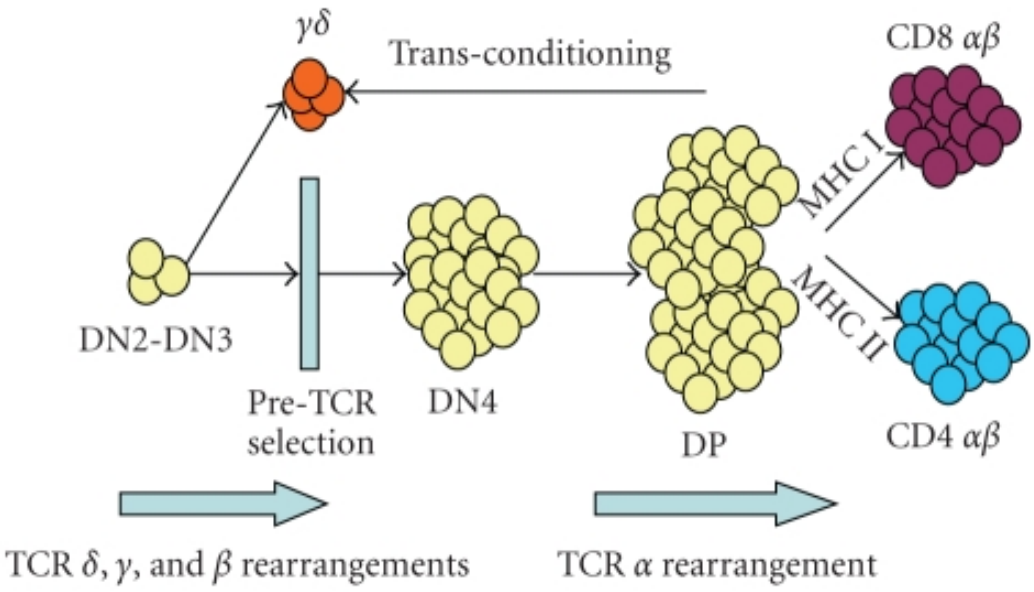

Fig. 4. Progress through different DN stages and rearrangement of TCR chains direct developing thymocytes to become either αβ or γδ T cells (Urban et al. 2010)

All chain proteins are encoded by genes with variable segments, but unlike αβ T cells, the available germ line repertoire of γ and δ genes is very small (Beetz et al. 2008). Nevertheless, other non-genetical mechanisms, such as joining and insertions assure that the combinational diversity of γδ TCR is at least as large as the one of αβ TCR. Shaping of this TCR repertoire takes place in childhood and the distribution varies even in healthy adult individuals tissue-specifically throughout life.

3.1.2 Vγ9Vδ2 T cells

Human γδ T cells can be subdivided in two main populations based upon δ chain expression (Urban et al. 2010). T cells expressing Vδ1 chain with different γ elements are often expressed in mucosal tissues like intestinal epithelium, where they are thought to maintain tissues integrity in case of damage or infection (Urban et al. 2010, Beetz et al. 2008). T cells that express Vδ2 gene are almost exclusively paired with Vγ9 chain, and despite the potential for diversity, Vγ9Vδ2 T cells account for more than 60 % of the total γδ T cell population in the circulating blood. This suggests either restricted chain pairing or a selective chronic expansion of this cell population (Urban et al. 2010). Studies that have compared the γδ genotypes derived from thymus and peripheral blood have found that thymic clones possessed nearby all possible Vγ-Vδ combinations at even amounts. Besides disproving restricted chain pairing theory, studies have also shown that nearly all peripheral γδ T cells acquire memory markers by the time individual reaches the age of two years. These results suggest that overrepresentation of Vγ9Vδ2 T cell subset is due to selective activation and expansion in response to environmental micro-organisms.

Overall, the Vγ9Vδ2 pairing is only present in T cells of humans and primates, which suggests that the role of Vγ9Vδ2 T cells in immune defense is quite specialized (Urban et al. 2010). It is not clear, how Vγ9Vδ2 T cells are rapidly and selectively activated by self-antigens, like isopentenyl diphosphate (IPP) produced in all mammalian cells, without causing any severe autoimmune reactions. More about γδ T cells activation and antigens will be discussed in the Chapter 3.2.

3.2 Activation and cytotoxic/effector functions of γδ T cells

As mentioned before, human γδ T cells form the interface between adaptive and innate immunity (Abbas et al. 2007). Rapid recognition of extensive number of antigens and lack of classical MHC restriction refer to innate immune system, whereas abilities for clonal expansion after antigen presentation and memory-type expansion during reinfection are features of adaptive immunity (Urban et al. 2010, Morita et al. 1995). In human infections, marked expansion of Vγ9Vδ2 T cells has been described at least with tuberculosis, leprosy, malaria, salmonella and streptococcus pneumonia (Casetti and Martino 2008). Activated γδ T cells have strong cytotoxic effector activity and they also produce various cytokines and chemokines like TNF-α and INF-γ (Beetz et al. 2008, Kabelitz et al. 2007). Recently, human γδ T cells have also been noted to have an important role in the initiation of adoptive immune responses. More precisely, γδ T cells seem to have capability to process complex protein antigens and then like APCs, present these antigens to other T cells.

3.2.1 Antigens

It has been shown that γδ T cells can recognize a variety of bacteria and parasites in a TCR dependent manner (Morita et al. 1995). In fact, most of the Vγ9Vδ2 TCRs recognize the phosphorylated intermediates from the bacterial isoprenoid biosynthesis pathway. This group of relevant antigens has been identified as nonpeptidic low molecular weight molecules called phosphoantigens (Fig. 5). All these phosphoantigens possess different potency but similar specificity for human Vγ9Vδ2 TCR (Urban et al. 2010, Caccamo et al. 2008). The most potent natural antigen by far is (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), a metabolite produced by bacteria or parasites through Rohmer pathway (Vantourout et al. 2009, Caccamo et al. 2008). Besides natural bacterial antigens, it is also possible to activate Vγ9Vδ2 TCR by number of strong and selective synthesized compounds like ethyl pyrophosphate, bromohydrin pyrophosphate (also known as Phosphostim) or 3-formyl-1-butyl pyrophosphate (Fig. 5). Some of these compounds are currently used in clinical trials, which investigate *in vivo* immunomanipulation of Vγ9Vδ2 T cells by phosphoantigens, and their possible use in the treatment of cancers.

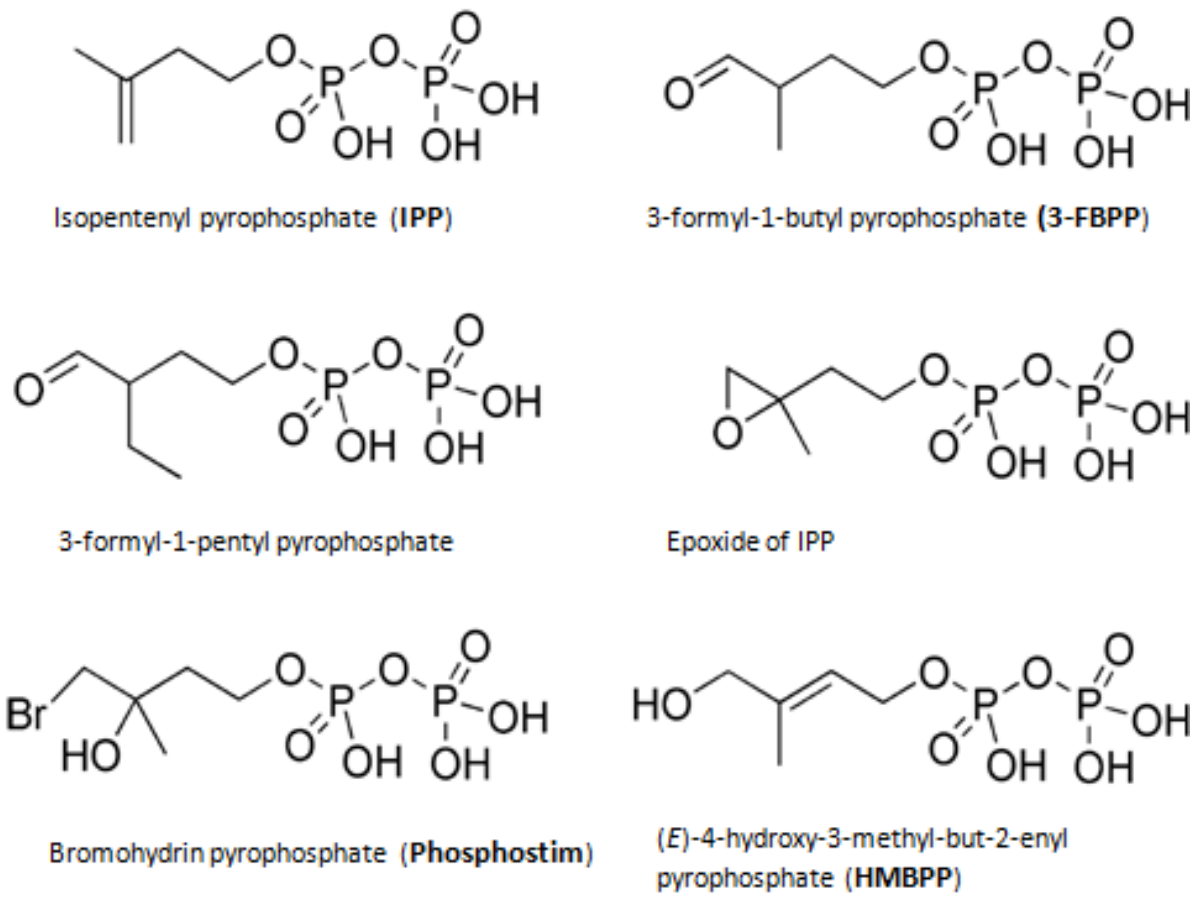

Fig. 5. Structures of some natural and synthetic phosphoantigens (Modified from Caccamo et al. 2008)

Instead of Rohmer pathway, eukaryotic cells use the mevalonate pathway for isoprenoid biosynthesis (Casetti and Martino 2008, Kabelitz et al. 2007). This pathway is essential for mammalian cells in the sterol synthesis, cell growth and membrane integrity. Mevalonate pathway generates IPP, which is also recognized by Vγ9Vδ2 T cell receptor, but only at 1000–10 000 fold higher concentrations than the HMBPP or the synthetic compounds. However, it has been found that certain tumor cell lines, like Daudi, naturally produce high concentrations of IPP, and are therefore sensed by the Vγ9Vδ2 TCR as tumor cells. And furthermore, certain drugs, like nitrogen-containing bisphosphonates, can manipulate the intracellular levels of IPP but this will be discussed later in the Chapter 4.3.2.

3.2.2 Mechanism of activation

The exact mechanism, that leads to activation Vγ9Vδ2 TCR by phosphoantigens remains still unclear, but the recognition does not require antigen entry and processing, or intracellular loading into an antigen presenting molecule (Morita et al. 1995). However, the activation seems to be dependent on direct cell-cell contact with APCs of human origin. These presenting cells can be either tumor cells or peripheral blood mononuclear cells, such as monocytes and macrophages (Stresing et al. 2007, Green et al. 2004). The cell-cell contact required for the activation suggests that phosphoantigens either induce structural modification of TCR, or that they are presented by unidentified surface molecules (Casetti and Martino 2008).

Besides phosphoantigens and TCR, some other ligands and receptors have been associated with the activation of γδ T cells. For example NK cell receptor (NKG2D) and TLR are shown to be involved in target cell recognition (Fig. 6) (Bonneville et al. 2010, Casetti and Martino 2008). NKG2D interacts with the stress inducible MICA/MICB proteins, which are frequently expressed in tumor cells. Also ATP synthase molecule expressed on the cell surface has been suggested to involve in the activation of γδ T cells (Clézardin 2011, Vantourout et al. 2010). This molecule will be discussed further in the Chapter 4.5.1. Based on the findings of these several γδ T cell ligands, researchers suggest that γδ T cells can recognize additively several separate receptors and ligands, and that these costimulatory signals should be taken into account when activating Vγ9Vδ2 T cells with phosphoantigens (Stresing et al. 2007).

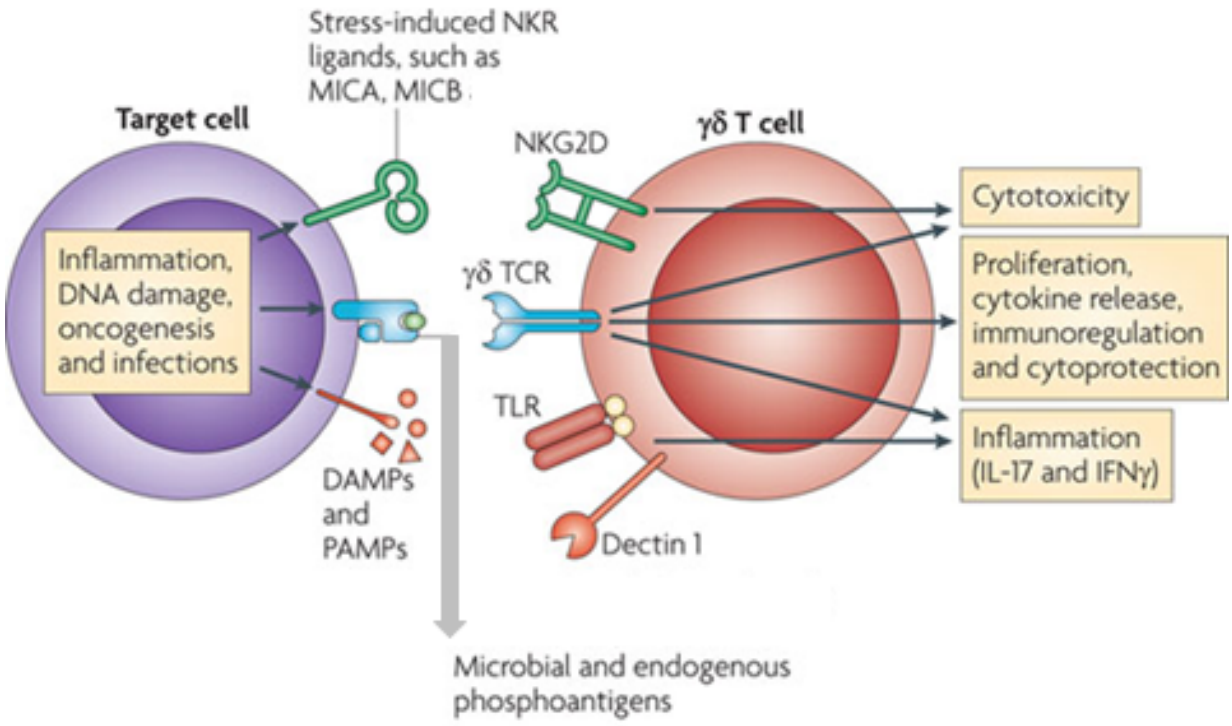

Fig. 6. γδ T cells can recognize separately, additively or synergistically three sets of stress-induced stimuli: TCR ligands (such as phosphoantigens), various cell surface molecules like MICA and MICB that bind to natural killer receptors such as NKG2D, and danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) recognized by pattern recognition receptors (TLRs and dectin 1) (Bonneville et al. 2010).

3.2.3 Human γδ T cell functions

Continuous cross-talk between γδ T cells and myeloid cells indicates that Vγ9Vδ2 T cells have an important role as an integral component of the innate and adaptive immune system (Casetti and Martino 2008). The broad array of effector functions of γδ T cells reflects their involvement in diverse physiopathological processes. Vγ9Vδ2 T cells are shown to express APC functions, and they are strongly involved in DC maturation, induced by cytokines produced upon activation.

γδ T cells can also kill infected, activated or transformed cells through pathways that involve the production of pro-inflammatory chemokines and cytokines, and the release of cytotoxic effector molecules like perforine and granzyme (Bonneville et al. 2010). These secreted chemokines and cytokines (e.g. TNF-α and IFN-γ) inhibit tumor cell growth and angiogenesis, and lead to stimulation of other immune cells like NK and NK T cells,

macrophages and αβ T cells (Casetti and Martino 2008). Furthermore, Vγ9Vδ2 T cells can downmodulate innate and adaptive effector cells through the production of immunosuppressive cytokines (TGF-β and IL-10) (Bonneville et al. 2010). They also contribute to tissue healing and epithelial cell regeneration through local release of epithelial cell growth- and survival factors, and recruitment of innate effector cells.

The implication of Vγ9Vδ2 T cells in the immune recognition of tumors has been demonstrated in different *in vitro* and *in vivo* observations (Kabelitz et al. 2011). γδ T cells have been consistently isolated and identified from various types of human cancer cell lines (colorectal, breast, prostate, ovarian and renal cell carcinoma). These Vγ9Vδ2 T cell lines and clones can recognize and kill not only the autologous tumor, but generally also a variety of related tumors, supposedly due to the identification of shared ligands. Studies have also demonstrated that activated Vγ9Vδ2 T cells from healthy donors have more cytotoxic activity against tumor cell lines than conventional αβ T cells. In addition, Vγ9Vδ2 T cells have shown anti-tumoral effects against tumors in mice (Malkovska et al. 1994) and it has been shown that Vγ9Vδ2 T cells can recognize a variety of cell lines like Daudi (Burkitt lymphoma) and RPMI 8826 (myeloma) (Fisch et al. 1990, Selin et al. 1992).

The ability of Vγ9Vδ2 T cells to develop immunological memory remains yet debated (Casetti and Martino 2008). Accelerated expansion of Vγ9Vδ2 T cells after a secondary infection reflects the occurrence of memory helper T responses, which can be interpreted as a demonstration of Vγ9Vδ2 T cell memory. However, the development of memory state may be quite different from that of conventional T cells, due to the ubiquitous natural phosphoantigens.

4 BISPHOSPHONATES

Bisphosphonates (BPs) are synthetic analogues of the naturally occurring pyrophosphate molecule (PP_i), which is characterized by its P-O-P backbone (Fig. 7) (Fleisch 2002). Instead of oxygen, the BPs have a carbon atom in their backbone, which makes them more resistant to hydrolysis. The bisphosphonates, also called as diphosphonates, have been used in textile industry for over a century, because of their ability to inhibit calcium carbonate precipitation. Only 40 years ago it was discovered, that pyrophosphate and its analogues have a biological activity. Fleisch et al. (1970) found that pyrophosphate inhibited calcium phosphate dissolution *in vitro* and prevented ectopic calcification *in vivo*. It was also discovered that pyrophosphate had no effect on normal mineralization and on bone resorption *in vivo*, and it was assumed that the compound was destroyed by phosphatases. Because of this, the group started to look for more stable analogues of pyrophosphate that might also have the antimineralisation properties, but would be resistant to hydrolysis (Russell 2011). Success was achieved with BPs, which had high affinity to bone mineral and were shown to prevent experimentally-induced pathological calcification (Fleisch et al. 1970).

4.1 Structure-activity relationship

The basic P–C–P structure of BPs allows many possible variations, and even small changes can alter the biological and physiochemical properties (Russell 2011, Fleisch 2002). The P-C-P backbone is responsible for the compound to bind in hydroxyapatite crystals of the bone. Variations in the structure are based on substitution of two side chains, referred to as R_1 and R_2, which are covalently bound to the central carbon atom (Fig. 7). The presence of a hydroxyl group at the R_1 position usually increases the affinity for calcium and thus bone mineral (Russell 2011). Stronger affinity is due to the ability of BPs to chelate calcium ions by tridentate rather than bidentate binding.

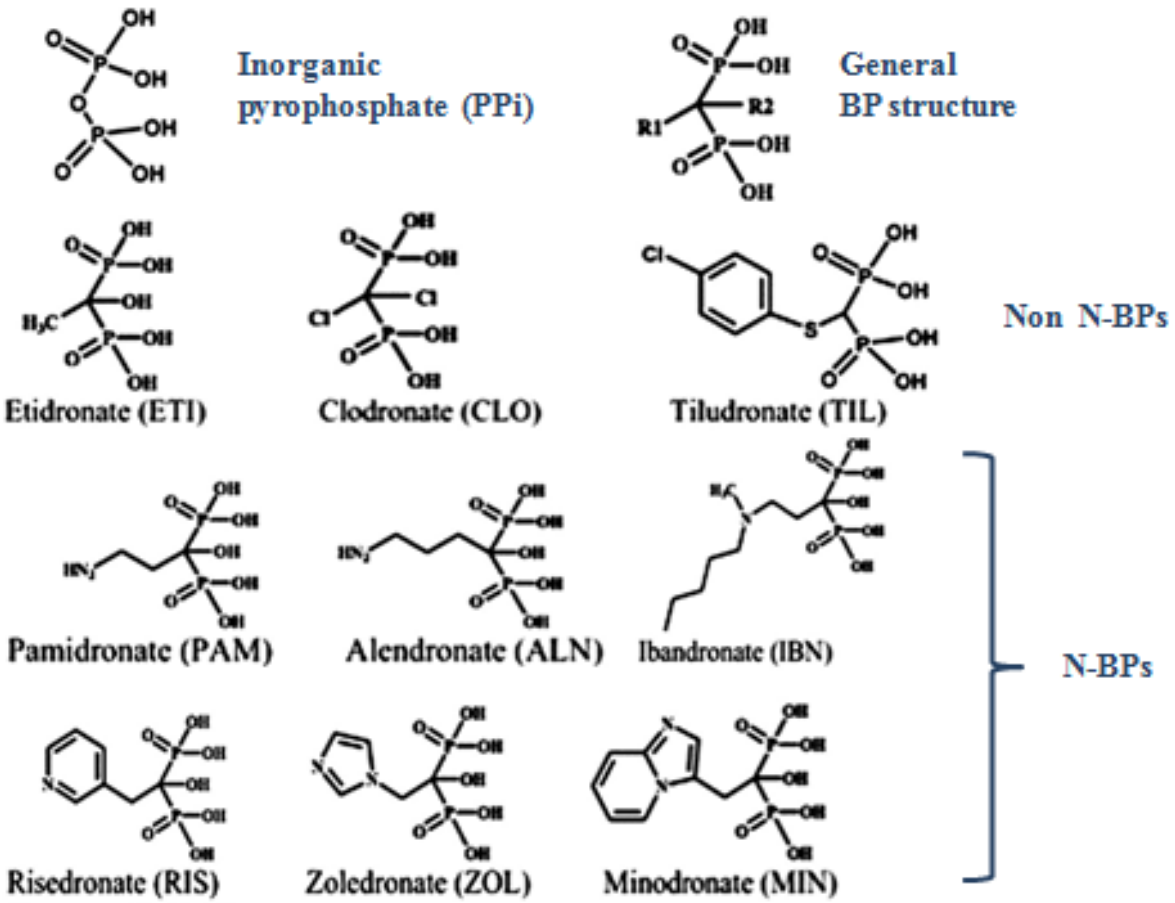

Fig.7. Structures of PP_i and some bisphosphonates used for clinical applications (Modified from Graham and Russell 2011)

The ability of BPs to reduce osteoclast-mediated bone resorption requires P-C-P structure but is also dependent on the structure of the R_2 side chain (Russell 2011). First studied BPs were clodronate and etidronate, and as they turned out success, more potent anti-resorptive BPs were created by changing the R_2 chain but keeping the R_1 chain unaltered (Fig. 7). In particular, BPs containing a basic primary nitrogen atom in an alkyl chain, like pamidronate and alendronate, were found to be significantly more potent without considerable difference in the inhibition of mineralization. The third generation of BPs, such as risedronate (RIS) and zoledronic acid (ZOL), contained a nitrogen atom within heterocyclic rings, and they were found to be even 1000-10 000 fold more effective than the simple BPs as inhibitors of bone resorption. Hence, for maximal potency, it is apparent that nitrogen atom must be placed in R_2 side chain, a critical distance away from the P-C-P group, and in specific spatial configuration.

4.2 Pharmacokinetics

A great number of different BPs has been investigated in humans to find the best effects in the inhibition of bone resorption (Fleisch 2002). Eight different compounds (Fig.7) are currently in clinical use (Minodronate only in Japan), and they are all indicated for one or several bone-related diseases such as osteoporosis, Paget's disease, cancer associated hypercalcemia and bone metastasis (Duodecim Lääketietokanta 2011). BPs can be administered to patients by oral or intravenous route, even though they are poorly absorbed after oral administration (Russell 2011). Based on the urinary recovery ratio after oral and intravenous administration, the oral bioavailability was about 0.7 % for alendronate, 0.3 % for pamidronate, and 3-7 % for etidronate (Lin 1996). BPs are also highly charged molecules and no intestinal transporters have been identified. BPs tend to form complexes with a number of polyvalent metallic ions (e.g. Al^{3+}, Ca^{2+}, Fe^{2+}, Mg^{2+}), which may lead to impaired absorption via paracellular transport (Stockley's Drug Interactions 2011, Lin 1996).

Once absorbed or injected in the blood stream, BPs exit the circulation rapidly and approximately 50 % of the dose accumulates to bone mineral surfaces (Lin 1996). BPs bind preferentially to sites of ongoing disintegration and regeneration of bone, and therefore the distribution is not homogeneous (Russell 2011, Lin 1996). Much of the efficacy and safety of this drug class is accounted for the accurate targeting to mineral surfaces with osteoclastic resorption (Rogers et al. 2011). Accurate targeting brings BPs to close extracellular contact with osteoclasts and osteocytes, but prevents prolonged exposure to most other cell types. The acidic pH in the bone resorption site causes the dissociation of BPs from the skeleton, after which osteoclasts uptake BPs by fluid-phase endocytosis.

Two important pharmacological properties of BPs are the progression of anti-resorptive effect with time and the meaning of total dose administered (Russell 2011). When BPs are given continuously, the inhibition of bone resorption reaches a steady state level instead of becoming progressively lower, because BPs accumulate in bone and seem to be inactive as long as they remain buried there. Also, it appears that equal inhibition of bone resorption is achieved regardless of whether the BP is given in small frequent doses or in larger infrequent doses. This fact has been used as a basis when developing bisphosphonate dosing regimens in man. BPs are not metabolized into inactive products and no drug derivates can be detected from the urine. Some BPs can undergo intracellular conversion to ATP derivates, but that is related to their mechanism of action, and will be discussed in the next chapter.

4.3 Mechanism of action

It is widely accepted that bisphosphonates exert their major effects on mature osteoclasts by affecting on their recruitment, differentiation and resorptive activity (Russell 2011). Up-to-date information has since shown that BPs might also inhibit bone resorption by preventing the formation of new osteoclasts from precursor cells. BPs have also been shown to disrupt the cytoskeleton of the osteoclasts and to induce apoptosis. After 40 years of clinical use, it is clear that bisphosphonates can be divided into two distinct groups according to their biochemical and molecular mechanisms of action (Rogers et al. 2011).

4.3.1 Non-nitrogen containing bisphosphonates

The first generation BPs, such as clodronate and etidronate, seem able to most closely mimic pyrophosphate (Russell 2011, Fleisch 2002). These compounds do not contain nitrogen moiety in their R_2 side chain. Once released from bone mineral surfaces and accumulated in osteoclasts, non-nitrogen-containing bisphosphonates (non-N-BPs) are metabolized into non-hydrolyzable cytoxic analoques of ATP (Rogers et al. 2011). The incorporation appears to be brought about by Type II class of aminoacyl-tRNA synthases (Rogers et al. 1996). When non-N-BPs are converted into AppCp-type nucleotides, they accumulate to high concentrations in the cytosol of osteoclasts, or other cell types that can effectively internalize BPs by endocytosis (Rogers et al. 2011). Often the intracellular AppCp accumulation eventually leads to osteoclast cell death, probably by interference with mitochondrial ATP translocases (Fig. 8) (Russell 2011).

In the light of current evidence, the group of non-N-BPs appears to act essentially as prodrugs converted to active drugs only after the intracellular uptake by osteoclast (Rogers et al. 2011). Etidronate was the first investigated BP and it, as well as clodronate, requires relatively high daily doses to inhibit bone resorption (Fleisch 2002). This dose is in rat more than 1mg/kg parentally every day, which is close to impair normal mineralization of bone. Therefore, requirements emerged for new BP compounds with greater efficacy but without higher restraint of mineralization.

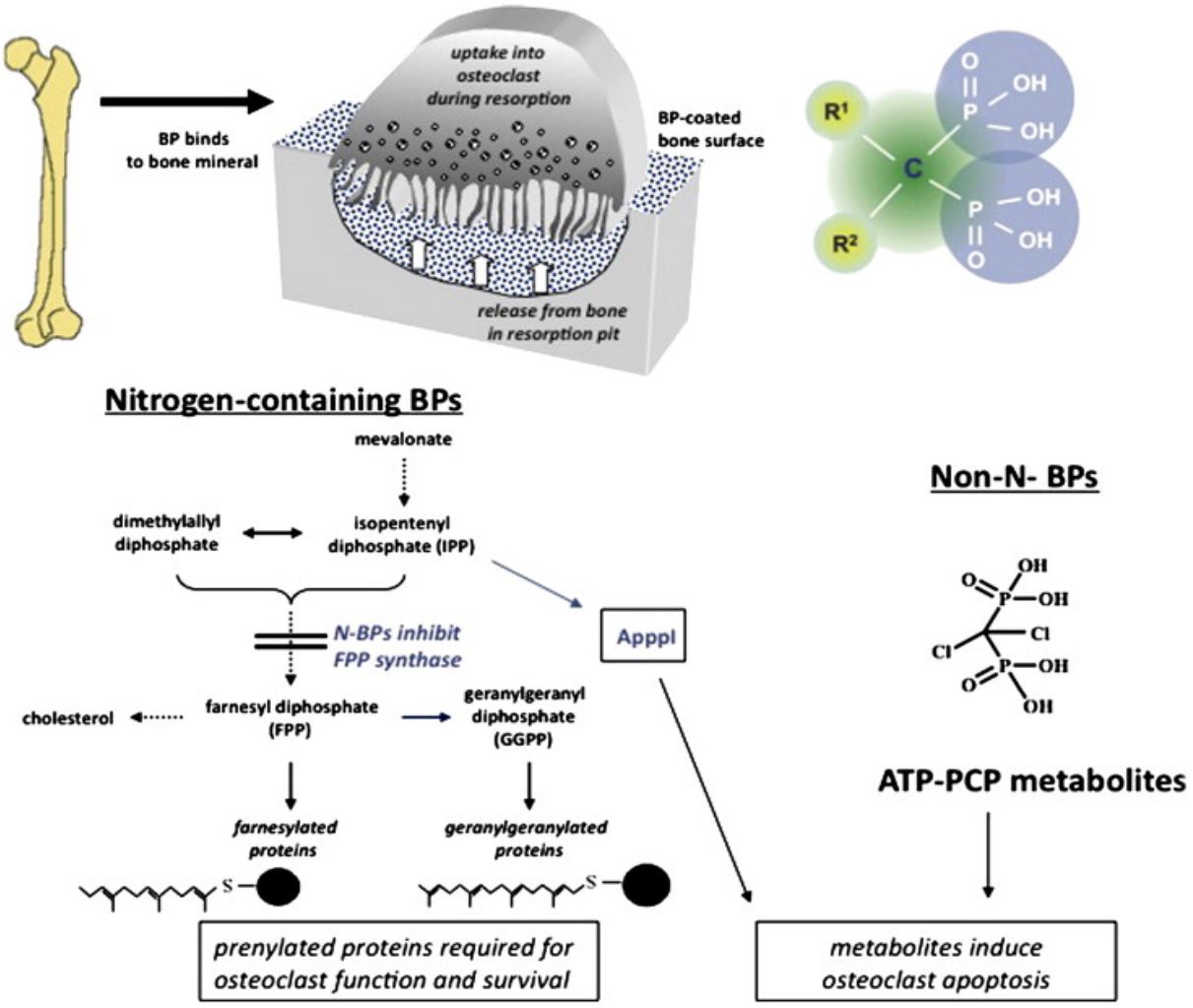

Fig. 8. The cellular and biochemical mechanisms of action of bisphosphonates (Russell 2011)

4.3.2 Nitrogen containing bisphosphonates

Nitrogen-containing bisphosphonates (N-BPs); pamidronate, alendronate, minodronate, risedronate, ibandronate and zoledronic acid, are up to several orders of magnitude more potent than non-N-BPs at inhibiting bone resorption (Rogers et al. 2011). N-BPs are not metabolized into AppCp-type nucleotides like non-N-BPs, but they inhibit the key enzymes of the mevalonate pathway (Roelofs et al. 2006, Luckman et al. 1998). Luckman et al. (1998) demonstrated, by using J774 macrophages, that N-BPs can inhibit the farnesyl diphosphate synthase (FPPS), an upstream enzyme of the mevalonate pathway (Fig 8).

Inhibition of protein prenylation

The primary function of the mevalonate pathway is the production of cholesterol as well as the synthesis of proteins with isoprenyl (farnesyl and geranylgeranyl) groups (Rogers et al. 2011, Russell 2011). Hence, the inhibition of FPPS directly leads to decrease in post-translational modification of isoprenoid lipids farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) (Fig. 8). These two isoprenoid lipids are shown to be essential for the prenylation of small GTPase signaling proteins, such as Ras, Rho, Rac, Cdc42 and Rab families (Rogers et al. 2011, Roelofs et al. 2006). Prenylation is required for the correct function of these signaling proteins, as it anchors the proteins in cell membranes and allows the interaction with other regulatory proteins. Prenylated small GTPases are essential signaling proteins that regulate many osteoclast functions like cytoskeletal arrangement, membrane ruffling and trafficking of intracellular vesicles. Disruption of their activity leads eventually to induction of cell death by apoptosis and could therefore account for most of the various described effects of N-BPs on osteoclasts.

In addition, also other inhibitors of the mevalonate pathway, such as statins, have a capability to mimic the effects of N-BPs (Rogers et al. 2011, Thompson and Rogers 2004). Statins are able to block the upstream enzyme hydroxymethyl-glytaryl-coenzyme-A (HMG-CoA), and thus lead to decrease in FPP and GGPP levels. Luckman and his group (1998) also showed with J774 cells, that alendronate-induced apoptosis could be partially overcome by adding components of the mevalonate pathway, especially FPP and GGPP.

Accumulation of IPP and synthesis of ApppI

It has been more recently discovered that inhibition of FPP synthase causes also accumulation of the upstream substrates isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the metabolites immediately upstream of FPPS in the mevalonate pathway (Fig. 9) (Rogers et al. 2011). The accumulation of IPP and DMAPP in cells, that have internalized N-BP, leads to generation of new metabolites ApppI (Mönkkönen et al. 2006) and ApppD (Jauhiainen et al. 2009). ApppI, or triphosphoric acid 1-adenosin-5’-yl ester 3-(3-methylbut-3-enyl) ester, is a metabolite in which the isopentenyl moiety is linked to AMP (Fig. 9).

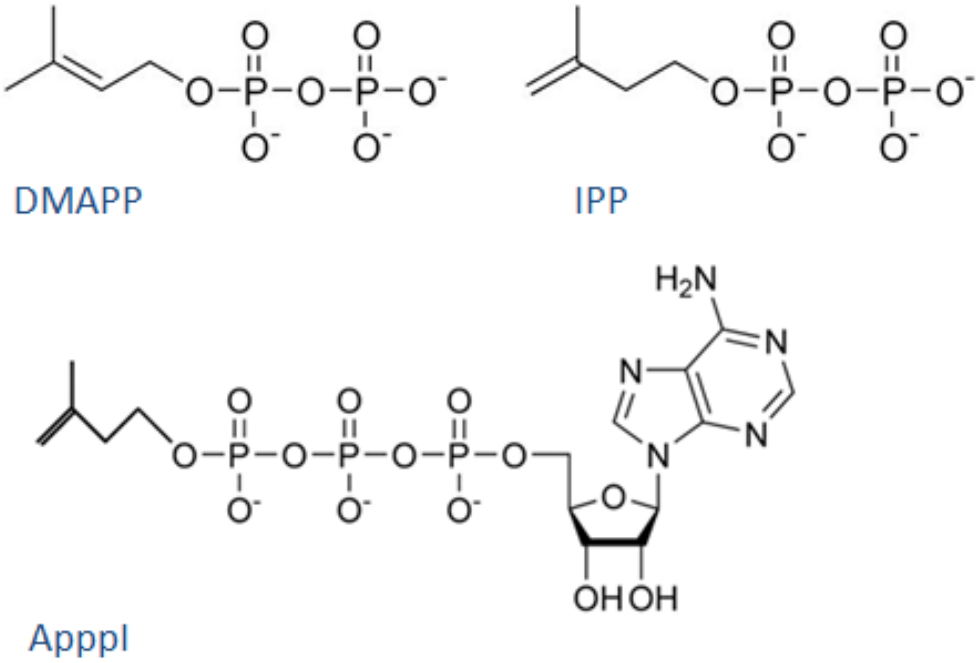

Fig. 9. Structures of endogenous mevalonate pathway metabolites

ApppI formation from IPP is probably catalyzed by the same enzyme family as the ATP analogues of non-N-BPs (Type II class of aminoacyl-tRNA synthases), but ApppI does not contain a bisphosphonate structure (Mönkkönen at al. 2006, Rogers et al. 1996). ApppI can induce direct target cell apoptosis through blockade of the mitochondrial ADP/ATP translocase (Mönkkönen et al. 2006). Current knowledge therefore suggests that N-BPs induce osteoclast apoptosis both via inhibition of protein prenylation of small GTPases and through the formation of the cytotoxic adenine nucleotide derivate, ApppI (Rogers et al. 2011).

4.4 Anti-tumor activity of bisphosphonates

Because of their capability to preserve bone tissue, the BPs have become a widely used treatment for tumor-associated bone diseases (Stresing et al. 2007). In the past few years, it has become increasingly clear that N-BPs have also direct and indirect anti-tumoral activities. These anticancer properties take effect on tumor cells of various origins by interaction with monocytes, macrophages, endothelial cells and tumor cells (Clézardin 2011, Stresing et al. 2007). The direct anti-tumor activity has been observed *in vitro*. Reduction of skeletal tumor burden and inhibition of bone metastases formation have been observed *in vivo*. However, whether these effects are caused by a direct action of N-BPs on tumor cells or indirectly through inhibition of bone resorption, are still debated.

4.4.1 Indirect effects

Extensive preclinical data suggests that BPs are able to reduce skeletal tumor burden and to inhibit the formation of bone metastases in animal models by effecting on the microenvironment of bone tissue (Stresing et al. 2007). In animal models of bone metastasis, the anticancer activity of N-BPs has been attributed to their ability to inhibit osteoclasts and thereby reduce the number of released growth factors. In addition of being effective against osteolytic changes, N-BPs have also been shown for example to impair some prostate cancer-induced osteoblastic changes (Corey et al. 2003) or to target directly to bone tumors such as osteosarcoma, and inhibit tumor growth and progression (Ory et al. 2005). In addition, N-BPs have been shown to reduce cytokine production of the cancer cells and prevent their spreading to bone by inhibiting cancer cells adhesion to the extracellular matrix *in vitro* (Stresing et al. 2007). The potency of N-BPs to prevent cancer cell adhesion to bone corresponds to their relative ability to inhibit osteoclast resorption *in vivo*.

Several N-BPs have been reported to interfere with tumor cells angiogenic process by interfering with the proliferation or mobilization of bone marrow-derived endothelial progenitor cells (Clézardin et al. 2011, Stresing et al. 2007). Several N-BPs have been reported to reduce endothelial cell proliferation and migration, and to decrease capillary-like tube formation by human umbilical vein endothelial cells *in vitro* (Stresing et al. 2007). *In vivo* N-BPs inhibit experimental angiogenesis in models such as the rat aortic ring or chicken egg membrane assays (Clézardin et al. 2011). However, often doses used to inhibit angiogenesis in experimental models are too high to be comparable with approved clinical

dosing regimens used to treat patients. Therefore, it is no surprise that for example clinical doses of ZOL had no effect on vascularization in rat models of bone healing (Biver et al. 2010). It has also been proposed that the high bone mineral affinity of N-BPs leads to insufficient exposure of bone marrow endothelial cells to the N-BP when compared to *in vitro* models. (Clézardin et al. 2011).

4.4.2 Direct effects

N-BPs are shown to exhibit direct anti-tumor capacity associated with programmed cell death (Clézardin 2011). The main mechanism, through which N-BPs induce apoptosis, appears to be the inhibition of FPPS in the mevalonate pathway (Stresing et al. 2007). Inhibition of FPPS prevents the prenylation of small GTPases leading to downregulation of their signaling as discussed earlier (Chapter 4.3.1, Fig. 8). The exact mechanism through which apoptosis is induced can however vary according to the cell type and BP used.

The use of N-BPs together with cytotoxic drugs is an established principle in cancer therapy, because of the synergistic interactions of these drug combinations (Ottewell et al. 2009, Stresing et al. 2007). *In vitro* studies have shown the potential for N-BPs to induce direct antitumor effects including inhibition of tumor cell growth, induction of apoptosis, inhibition of adhesion and invasion and antiangiogenic activity. ZOL seems to be the most promising candidate for the combination treatments, as it has been shown to synergistically increase cancer cell death when combined for example with doxorubicin, paclitaxel or cyclophosphamide in breast cancer cells or with paclitaxel, etoposide, cisplatinum and irinotecan in lung cancer cells.

The potential cooperative anti-tumor effects of clinically relevant doses of N-BPs *in vivo* are investigated in considerably smaller number of studies. For example Ottewell et al. (2008) showed that treatment with doxorubicin (2 mg/kg) followed by single injection of ZOL (100 ug/kg) reduced intraosseous tumor growth in the BO2 model of breast cancer bone metastasis. Also they have shown that 6-week course with weekly administration of doxorubicin, followed 24 h later by ZOL, was able to cause substantial inhibition of MDA-MB-436 breast tumor burden in bone in an *in vivo* model (Ottewell et al. 2009). This data was also the first to show the specific molecular pathways, by which this combination treatment induces tumor cell apoptosis and inhibits proliferation compared to administration of the single agents.

As mentioned before, doubt exists whether some anti-tumoral effects of N-BPs are caused by their direct action on tumor cells, or indirectly through inhibition of bone resorption (Clézardin et al. 2011). Several researcher groups have therefore used different structural phosphonocarboxylate analogues of RIS to study these mechanisms and to determine whether the high affinity of N-BPs for bone mineral limits their anti-tumoral potential. These analogues posses either lower bone mineral affinity or different potencies to inhibit FPPS activity (Stresing et al. 2011, Fournier et al. 2010 and 2008). In their experiments, Fournier et al. (2008) demonstrated that weak bone mineral affinity led to release of higher concentration of BP near the bone mineral surface, and reduction of skeletal tumor growth at a dosage that does not inhibit osteolysis. Therefore, it can be assumed that direct anti-tumor activity takes place and that the high affinity of bisphosphonates for bone mineral might limit their direct anti-tumor potential *in vivo*.

4.5 Immunomodulatory effects

Distinct feature of N-BPs is to cause flu-like symptoms in patients (Russell 2011, Dieli et al. 2007). These acute phase responses are sometimes called also 'post-dose' symptoms and they occur in most cases few days after the first exposure to N-BP, especially with intravenous administration. These symptoms have been attributed to pro-inflammatory cytokines, which are released by IPP-activated γδ T cells. Indeed, dosing N-BPs causes the inhibition of FPPS in peripheral blood mononuclear cells (PBMCs) and leads to accumulation of IPP, which is then presented to Vγ9Vδ2 T cells (Stresing et al. 2007). The triggering of γδ T cell activation leads consequently to the release of cytokines like IL-2 and INF-γ, leading to flu-like symptoms.

4.5.1 Activation of Vγ9Vδ2 T cells by N-BPs

As noted, N-BPs are able to block the FPPS (Chapter 4.3.1, Fig. 8), and thereby induce accumulation of IPP and DMAPP in monocytes from human peripheral blood mononuclear cells (Gober et al. 2003). Increased amount of IPP leads to proliferation of Vγ9Vδ2 T cells from PBMCs (Benzaid and Clézardin 2010). In contrast, statins are able to block the HMG-CoA reductase and thereby inhibit the production of IPP and further prevent the expansion of γδ T cells (Caccamo et al. 2008, Thompson and Rogers 2004). Also other metabolites of the mevalonate pathway, FPP and GGPP, can stimulate proliferation of Vγ9Vδ2 T cells, but their potency is substantially lower compared to IPP (Benzaid and Clézardin 2010). The cytotoxic metabolite ApppI does not appear to have intrinsic stimulatory activity on Vγ9Vδ2 T cells, though it is undoubtedly present in the Vγ9Vδ2 cell-sensitive tumors (Vantourout et al. 2009).

Vantourout et al. (2009) demonstrated that in the absence of APCs, optimal activation of Vγ9Vδ2 T cells with ApppI requires addition of cleavage enzyme. This enzyme, nucleotides pyrophosphatase (NPP), cuts the O-P bond of ApppI leading to release of IPP and AMP (Fig 10). Also HPLC analysis revealed that latent phosphoantigenic activity is stored in Vγ9Vδ2 cell-sensitive Daudi Burkitt's lymphoma tumors and can be activated by NPP. Therefore it has been suggested that ApppI serves as an inactive storage form of phosphoantigen, which may then bind to carrier proteins and remain protected from degradation, but which requires conversion to IPP in order to take effect.

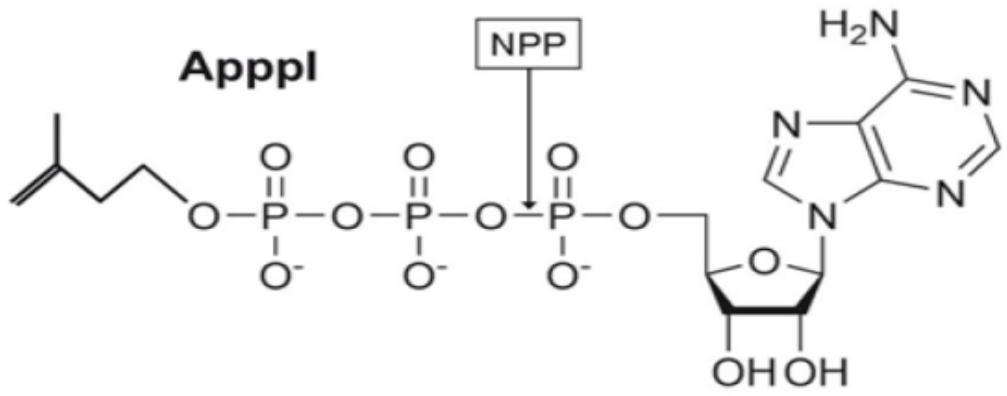

Fig.10. Structure of ApppI and the cleavage site for NPP (Vantourout et al. 2009)

By far, researchers have not succeeded in crystallizing the Vγ9Vδ2 T cell receptor with the phosphoantigens (IPP or HMBPP), but according to present knowledge, at least the bacterial phosphoantigen (HMBPP) is presented to TCR by a specific cell surface antigen–presenting molecule (Benzaid and Clézardin 2010). Therefore it is unlikely that other phosphoantigens would directly bind to Vγ9Vδ2 T cell receptor. One suggestion is that a particular cell surface enzyme, ATP synthase (AS), might be involved in activation of Vγ9Vδ2 T cells in a TCR dependent manner (Clézardin 2011, Vantourout et al. 2010). In this theory, IPP would form a complex with the cell surface AS in order to be recognized by the TCR (Fig. 11).

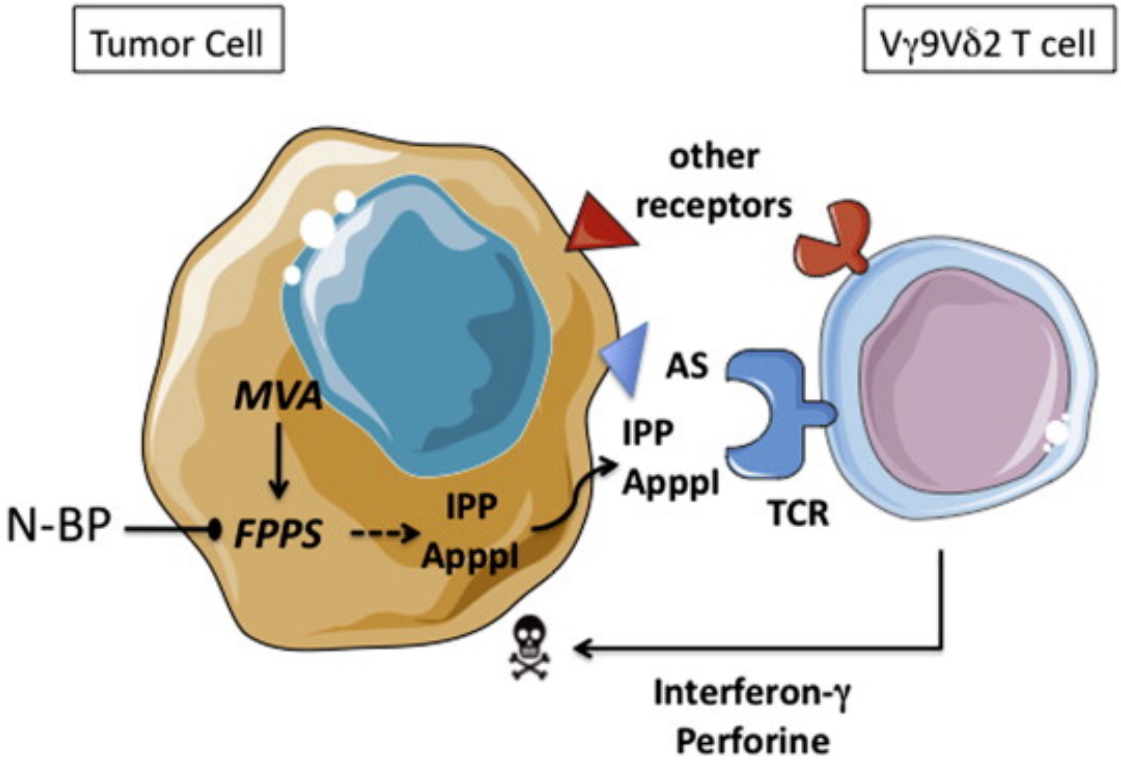

Fig.11. Activation of human Vγ9Vδ2 T cells by N-BP treated cancer cells. N-BP is internalized by cancer cells and inhibits FPPS, causing accumulation of the endogenous metabolites IPP and ApppI. Both metabolites are then released by cancer cells and could bind to a cell surface antigen-presenting molecule such as ATP synthase (AS), in order to be recognized by the Vγ9Vδ2 T cell receptor (TCR). Recognition of IPP/ApppI by Vγ9Vδ2 TCR leads to activation of γδ T cells and release of interferon-γ and perforin, which induce cytotoxicity against cancer cells. (Clézardin 2011).

4.5.2 Cytotoxic effects of N-BP activated γδ T cells and their role in the anti-tumor immune response

Despite the various and yet partially unclear functions of the receptors, it is a foregone conclusion that the internalization of N-BPs by cancer cells causes intracellular accumulation of IPP and ApppI (Clézardin 2011, Benzaid et al. 2011). These mevalonate pathway metabolites are then cell surface exposed and presented to Vγ9Vδ2 T cells. Thereafter, the activation of Vγ9Vδ2 TCR by ligation leads to proliferation of the immune cells and efficient killing of tumor cells *in vitro*. Yet, the significance of these findings *in vivo* remains largely unknown.

Preclinical findings

Several N-BPs like pamidronate, ibandronate, alendronate, risedronate and zoledronic acid have shown to induce a significant expansion of γδ T cells, both *in vitro* and *in vivo* (Clézardin et al. 2011, Stresing et al. 2007). Vγ9Vδ2 T cells, purified from human PBMCs and activated by N-BPs, have demonstrated potential antitumor effects *in vitro* in several studies, and they have found to maintain that activity upon adoptive transfer into immunodeficient mice baring human tumors (e.g. lung cancer, bladder cancer, pancreatic adenocarcinoma, melanoma, leukemia). Antitumor activity of Vγ9Vδ2 T cells against different tumor cells treated with N-BPs varies significantly, but also the vulnerability of different tumor cell lines to Vγ9Vδ2 T cells varies.

Benzaid et al. (2011) demonstrated that Vγ9Vδ2 T cells were able to infiltrate and inhibit tumor growth in ZOL treated mice bearing subcutaneous breast xenografts. This inhibition was significally higher with cancer cells known to produce high IPP/ApppI levels after ZOL treatment than with cancer cells that did not produce IPP/ApppI. Therefore the group has suggested that IPP and ApppI are chemotactic factors for Vγ9Vδ2 T cells, and that cancers producing high IPP/ApppI levels after N-BP treatment would be most vulnerable for the γδ T cell-based immunotherapy. One suggestion for effective T cell-mediated immunotherapy involves a double strategy to exploit Vγ9Vδ2 T cells (Stresing et al. 2007). This kind of treatment would include first an infusion of N-PB together with IL-2 to stimulate patients' own γδ T cells, followed by an adoptive cell transfer of *in vitro* expanded Vγ9Vδ2 T cells.

Clinical findings

Dieli et al. (2003) reported that ZOL, combined with IL-2, is able to activate Vγ9Vδ2 T cell effector functions in patients with solid tumors. In their report, the authors found that intravenous ZOL treatment lead to long-term and substantial Vγ9Vδ2 T cell specialization towards INF-γ producing effector phenotype. Also, the same group has clearly shown that combination treatment with IL-2 and ZOL leads to better results that monotherapy with ZOL alone (Dieli et al. 2007). Indeed, whereas only two of nine patients treated with ZOL alone achieved a clinically relevant response, almost two of three patients treated with ZOL + IL-2 showed a correlation with tumor response and absolute numbers of γδ T cells. Moreover, the group has also concentrated in assessing the potential toxicity of the drugs and found no severe adverse effects or consequences of administrating ZOL *in vivo* in the presence or absence of IL-2.

In general, Dieli's group (2003) has identified several diverse subsets of human γδ T cells: Central Memory cells (CM), Effector Memory cells (EM), and Terminal Effector cells (TE). CM γδ T cells are highly proliferative whereas EM γδ T cells produce INF-γ, and TE γδ T cells express cytotoxic functions. Based on the described effects, Dieli et al. (2003) concluded that *in vivo* treatment with ZOL induces Vγ9Vδ2 T cells to mature toward an INF-γ-producing effector phenotype, which is able to induce more effective antitumor responses.

Also, Santini et al. (2009) conducted an observational perspective study to evaluate the immunomodulating properties of a single-dose ZOL (4 mg) on Vγ9Vδ2 T cells in disease free breast cancer patients. They found that ZOL was not able to induce significant changes in the absolute numbers of the Vγ9Vδ2 T cells during the treatment. However, ZOL did induce redistribution among the γδ T lymphocytes subsets. First, the number of peripheral blood CM γδ T cells decreased significally, whereas the amount of EM and TE γδ T cells increased. Later, after two and three months, the number of peripheral blood EM and TE γδ T cells also decreased, which suggests that ZOL has the ability to activate effector subsets and stimulate their migration into peripheral tissues where they are most effective. Another interesting finding of this group is the long-lasting immunomodulating capacity of a single infusion of ZOL. Because of the very short plasmatic half-life of ZOL, the persistent effects must be explained otherwise. Santini et al. (2009) suggest that ZOL accumulates in the bone where it has the half-life of 150-200 days. Afterwards, ZOL is constantly released from the bone and is able to stimulate the activation of peripheral blood Vγ9Vδ2 T cells.

However, in these and other studies, there appears to be always one subset of patients, who express no changes in the kinetics of the γδ T cells (Santini et al. 2009). The immunological unresponsiveness of some patients is most likely related with their clinical characteristics, but yet no significant correlation has been found and the different behavior remains unanswered. Overall, the fact that the clinical outcome in several studies has been influenced by the type of regimen used to activate γδ T cells strongly suggests that γδ T cell activation is at least partially causal to the anti-tumoral immune response (Dieli et al. 2007). The combined clinical findings regarding the Vγ9Vδ2 T cells suggest that N-BPs, especially ZOL, have immunomodulating properties, which need to be investigated further.

II EXPERIMENTAL PART

Cytotoxic potency of Vγ9Vδ2 T cells against N-BP-treated breast cancer cells

5 INTRODUCTION

Nitrogen-containing bisphosphonates (N-BPs) have become a standard treatment of different bone loss disorders such as postmenopausal osteoporosis and cancer treatment-induced bone loss (Clézardin 2011). In addition to these traditional applications, N-BPs have provided strong evidence of both direct and indirect anti-tumor properties. These anticancer properties result from inhibition of FPPS, which interferes in the functions of mevalonate pathway, and prevents prenylation of small GTPase signaling proteins, such as Ras, Rho and Rab families. The most noted effect of N-BPs is directed towards bone, where it has been found to inhibit the release of bone-derived growth factors during bone resorption. This makes the bone marrow a less favorable environment for cancer cell colonization. In addition, current preclinical data suggests that N-BP treatment can also inhibit tumor cell invasion, proliferation, adhesion and angiogenesis both *in vitro* and *in vivo* with concentrations that reflect the clinical reality (Clézardin 2011, Stresing et al. 2007). Furthermore, combination of N-PBs and cytotoxic agents has resulted several profitable anti-tumor effects, for example in animal models of skeletal lesions.

In addition, current knowledge states, that N-BPs represent some immunomodulatory properties, which can be exploited in therapeutic applications (Clézardin 2011, Stresing et al. 2007). For example, treatment of human PBMCs with pharmacologically relevant concentrations of N-BPs, such as RIS and ZOL, has been found to induce proliferation of Vγ9Vδ2 T cells when cultured *in vitro* (Roelofs et al. 2009). Recently, this expansion was confirmed *in vivo* after intraperitoneal injection of human PBMCs, ZOL and IL-2 into SCID-NOD mice (Benzaid et al. 2011). Proliferation of Vγ9Vδ2 T cells is explained by the internalization of ZOL in antigen presenting cells and inhibition of the activity of FPPS in their mevalonate pathway. This inhibition leads to intracellular accumulation of IPP, which is an agonist of the Vγ9Vδ2 T cell receptor (Roelofs et al. 2009). Also the ATP analogue ApppI, converted from IPP (Mönkkönen et al. 2006) can act as a phosphoantigen and trigger Vγ9Vδ2 T cell proliferation from PBMCs (Vantourout et al. 2009). Debate exists, whether ApppI really has any stimulatory activity on Vγ9Vδ2 T cells as such or does it require conversion to IPP to activate γδ T cells, which would make ApppI only an inactive storage form of phosphoantigen (Vantourout et al. 2009).

The anti-tumor activity of Vγ9Vδ2 T cells against different tumor cells treated with N-BPs varies significally, and interestingly also IPP/ApppI production varies between different cell lines (Benzaid et al. 2011, Mönkkönen et al. 2008). It is therefore assumed, that Vγ9Vδ2 T cells sense these mevalonate pathway metabolites as a tumor phosphoantigens (Vantourout et al. 2009). Besides the activation of Vγ9Vδ2 T cells, IPP and ApppI might also be able to stimulate their migration (Benzaid et al. 2011). After demonstrating the chemoattractant effect of conditioned medium from ZOL treated breast cancer cell, Benzaid et al. (2011) also found that both pure IPP and ApppI were strong stimulators of the Vγ9Vδ2 T cell migration. In addition, the group measured the amounts of several cytokines known to attract T cells from ZOL-treated and non-treated cancer cells. ZOL-treatment did not increase the cytokine production so the effect of cytokines in Vγ9Vδ2 T cell migration can be ruled out.

Overall, there is abundant evidence, that N-BP-treated tumor cells, which overproduce IPP/ApppI are able to cause activation of Vγ9Vδ2 T cells, even though the exact mechanism of action remains still unclear. It seems that the activation of Vγ9Vδ2 T cells is dependent on direct cell-cell contact with antigen-presenting cells (cancer cells or PBMCs) from human origin (Stresing et al. 2007). Because it is unlikely that IPP or ApppI would directly bind to Vγ9Vδ2 T cell receptor, it has been suggested that IPP is complexed for example with cell surface ATP synthase in order to be recognized by the TCR (Clézardin 2011). This cell surface ATP synthase has been found on the plasma membrane of several tumor cells and its expression has been related to anti-tumor activity of Vγ9Vδ2 T lymphocytes, as well as its deficiency has been associated with missing phosphoantigen responses (Vantourout et al. 2010, Mookerjee-Basu et al. 2010).

Aims

In this work, our first objective was to complete data from prior studies and measure both intracellular IPP/ApppI accumulation and cytotoxic effect of Vγ9Vδ2 T cells against RIS-treated ZR 75-1 and BT-474 breast cancer cell lines. We also determined if there is a correlation between IPP/ApppI production of different breast cancer cell lines and their vulnerability to Vγ9Vδ2 T cells.

In preceding studies Benzaid et al. (2011) demonstrated that conditioned medium from ZOL treated breast cancer cell was able to act as a chemoattractant and induce migration of Vγ9Vδ2 T cells to tumor cells. Using mass spectrometry, they were also able to detect substantial IPP levels in conditioned medium from ZOL-treated T47D and MCF-7 cells, but not B02 cells. To confirm further, whether extracellulary produced IPP/ApppI is responsible for attracting Vγ9Vδ2 T cells, we wanted to produce and analyze conditioned medium from ZOL and RIS-treated T47D, MCF-7 and B02 cancer cells.

After having confirmed detectable IPP/ApppI amounts in supernatants of the cells, we planned to perform migration assays in order to see, if conditioned medium from bisphosphonate-treated cancer cells induces Vγ9Vδ2 T cell migration and whether this can be prevented by adding cleavage enzymes to the conditioned medium. However, because mass spectrometry measurements detected no IPP or ApppI in the conditioned media, these planned assays were dropped and only pure IPP was tested.

Our final aim was to study whether a possible antigen-presenting enzyme, cell surface ATP synthase, is expressed in our three breast cancer cell lines (B02, T47D, MCF-7), and if there exists a correlation between ATP synthase expression and Vγ9Vδ2 T cell-mediated cytotoxicity.

6 MATERIALS AND METHODS

6.1 Cell culture, Drugs and Reagents

Breast cancer cell lines were selected on the grounds to complete some, at that time unpublished data (Benzaid et al. 2011). Cell lines (T47D, MCF-7, Hep-G2, ZR 75-1 and BT-474) were obtained from American Type Culture Collection (ATCC)-LGC Promochem (Molsheim, France). Human estrogen receptor-negative B02 cell line, a subpopulation of MDA-MB-231 (ATCC), was prepared as previously described (Peyruchaud et al. 2001).

T47D, B02, HeLa and Hep-G2 cells were cultured in DMEM Low Glucose (1g/l) with L-Glutamine (PAA laboratories, Austria), supplemented with 10 % fetal bovine serum (FBS) (Invitrogen, Paisley, UK), 1 % penicillin/streptomycin (PAA laboratories, Austria) and 1 % fungizone (Invitrogen). MCF-7 cells required addition of 0.01 mg/mL bovine insulin, and they were cultured in EMEM with Earle's balanced salt solution, non-essential amino acids and sodium puryvate, (ATCC) with same remainder supplements. ZR-75-1 cells were cultured in RPMI-1640 with HEPES, sodium puryvate and L-glutamine (ATCC) and BT-474 cells in Hybri-Care Medium (ATCC) with 5 % $NaHCO_3$, both also supplemented with 10 % FBS, 1 % penicillin/streptomycin and 1 % fungizone. Cells were cultured under standard conditions and maintained in a humidified incubator in an atmosphere of 5 % CO_2 at 37°C.

Zoledronic acid (ZOL) [2-(imidazol-1-yl)-hydroxyethylidene-1.1-bisphosphonic acid, disodium salt, 4.75 hydrate] was provided by Novartis Pharma AG (Basel, Switzerland) and risedronate (RIS) [2-(3-pyridinyl)1-hydroxyethylidene-bisphosphonic acid] by Procter and Gamble Pharmaceuticals (Cincinnati, Ohio, USA). Recombinant IL-2 was obtained from Chiron (Horsham, UK), ATP from Sigma Aldrich, and IPP from Isoprenoid (USA). Phosphate buffered saline (PBS) was from PAA Laboratories (Austria) and 10 mg/mL bovine serum albumin (BSA) from Promega (Madison, WI, USA). Cleavage enzymes NPP and apyrase were gained from Sigma Aldrich. ApppI was synthesized as previously described (Mönkkönen et al. 2006).

6.2 Separation of PBMCs from blood samples and in vitro Vγ9Vδ2 T cell Expansion

Many N-BPs are known to stimulate expansion of Vγ9Vδ2 T cells in vitro (Sanders et al. 2004). Benzaid and her group tested Vγ9Vδ2 T cell proliferation from human PBMCs with increasing concentrations of ZOL and found time- and dose-dependent expansion of Vγ9Vδ2 T cells, which reached a plateau after 14 days of treatment with 10 μM ZOL. To confirm that expanded cells were Vγ9Vδ2 T cells, they analyzed proliferation by flow cytometry at day 0 and day 14, after immunostaining with fluorochrome-conjucated antibodies. In our experiments we trusted this previous data (Benzaid et al. 2011) and cultured PBMCs according to their protocol.

Blood was donated by healthy volunteers in Edouard Herriot hospital. PBMCs were isolated by Ficoll-Paque density gradient centrifugation, using LSM (Lymphocyte separation medium; sodium diatrizolate and ficoll, MP Biomedics LLC). PBMCs were cultured in RPMI-1640 + Glutamax^{TM}-I 1x (Invitrogen), supplemented with 10 % FBS and 1 % Penicillin/Streptomycin. To expand the Vγ9Vδ2 T cells, PBMCs were seeded in 12-well plates (2 x 10^6 cells/well) and exposed to 10 μM ZOL or RIS, and 100 U/mL IL-2 for 12 - 14 days. Medium was changed, and fresh IL-2 was added every 2-3 days.

After the expansion, all cells were collected, counted and stained with antibodies (Anti-TCR g/d MicroBead Kit human, Miltenyi Biotech GmbH). Cells were first centrifuged, then resuspended in (40 μL/10^7 cells) buffer solution prepared from cold PBS with 0.5 % BSA and 2 mM EDTA and stained with Anti-TCR γ/δ Hapten Antibody (10 μL/10^7 cells). After 10 minutes incubation at +4°C, buffer (30 μL/10^7 cells) and MACS Anti-Hapten MicroBeads-FITC –antibody (20 μL/10^7 cells) were added and cell suspension was incubated for additional 15 minutes at +4°C. After the second incubation, cells were washed and finally resuspended in 1 mL of buffer. Magnetically labelled populations of Vγ9Vδ2 T cells were purified by positive selection of TCR γ/δ^+ cells, using immunomagnetic cell sorter (AutoMACS® Pro Separator, Miltenyi Biotec, Bergish Gladbach, Germany).

6.3 Intracellular IPP/ApppI Analysis

To determine N-BP-induced IPP and ApppI production in breast cancer cells *in vitro*, ZR 75-1 and BT-474 breast cancer cells were seeded in 6-well plates (1×10^6 cells/well) and cultured in complete medium overnight. After, the cells were exposed for 1 hour to 25 µM RIS and left to recover in complete medium for an additional 18 hours. The wells were also exposed continuously to 25 µM RIS or vehicle (medium) for 19 hours. T47D, MCF-7 and B02 cells were seeded in Petri dishes (2.5 x 10^6 cells/dish), and treated either with 25 µM RIS or 25 µM ZOL. Also instead of complete medium, these cells were kept in DMEM with 0.25 % of BSA. Otherwise the treatment protocol and incubation times remained the same.

After 19 hours, the cells were washed twice with cold PBS, scraped, pelleted, and extracted using mixture (3:2) of ice-cold acetonitrile and water containing 0.25 mM sodium fluoride (NaF) and 0.25 mM sodium vanadate (Na_3VO_4) to prevent degradation of IPP and ApppI. After pelleting again (14000 rpm, 2 min), supernatant was transferred to Eppendorf tube, frozen in -80°C, and sent to Kuopio for measurements of intracellular accumulation of IPP/ApppI (measurements were carried out in University of Eastern Finland in March 2011). The molar amounts of IPP and ApppI were determined by HPLC-ESI-MS as previously described (Mönkkönen 2005).

To quantify total protein amount in the samples, pellets were digested in 1 M NaOH at 60°C in water bath for two hours, cooled, mixed and diluted 5 or 9 times. Standard curves (20-200 µg/mL or 20-400 µg/mL) were prepared from BSA stock (1 mg/mL in 1 M NaOH), which was kept 15 minutes at 60°C. Samples and standards were pipetted in duplicates to 96-well plate, and Bradford reagent was added to form a protein-dye complex, which causes a shift in the absorption maximum, and allows concentration of proteins to be determined. Optical density was measured at 570 nm using a 96-well plate reader (Bio-Rad microplate reader, model 680). The intracellular amounts of IPP and ApppI were then reported as a picomol / mg of total protein.

6.4 Cytotoxicity Assays

To determine if there is a correlation between different breast cancer cell lines vulnerability to Vγ9Vδ2 T cells and their IPP/ApppI production, we compared several cytotoxicity assays (unpublished data from Benzaid et al. and data from this work). All these cytotoxicity assays had been performed using MTT colorimetric assay, which measures viability of the cancer cells. Besides MTT assay, there are also other tests to evaluate the cytotoxicity of Vγ9Vδ2 T cells, and in order to evaluate this cytotoxic effect more completely, we wanted to conduct alongside with the MTT assay also FACS-based CD107 assay. This technique has been previously used by Mittendorf et al. (2005), and instead of assessing cancer cells death it measures Vγ9Vδ2 T cells activity by detection of the degranulation marker CD107a on the cells surface.

MTT-assay

BT-474 and ZR 75-1 cells were seeded at a density of 20000 cells/well in 96-well plates. After overnight incubation the cells were washed and pulse-treated for 1 hour with vehicle (medium) or increasing concentrations of RIS (1, 5, 10, 15, 25 μM). Cells were then washed to remove PBS and incubated in medium for 18 hours. T47D breast cancer cells were used as a positive control, and treated either with vehicle or 25 μM RIS.

After 18 h breast cancer cells were cultured with fresh Vγ9Vδ2 T cells at Target:Effector ratio 1:20 for 4 or 24 hours. After incubation, cells were washed and the viability of cancer cells was assessed by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide, Merck KGaA, Germany) assay as described previously (Fournier et al. 2002). MTT (0.5mg/mL) is transformed by mitochondrial dehydrogenases in living cells to a blue formazan product. After incubation for 1 hour at 37 °C, cells were solubilized in 20% DMF-SDS solution (N,N-Dimethylformamide and Sodium Dodecyl Sulfate, Sigma-Aldrich, USA), pH 4.7, and incubated for 5 hours at 37 °C to dissolve the blue formazan. Optical density was measured at 570 nm using a 96-well plate reader (Bio-Rad microplate reader, model 680). Results were presented as a viability percentage after 4 or 24 hours co-incubation with γδ T cells.

CD 107-assay

For the CD107 assay, three human breast cancer cell lines (T47D, MCF-7 and B02) were seeded in 96-well plates (T47D and MCF-7: 20000 cells/well, B02: 7000 cells/well) and cultured overnight (in order to the cells to become adherent on the bottom of the wells). Prior to their use in assay, cancer cells were treated for 1 h with 1, 10 or 25 μM ZOL or control (medium), washed and incubated in medium for 18 hours.

On the day of the assay, supernatant was removed from the wells and Vγ9Vδ2 T cells were added at Target:Effector ratio 1:20. Control wells containing only Vγ9Vδ2 T cells or Vγ9Vδ2 T cells with antibodies were also set up for the assay. 1/25 diluted CD107a -antibody (PE Mouse Anti-Human CD107a, BD Biosciences Pharmingen) was also added in the wells at the same time as effector cells. Plates were centrifuged for one minute (1080 rpm) to facilitate immediate contact between the effector and the target at the bottom of the wells and incubated at 37 °C for 4 or 24 hours.

After the incubation time, cells were collected from plates, washed, resuspended in PBS, stained with 1/15 diluted Vδ2 –antibody labeled with fluorochrome (Anti-TCR Vδ2-FITC, IOTest®, Beckman Coulter, Marseille, France) and incubated for 35 minutes in the dark. Cells were washed again, resuspended in 500 μL of PBS and analyzed with DIVA software of BD FACSCanto II flow cytometer (BD Biosciences, San Jose, USA).

6.5 Extracellular IPP/ApppI analysis and Transwell Migration Assay

IPP/ApppI analysis

T47D, MCF-7 and B02 breast cancer cells were seeded in Petri dishes at 2.5 x 10^6 cells/ dish overnight. Cells were pulse-treated with 25 µM ZOL or RIS for 1 h or 19 h as previously described in Chapter 6.3. After, the conditioned medium was collected and incubated with cleavage enzymes NPP (0.02 or 0.2 U/mL) and apyrase (0.025 or 0.2 U/mL) for 1 hour in water bath. Pure ATP solution (100 µM) was used as a control, and also incubated with both concentrations of NPP and Apyrase.

A moiety of the conditioned medium was treated with 25 mM NaF and $NaVO_4$, to prevent degradation of IPP and ApppI, and sent to Kuopio to be analyzed by HPLC-ESI-MS. The other half of the conditioned medium was preserved in our laboratory for possible later use in migration assay.

Migration assay

Before migration assays, Vγ9Vδ2 T cells were expanded from PBMCs and purified by immunomagnetic cell sorting as described in Chapter 6.2. Purified populations were resuspended in assay buffer (RPMI-1640 + 0.25 % BSA). Migration assays were performed with 24-well Transwell® plates (Corning Incorporated, NY, USA) with 5 µm pore polycarbonate membrane. Subject solution (600 µL) was placed in the lower chamber of Transwell inserts and Vγ9Vδ2 T cell suspension (3x 10^6 cells/100 µL) in the upper chamber. The solutions examined were assay buffer (control), 0.025 U/mL apyrase, 0.5 µM and 1.0 µM IPP with or without 0.025 U/mL apyrase and 200 ng cytokine MCP-1 (Recombinant Human MCP-1, Shenandoah Biotechnology). Cleavage enzyme apyrase is able to degrade IPP by cutting the P-O bond of the molecule, and it was used to see whether degraded IPP induces T cell migration or not. After incubation period of 24 hours at 37°C, cells from the lower chamber were counted as the percentage of cells migrated compared to assay buffer.

6.6 Detection of Cell Surface ATP synthase

To be able to perform detection assays by flow cytometer, we first needed to determine suitable concentrations for the ATP synthase α- and β-subunit antibodies using two cancer cell lines (Hep-G2 and HeLa). These cell lines are known to express cell surface ATPase (Vantourout et al. 2010). Alongside the flow cytometer assay we also extracted proteins from the cells for western blot. This was made because we wanted to confirm, that primary antibodies are really working and able to detect ATP synthase. Although, in western blot the antibodies recognize the total ATPase in cells, expressed both in mitochondria and on cell surface. The reason for using western blot was to be sure that our detection problems were not due to unfunctioning commercial antibodies.

Flow Cytometry

Two cancer cell lines, HeLa and HepG2 were used when defining suitable concentrations for primary antibodies. Cell suspensions (6.6x 10^5 or 1x 10^6 cells/tube) were stained with increasing concentrations of primary antibodies (ATP synthase subunit-α monoclonal antibody, Molecular Probes, Invitrogen, Camarillo, CA, USA or subunit-β monoclonal antibody, Molecular Probes, Invitrogen, Eugene, Oregon, USA), with PBS (negative control) or with isotypic control antibody (Mouse IgG1 isotype control antibody, Immunotech). Concentrations tested were 1, 2, 5, 10, 20 and 40 µg/mL, isotypic antibody was used at 10 or 20 µg/mL.

After the addition of primary antibodies, cells were incubated for 35 minutes in +4°C, washed twice with cold PBS and resuspended in PBS. Secondary antibody (fluorescein $F(ab')_2$ fragment of goat anti-mouse IgG (H+L), Molecular Probes, Invitrogen, Oregon, USA) was added at concentrations 1, 10 or 20 µg/mL. After another incubation of 35 min in +4°C, cells were washed, protected from light, fixed in formaldehyde (PBS with 2 % of formaldehyde and 5 % of FBS) and analyzed with DIVA software of BD FACSCanto II flow cytometer (BD Biosciences, San Jose, USA).

Western Blot

To quantify total protein concentration, HeLa and Hep-G2 cell suspensions were diluted in lysis buffer and treated with Bio-Rad DC™ Protein Assay Reagents (Bio-Rad Laboratories, California, USA). Standard curve (0.1 – 1.6 mg/mL) was prepared from 10 mg/mL BSA. Samples were pipetted in triplicates and standards in duplicates to 96-well plate, and optical

density was measured at 750 nm using a 96-well plate reader (Bio-Rad microplate reader, model 680).

Protein samples for gel electrophoresis were prepared from HeLa and Hep-G2 cells using lysis buffer + protein inhibitor, NUPAGE® LDS Sample Buffer (4x) (Invitrogen, Carlsbad, USA) and NuPAGE® Sample reduction agent (10x) (Invitrogen, Carlsbad, USA). Samples were heated at 70°C for 10 minutes, in order for polypeptides to maintain in a denatured state, and then put on ice. Gel electrophoresis was performed with pre-cast SDS-polyacrylamide gels (NuPAGE® 4-12 % BT 1.5 mm thick gel), NuPAGE® MES SDS Running buffer (20x) and NuPAGE® Antioxidant (Invitrogen, Carlsbad, USA). Protein markers (Novex® Sharp Pre-Stained Protein Standards) and samples (17 µg HeLa, 20 and 40 µg Hep-G2) were then transferred from gels onto transfer membranes (Immobilon™ - P transfer membrane with pore size 0.45 µm, Millipore, Bedford, USA). Electroblotting was made using cold transfer buffer (25 mM Tris, 192 mM Glysine, 10 % Methanol). After transferring the proteins, membranes were incubated in red Ponceau liquid, washed with water and washing solution (TBS-Tween) and then incubated in TBS-Tween 5 % milk overnight at +4°C.

Two membranes were applied, one for α-antibody and another for β-antibody and control background without primary antibody. For immunodetection, membranes were first incubated for 1 hour in TBS-Tween 5 % milk with primary antibodies (ATP-synthase subunits α and β) at concentration 1 µg/mL. Membranes were then washed with TBS-Tween and incubated for 1 hour with secondary antibody (ECL™ Anti-mouse IgG Horsedish Peroxide linked whole antibody from sheep, GE Healthcare, UK) used at a dilution of 1/2000. Amersham™ ECL™ Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK) were used to develop images on autoradiography film (Amersham Hyperfilm™ ECL High performance chemiluminescence film, GE Healthcare, Buckinghamshire, UK).

7 RESULTS AND DISCUSSION

7.1 Intracellular IPP/ApppI Analysis

The first cell lines tested were 1 hour RIS-treated BT-474 and ZR 75-1. After 18 hours of drug removal, IPP/ApppI accumulation was low in BT-474 cells and undetectable in ZR 75-1 cells (Fig. 12). Also after 19 hours of continuous RIS-treatment only BT-474 cells were producing high IPP/ApppI levels, whereas ZR 75-1 cells expressed low or undetectable amounts. These findings are well in accordance with preceding studies, where IPP/ApppI accumulation was measured in the same cell lines after 1 or 19 hours of ZOL treatment (Benzaid 2009). After the treatment with 25 µM ZOL, only BT-474 cells were able to produce moderate (after 1 h) or high (after 19 h) IPP levels, whereas the accumulation in ZR 75-1 cells was low or undetectable. When comparing these two data, IPP concentrations (pmol/mg protein) in BT-474 cells were overall lower after RIS treatment when compared to ZOL treatment, whereas ApppI concentrations were similar in both RIS- and ZOL-treated BT-474 cells.

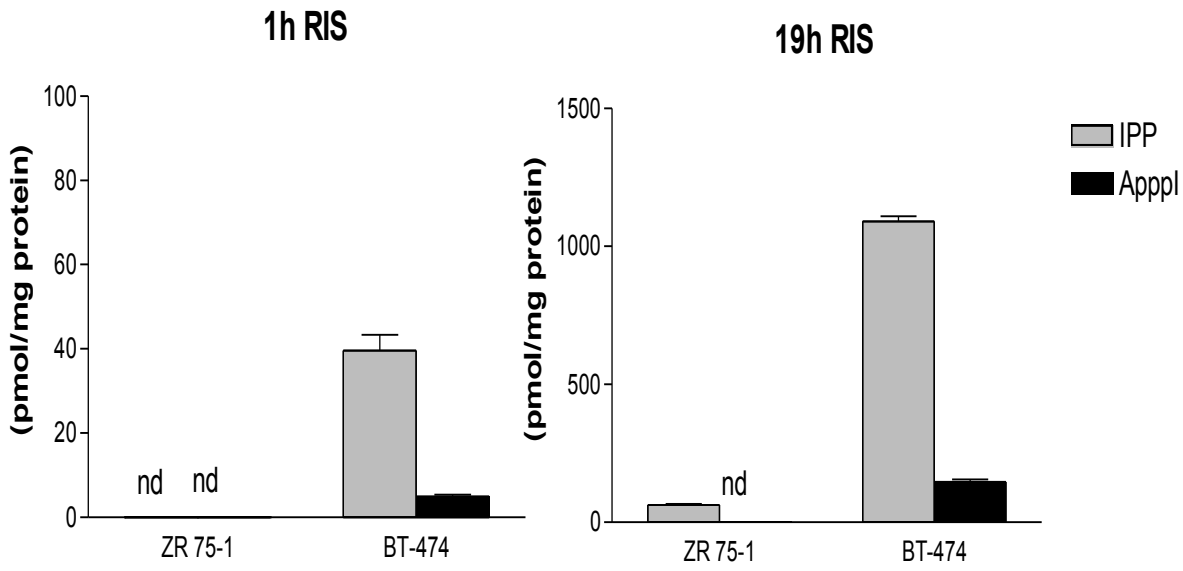

Fig. 12. Intracellular accumulation of IPP/ApppI in ZR 75-1 and BT-474 cells at 18 h after a one hour incubation with 25 µM RIS (left), or after 19 hours of continuous incubation with 25 µM RIS (right) (nd= not detected).

Next, three different breast cancer cell lines, T47D, MCF-7 and B02, treated either with ZOL or RIS were compared. The cells were cultured with 0,25 % of BSA instead of 10 % FBS, and thus the results are not comparable with BT-747 and ZR 75-1 cells. Low concentration of BSA was used because part of the conditioned medium was supposed to be used in the transwell migration assays, and in this assay 10 % FBS would have disturbed the function of cleavage enzymes, NPP and apyrase. However, three cell lines used are comparable with each other.

As was to be expected, remarkable variation in IPP/ApppI accumulation between the cell lines was detected. After both RIS and ZOL treatment there was a high IPP/ApppI accumulation in T47D and MCF-7 cells, whereas IPP/ApppI levels produced by B02 cells were either very low or undetectable (Fig. 13). Benzaid et al. (2011) suggest that variations in endocytosis between cancer cell lines might explain differences in N-BP induced IPP/ApppI production. Based on experiments with ^{14}C -labeled ZOL, they suggest that IPP/ApppI production in breast cancer cells depends both on cellular uptake of N-BPs and on HMG-CoA reductase expression, which represents the activity of the mevalonate pathway. However, other groups have not been able to confirm, that cellular uptake could explain the differences between cancer cell lines IPP/ApppI production (Mönkkönen et al. 2008).

Also, IPP/ApppI levels were noticed to fluctuate depending on the potency of N-BP used (Fig. 13). ZOL-treated T47D and MCF-7 cells seemed to produce higher IPP and ApppI concentrations than RIS-treated T47D and MCF-7 cells. Preceding studies have found that IPP/ApppI production correlates with the potency of N-BPs to inhibit FPPS activity in cells (Mönkkönen et al. 2006). Our data is well in accordance with these findings, for ZOL has been found to be more potent inhibitor of FPPS than RIS (Dunford et al. 2001).

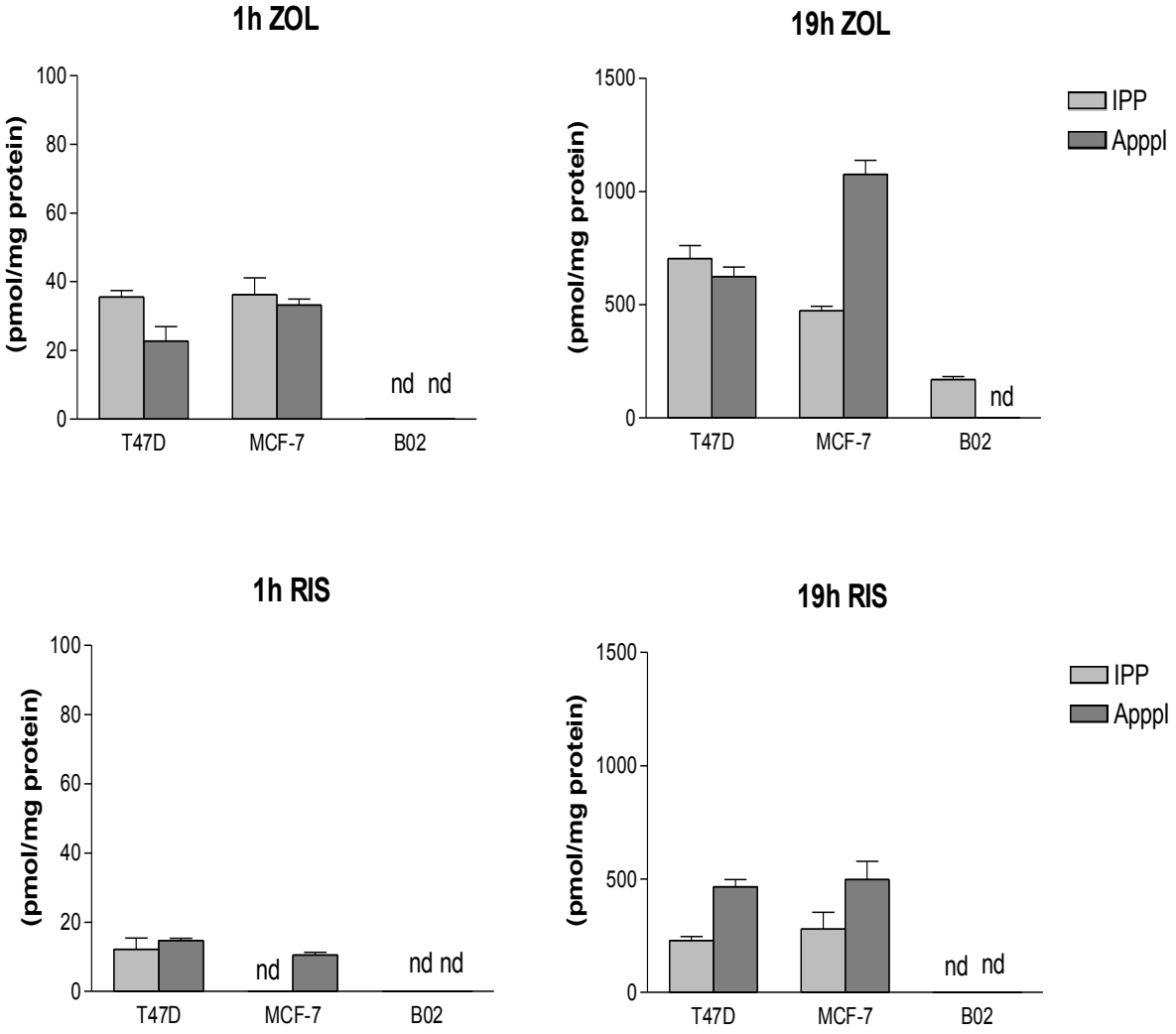

Fig. 13. Intracellular accumulation of IPP/ApppI in T47D, MCF-7 and B02 cell lines at 18 h after a one hour incubation with 25 µM ZOL or RIS (left), or after 19 hours of continuous incubation with 25 µM ZOL or RIS (right) (nd= not detected).

7.2 Cytotoxicity Assays

Cytotoxic effect of human Vγ9Vδ2 T cells against different cancer cell lines can be measured by direct or indirect tests. Direct, like MTT colorimetric test and ^{51}Cr- radioactive test, measure the viability of cancer cells, whereas indirect test measures the activity of T-lymphocytes (Mittendorf et al. 2005). From these assays, the most relevant and commonly used is still ^{51}Cr release assay, despite the prolonged manipulations required to perform it. However, because of the lack of premises where to work with radioactive material, MTT assay has been the principal choice in our research.

MTT -assay

The data obtained from MTT assays were statistically analyzed by one-way ANOVA, and the cell viability was counted as a percentage compared to assay buffer. One hour incubation with different concentrations of RIS without Vγ9Vδ2 T cells did not affect the cancer cells viability in any of the cell lines (Fig. 14 and 15). As expected, 4 hours co-culture with purified human Vγ9Vδ2 T cells led to statistically significant cancer cells death in our positive control cells (T47D), and some cytotoxicity was also detectable in ZR 75-1 cells treated with 25 μM of RIS. Then again, no cytotoxic effect could be detected in this cell line after 24 hours incubation with Vγ9Vδ2 T cells. In BT-474 cells, no statistically significant cytotoxicity could be seen after 4 hours incubation. However, after 24 hours there was a clear decrease in the cancer cells viability with 25 μM of RIS.

Our results are in accordance with the previous research by Benzaid (2009), where the cytotoxic potency of Vγ9Vδ2 T cells against BT-474 and ZR 75-1 cancer cell lines was examined by using ZOL. ZOL-treatment alone was not able to induce death of cancer cells. However, significant decrease in the viability of BT-474 cells was seen after 4 hours and especially after 24 hours of co-incubation with Vγ9Vδ2 T cells. This effect was significant only with the highest concentration (25 μM) of ZOL. Similarly to our results, in the experiments by Benzaid (2009) treatment with ZOL and co-incubation with Vγ9Vδ2 T cells (either 4 or 24 hours) were not able to induce any significant changes in the viability of ZR 75-1 cells.

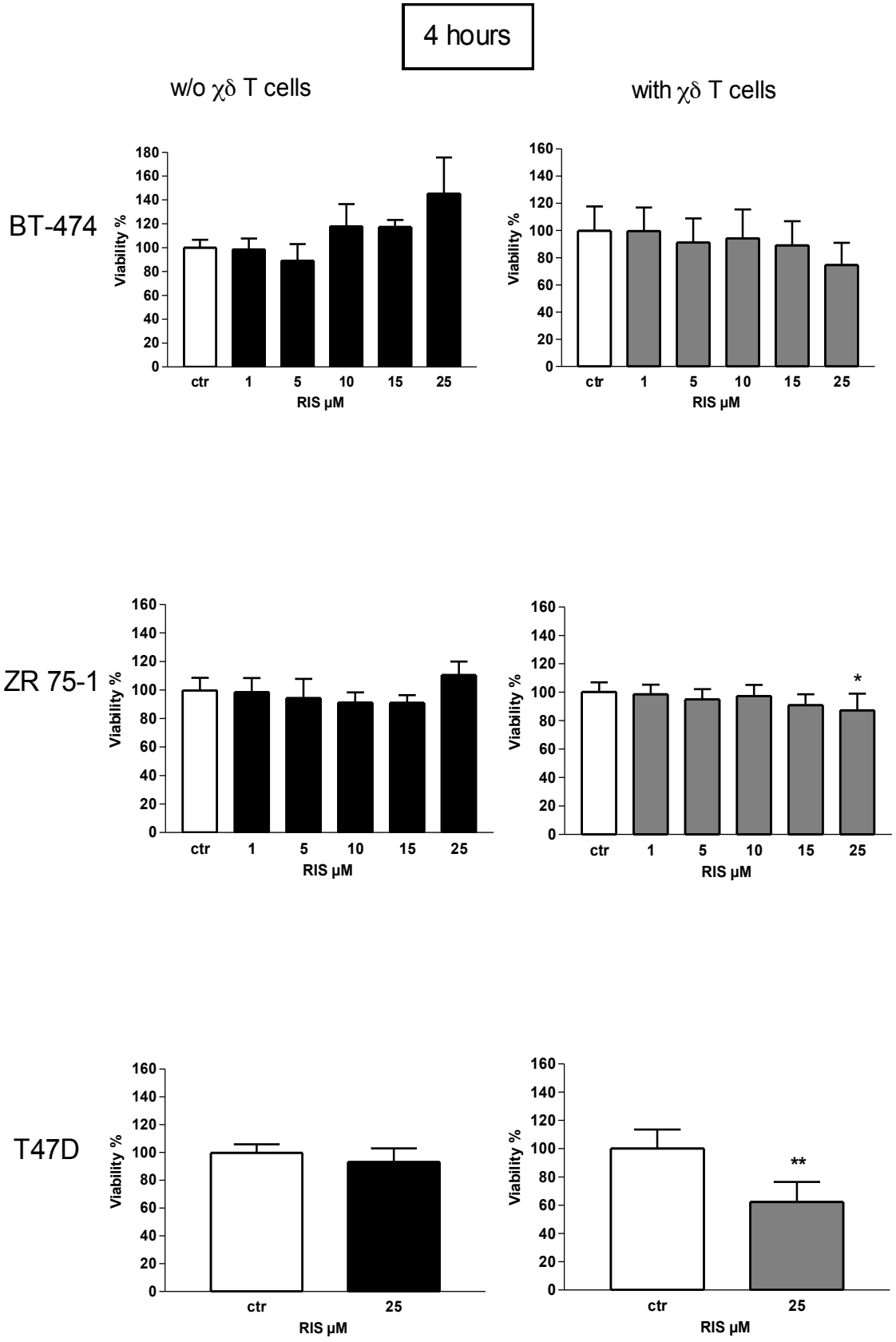

*Fig. 14. The viability (% compared to assay buffer) of RIS-treated cancer cells after 4 hours co-culture alone or with γδ T cells (mean + SD ; *, P<0,05 ; **, P<0,01)*

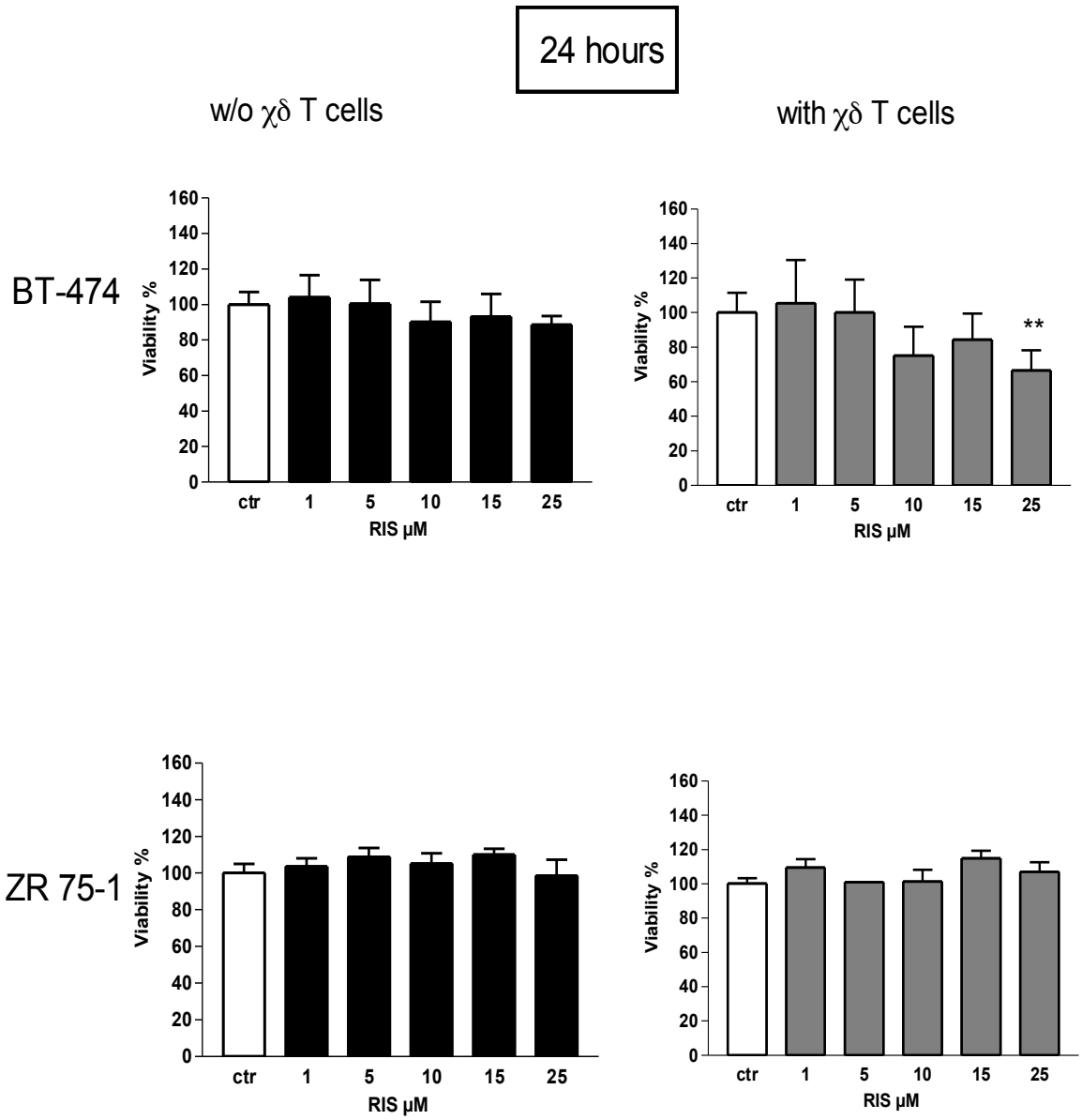

*Fig. 15. The viability (% compared to assay buffer) of RIS-treated cancer cells after 24 hours co-culture alone or with γδ T cells (mean + SD ; **, P<0,01)*

BP-induced IPP/ApppI accumulation in human breast cancer cells correlates with Vγ9Vδ2 T cell-mediated cancer cell death in vitro

As number of evidence suggest the possible correlation between IPP/ApppI production of different cancer cell lines and the cytotoxic effect of γδ T cells, we summarized the data in Fig. 16 to demonstrate this possible correlation. The data of both IPP/ApppI production and cytotoxicity have been collected from different experiments performed during last two years (the data from this study, from Benzaid et al. 2011, Benzaid 2009 and Benzaid et al. unpub.) so the conditions are not the same in all the cases. For example, Vγ9Vδ2 T cells were separated from blood samples of different donors. Therefore their proliferation and cytotoxic potency are variable, and hence the cytotoxicity percentages are not truly comparable. However, the data obtained from various IPP/ApppI measurements are comparable, as all the cell lines were cultured in same conditions with 10 % FBS and treated with N-BP for 1 h only, after which they were incubated in medium for 18 h. One hour treatment with N-BP was chosen because it supports the clinical relevance of these findings. When administered *in vivo*, N-BPs stay in the bloodstream approximately one hour and then accumulate rapidly to bone minerals.

Although the cytotoxicity percentages are not totally comparable, a clear positive correlation between IPP/ApppI production and Vγ9Vδ2 T cell-mediated cytotoxicity can be seen (Fig. 16). These findings support the theory, that cancer cells which have high IPP/ApppI accumulation capacity are likely to be the most vulnerable to the effects of N-BP based Vγ9Vδ2 T cell therapy (Benzaid et al. 2011). Furthermore, in all the cases ZOL seems to be more potent inducer of both IPP/ApppI production and the cytotoxic effect when compared to RIS.

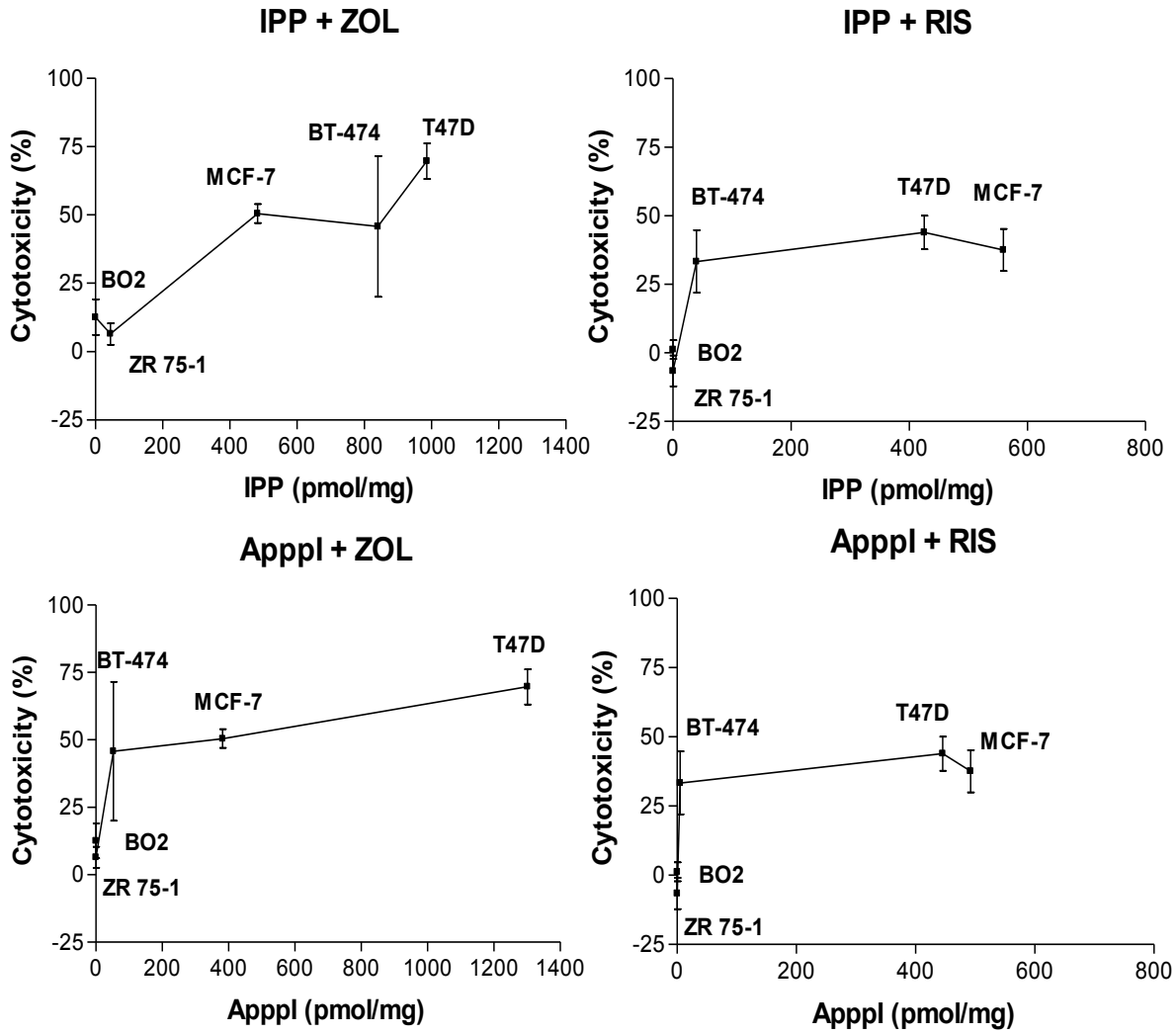

Fig. 16. Correlation between IPP/ ApppI production of the cancer cells and their vulnerability to cytotoxic effects of N-BP treated Vγ9Vδ2 T cells. Cytotoxicity (% compared to assay buffer) was measured by MTT assay. B02, MCF-7, T47D, ZR 75-1 and BT-474 cell were pretreated with 25 µM ZOL or RIS for 1 hour and cultured without drug for 18 hours. After incubation, cells were co-cultured with purified Vγ9Vδ2 T cells for 24 hours. Identification of intracellular IPP/ApppI was carried out by HPLC-ESI-MS. The data were obtained from different experiments (from this study, Benzaid et al. 2011, Benzaid 2009 and Benzaid et al. unpub.).

CD 107-assay

In order to evaluate T cell-mediated cytotoxicity more precisely, FACS-based CD107 assay was conducted alongside with the MTT assay. This is a technique that measures the activity of the effector cells, but not the viability of the target cells (Mittendorf et al. 2005). To be precise, Vγ9Vδ2 T cell activity is measured by the detection of the degranulation marker CD107a on the cell surface.

When Vγ9Vδ2 T cells are activated, they produce more lysosomes that contain cytotoxic mediators such as INF-γ, granenzymes and perforin (Mittendorf et al. 2005). Stimulation of T cell receptor (TCR) results in degranulation and release of the cytotoxic mediators once these lysosomes fuse with the T cells plasma membrane (Fig. 17). The core within the granule contains many lysosome-associated membrane glycoproteins, including CD107a, which also becomes mobilized to cell surface during degranulation. Identification and quantification of this CD107a with a specific antibody by flow cytometry allows for more accurate specifications between the activity of the Vγ9Vδ2 T cells and the viability of the cancer cells. Hence, to confirm that Vγ9Vδ2 T cells are responsible for the cancer cell death observed in MTT assay, an increase of surface CD107a expression on effector cells should be perceivable.

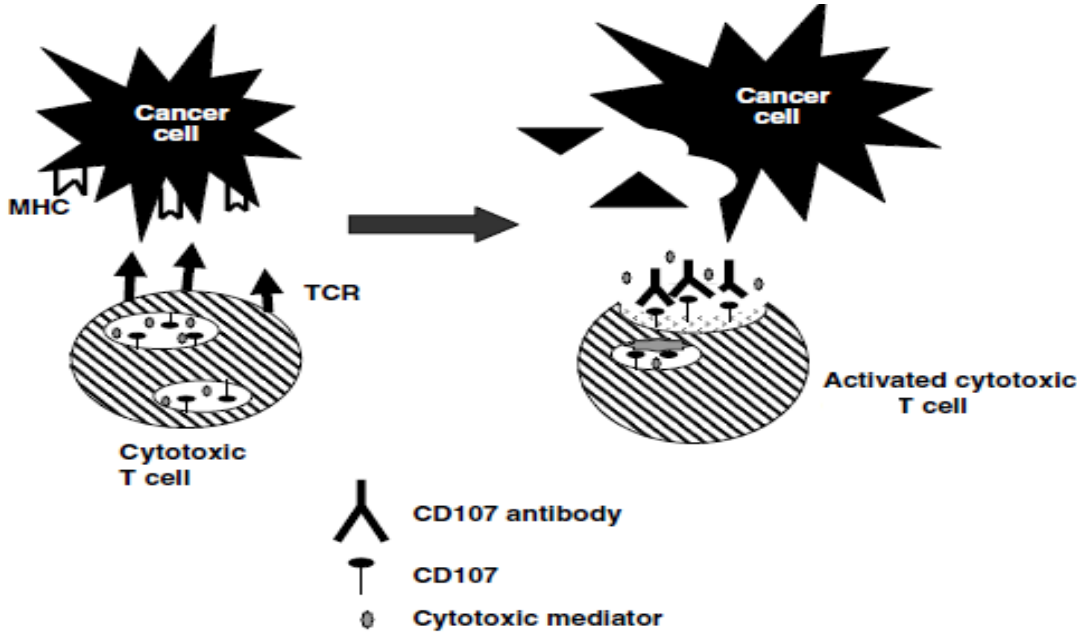

Fig. 17. Stimulation of T cell receptor (TCR) results in degranulation and release of the cytotoxic mediators and CD107 on the T cells plasma membrane (Mittendorf et al. 2005)

In our assay, flow cytometer was able to detect both labelled Vγ9Vδ2 T cells and CD107a antibody. However, the problem was that all the samples gave exactly the same CD107a intensity as was observed with the positive control, therefore no applicable results could be obtained.

The most probable reason for inconclusive data could be the possible activation of Vγ9Vδ2 T cells by N-BPs already before the assay. When PBMCs were first separated from blood samples and cultured to expand the Vγ9Vδ2 T cells, they were exposed to 10 μM ZOL and 100 U/mL IL-2 for 14 days. This ZOL treatment might be responsible for immature T cell stimulation to effector cells, degranulation of lysosomes and hence expression of CD107a on the cell membrane. Thereafter, the cancer cells, treated with different concentrations of ZOL, were not able to activate Vγ9Vδ2 T cells any further, and CD107a expression remained the same in all the samples.

7.3 Extracellular IPP/ApppI analysis and Transwell Migration Assay

IPP/ApppI analysis

First, we checked the function of our cleavage enzymes using ATP (100 µM) as a control. Both NPP and apyrase were able to degrade ATP so the enzymes were functional (table 1). However, no detectable amounts of IPP or ApppI could be measured in any of the samples. The fact that none of the cell lines did excrete IPP or ApppI into the conditioned medium was very strange, because previously these phosphoantigens had been detected using the same method (Benzaid et al. 2011). Extracellular IPP has also been detected in the supernatant of N-BP treated dendritic cells (Castella et al. 2011). These previous results are in line with our hypothesis suggesting that N-BP treated cancer cell lines are able to release IPP in the media. According to some theories, IPP can bind to carrier protein when it is cell surface exposed. It might be possible that if this carrier protein has been degraded during storage and transportation to Kuopio, IPP/ApppI could have been left undetected. Phosphatase inhibitors were already added in the conditioned media, but addition of some protease inhibitors might also be worth trying in the future experiments.

Table 1. ATP concentrations after NPP and apyrase treatments, detected by mass spectrometry. ATP was used as a control to confirm the functionality of the cleavage enzymes, because it can attract Vγ9Vδ2 T cells and is also demerged by both NPP and apyrase.

N° tube	Echantillons	ATP (µM)
ATP-1	CTR	3,138
ATP-2	0,025U/ml Apyrase	< 0.1
ATP-3	0,2U/ml Apyrase	< 0.1
ATP-4	0,02U/ml NPP	< 0.1
ATP-5	0,2U/ml NPP	< 0.1

Migration assay

Because of the lack of IPP and ApppI in conditioned medium, we performed migration assays with commercial IPP and cleavage enzyme apyrase (Fig. 18). Transwell plates used in the migration assays had pore size of 5 µm whereas the size of Vγ9Vδ2 T cells is approximately 7-8 µm. Narrow pore size was essentially important in order to prevent the passive drifting of the Vγ9Vδ2 T cells through the pores.

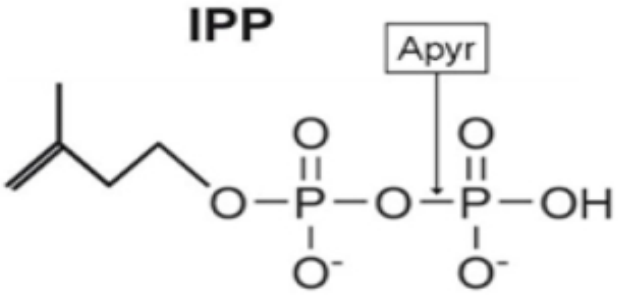

Fig. 18. Structure of IPP and ApppI, and the cleavage sites for nucleotides pyrophosphatase (NPP) and apyrase (Vantourout et al. 2009).

In the first migration assay, we could see that 1 μM IPP was able to induce significant Vγ9Vδ2 T cell migration compared to assay buffer, whereas 0.5 μM IPP was not (Fig. 19, Assay 1). Also migration was clearly decreased, when 1 μM IPP was incubated with apyrase. Although, we confirmed that apyrase was working, it did not seem to be able to block migration totally, which could be due to too short incubation time before starting the assay. In future experiments, one possible mean to confirm that all IPP has been cleaved before Vγ9Vδ2 T cells start to migrate would be to put IPP + apyrase tubes in water bath for one hour before starting the migration assay.

For the second migration assay we added also non-phospoantigenic chemoattractant, cytokine MCP-1 to act as a control. However, MCP-1 was not able to induce detectable migration (Fig. 19, Assay 2), which makes us to question either functionality of this cytokine or the uniform quality of the transwell plates. Results from 1 μM IPP are here well in line with the previous tests where it was also able to induce 1.5 times higher migration of Vγ9Vδ2 T cells than assay buffer (Benzaid et al. 2011). In our second assay, as well as in the first one, apyrase is able to reduce the chemoattractive effect of 1 μM IPP (Fig. 19).

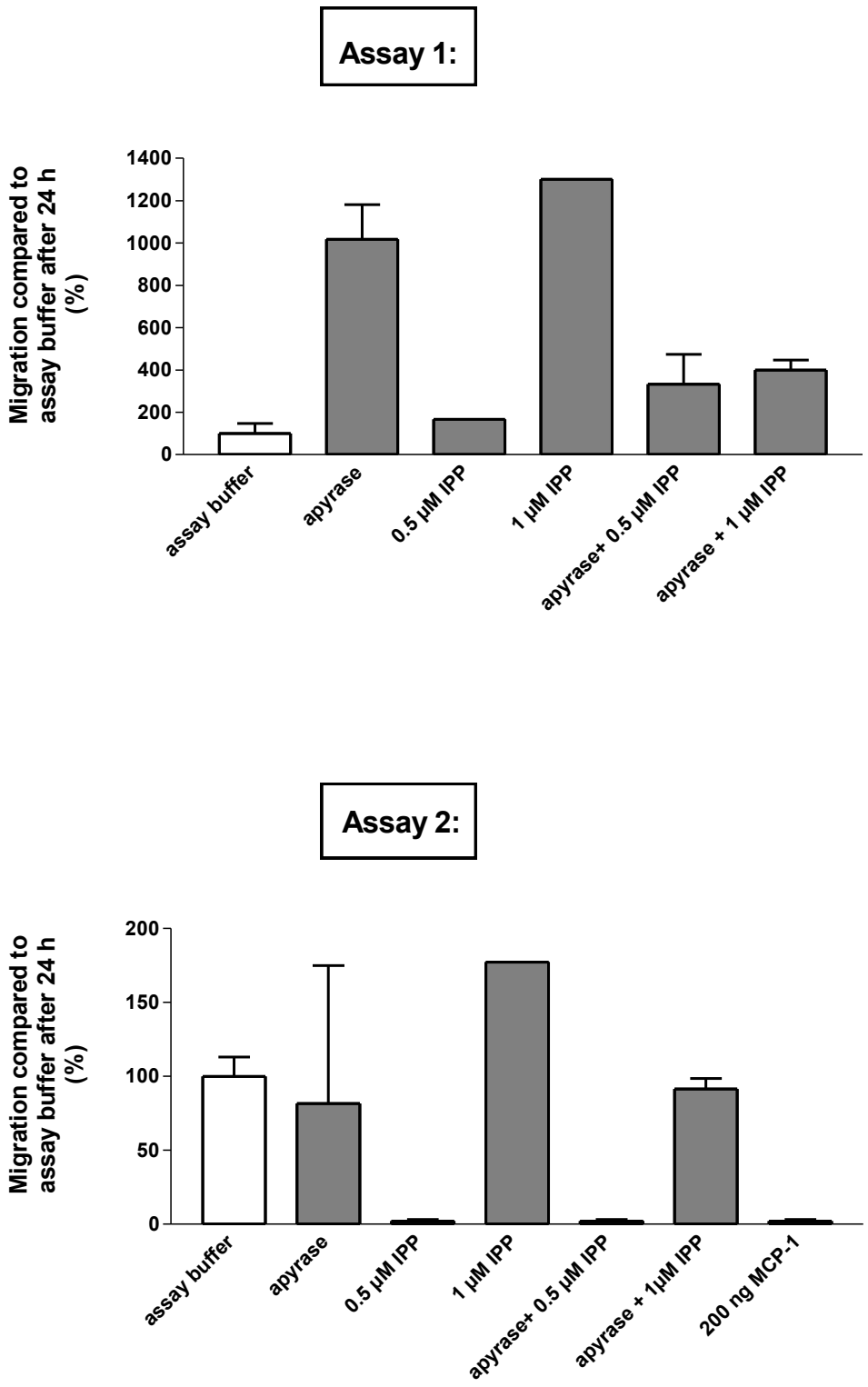

Fig. 19. IPP-induced chemotactic migration of Vγ9Vδ2 T cells, and the effect of cleavage enzyme apyrase. Assays 1 and 2 were conducted separately, with different donors of Vγ9Vδ2 T cells, and only Assay 2 included cytokine MCP-1.

7.4 Detection of Cell surface ATP synthase

Flow Cytometry

In order to investigate whether our three cell lines (B02, T47D and MCF-7) expressed ATP synthase on their surface, we first defined the suitable concentrations for the antibodies, for both flow cytometry and western blot. For this, we used two cell lines (Hep-G2 and HeLa cells) known to express ATP synthase (Mookerjee-Basu et al. 2010, Vantourout et al. 2010). However, despite the several experiments performed with different antibody concentrations, we were not able to detect ATPase subunits α or β from either Hep-G2 or HeLa cells by flow cytometry (Fig. 20).

Also, other groups have encountered the same detection problem, and one hypothesis is that cell surface ATPase is only partially accessible to antibodies due to tight interactions with surrounding surface proteins (Mookerjee-Basu et al. 2010). At least, high expression of MHC-I molecules on cell surface has been noted to hamper the detection of ATP synthase epitopes.

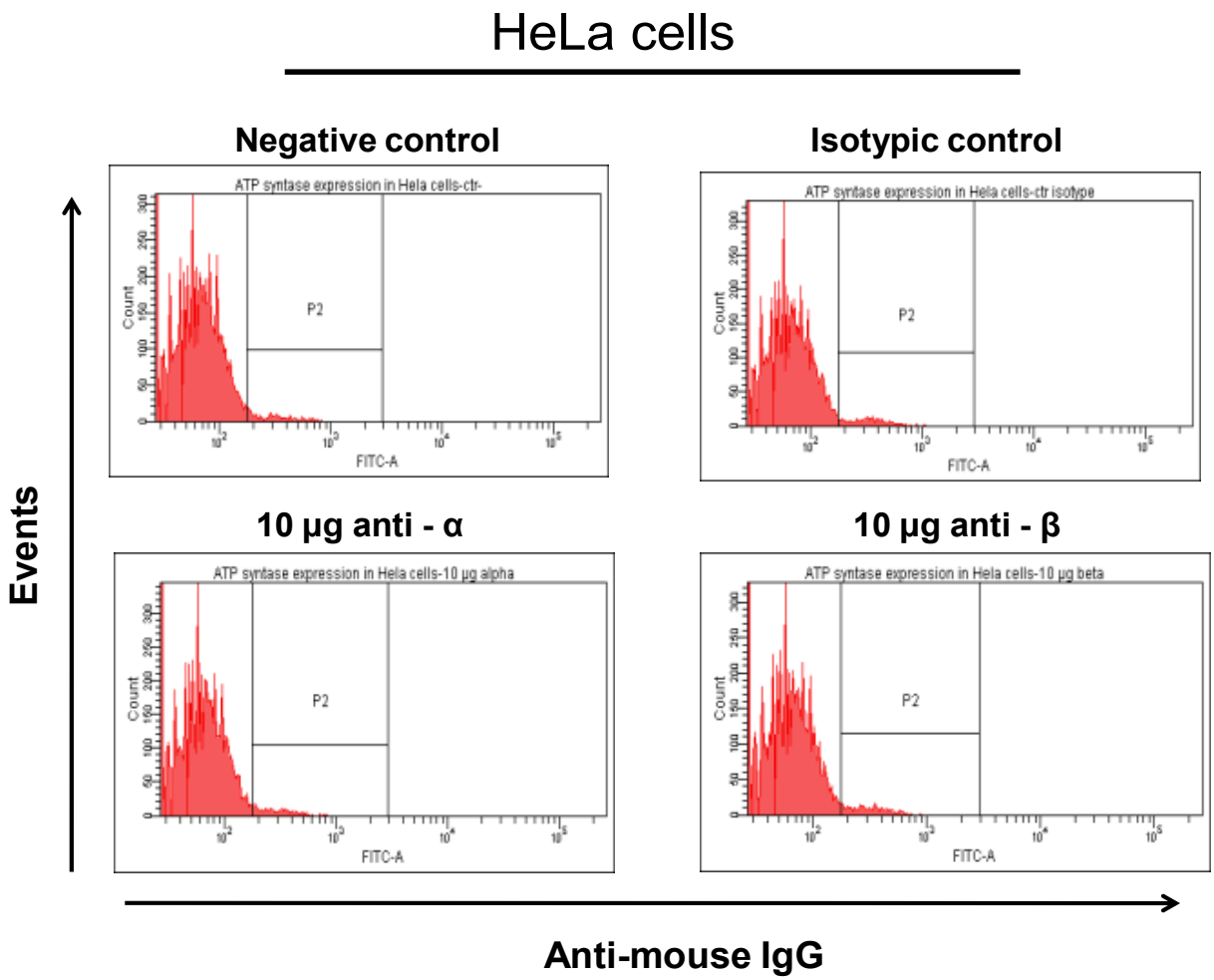

Fig. 20. Cell surface ATP synthase subunits α and β were not detected on either Hep-G (data not shown) or HeLa cells by flow cytometry. Tested concentrations varied between 1 to 40 µg of antibodies.

Western Blot

Western blotting is a standard laboratory procedure to verify the expression and relative amounts of a protein present in different samples, and analyze the results of co-immunoprecipitation experiments (Penna and Cahalan 2007). Here, we used western blot to confirm the functionality of the selected antibodies.

Before starting the immunoblotting, we needed to quantify protein amounts in our cell extracts. Based on our standard curves, Hep-G2 cell suspension contained 2,58 mg protein/mL and HeLa cells 0,72 mg/mL. Protein quantities used were HeLa 17 µg/mL, Hep-G2 20 and 40 µg/mL. Our target protein, ATP-synthase, was attempted to detect by probing it with specific primary subunit α and β monoclonal antibodies.

After unbound primary antibodies had been washed away, the membranes were exposed to secondary antibody, directed against the species-specific portion of the primary antibody. A chemiluminescent agent was used as a substrate that would luminescence when exposed to HRP on the secondary antibody, and the light was detected by photographic film. Cells, which were stained only with secondary antibody, were used as a background (Fig. 13 A). When second membrane was compared to protein markers, we were able to detect β-subunit in both HeLa and Hep-G2 cells (Fig. 13 B) between 50 and 60 kDa. However, no bands indicating the presence of ATP synthase α-subunit were visible in the first membrane. The question why α-subunit was not detected remained unclear for us and requires further examinations. The molecular weight of α-subunit was 55 kDa and of β-subunit 52 kDa.

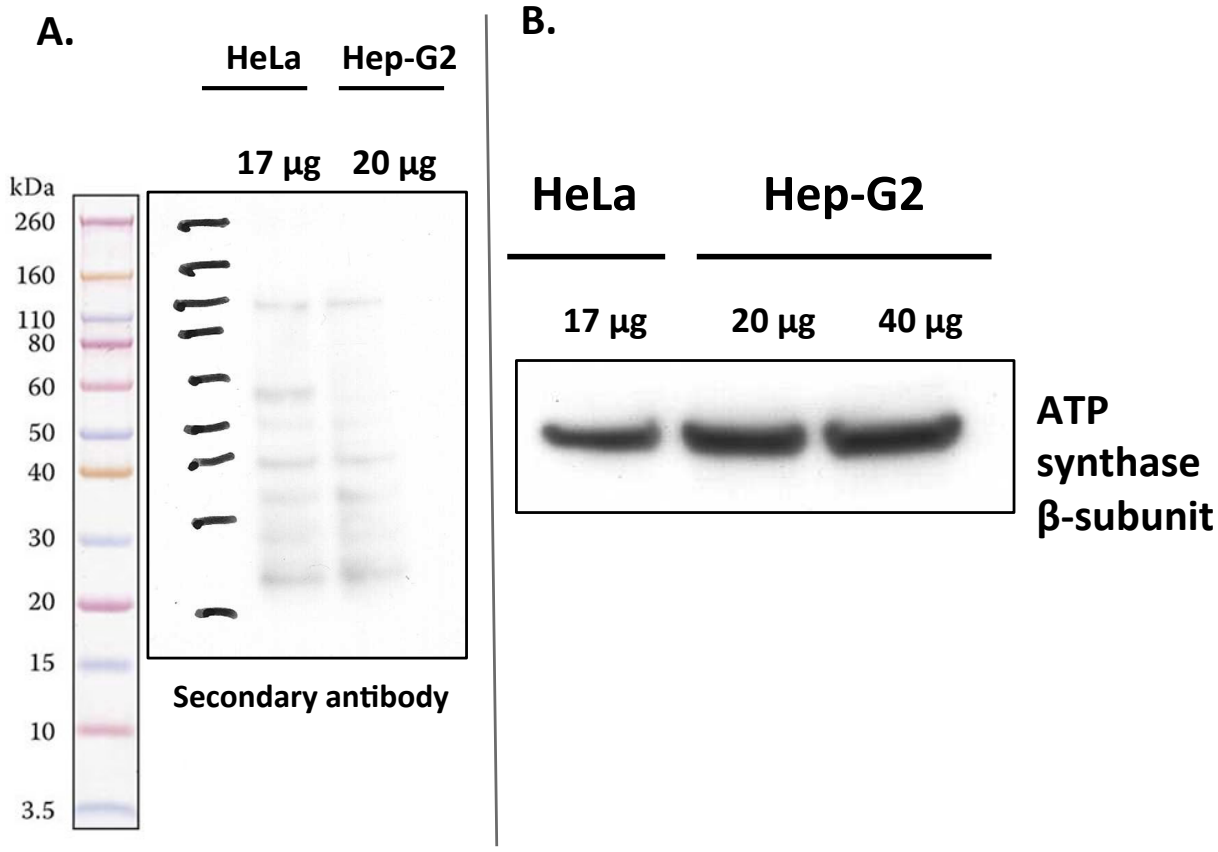

Fig. 21. A) The apparent molecular weights of Novex® Sharp Pre-Stained Protein Standard and secondary antibody (background). B) ATP synthase beta subunit was detected between 60 and 50 kDa. Size of alpha F-1 = 55 kDa, beta F-1 = 52 kDa.

8 CONCLUSIONS

Several observations *in vitro* and *in vivo* have laid an interesting foundation for examining the potential of γδ T cell-based tumor immunotherapy. Human peripheral blood Vγ9Vδ2 T cells, that appear to combine properties of both adaptive and innate immunities, can be readily and selectively activated either by low molecular mass phosphoantigens, such as HMBPP and IPP, or agents that provoke IPP accumulation. Nitrogen-containing bisphosphonates are found to induce intracellular IPP/ApppI accumulation in cancer cells and extensive data are available on the direct antiangiogenetic, antiosteolytic and proapoptotic properties of the compounds (Clèzardin 2011). Besides the activation of γδ T cells, N-BPs exert indirect anti-tumor effects by making the bone less favorable environment for cancer cell growth and interfering with the functions of bone marrow-derived cells that would enable the formation of metastasis.

Our aims were to study intracellular IPP/ApppI accumulation in RIS or ZOL treated cancer cells and cytotoxic effect of Vγ9Vδ2 T cells against the cancer cells. In accordance to with preceding studies (Benzaid et al. 2011), we found a correlation between IPP/ApppI production of different N-BP-treated breast cancer cells and their vulnerability to Vγ9Vδ2 T cells. Therefore, it seems that cancer types producing high IPP/ApppI levels after N-BP treatment would benefit mostly from the γδ T cell-based therapies. On the other hand, we were not able to confirm that cancer cells secrete IPP or ApppI in the conditioned medium, and therefore further studies are required to confirm that extracellular IPP/ApppI would promote the migration of Vγ9Vδ2 T cells to tumors. In addition, we were unable to confirm that high IPP/ApppI production and Vγ9Vδ2 T cell activation of certain cancer cell lines would be related to cell surface ATP synthase expression as previously suggested (Vantourout et al. 2010). The detection of ATP synthase from plasma membrane seems to be very challenging technique and definitely needs to be optimized further.

Despite the many exciting possibilities of exploiting N-BP-activated Vγ9Vδ2 T cells in the clinical practice, it should be remembered that γδ T cells are a part of tightly regulated multicellular immune system, which includes multiple signaling pathways and regulatory cells. Manipulation of these signaling events by synthetic compounds carries always a risk of unexpected adverse reactions, especially with cancer patients whose immune system is functionally compromised by previous treatments and the long-lasting tumor exposure. Also,

it has become clear that the successful clinical outcome of these therapies depends much on the expansion and differentiation status of the activated Vγ9Vδ2 T cells (Santini et al. 2009). In conclusion, the future studies should mostly address the optimization of doses, schedules and synergistic effects of N-BPs in cancer immunotherapies. The combinations of innovative γδ T cell-based therapies with traditional approaches, such as chemotherapy, seem to be the most promising strategies by far.

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