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TITOLO TESI

Impaired immune pro-inflammatory cytokine profiling in prostate cancer patients upon induction using peptides within polyomavirus BK LTag-p53 binding regions

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Introduzione generale

Il carcinoma prostatico rappresenta la seconda causa di morte piu'importante nel mondo tra tutti i tumori dell'uomo. Circa un terzo dei nuovi casi di tumore diagnosticati annualmente nei soggetti maschi adulti, sono carcinomi prostatici. Un modello attuale riguardante l'insorgenza e la progressione del carcinoma prostatico dovrebbe includere il potenziale contributo dell'infiammazione come stimolo iniziale di lesioni preneoplastiche. Il virus del polioma umano BK è stato associato a stadi iniziali dei tumori dell'apparato urogenitale e si è postulato che il virus stesso giochi un ruolo importante nella patogenesi del carcinoma prostatico. La capacità oncogenica del virus BK è fortemente associata all'attività della sua principale proteina regolatoria, l'antigene tumorale Large T antigen, (L-Tag) data la capacità che ritiene la stessa di legare e inattivare i prodotti di geni oncosoppressori.

Il coinvolgimento della proteina regolatoria L-Tag del virus BK nell'alterazione di pathway enzimatici importanti del ciclo cellulare insieme con l'identificazione e l'espressione di sequenze della proteina regolatoria L-Tag in tessuti preneoplastici prostatici ci ha indotto ad investigare il ruolo che potrebbe assumere questo antigene virale come bersaglio della sorveglianza dello stato immunitario nei pazienti con carcinoma prostatico. Nostri precedenti risultati suggeriscono che determinati peptidi della proteina regolatoria L-Tag associati all'antigene HLA-A*0201, che posizionano all'interno di regioni del L-Tag responsabili per il legame con la proteina p53, possono efficientemente essere usati per testare la risposta immune in soggetti HLA-A*0201 positivi e sieropositivi per il virus BK. In questo studio proponiamo di caratterizzare più ampiamente la risposta immune contro peptidi della proteina regolatoria L-Tag in pazienti affetti da carcinoma prostatico e iperplasia benigna della

prostata in confronto a soggetti sani dello stesso sesso. Lo scopo principale è quello di testare l'ipotesi riguardo alla quale un'inefficiente risposta immune contro antigeni, espressi da virus oncogeni presenti nel tratto urogenitale può definire un ruolo della sorveglianza dello stato immunitario contro la proteina regolatoria del virus BK nell'ambito della trasformazione tumorale dell'organo prostata e relativa progressione neoplastica. In aggiunta, vogliamo testare se una stimolazione sistematica di cellule di pazienti affetti da carcinoma prostatico usando peptidi della proteina regolatoria L-Tag ritenuti immunogeni in quanto derivanti da parti dell'antigene deputate al legame con proteine di geni oncosoppressori e prevalentemente espressi in cellule tumorali, possa implementare la risposta immune cellulo-mediata. Vogliamo dunque ampiamente analizzare le caratteristiche funzionali della risposta immune contro il virus BK in pazienti affetti da carcinoma prostatico.

1. General introduction

Prostate cancer (PCa) is a leading cause of cancer death in men. Nearly one third of annually new diagnosed cancers are prostate tumors. A contemporary model for prostate cancer induction and progression should include the potential contribution of inflammation to the development of preneoplastic or neoplastic lesions.

Human Polyomavirus BK (BKV) has been associated to pre-early stages of cancer in the urinary tract and it is postulated to play an important role in the pathogenesis of prostate cancer.

The BKV oncogenic effect appears to be highly associated to the activity of its main regulatory protein Large Tumor antigen (L-Tag) because of this antigen capability to bind and inactivate the products of tumor suppressor genes.

The involvement of BKV L-Tag in the alteration of critical pathways of the human cell cycle together with the detection and expression of BKV L-Tag sequences in preneoplastic prostate tissues prompted us to investigate the role of this viral antigen as target of cellular immune surveillance in prostate cancer.

Our previous results suggest that specific HLA-A*0201-associated BKV L-Tag candidate peptides nesting within regions responsible for L-Tag binding to p53 could efficiently be used to test the immune response in HLA-A*0201+ BKV seropositive donors.

In this study we propose a comprehensive characterization of an epitope specific T cell response against BKV L-Tag in BKV-experienced patients bearing either PCa or benign prostate hyperplasia (BPH), as compared to gender-matched healthy donors. The specific aim of this study is to test the hypothesis whether an inefficient immune responsiveness to antigens specifically expressed by oncogenic viruses

located in the urinary tract may define a role for BKV L-Tag immune surveillance in organ specific tumorigenesis and subsequent neoplastic progression. Furthermore, we want to test if a systemic boosting of T cells from prostate cancer patients using immunogenic peptides within BKV L-Tag regions prevalently binding products of tumor suppressor genes (i.e. p53) and highly expressed in cancer cells would implement a T cell immune response in favor to a pro-inflammatory immune activity. We thus want to fully characterize the functional features of BKV specific T cell immune response elicited by L-Tag in patients bearing PCa.

2. Polyomavirus BK

2.1. Etiology, epidemiology and clinical manifestation

Human polyomavirus BK is a DNA virus belonging to the taxonomic family Polyomavirinae that comprises 13 distinct viruses with a common ancestor that exhibit a limited host range of host species to infect (Cole 1996). Human polyomaviruses are BKV, JCV, and the newly discovered polyomaviruses WUV (Washington University polyomavirus) (Gaynor, Nissen et al. 2007), KIV (Karolinska Institute polyomavirus) (Allander, Andreasson et al. 2007), and MCV (Merkel cell carcinoma polyomavirus or MCPyV) (Feng, Shuda et al. 2008). They share approximately a 70-75% genome homology.

Human Polyomavirus BK has been first identified in 1979 isolated from urine of a Sudanese patient (with initial B.K.) suffering from ureteral stenosis after kidney transplantation (Gardner, Field et al. 1971). The BK virus is ubiquitous in the human population worldwide (Padgett and Walker 1976) establishing a life-long latent infection, except in some populations of Brazil, Paraguay and Malaysia (Brown, Tsai et al. 1975). Serological evidence indicates that nearly 90% of individuals are infected by early childhood, although a decrease in this rate (70-80%) during the human lifespan is reported (Taguchi, Kajioka et al. 1982).

After primary infection the virus disseminates and establishes a latent infection in renal tubular epithelial cells and urothelial cell layers, the latter representing the principal site of viral latency (Imperiale 2000; Hirsch and Steiger 2003). It is also possible to detect BKV in liver, stomach, lung, lymph nodes (Israel, Martin et al. 1978; Pater 1980) and lymphocytes (Dorries, Vogel et al. 1994; Degener, Pietropaolo

et al. 1997). The presence of BKV in tonsillar tissue (Goudsmit, Wertheim-van Dillen et al. 1982) suggests for respiratory tract as the initial site of viral replication. However, primary infection is thought to be asymptomatic although no specific upper respiratory symptoms have been noted in some individuals (Shah 1996). The route of transmission may thus occur via respiratory secretions or exposure to urine (Major 2001). Spontaneous reactivations and low-level replication with shedding into urines is observed in 5-20% of healthy individuals (Hirsch 2005).

Usually polyomavirus cause persistent subclinical infections in humans and BKV infection rarely leads to clinical manifestation. Mild pyrexia, malaise, vomiting, respiratory illness, pericarditis and hepatic dysfunctions have been reported with primary infection (Fioriti, Videtta et al. 2005). Reactivation in immune competent individuals with intermittent low-level urinary replication (BKV loads of $\leq 10^6$/ml) and urinary viral shedding has been detected in 5% of infected subjects (Shah, Daniel et al. 1997). However, when the immune system is compromised, as following solid organ and bone marrow transplantation, HIV infection, chemotherapy or pharmacologic immune suppression, rate and level of BKV replication increase and may lead to organ disease (Gardner, MacKenzie et al. 1984; Leung, Suen et al. 2001; Hirsch, Knowles et al. 2002).

Polyomavirus-associated nephropathy (PVAN) is the most challenging infectious in immune compromised kidney transplanted patients that leads to renal allograft dysfunction and graft loss (Hirsch and Steiger 2003; Hirsch 2005). BKV viral shedding in the urine is a useful test to exclude PVAN in kidney recipients (Comoli, Hirsch et al. 2008), although distinguishing between asymptomatic viral shedding in the urine of immune competent subjects (0.3%) and BKV disease (10%-45%) caused

by viral reactivation (PVAN, ureteric stenosis, hemorrhagic cystitis) remains difficult (Fioriti, Videtta et al. 2005).

Recent investigations have pointed out the role of viral non-coding control region (NCCR) variants in viral host cell permissiveness, rate of viral replication and type of disease diagnosed in BKV infected subjects (Gosert, Rinaldo et al. 2008).

2.2. Viral structure and mechanisms of viral replication

Human polyomavirus BK (BKV) is an on average 5,071 base pair (bp) closed circular double-stranded (ds)DNA virus (5,153 bp for Dunlop strain (Dhar, Lai et al. 1978; Seif, Khoury et al. 1979) (Figure 1); 4,963 bp for strain MM (Tavis, Walker et al. 1989); 5,098 bp for strain AS (Yoshiike 1986)). BKV DNA is packaged as circular mini-chromosome in a complex with histone proteins (H2A, H2B, H3 and H4) and enclosed by a non-enveloped icosahedral capsid composed of three viral proteins (VP1, VP2, and VP3) (Eash, Manley et al. 2006).

The genome of BKV consists of the hypervariable non-coding control region (NCCR) located between the genetically conserved coding regions for early genes and late genes. The NCCR drives viral gene expression. It contains, beyond the relevant cellular transcription factor binding sites, the origin for viral DNA replication (*ori*), promoter and enhancer for transcription of early and late genes and T antigens (Tags) binding sites (Cole 2001; Moens 2001). The coding regions for early genes (L-Tag and small tumor antigen, s-tag) are transcribed in a counter-clockwise direction while coding regions for late genes (agnoprotein, major structural protein VP1 and the two minor structural proteins VP2 and VP3) in a clockwise direction (Lednicky and Butel 1999) (Figure 1). All proteins from each of the two coding regions are derived from a

common precursor mRNA by alternate splicing. In particular, the early region is the first part of the viral genome to be transcribed and translated since encodes the two viral lifecycle regulatory L-Tag and s-tag antigens. L-Tag has been identified early on as a key regulatory molecule interacting with infected cell cycle (Fanning and Knippers 1992). Differently, the role of s-tag in the lifecycle of polyomavirus is unknown and it has been given an ancillary role for L-Tag activity, such as increasing virus yield in permissive cell infection (Rundell and Parakati 2001).

The infection cycle of BKV is divided into two phases: early and late. The first phase comprises viral entry and DNA replication while the second phase consists on assembly of new virions and release of viral progeny. Viral entry is governed by caveolae-mediated endocytosis (Eash, Querbes et al. 2004) that allows viruses to reach the nucleus of infected cells where their replication takes place (Drachenberg, Papadimitriou et al. 2003). Upon early viral gene transcription, L-Tag accumulation initiates viral DNA replication in the nucleus of infected cells by binding the NCCR *ori* region, promoting unwinding of DNA and recruiting the host cellular DNA polymerase-α and replication proteins (Imperiale 2007). Late genes expression encoding for structural proteins (VP1, VP2, and VP3) as well as expression of the regulatory agnoprotein, will follow. Viral assembly is achieved in the nucleus. The three viral structural proteins (VP1, VP2, and VP3) assemble with the replicated viral DNA to form virions that are released upon cell lysis (White and Khalili 2004).

3. Virus-mediated cell transformation

In the host species permissive cells, polyomavirus BK spreads by lytic infection. However, in non permissive cells, in the context of an abortive infection, it leads to cellular transformation (White and Khalili 2004; White and Khalili 2005), although in the presence of activated oncogenes, such as c-Harvey-ras (Pagnani, Corallini et al. 1988), adenovirus E1A, c-rasA, c-myc (Corallini 2001). In particular, the two BKV early gene products L-Tag and s-tag play a relevant role in cell immortalization and neoplastic transformation (Imperiale 2000; Imperiale 2001).

As seen above, the role of L-Tag in the viral lifecycle is relevant for productive infections while the role of s-tag in this context is less unclear (Shenk, Carbon et al. 1976). Differently, L-Tag and s-tag cooperate in the transformation of infected cells (Martin, Setlow et al. 1979; Martin, Setlow et al. 1979; Porras, Bennett et al. 1996; Yu, Boyapati et al. 2001). L-Tag is a nuclear phosphoprotein that promotes cellular transformation by interacting with products of tumor suppressor genes: retinoblastoma (Rb) gene product (pRb), Rb family members p107 (Ewen, Xing et al. 1991) and p130 (pRb2) (Hannon, Demetrick et al. 1993), and wild-type p53 (Bocchetta, Eliasz et al. 2008), thus interfering with physiological check points of cell cycle (Tognon, Corallini et al. 2003; Ahuja, Saenz-Robles et al. 2005). L-Tag-tumor suppressor gene products (thereafter referred as pRbs) binding modulates cellular signaling pathways that stimulate progression of infected cells into S phase in order to optimize the environment for viral replication (Fioriti, Videtta et al. 2005; White and Khalili 2006). Therefore, this mechanism is used by the virus to keep infected cells alive during productive infection but in the context of non permissive cells it turns out into neoplastic transformation (Imperiale 2000).

In details, L-Tag binds to pRb, p107, p130 competing with the transcription factors E2Fs (E2F-1 to E2F-8) for the re-phosphorylation of pRb, thereby interfering with E2Fs control activity and inducing infected cells to enter the cell cycle (S phase). In fact, pRbs-E2Fs binding (E2F1, 2, 3a and 3b for pRb; E2F4 and E2F5 for p107 and p130, (Weinberg 1995; Weinberg 1996) maintains pRb in a hypophosphorylated form in G0 and its phosphorylation during G1 to S progression is due to actions of mitogenic signals through cyclin-dependent kinases (Cobrinik 2005; White and Khalili 2006). However, L-Tag-pRb, p107, and p130 interactions are limited due to the low expressed levels of the BKV regulatory protein. Nevertheless, L-Tag alters phosphorylation patterns of pRbs members inducing serum-independent growth of infected cells (Harris, Chang et al. 1998; Harris, Christensen et al. 1998). Relevant is also the notion that polyomavirus L-Tag-pRbs binding is not stable during cell cycle phases (Ludlow, Shon et al. 1990).

Sequestration of p53 by L-Tag in the cytoplasm of infected cells represents the main step for their malignant transformation. The latter requires p53 inactivation to prevent: a) cell cycle arrest; b) DNA repair; c) cell apoptosis (Pipas and Levine 2001). The tumor suppressor p53 is able to mediate cell cycle arrest by inducing p21 expression, a cyclin-dependent kinase inhibitor (Xiong, Hannon et al. 1993) that in turn causes cell arrest. It also drives to DNA repair in genetic damaged cells thus avoiding propagation of genetic mutations or aberrations to daughters' cells (Bocchetta, Eliasz et al. 2008). L-Tag-p53 binding stabilizes the protein but inactivates its tumor suppressor function, thus mimicking the phenotypic effects caused by mutations in the p53 gene (Figure 2). The coexistence of BKV L-Tag and wild-type p53 in the cytoplasm of infected cells (Das, Shah et al. 2004) is a key point

for supporting a BKV intervention in the genesis of human cancers (Das, Wojno et al. 2008).

4. Association of BKV with human cancers

4.1. Background

The role of human polyomavirus in human cancers is still debated. Recent investigations have associated polyomavirus with the outgrowth of specific cancer types including colorectal cancer (Enam, Del Valle et al. 2002; Casini, Borgese et al. 2005), glioblastomas (Tognon, Casalone et al. 1996; Del Valle, Delbue et al. 2002), mesotheliomas (Carbone, Rizzo et al. 1997), prostate cancer (Das, Shah et al. 2004), lymphomas (Engels, Rollison et al. 2005; Rollison, Engels et al. 2006). Complete viral polyomavirus DNA genome or fragments containing the early region are able to transform embryonic fibroblasts and cells cultured from kidney or brain of mouse, rat, hamster, rabbit and monkey (Corallini 2001; Tognon, Corallini et al. 2003). It has rendered polyomavirus as prototypes of DNA tumor virus well amenable to studies in experimental models. However, the possible role for polyomavirus BK in human cancers is still controversial.

In human, transformation of human cells by BKV is not efficient and often abortive (Tognon, Corallini et al. 2003) although BKV-infected or transfected cells show morphological alterations and increased lifespan (Grossi, Caputo et al. 1982). A complete transformed phenotype could be seen in human infected cells when they are *in vitro* transfected with recombinant DNA containing BKV early region genes (L-Tag) and oncogenes, such as c-rasA (pBK/c-rasA) or c-myc (pBK/c-myc), as seen in human embryo fibroblasts (Pater and Pater 1986) and adenovirus E1A (pBK/12-E1A) in human embryonic kidney (HEK) cells (Vasavada, Eager et al. 1986).

So far, several scientific works examining the presence of BKV in human cancers using different techniques as polymerase chain reaction (PCR), immunohistochemistry (IHC), *in situ* hybridization (ISH) are available in the literature. Among them, some reported either BKV DNA, or viral RNA or proteins presence in the tumor specimens tested (Pagnani, Corallini et al. 1988; Imperiale 2000; Tognon, Corallini et al. 2003; White and Khalili 2004). In contrast, some other reports found no evidence for BKV in human tumors (Weggen, Bayer et al. 2000; Knoll, Stoehr et al. 2003; Bergh, Marklund et al. 2007; Lau, Lacey et al. 2007; Rollison, Sexton et al. 2007).

4.2. BKV and Prostate Cancer

The prostate is an accessory reproductive male gland located next to and under the bladder completely surrounding the urethra. The gland normal functioning depends on androgen hormones activity such as testosterone. Prostate cancer (PCa) is the second leading cause of cancer death in older men (Gonzalgo and Isaacs 2003) with an incidence rate of 300.000 new diagnosed cases per year (ranging from 119.9% in Northern America to 1.8% in China) and a worldwide mortality rate of 12.6% (range: 28% in the Caribbean to 1% in China) (Parkin, Bray et al. 2005; Jemal, Siegel et al. 2007). Risk factors associated with increased incidence rate are: i) advanced age with an average age of 72 years at first diagnosis due to a longer life expectancy (the tumor is almost rare in 40-45 years old younger men); ii) family history of PCa (9% of PCa, genetically passed on in an autosomal dominant fashion); iii) race (African Americans develop PCa 50% more frequently than their Caucasian counterparts at the same age); iv) hormones activity (androgens and insuline-like

growth factor); v) lower rate of daily dietary fibers and higher rate of fat (Littrup 1997; Brawley 1998; Brawley, Knopf et al. 1998).

Prostate adenocarcinoma is the most common histological type among prostate cancers. It usually arises from the active glandular epithelium along the periphery of the gland. PCa is an organ confined low risk cancer with low aggressive potential (usually, only 1 out of 6 PCa are lethal cancers; Gleason score 8 to 10 at first diagnosis, 50% survival rate at 5 years). It has been reported that PCa at prostate intraepithelial neoplasia (PIN) stage (tumor size >0.5 cm^3; Gleason score 2 to 4) progress to the level of overt cancer (tumor size of 3-4 cm^3) in about 10 years, and metastasize (preferentially in bone and brain) in about 15 years (Pound, Partin et al. 1999). Improvement of pre-emptive screenings (Prostate Specific Antigen; PSA test) has reduced the frequency of localized invasive overt cancers in patients at the time of first diagnosis with a dramatic shift toward a "watchful waiting" treatment of choice. The latter has dramatically limited the development of new research strategies owing to the intrinsic properties of PCa that render this tumor a difficult target (Schlomm, Erbersdobler et al. 2007). It has thus prompted researchers to focus on the identification of early stages of the tumor to better understand the mechanism responsible for PCa onset and progression. The proliferative inflammatory atrophy (PIA) of the prostate has recently gained relevance as potential precursor of prostatic intraepithelial neoplasia (PIN) and overt PCa (De Marzo, Platz et al. 2007), in particular owing to the prevalence of PIA in the peripheral zone of the organ, where usually histological transitions between PIA and PIN occur (De Marzo, Marchi et al. 1999; Putzi and De Marzo 2000) (Figure 3).

The proliferative nature of the prostate atrophy together with low rate of mutations detected in the tumor suppressor genes Rb1 and p53 of prostate cancer cells (Dong 2006), as compared to other tumors, has suggested for the involvement of an infectious agent that establishes a persistent subclinical infection in the urinary tract, such as polyomavirus BK (Das, Wojno et al. 2008). To sustain this hypothesis there are both the role played by human papillomavirus (HPV) oncoproteins E6 and E7 for the genesis of cervical cancer (Dyson, Howley et al. 1989; Choo and Chong 1993) and the discovery of BKV L-Tag-p53 colocalization to the nuclei of bladder carcinoma cells (Geetha, Tong et al. 2002). On this regard, in an interesting study, Weinreb et al identified a population of patients with polyomavirus infected "decoy" cells in the urine (Weinreb 2006). The data correlate to the incidence of bladder cancer in those "decoy" cells positive patients thus leading to an association between polyomavirus and bladder cancer. In addition, Monini et al. demonstrated the presence of BKV sequences by PCR in more than 50% of both human urinary tract normal and tumor tissues, including prostate (Monini, Rotola et al. 1995). Immunohistochemical analysis of normal and atrophic epithelial prostatic cells using monoclonal antibodies specific to BKV L-Tag has shown that 60% of prostate tissues from healthy subjects are BKV positive as well as 71% of specimens from PIA. In particular, dual staining for L-Tag and p53 has been detected either in the nucleus or in the cytoplasm of PIA specimens but BKV L-Tag and p53 colocalization to the cytoplasm has been particularly seen in prostate cancer cells, while in L-Tag-negative tumors p53 staining was nuclear (Das, Shah et al. 2004). More recently, the same group has demonstrated that the p53 inactivation occurs in atrophic cells expressing wild-type p53 (Das, Wojno et al. 2008), finding promptly supported by other groups (Bocchetta, Eliasz et al. 2008).

Imperiale's group investigation fosters BKV as a cofactor in the etiology of PCa at early stages based on the following "hit-and-run" model: BKV infection of normal epithelial cells drive into PIA. At this level, expression of BKV is evident. The sequestration of wild-type p53 induces infected cells to accumulate enough mutations to growth clonally without control. Both the dilution of BK viral episomes due to lack of replication and the immune selection of BKV infected cell against L-Tag are responsible for the loss of BKV in tumor cells (Das, Wojno et al. 2008). Although a low frequency of BKV L-Tag expression is reported by some authors (Zambrano, Kalantari et al. 2002; Balis, Sourvinos et al. 2007; Bergh, Marklund et al. 2007; Lau, Lacey et al. 2007)**,** their data cannot exclude the possibility for a "hit-and-run" mechanism of BKV oncogenisis. Moreover, because of NCCR hypervariability in tissue cultures (Rubinstein, Schoonakker et al. 1991; Hanssen Rinaldo, Hansen et al. 2005), genetically rearranged BKV variant might exist in the urinary tract and be responsible for neoplastic transformation in prostate cells (i.e. URO-1) (Shinohara, Matsuda et al. 1993). Thus, given the results obtained with prostate and bladder carcinomas, along with the kidney being the main site for BKV persistence, tumors of the urinary tract are the most logical target sites for an etiological association with BKV.

5. BKV L-Tag and cellular immune response

Since L-Tag mediated inactivation of p53 has been suggested to represent a critical step for BK viral oncogenicity, (Imperiale 2001; Tognon, Corallini et al. 2003) this antigen has been identified as an important target for cancer immunity in murine models (Degl'Innocenti, Grioni et al. 2005; Otahal, Hutchinson et al. 2005). Early studies in SV40 infected mice have reported on conserved CD8+ T cells immune responses against SV40 L-Tag (Mylin, Schell et al. 2000).

Within the BK virus-cell interaction modalities, L-Tag is thus the most highly expressed BKV antigen in non permissive infected cells and might encompass viral peptides generated by the MHC antigen processing pathway and, possibly, targeted by specific T cells epitopes. Moreover, the L-Tag regions required for viral transformation, (pRbs domain aa91-120; p53 domains aa251-470 and aa521-640; almost 80% of the entire sequence) are less likely to be mutated or lost, thus genetically conserved among polyomavirus strains (Velders, Macedo et al. 2001).

So far, cellular immune response against BKV reactivation has been limited to hematopoietic stem cell or solid organ (i.e. kidney) transplantations due to the relevant association between BKV reactivation upon immune suppression and polyomavirus-associated nephropathy (PVAN) inducing allograft failures (Purighalla, Shapiro et al. 1995). However, it has become more than ever evident, among immunologists and virologists, the principal role of T cell immune response in balancing BKV reactivation in latently infected cells since it has been seen that BKV-specific humoral immune response alone is inefficient in controlling virus reactivation and spreading and in containing BKV-related diseases (Comoli, Binggeli et al. 2006; Comoli, Hirsch et al. 2008). The finding has been of greater importance when a

correlation between reductions of immunosuppressive treatments in PVAN transplanted recipients and decrease of BKV viral load in both plasma and urine along with an increase of frequency of IFN-γ-secreting lymphocytes was proved (Comoli, Azzi et al. 2004).

To gain further insight into BKV specific cellular immune response, it is important to define a specific pattern of T cell response to BKV antigens. Several studies have been devoted to the identification of HLA class I specific immunogenic epitopes within either BKV capsid proteins VP1 and VP2 or the regulatory protein L-Tag (Zhu, Rice et al. 1992; Krymskaya, Sharma et al. 2005; Li, Melenhorst et al. 2006; Provenzano, Bracci et al. 2006; Randhawa, Popescu et al. 2006). Taking advantage of VP1 and L-Tag overlapping peptide pools and using as readout IFN-γ protein release measured by ELIspot or flow cytometry assays, a relevant CD4+ and CD8+ T cell immune response has been observed in transplanted patients beyond a mandatory HLA restriction (Hammer, Brestrich et al. 2006). However, in spite of the use of this technically sound approach, the characterization of an epitope specific T lymphocyte response against immunogenic BKV peptides, in BKV-related diseases (either PVAN or urinary tract malignancies) is still demanding.

Cytotoxic T lymphocytes (CTLs) specific for antigens from viruses responsible for persistent infections are frequently characterized by specific phenotypic profiles. For instance, Epstein-Barr virus (EBV) or Cytomegalovirus (CMV) specific CTL responses are elicited by distinct subset of memory T cells reported as early effectors for EBV (CD45RA-/(+)/CCR7+) or late effectors for CMV (CD45RA+/CCR7-) (Appay, Dunbar et al. 2002). In a recent study, Provenzano et al indicated that BKV specific CTL responders to portions of L-Tag exquisitely belong to a CD45RA+/CCR7+(-) CD8 T cell population (Provenzano, Bracci et al. 2006).

Notably, the study demonstrated for the first time that BKV seropositive subjects mount a powerful CTL response towards epitopes encompassed by highly phylogenetically L-Tag conserved regions implicated in the p53 mediated control of the cell cycle of host cells (Provenzano, Bracci et al. 2006).

6. Study design

6.1. Background

Polyomavirus BK (BKV) oncogenisis is due to the ability of the Large Tumor antigen (L-Tag) to regulate critical pathways of human cell cycle when BKV infects non permissive cells. Thus, owing to L-Tag expression in tumor cells, it has been identified as an important target for immune surveillance in cancer. In prostate, cytoplasmic colocalization of BKV L-Tag and p53 has been detected at pre-cancerous lesions stages such as proliferative inflammatory atrophy (PIA). It prompted us testing whether an inefficient immune response against L-Tag-p53 binding regions might define a role for BKV immune surveillance in the progression of prostate cancer (PCa).

Eighty-two male patients (39 benign prostate hyperplasia (BPH) and 43 PCa) and 10 healthy gender-matched donors were enrolled. Detection of BKV specific IgG in patients and donors sera was carried out by ELISA using recombinant L-Tag and capsid VP1 proteins purified from baculovirus expression system. BKV and JCV DNA was detected in surgically excised prostate specimens by using quantitative real time (qrt)PCR specific for genetically conserved regions in either BKV or JCV L-Tag. Peripheral blood mononuclear cells (PBMCs) from HLA-A*0201 patients were ex vivo stimulated with HLA-A*0201-restricted peptides within L-Tag-p53 binding domains. Quantitative rt-PCR gene expression for a panel of 6 cytokines was used as readout. Level of CD4+ and CD8+ T regulatory cells through FoxP3 expression was determined in peptide expanded PBMCs of PCa patients. Correlations between the

extent of cytokine gene expression, BKV serology and clinical features of our patients were analyzed.

6.2. Material and methods

6.2.1. Patients and cell sampling

Eighty-two men who had been diagnosed for benign prostate hyperplasia (BPH; n=39) or prostate cancer (PCa; n=43) at the Department of Urology of the Basel University Hospital (Basel, Switzerland) in 2007 were enrolled in the study upon informed consent approved by the Ethical Committee (EKBB) of Basel. BPH patients underwent either transurethral resection (TUR) or potassium titanyl phosphate laser vaporization (KTP) of the prostate, while PCa patients underwent either TUR of prostate, Laparoscopic radical prostatectomy (LRP) or Endoscopic Extraperitoneal Radical Prostatectomy (EERP). The relevant clinical data were collected by retrospective review of the patients' files.

Peripheral blood mononuclear cells (PBMCs) from venous blood of patients were separated by Ficoll-Hypaque density gradient centrifugation. T lymphocyte were cultured in RPMI medium supplemented with 100μg/ml Kanamycin, 10mM Hepes, 1mM sodium pyruvate, 1mM Glutamax and nonessential amino acids (all from GIBCO Paisley, Scotland), thereafter referred to as complete medium supplemented with 5% human serum (Blutspendezentrum Universitätsspital Basel Switzerland). Cells were subsequently plated in complete medium with 5% human serum in 24-well plates at a final concentration of $1x10^6$ cells/ml and were co-cultured (37°C, 5% CO_2 atmosphere) with irradiated (750 sec in a gamma ray irradiator equipped with a ^{137}Cs radiation source emitting 100 rad/min) autologous antigen presenting cells (APCs) previously pulsed for 2h with peptides at a final concentration of 10μg/ml either for priming or for re-stimulation rounds. Recombinant human (rh) IL-2 (Hoffmann-

LaRoche, Basel, Switzerland) was added to the cultures at 1ng/ml, 1ng/ml and 5ng/ml, on days 3, 7 and 10, respectively, and cells were re-stimulated with specific peptide in the presence of irradiated APCs on day 7 of culture. Control cultures were performed in the absence of antigenic peptides.

6.2.2. Patient serology by using Enzyme immunoassay (EIA).

Detection of BKV specific antibodies in donor and patients sera was performed by EIA as reported in (Leuenberger, Andresen et al. 2007). Optical densities (ODs) were measured using an automated plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 492 nm. Equivalents of 1.0 pmol of antigen/well, serum dilutions of 1:400, and dilutions of anti-IgG antibodies of 1:10,000 were found to yield minimal background. Affinity-purified GST was run as a negative control and subtracted from the GST-BKV L-Tag domain 1 (LTD1), GST-BKV VP1, and GST-BKV agnoprotein signals. For every sample, the OD was determined by subtraction of the GST background applied in parallel. The cut-off was defined as two standard deviations the GST background level taken as the negative control. Therefore, all OD values of <0.02-0.04 were considered negative. Values from 0.04 to 0.1 were classified as borderline positive and values of >0.1 as positive. For confirmatory testing, we included three selected samples as references, which were negative, borderline positive and strongly positive, indicating variation coefficients of <10%, which can cause minimal shifts in the groups.

6.2.3. Quantitative PCR for BKV DNA detection.

Measurements of BKV viral load in tissue specimens were performed according to standard real-time PCR protocols. Briefly, cells from tissue samples were scraped under sterile conditions, incubated at 95°C for 10 min, transferred to ice, and then treated with 5 µl of proteinase K (5 mg/ml) at 55°C for 3 to 5h, followed by enzyme inactivation at 95°C for 10 to 15 min. DNA was isolated with the QIAamp DNA minikit (QIAGEN, Hilden, Germany) and quantified by real-time PCR using an ABI Prism 7900 HT sequence detector (Applied Biosystems, Rotkreuz, Switzerland), as described previously. The following primers were used for BKV L-Tag antigen amplification: PYV.for (5′-TAGGTGCCAACCTATGGAACAGA-3′) and PYV.rev (5′-GAAAGTCTTTAGGGTCTTCTACC-3′). The BKV probe was 5′-FAM-CATTAAAGGAACTCCACCAGGACTCCCACTC-TAMRA-3′.

6.2.4. Peptide synthesis and *ex vivo* induction using qrt-PCR assay

Peptides used in this study were synthesized by Princeton Biomolecules (Langhorne, PA, USA) with a purity ranging from 90% to 100%, as analyzed by high-performance liquid chromatography (HPLC), dissolved in 100% DMSO and stored at -70°C until use.

Ex vivo induction of peptide-specific responses was attempted as follows. Briefly, PBMCs isolated from venous blood by Ficoll-Hypaque density gradient centrifugation, were incubated in 96 U bottom well plates at the concentration of 2×10^5 cells in 200 µl total complete medium supplemented with 5% human serum. After an overnight resting, cells were both peptide-stimulated (1 µM) or left unstimulated and 3 hours after they were harvested for RNA extraction (RNeasy® Mini Kit

Protocol, Qiagen, Basel, Switzerland) and cDNA synthesis (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qrt-PCR) assays were performed as previously described and conducted on an ABI prism™ 7500 FAST sequence detection system using TaqMan® Universal PCR Master Mix Reagents Kit (Applied Biosystems, Rotkreuz, Switzerland) and sets of primers and probes from cytokine genes (IFN-γ, IL-2, IL-4, and IL-10) already extensively utilized. Beta actin (β-actin) was used as endogenous reference gene.

Normalized data were subsequently presented as relative quantification. The $2^{-\Delta\Delta Ct}$ method [$\Delta\Delta C_T = (C_T,$ cytokine $- C_T$ β-actin$)_{induction} - (C_T,$ cytokine $- C_T,$ β-actin$)_{baseline,}$ where C_T is the mean cycle times of the triplicate well readings] was utilized to compute the fold change of cytokine gene expression after peptide induction relative to baseline (unstimulated cells), normalized to an endogenous reference gene (β-actin).

6.2.5. Quantification of regulatory T cells.

For the quantification of $FoxP3^+$ $CD25^+$ regulatory T cells, in vitro expanded PBMCs were immunostained with anti-CD3 FITC, either anti-CD4 PerCP or anti-CD8 PerCP, anti-CD25 APC, (BD Biosciences), and anti-FoxP3 PE antibodies. FoxP3 Fix/Perm buffers were used for the intracellular staining of FoxP3 according to the manufacturer's protocol (eBioscience). Data were acquired on a FACSCalibur flow-cytometer equipped with Cellquest software (Becton Dickinson, San Jose`, CA, USA).

6.2.6. Statistical analysis

Statistical analysis was performed with Prism 4 (GraphPad) software. Data were reported as mean ± standard deviation (SD) or median and ranges where appropriate. Categorical markers were analyzed by Pearson`s chi-square test. A two tailed paired T test was used to calculate P-values. Differences were considered significant at $P<0.05$, CI 95%.

6.3. Results

6.3.1. Detection of BKV in BPH and PCa tissue specimens

The first aim was to detect the presence of BKV in surgically excised PCa specimens, as compared to BPH specimens. Twenty-one out of 43 PCa as well as 33 out of 39 BPH tissue specimens were analyzed. L-Tag BKV DNA was detectable in either tissue specimens from PCa (n=12/21, 57.2%) or from BPH (n=15/33, 45.5%) patients. The extent of DNA copies was not significantly different among both lesions (BPH median=264, range 60-673, PCa median=467, range 132-1576, p=0.61). However, to strengthen the specificity of BKV in prostate specimens, detection for JCV was also carried out. Of relevance was the negligible detection of JCV in specimens of both BPH (n=1/33, 0.03%) and PCa (n=0/21, 0.00%) patients, having the positive BPH patient a DNA detection below the limit of 300 copies/ml (Figure 4).

6.3.2. No detectable humoral immune response to L-Tag in patients bearing BKV+ specimens

To determine whether L-Tag could be the main target for the identification of competent immune surveillance in prostate cancer, we detected BKV serology in all BPH and PCa patients enrolled either against L-Tag or VP1, as compared to gender-matched healthy donors. We first confirmed the strongest positive humoral (IgG) response to VP1 for all three cohorts of subjects studied (HD: mean ± SD=0.083±0.073, median=0.045, range=0.03-0.2; BPH: mean ± SD=0.129±0.132, median=0.075, range=0.03-0.7; PCa: mean ± SD=0.124±0.082, median=0.105, range=0.03-0.43). In contrast, humoral IgG response against L-Tag was lower but

only significant in both BPH and PCa patients (HD: 0.062±0.023, median=0.05, range=0.04-0.1, p=0.55; BPH: 0.065±0.053, median=0.045, range=0.02-0.25, p=0.03; PCa: 0.057±0.035, median=0.05, range=0.02-0.15, p<0.0001) (data not shown). Indeed, we specifically detected anti-VP1 IgG production above the cut-off for strongly positive values (<0.1) in 19 out of 33 BPH (57%) and 17 out of 41 PCa (42%) patients as compared to that against L-Tag in BPH (29/33; 89%) and PCa (38/41; 92%) patients (Figure 5).

Stratifying our cohort of patients based on their positive specimens for L-Tag BKV DNA detection, we noted that all BPH (n=9/9, 100%) and PCa (n=9/10, 90%) patients bearing BKV+ specimens clustered in the range above the cut-off for strongly positive values of 0.1 for L-Tag serology, as compared to VP1 serology for both PCa (p=0.015) and BPH (p=0.048) BKV+ patients (data not shown).

6.3.3. Cytokine gene profiling in HLA-A*0201 BPH and PCa patients upon BKV L-Tag$_{579}$ and L-Tag$_{406}$ peptides induction

Among all patients, we respectively recruited 20 HLA-A*0201 positive PCa (46.5%) and 19 HLA-A*0201 positive BPH (48.7%) patients. Freshly isolated PBMCs from both cohorts of HLA-A*0201+ patients were either 3-hour *ex vivo* stimulated using in turn L-Tag$_{406}$ and L-Tag$_{579}$ peptides or left unstimulated. A panel of 6 cytokines was used as readout. Pro-inflammatory cytokine gene expression (IFN-γ and TNF-α was barely detected in PCa compared to BPH (p=0.05). Unexpectedly, levels of immune regulatory activity, as defined by IL-10 and TGF-β1 gene expression, were higher detected in PCa patients (median 4.94fold, range 1.68-10.8), as compared to BPH patients (median 1.8fold, range 0.21-3.89). Negligible was the

cytokine gene expression for lytic machinery components perforin and granzyme B (>2fold for both group of patients upon both peptides' induction; Figure 6).

An IL-10/IFN-γ ratio upon both L-Tag$_{406}$ and L-Tag$_{579}$ peptides was better correlating with IgG titer against L-Tag (L-Tag$_{406}$ r=0.71, p=0.002; L-Tag$_{579}$ r=0.61, p=0.001, Figure 7) than IgG titer against VP1 (LTag$_{406}$ r=0.162, p=0.46; LTag$_{579}$ r=0.368, p=0.25) in PCa, while there were no correlations between the immune regulatory trend and IgG titer against L-Tag or VP1 in BPH patients upon both peptides induction (LTag$_{406}$ r=-0.67, p=0.32; L-Tag$_{597}$ r=-0.11, p=0.66 for PCa patients and L-Tag$_{406}$ r=-0.32, p=0.54; L-Tag$_{579}$ r=-0.17, p=0.24 for VP1, respectively; data not shown).

6.3.4. Immune regulatory activity in PCa patients upon both L-Tag$_{406}$ and L-Tag$_{579}$ peptides induction

Due to recent investigations aiming at defining the involvement of regulatory T cells in mediating immune suppression in cancer, in particularly prostate cancers, we tested the expression of FoxP3 in either CD4+ CD25+ or CD8+ CD25+ T cells upon LTag$_{406}$ and LTag$_{579}$ peptide *in vitro* stimulation of PBMCs from 4 PCa patients. While both peptides were able to fairly increase the number of CD4+ CD25+ FoxP3+ cells upon induction (LTag$_{406}$ positive cells: 60.71±0.3 to 63.95±0.7, increase 5% p=0.02; LTag$_{579}$ positive cells: 48.61±1.2% to 54.75±1.7, increase 11%; p=0.06), only LTag$_{406}$ increased CD8+ CD25+ FoxP3+ cells in peptide-expanded PBMCs from PCa patients (48.23±0.4 to 62.48±1.4, increase 23%; p=0.005), as compared to LTag$_{579}$ positive cells (24.19±4.6 to 23.46±3.6; p=0.8). One representative PCa patient is depicted in Figure 8.

6.3.5. BKV L-Tag specificity for peptide $LTag_{406}$

Testing possible homologies within human polyomaviruses, we noted that peptide $LTag_{579}$ (LLLIWFRPV) shares a 98 to 100% homology in almost 100% of BKV and JCV strains and 78% in all SV40 strains. Differently, peptide $LTag_{406}$ (VIFDFLHCI) shares a 78% homology in almost 100% BKV and JCV strains and 56% in all SV40 strains. In details, two amino acid substitutions in position 3 (Tyrosine vs Phenylalanine) and position 8 (Cysteine vs Lysine) in the JCV L-Tag and 2 more in the SV40 L-Tag (position 2: Isoleucine vs Valine and position 9: Isoleucine vs Methionine) were respectively found.

6.3.6. Clinical relevance of the study

In order to define clinical relevance of our study, clinical features of PCa patients (PSA levels and Gleason score) were compared to BKV+ specimens, IgG serology and immunologic trends. Due to lack of correspondence between IgG serology and DNA detection in surgically excised tissue specimens among all HLA-A*0201 patients, the PCa cohort selected for this study was of 15 PCa patients. Upon $LTag_{579}$ induction, 4 PCa patients bearing BKV+ specimens (50% of those BKV+ (4/8) and 26.7% of all tested (4/15)) share immune regulatory pattern (IL-10/IFN-γ) and higher Gleason scores (≥7). Upon $LTag_{406}$ induction, 5 PCa patients bearing BKV+ specimens (63% of those BKV+ (5/8) and 33.4% of all tested (5/15)) share immune regulatory pattern and higher Gleason scores. More relevantly, patients showing an

increase of CD8+ CD25+ FoxP3+ cells upon L-Tag$_{406}$ stimulation are prevalently those with both BKV+ specimens and immune regulatory profiling (IL-10/IFN-γ).

7. Discussion

In this study we aimed at testing the hypothesis whether an inefficient immune response against L-Tag-p53 binding regions might define a role for BKV immune surveillance in organ specific tumorigenesis and subsequent neoplastic progression, as particularly in prostate cancer. Our previous data support the concept that CTL immune responses against viral antigens can be effectively expanded by reactivating T cell memory peptide-specific cells from virus-seropositive subjects (Provenzano, Panelli et al. 2006). Moreover, our previous results suggest that specific HLA-A*0201-associated BKV L-Tag candidate peptides could efficiently be used to test the immune response in HLA-A*0201+ BKV seropositive donors (Provenzano, Bracci et al. 2006). Indeed, in spite the strong immunogenic potential of both HLA-A*0201-restricted L-Tag$_{406}$ and L-Tag$_{579}$ peptides, as described in this study (Provenzano, Bracci et al. 2006), it seems that they function as inducers of immune regulatory activity in BKV seropositive PCa. Among them, L-Tag$_{406}$ turned out to be the candidate HLA-A*0201 restricted peptide for immune regulatory activity. Before focusing more in detail into the role of BKV L-Tag peptides in regulating immune responses in PCa patients, a first important result of our study was the confirmation of the strong immune response mounted by either BKV seropositive subjects or BKV seropositive patients against proteins of the viral capsid, such as VP1, as compared to that has been seen against regulatory proteins (i.e. L-Tag). This discrepancy could be ascribed to: i) the status of "viral latency" accompanied by smouldering reactivations of the virus inducing to formation of new viral progeny spreading in the circulation and infecting other organs; ii) the relevant low extent of expression of L-Tag in PCa, being L-Tag expression more peculiar for earlier stages of the disease. Although the speculation prompting at explaining the lower titer rate of

specific antibodies against L-Tag detected in PCa, the finding remarks the fact that it is hard to define a role for BKV in the genesis of PCa only based on L-Tag serology in PCa patients (Newton, Ribeiro et al. 2005).

Of note is the finding that more than 50% of PCa patients bear positive BKV L-Tag tissue specimens. In this context, more interesting is also the possibility to positively correlate PCa patients bearing BKV L-Tag positive specimens with the impaired humoral immune response defined in the patients bloodstream. Among the two immunogenic HLA-A*0201 restricted peptides used for the analyses of the immune reactivation in PCa patients, peptide L-Tag$_{406}$ stimulation better induce to an impairment of pro-inflammatory and lytic machinery component activity in favor to an immune regulatory function in PCa, as compared to BPH. Moreover, almost 50% of PCa patients upon peptide L-Tag$_{406}$ induction show evidence of enhanced immune regulatory trend and higher Gleason score with the concomitant expansion of CD8+, CD25+, FoxP3+ T cells from the same patients (Kiniwa, Miyahara et al. 2007). The possibility to attribute the PCa patients' immune regulatory pattern, as characterized by L-Tag$_{406}$ activity, to the polyomavirus BK is in the amino acid structure of the peptide. The homology between BKV and JCV L-Tag is of 78%, and even lower, about 56%, with SV40 L-Tag. It thus defines the possibility for L-Tag to have some regions with highly conserved structure among polyomavirus strains, such as the one nesting L-Tag$_{596}$, and some other regions with higher rate of amino acids substitutions, such as the one nesting L-Tag$_{406}$. Although peptide L-Tag$_{406}$ substitutions do not consider amino acids in their position as anchor residues responsible for peptide binding to HLA-A*0201 determinant, they unquestionably confer to L-Tag$_{406}$ to exquisitely belong to BKV L-Tag.

The implementation of these studies might set the stage for original controlled investigations on mechanisms allowing BKV involvement in prostate cancers from active immune responses identifiable at early stages of prostate cancer and might provide sound scientific bases for the application of a wide range MHC peptide-vaccination in either preventive or therapeutic settings.

8. Bibliography

- Ahuja, D., M. T. Saenz-Robles, et al. (2005). "SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation." Oncogene 24(52): 7729-45.
- Allander, T., K. Andreasson, et al. (2007). "Identification of a third human polyomavirus." J Virol 81(8): 4130-6.
- Appay, V., P. R. Dunbar, et al. (2002). "Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections." Nat Med 8(4): 379-85.
- Balis, V., G. Sourvinos, et al. (2007). "Prevalence of BK virus and human papillomavirus in human prostate cancer." Int J Biol Markers 22(4): 245-51.
- Bergh, J., I. Marklund, et al. (2007). "No link between viral findings in the prostate and subsequent cancer development." Br J Cancer 96(1): 137-9.
- Bocchetta, M., S. Eliasz, et al. (2008). "The SV40 large T antigen-p53 complexes bind and activate the insulin-like growth factor-I promoter stimulating cell growth." Cancer Res 68(4): 1022-9.
- Brawley, O. W. (1998). "Prostate cancer and black men." Semin Urol Oncol 16(4): 184-6.
- Brawley, O. W., K. Knopf, et al. (1998). "The epidemiology of prostate cancer part II: the risk factors." Semin Urol Oncol 16(4): 193-201.
- Brown, P., T. Tsai, et al. (1975). "Seroepidemiology of human papovaviruses. Discovery of virgin populations and some unusual patterns of antibody prevalence among remote peoples of the world." Am J Epidemiol 102(4): 331-40.
- Carbone, M., P. Rizzo, et al. (1997). "Simian virus-40 large-T antigen binds p53 in human mesotheliomas." Nat Med 3(8): 908-12.
- Casini, B., L. Borgese, et al. (2005). "Presence and incidence of DNA sequences of human polyomaviruses BKV and JCV in colorectal tumor tissues." Anticancer Res 25(2A): 1079-85.
- Choo, K. B. and K. Y. Chong (1993). "Absence of mutation in the p53 and the retinoblastoma susceptibility genes in primary cervical carcinomas." Virology 193(2): 1042-6.
- Cobrinik, D. (2005). "Pocket proteins and cell cycle control." Oncogene 24(17): 2796-809.
- Cole, C., S.D. Conzen (2001). "Fundamental Virology." Knipe DM and Howley PM (eds). Lippincott Williams and Wilkins, Phipadelphia: 985-1018.
- Cole, C. N. (1996). "Fundamental Virology." Fields BN, Knipe DM, Howley PM (eds). Lippincott, Williams and Wilkins, Philadelphia: 917-946.
- Comoli, P., A. Azzi, et al. (2004). "Polyomavirus BK-specific immunity after kidney transplantation." Transplantation 78(8): 1229-32.
- Comoli, P., S. Binggeli, et al. (2006). "Polyomavirus-associated nephropathy: update on BK virus-specific immunity." Transpl Infect Dis 8(2): 86-94.
- Comoli, P., H. H. Hirsch, et al. (2008). "Cellular immune responses to BK virus." Curr Opin Organ Transplant 13(6): 569-74.

- Corallini, A., M. Tognon (2001). "Human polyomaviruses: Molecular and clinical perspectives " Fields BN, Knipe DM, Howley PM (eds). Lippincott Williams and Wilkins, Philadelphia: 917-946.
- Das, D., R. B. Shah, et al. (2004). "Detection and expression of human BK virus sequences in neoplastic prostate tissues." Oncogene 23(42): 7031-46.
- Das, D., K. Wojno, et al. (2008). "BK virus as a cofactor in the etiology of prostate cancer in its early stages." J Virol 82(6): 2705-14.
- De Marzo, A. M., V. L. Marchi, et al. (1999). "Proliferative inflammatory atrophy of the prostate: implications for prostatic carcinogenesis." Am J Pathol 155(6): 1985-92.
- De Marzo, A. M., E. A. Platz, et al. (2007). "Inflammation in prostate carcinogenesis." Nat Rev Cancer 7(4): 256-69.
- Degener, A. M., V. Pietropaolo, et al. (1997). "Detection of JC and BK viral genome in specimens of HIV-1 infected subjects." New Microbiol 20(2): 115-22.
- Degl'Innocenti, E., M. Grioni, et al. (2005). "Peripheral T cell tolerance occurs early during spontaneous prostate cancer development and can be rescued by dendritic cell immunization." Eur J Immunol 35(1): 66-75.
- Del Valle, L., S. Delbue, et al. (2002). "Expression of JC virus T-antigen in a patient with MS and glioblastoma multiforme." Neurology 58(6): 895-900.
- Dhar, R., C. J. Lai, et al. (1978). "Nucleotide sequence of the DNA replication origin for human papovavirus BKV: sequence and structural homology with SV40." Cell 13(2): 345-58.
- Dong, J. T. (2006). "Prevalent mutations in prostate cancer." J Cell Biochem 97(3): 433-47.
- Dorries, K., E. Vogel, et al. (1994). "Infection of human polyomaviruses JC and BK in peripheral blood leukocytes from immunocompetent individuals." Virology 198(1): 59-70.
- Drachenberg, C. B., J. C. Papadimitriou, et al. (2003). "BK polyoma virus allograft nephropathy: ultrastructural features from viral cell entry to lysis." Am J Transplant 3(11): 1383-92.
- Dyson, N., P. M. Howley, et al. (1989). "The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product." Science 243(4893): 934-7.
- Eash, S., K. Manley, et al. (2006). "The human polyomaviruses." Cell Mol Life Sci 63(7-8): 865-76.
- Eash, S., W. Querbes, et al. (2004). "Infection of vero cells by BK virus is dependent on caveolae." J Virol 78(21): 11583-90.
- Enam, S., L. Del Valle, et al. (2002). "Association of human polyomavirus JCV with colon cancer: evidence for interaction of viral T-antigen and beta-catenin." Cancer Res 62(23): 7093-101.
- Engels, E. A., D. E. Rollison, et al. (2005). "Antibodies to JC and BK viruses among persons with non-Hodgkin lymphoma." Int J Cancer 117(6): 1013-9.
- Ewen, M. E., Y. G. Xing, et al. (1991). "Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein." Cell 66(6): 1155-64.
- Fanning, E. and R. Knippers (1992). "Structure and function of simian virus 40 large tumor antigen." Annu Rev Biochem 61: 55-85.

- Feng, H., M. Shuda, et al. (2008). "Clonal integration of a polyomavirus in human Merkel cell carcinoma." Science 319(5866): 1096-100.
- Fioriti, D., M. Videtta, et al. (2005). "The human polyomavirus BK: Potential role in cancer." J Cell Physiol 204(2): 402-6.
- Gardner, S. D., A. M. Field, et al. (1971). "New human papovavirus (B.K.) isolated from urine after renal transplantation." Lancet 1(7712): 1253-7.
- Gardner, S. D., E. F. MacKenzie, et al. (1984). "Prospective study of the human polyomaviruses BK and JC and cytomegalovirus in renal transplant recipients." J Clin Pathol 37(5): 578-86.
- Gaynor, A. M., M. D. Nissen, et al. (2007). "Identification of a novel polyomavirus from patients with acute respiratory tract infections." PLoS Pathog 3(5): e64.
- Geetha, D., B. C. Tong, et al. (2002). "Bladder carcinoma in a transplant recipient: evidence to implicate the BK human polyomavirus as a causal transforming agent." Transplantation 73(12): 1933-6.
- Gonzalgo, M. L. and W. B. Isaacs (2003). "Molecular pathways to prostate cancer." J Urol 170(6 Pt 1): 2444-52.
- Gosert, R., C. H. Rinaldo, et al. (2008). "Polyomavirus BK with rearranged noncoding control region emerge in vivo in renal transplant patients and increase viral replication and cytopathology." J Exp Med 205(4): 841-52.
- Goudsmit, J., P. Wertheim-van Dillen, et al. (1982). "The role of BK virus in acute respiratory tract disease and the presence of BKV DNA in tonsils." J Med Virol 10(2): 91-9.
- Grossi, M. P., A. Caputo, et al. (1982). "Transformation of human embryonic fibroblasts by BK virus, BK virus DNA and a subgenomic BK virus DNA fragment." J Gen Virol 63(2): 393-403.
- Hammer, M. H., G. Brestrich, et al. (2006). "HLA type-independent method to monitor polyoma BK virus-specific CD4 and CD8 T-cell immunity." Am J Transplant 6(3): 625-31.
- Hannon, G. J., D. Demetrick, et al. (1993). "Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins." Genes Dev 7(12A): 2378-91.
- Hanssen Rinaldo, C., H. Hansen, et al. (2005). "Human endothelial cells allow passage of an archetypal BK virus (BKV) strain--a tool for cultivation and functional studies of natural BKV strains." Arch Virol 150(7): 1449-58.
- Harris, K. F., E. Chang, et al. (1998). "BK virus as a potential co-factor in human cancer." Dev Biol Stand 94: 81-91.
- Harris, K. F., J. B. Christensen, et al. (1998). "Novel mechanisms of E2F induction by BK virus large-T antigen: requirement of both the pRb-binding and the J domains." Mol Cell Biol 18(3): 1746-56.
- Hirsch, H. H. (2005). "BK virus: opportunity makes a pathogen." Clin Infect Dis 41(3): 354-60.
- Hirsch, H. H., W. Knowles, et al. (2002). "Prospective study of polyomavirus type BK replication and nephropathy in renal-transplant recipients." N Engl J Med 347(7): 488-96.
- Hirsch, H. H. and J. Steiger (2003). "Polyomavirus BK." Lancet Infect Dis 3(10): 611-23.
- Imperiale, M. J. (2000). "The human polyomaviruses, BKV and JCV: molecular pathogenesis of acute disease and potential role in cancer." Virology 267(1): 1-7.

- Imperiale, M. J. (2001). "Oncogenic transformation by the human polyomaviruses." Oncogene 20(54): 7917-23.
- Imperiale, M. J., E.O. Major (2007). "Polyomaviruses." Knipe DM, Howley PM, Griffin RA (eds). Lippincott Williams and Wilkins, Phipadelphia: 2263-2298.
- Israel, M. A., M. A. Martin, et al. (1978). "Evaluation of normal and neoplastic human tissue for BK virus." Virology 90(2): 187-96.
- Jemal, A., R. Siegel, et al. (2007). "Cancer statistics, 2007." CA Cancer J Clin 57(1): 43-66.
- Kiniwa, Y., Y. Miyahara, et al. (2007). "CD8+ Foxp3+ regulatory T cells mediate immunosuppression in prostate cancer." Clin Cancer Res 13(23): 6947-58.
- Knoll, A., R. Stoehr, et al. (2003). "Low frequency of human polyomavirus BKV and JCV DNA in urothelial carcinomas of the renal pelvis and renal cell carcinomas." Oncol Rep 10(2): 487-91.
- Krymskaya, L., M. C. Sharma, et al. (2005). "Cross-reactivity of T lymphocytes recognizing a human cytotoxic T-lymphocyte epitope within BK and JC virus VP1 polypeptides." J Virol 79(17): 11170-8.
- Lau, S. K., S. F. Lacey, et al. (2007). "Low frequency of BK virus in prostatic adenocarcinomas." APMIS 115(6): 743-9.
- Lednicky, J. A. and J. S. Butel (1999). "Polyomaviruses and human tumors: a brief review of current concepts and interpretations." Front Biosci 4: D153-64.
- Leuenberger, D., P. A. Andresen, et al. (2007). "Human polyomavirus type 1 (BK virus) agnoprotein is abundantly expressed but immunologically ignored." Clin Vaccine Immunol 14(8): 959-68.
- Leung, A. Y., C. K. Suen, et al. (2001). "Quantification of polyoma BK viruria in hemorrhagic cystitis complicating bone marrow transplantation." Blood 98(6): 1971-8.
- Li, J., J. Melenhorst, et al. (2006). "T-cell responses to peptide fragments of the BK virus T antigen: implications for cross-reactivity of immune response to JC virus." J Gen Virol 87(Pt 10): 2951-60.
- Littrup, P. J. (1997). "Prostate cancer in African-American men." Prostate 31(2): 139-41; discussion 142.
- Ludlow, J. W., J. Shon, et al. (1990). "The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation and binding to and release from SV40 large T." Cell 60(3): 387-96.
- Major, E. O. (2001). "Human polyomavirus." Fields BN, Knipe DM, Howley PM (eds). Lippincott Williams and Wilkins, Philadelphia: 2175-2196.
- Martin, R. G., V. P. Setlow, et al. (1979). "Roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells: studies with simian virus 40 double mutants." J Virol 31(3): 596-607.
- Martin, R. G., V. P. Setlow, et al. (1979). "The roles of the simian virus 40 tumor antigens in transformation of Chinese hamster lung cells." Cell 17(3): 635-43.
- Moens, U., P. Rekvig. (2001). "Human polyomaviruses: Molecular and clinical perspectives." Khalili K and Stoner GL (eds). Wiley-Liss, New York: 359-408.
- Monini, P., A. Rotola, et al. (1995). "DNA rearrangements impairing BK virus productive infection in urinary tract tumors." Virology 214(1): 273-9.

- Mylin, L. M., T. D. Schell, et al. (2000). "Quantitation of CD8(+) T-lymphocyte responses to multiple epitopes from simian virus 40 (SV40) large T antigen in C57BL/6 mice immunized with SV40, SV40 T-antigen-transformed cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes." J Virol 74(15): 6922-34.
- Newton, R., T. Ribeiro, et al. (2005). "Antibody levels against BK virus and prostate, kidney and bladder cancers in the EPIC-Oxford cohort." Br J Cancer 93(11): 1305-6.
- Otahal, P., S. C. Hutchinson, et al. (2005). "Inefficient cross-presentation limits the CD8+ T cell response to a subdominant tumor antigen epitope." J Immunol 175(2): 700-12.
- Padgett, B. L. and D. L. Walker (1976). "New human papovaviruses." Prog Med Virol 22: 1-35.
- Pagnani, M., A. Corallini, et al. (1988). "Co-operation in cell transformation between BK virus and the human c-Harvey-ras oncogene." Int J Cancer 42(3): 405-13.
- Parkin, D. M., F. Bray, et al. (2005). "Global cancer statistics, 2002." CA Cancer J Clin 55(2): 74-108.
- Pater, A. and M. M. Pater (1986). "Transformation of primary human embryonic kidney cells to anchorage independence by a combination of BK virus DNA and the Harvey-ras oncogene." J Virol 58(2): 680-3.
- Pater, M. M., A. Pater, et al. (1980). "Viruses in naturally occurring cancers." Essex M, Todaro G, zur Hausen H (eds). Cold Spring Harbor Laboratory Press, New York: 329-341.
- Pipas, J. M. and A. J. Levine (2001). "Role of T antigen interactions with p53 in tumorigenesis." Semin Cancer Biol 11(1): 23-30.
- Porras, A., J. Bennett, et al. (1996). "A novel simian virus 40 early-region domain mediates transactivation of the cyclin A promoter by small-t antigen and is required for transformation in small-t antigen-dependent assays." J Virol 70(10): 6902-8.
- Pound, C. R., A. W. Partin, et al. (1999). "Natural history of progression after PSA elevation following radical prostatectomy." JAMA 281(17): 1591-7.
- Provenzano, M., L. Bracci, et al. (2006). "Characterization of highly frequent epitope-specific CD45RA+/CCR7+/- T lymphocyte responses against p53-binding domains of the human polyomavirus BK large tumor antigen in HLA-A*0201+ BKV-seropositive donors." J Transl Med 4: 47.
- Provenzano, M., M. C. Panelli, et al. (2006). "MHC-peptide specificity and T-cell epitope mapping: where immunotherapy starts." Trends Mol Med 12(10): 465-72.
- Purighalla, R., R. Shapiro, et al. (1995). "BK virus infection in a kidney allograft diagnosed by needle biopsy." Am J Kidney Dis 26(4): 671-3.
- Putzi, M. J. and A. M. De Marzo (2000). "Morphologic transitions between proliferative inflammatory atrophy and high-grade prostatic intraepithelial neoplasia." Urology 56(5): 828-32.
- Randhawa, P. S., I. Popescu, et al. (2006). "Detection of CD8+ T cells sensitized to BK virus large T antigen in healthy volunteers and kidney transplant recipients." Hum Immunol 67(4-5): 298-302.

- Rollison, D. E., E. A. Engels, et al. (2006). "Prediagnostic circulating antibodies to JC and BK human polyomaviruses and risk of non-Hodgkin lymphoma." Cancer Epidemiol Biomarkers Prev 15(3): 543-50.
- Rollison, D. E., W. J. Sexton, et al. (2007). "Lack of BK virus DNA sequences in most transitional-cell carcinomas of the bladder." Int J Cancer 120(6): 1248-51.
- Rubinstein, R., B. C. Schoonakker, et al. (1991). "Recurring theme of changes in the transcriptional control region of BK virus during adaptation to cell culture." J Virol 65(3): 1600-4.
- Rundell, K. and R. Parakati (2001). "The role of the SV40 ST antigen in cell growth promotion and transformation." Semin Cancer Biol 11(1): 5-13.
- Schlomm, T., A. Erbersdobler, et al. (2007). "Molecular staging of prostate cancer in the year 2007." World J Urol 25(1): 19-30.
- Seif, I., G. Khoury, et al. (1979). "The genome of human papovavirus BKV." Cell 18(4): 963-77.
- Shah, K. V. (1996). "Polyomaviruses." Fields BN, Knipe DM, Howley PM (eds). Lippincott-Raven, Philadelphia: 2027-2043.
- Shah, K. V., R. W. Daniel, et al. (1997). "Investigation of human urine for genomic sequences of the primate polyomaviruses simian virus 40, BK virus, and JC virus." J Infect Dis 176(6): 1618-21.
- Shenk, T. E., J. Carbon, et al. (1976). "Construction and analysis of viable deletion mutants of simian virus 40." J Virol 18(2): 664-71.
- Shinohara, T., M. Matsuda, et al. (1993). "BK virus infection of the human urinary tract." J Med Virol 41(4): 301-5.
- Taguchi, F., J. Kajioka, et al. (1982). "Prevalence rate and age of acquisition of antibodies against JC virus and BK virus in human sera." Microbiol Immunol 26(11): 1057-64.
- Tavis, J. E., D. L. Walker, et al. (1989). "Nucleotide sequence of the human polyomavirus AS virus, an antigenic variant of BK virus." J Virol 63(2): 901-11.
- Tognon, M., R. Casalone, et al. (1996). "Large T antigen coding sequences of two DNA tumor viruses, BK and SV40, and nonrandom chromosome changes in two glioblastoma cell lines." Cancer Genet Cytogenet 90(1): 17-23.
- Tognon, M., A. Corallini, et al. (2003). "Oncogenic transformation by BK virus and association with human tumors." Oncogene 22(33): 5192-200.
- Vasavada, R., K. B. Eager, et al. (1986). "Adenovirus type 12 early region 1A proteins repress class I HLA expression in transformed human cells." Proc Natl Acad Sci U S A 83(14): 5257-61.
- Velders, M. P., M. F. Macedo, et al. (2001). "Human T cell responses to endogenously presented HLA-A*0201 restricted peptides of Simian virus 40 large T antigen." J Cell Biochem 82(1): 155-62.
- Weggen, S., T. A. Bayer, et al. (2000). "Low frequency of SV40, JC and BK polyomavirus sequences in human medulloblastomas, meningiomas and ependymomas." Brain Pathol 10(1): 85-92.
- Weinberg, R. A. (1995). "The retinoblastoma protein and cell cycle control." Cell 81(3): 323-30.
- Weinberg, R. A. (1996). "E2F and cell proliferation: a world turned upside down." Cell 85(4): 457-9.

- Weinreb, D. B. (2006). "BK virus and carcinoma of the prostate, kidney and bladder." Br J Cancer 94(12): 1948; author reply 1949-50.
- White, M. K. and K. Khalili (2004). "Polyomaviruses and human cancer: molecular mechanisms underlying patterns of tumorigenesis." Virology 324(1): 1-16.
- White, M. K. and K. Khalili (2005). "Expression of JC virus regulatory proteins in human cancer: potential mechanisms for tumourigenesis." Eur J Cancer 41(16): 2537-48.
- White, M. K. and K. Khalili (2006). "Interaction of retinoblastoma protein family members with large T-antigen of primate polyomaviruses." Oncogene 25(38): 5286-93.
- Xiong, Y., G. J. Hannon, et al. (1993). "p21 is a universal inhibitor of cyclin kinases." Nature 366(6456): 701-4.
- Yoshiike, K., K.K. Takemoto. (1986). "Studies with BK virus and monkey lymphotropic papovavirus." Salzman, NP (eds). Plenum Press, New York: 295-326.
- Yu, J., A. Boyapati, et al. (2001). "Critical role for SV40 small-t antigen in human cell transformation." Virology 290(2): 192-8.
- Zambrano, A., M. Kalantari, et al. (2002). "Detection of human polyomaviruses and papillomaviruses in prostatic tissue reveals the prostate as a habitat for multiple viral infections." Prostate 53(4): 263-76.
- Zhu, J., P. W. Rice, et al. (1992). "Transformation of a continuous rat embryo fibroblast cell line requires three separate domains of simian virus 40 large T antigen." J Virol 66(5): 2780-91.

9. Figures

Figure 1: Organization of the human polyomavirus BK genome

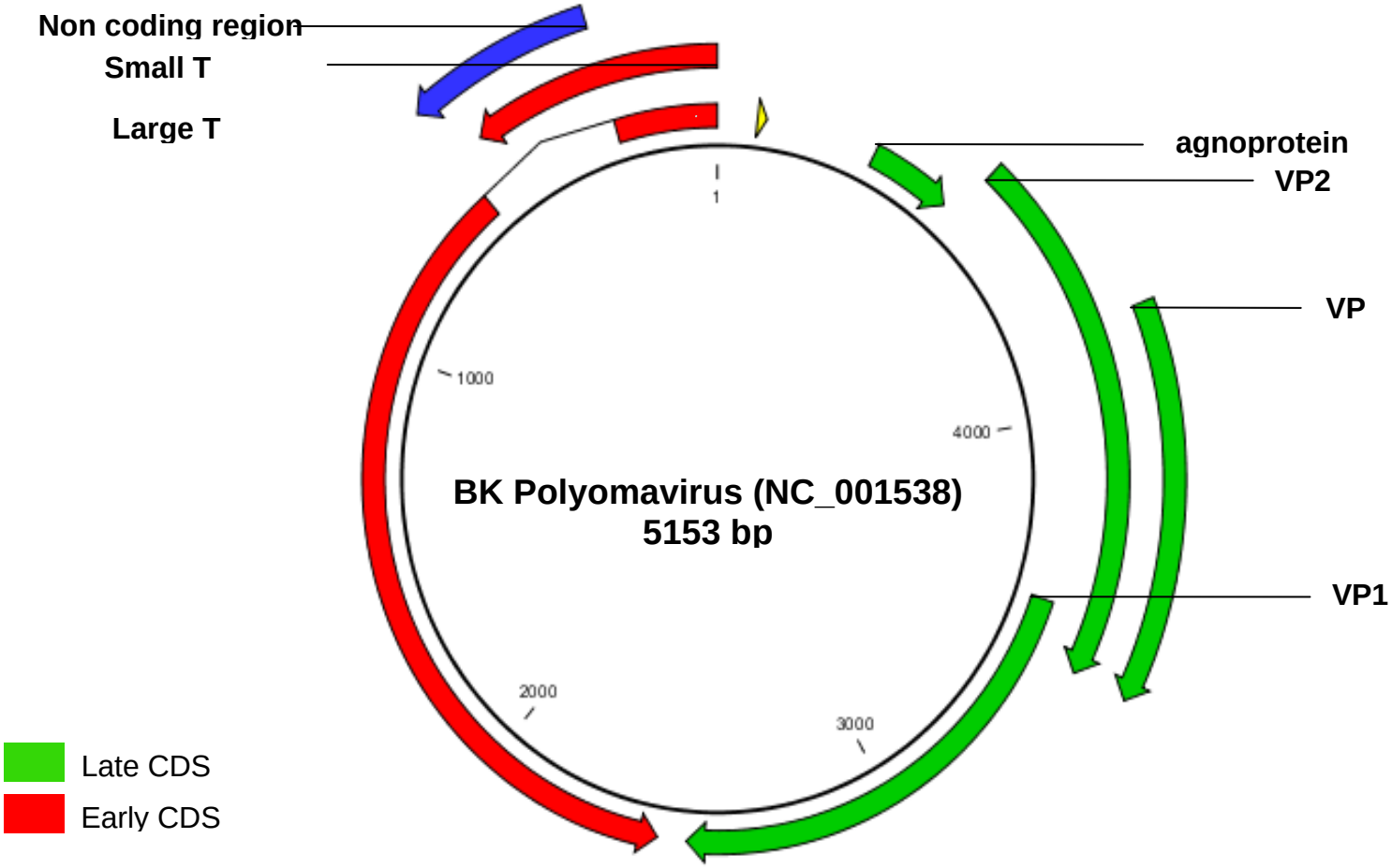


Figure 2: Interaction of BKV L-Tag with pRbs and p53

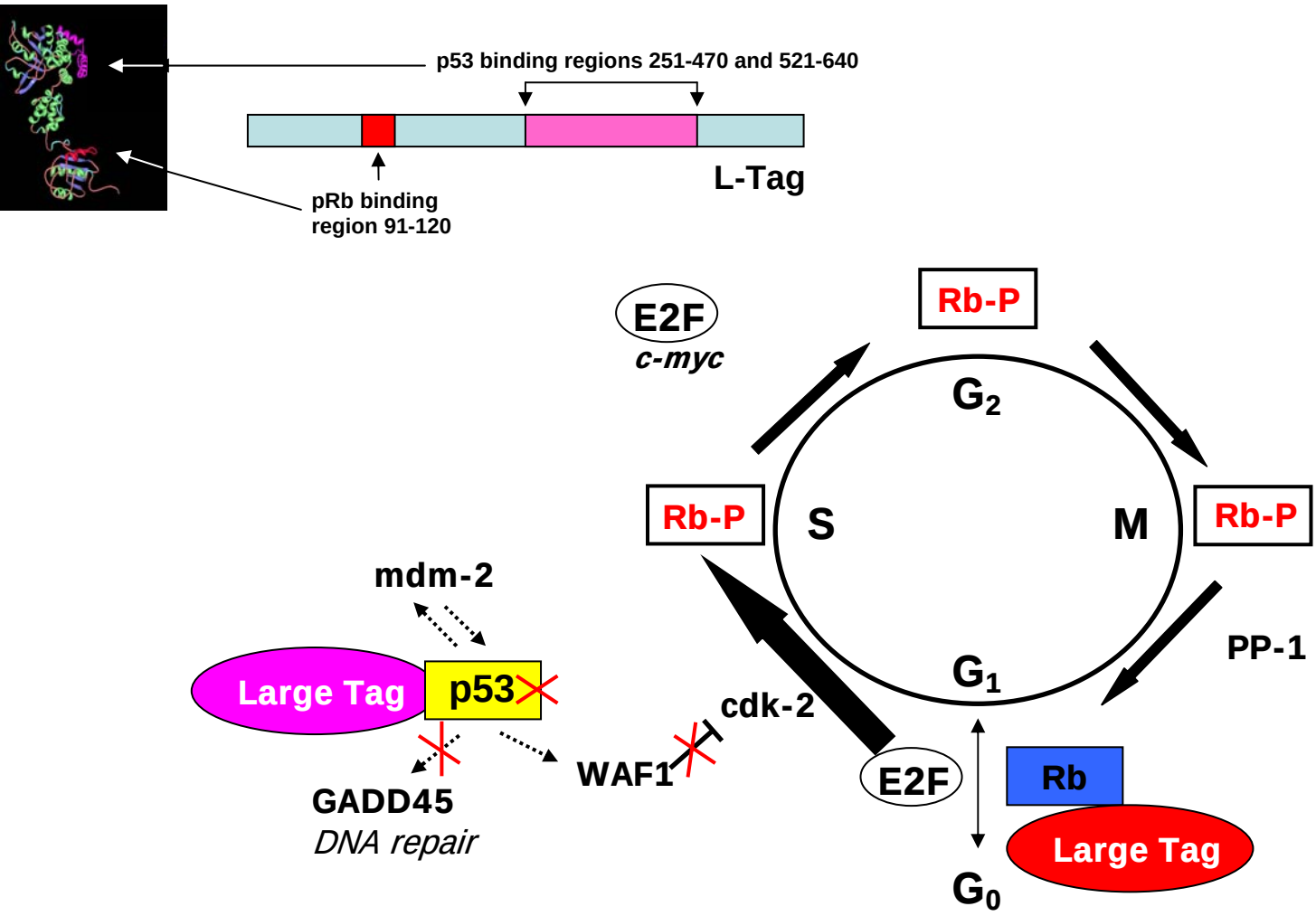


Figure 3: Pathological progression of prostate cancer

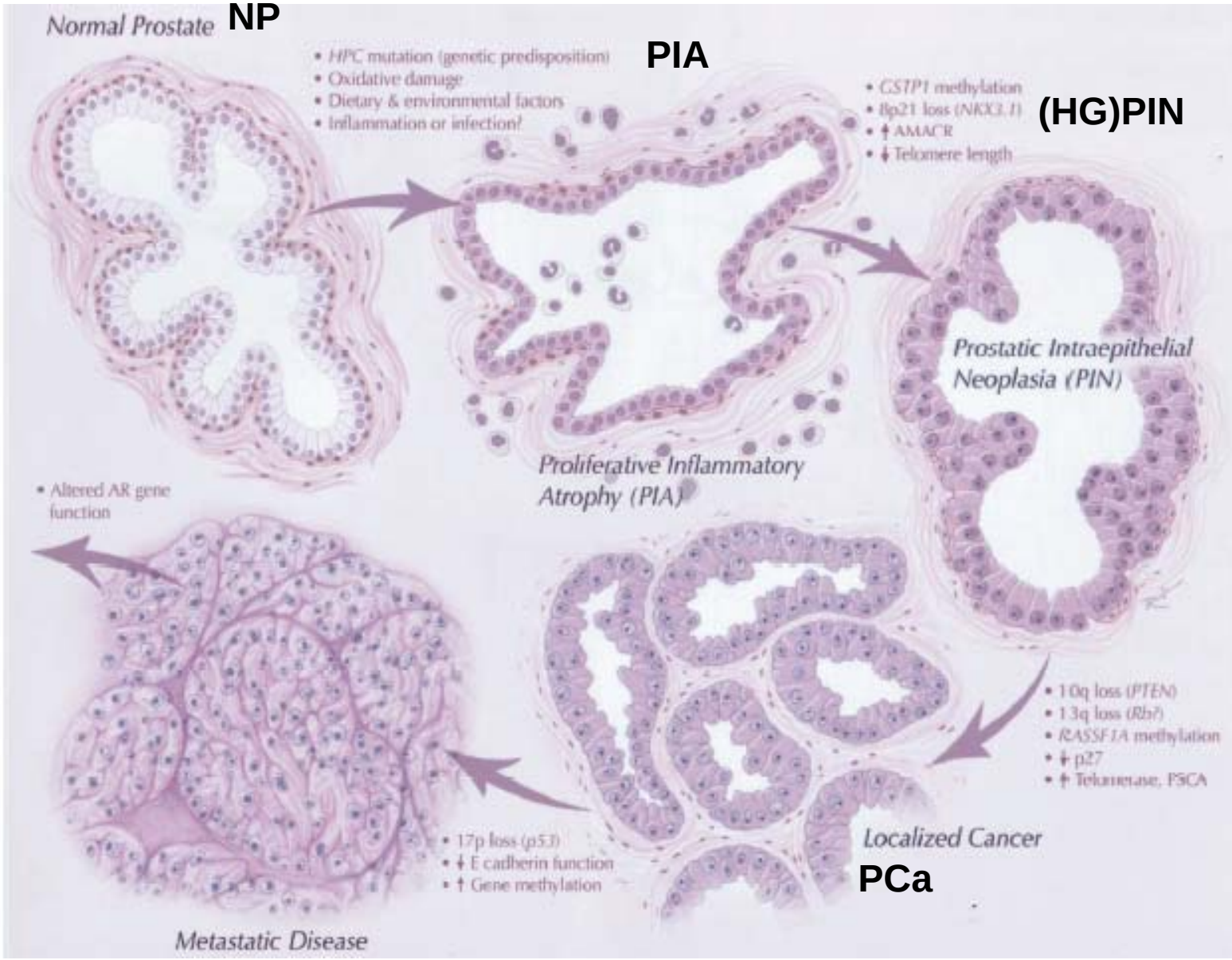


Figure 4: BKV and JCV L-Tag DNA detection in either BPH or PCa tissue specimens

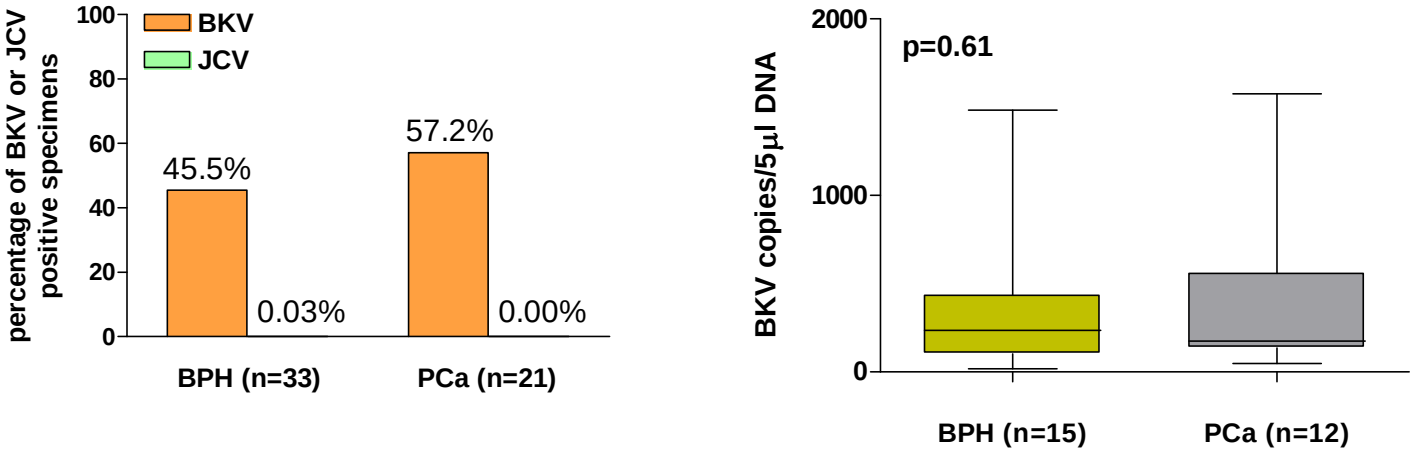


Figure 5: BKV VP1 and L-Tag serology in either BPH or PCa patients

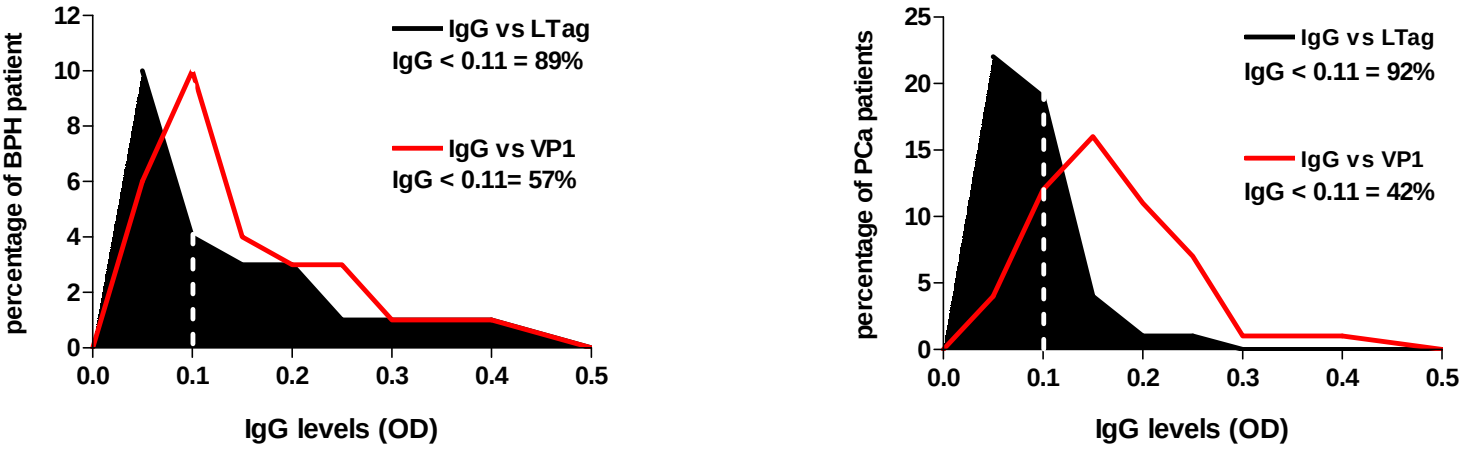


Figure 6: Cytokine gene profiling in HLA-A*0201 BPH and PCa patients upon L-Tag$_{579}$ and L-Tag$_{406}$ peptide induction

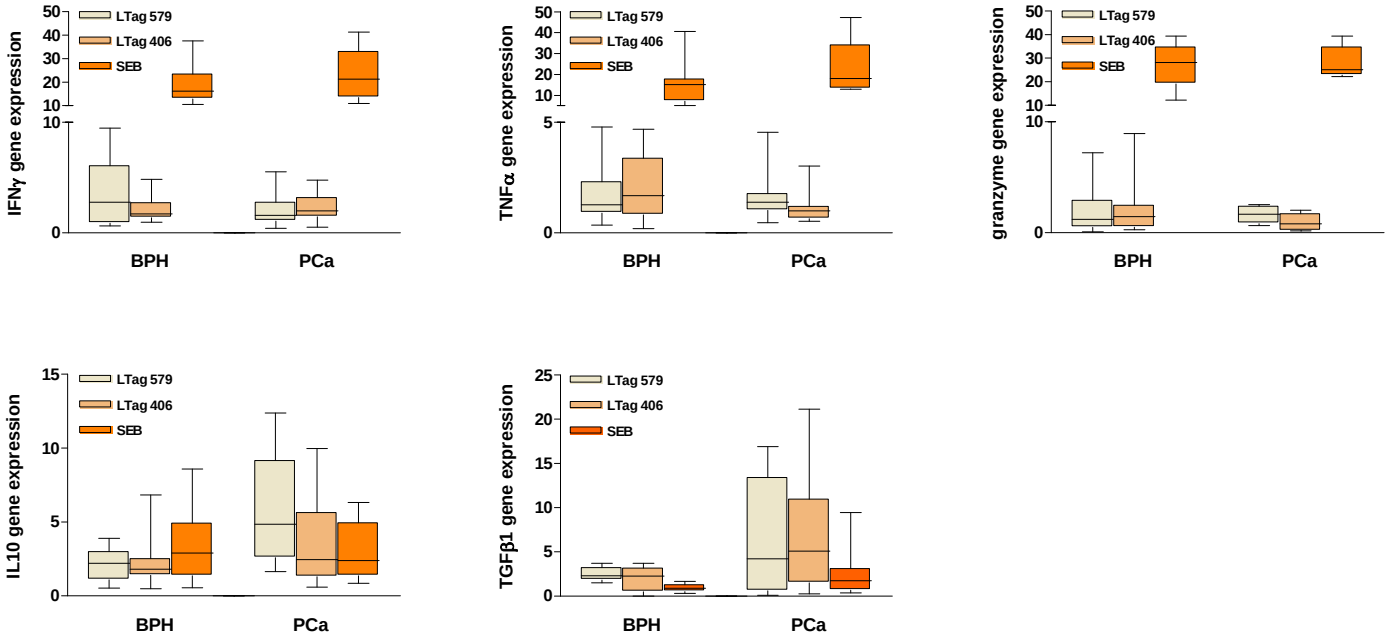


Figure 7: Correlation between L-Tag serology and immune regulatory trend in PCa patients upon L-Tag$_{579}$ and L-Tag$_{406}$ peptide induction

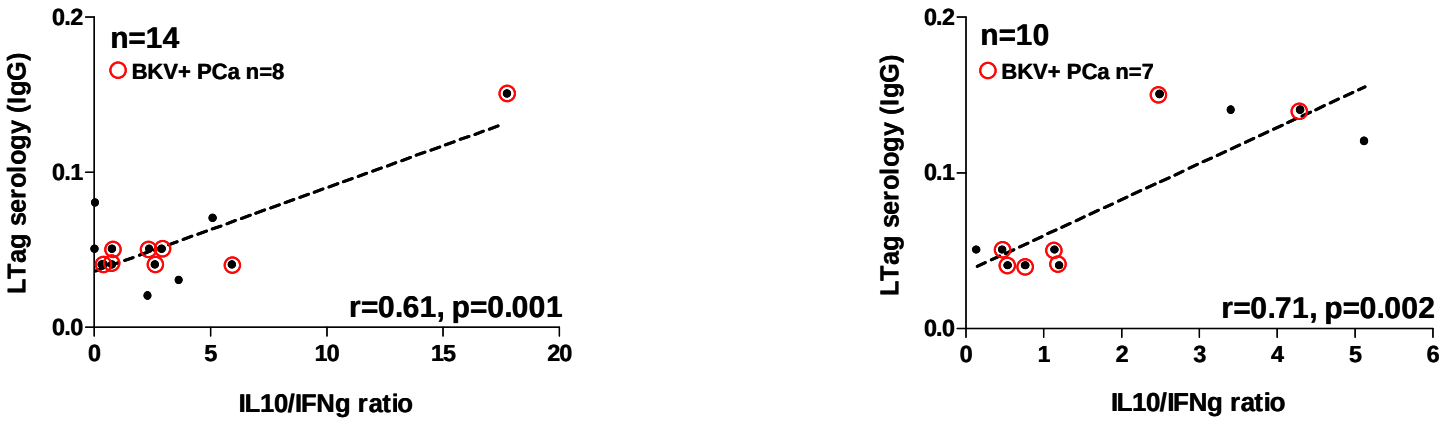
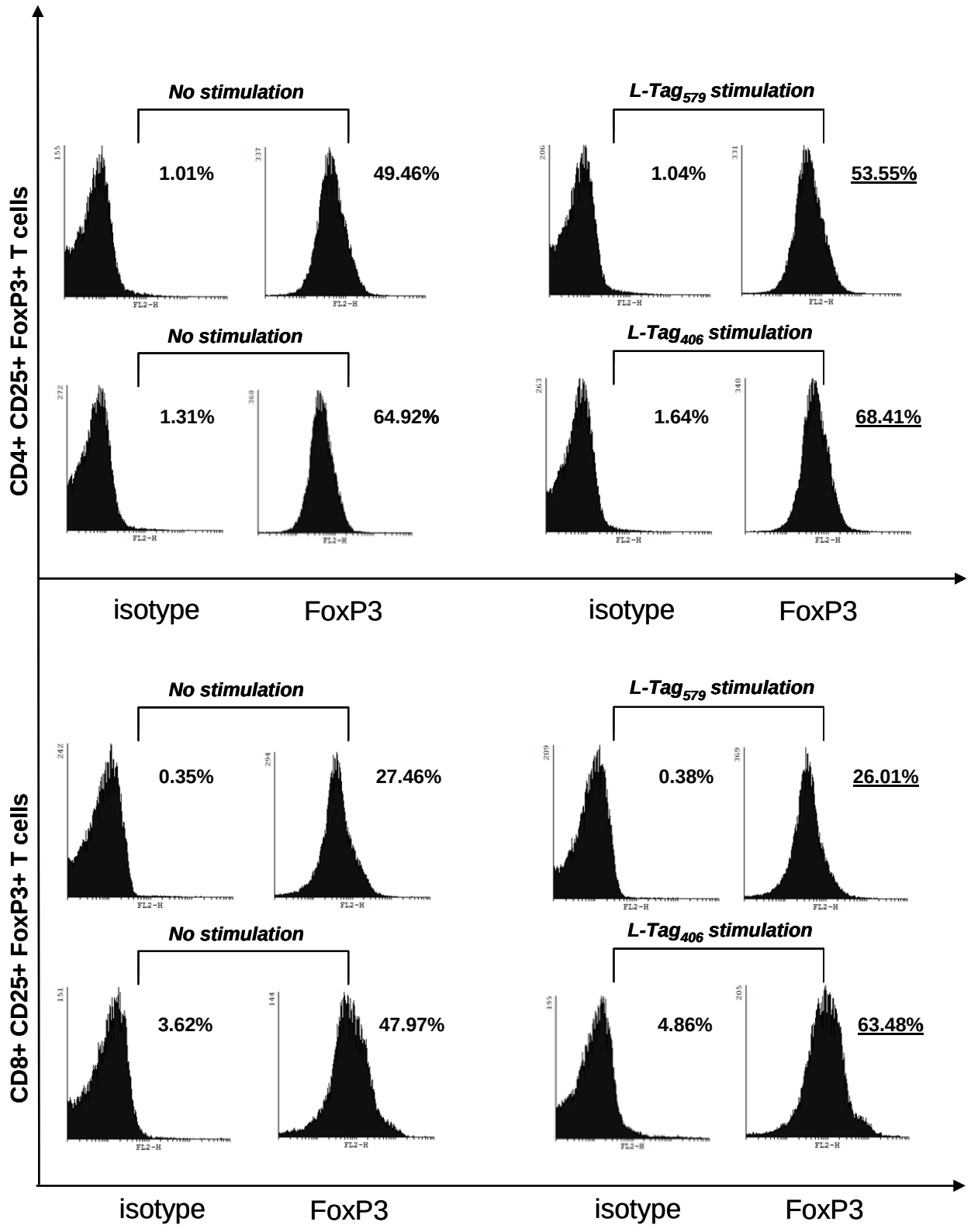


Figure 8: T regulatory cells expansion in PCa patients upon L-Tag$_{579}$ and L-Tag$_{406}$ peptide induction



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