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Review Article

Tumour Immunogenicity, Antigen Presentation, and Immunological Barriers in Cancer Immunotherapy

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Since the beginning of the 20th century, scientists have tried to stimulate the antitumour activities of the immune system to fight against cancer. However, the scientific effort devoted on the development of cancer immunotherapy has not been translated into the expected clinical success. On the contrary, classical antineoplastic treatments such as surgery, radiotherapy, and chemotherapy are the first line of treatment. Nevertheless, there is compelling evidence on the immunogenicity of cancer cells and the capacity of the immune system to expand cancer-specific effector cytotoxic T cells. However, the effective activation of anticancer T cell responses strongly depends on efficient tumour antigen presentation from professional antigen presenting cells such as dendritic cells (DCs). Several strategies have been used to boost DC antigen presenting functions, but at the end cancer immunotherapy is not as effective as would be expected according to preclinical models. In this review, we comment on these discrepancies, focusing our attention on the contribution of regulatory T cells and myeloid-derived suppressor cells to the lack of therapeutic success of DC-based cancer immunotherapy.

1. Introduction: Vaccines Infectious Diseases and Cancer

Not so long ago, I had a conversation with a colleague of mine about our differing research interests. He asked me why I was so much interested in cancer research and not that keen on infectious diseases. This particular question made me think about my motives for working in cancer research. Cancer is in many cases an "adult" noncontagious disease (with exceptions), while infectious diseases can attack anyone at any time. In addition, infectious agents are highly contagious and new ones arise from time to time [1, 2]. He argued that compared to cancer, infectious diseases are a much higher health burden worldwide. After this conversation I quickly looked at the statistics and according to Cancer Research UK (http://www.cancerresearchuk.org), there were about 7.6 million deaths from cancer in 2008. Then I looked at the deaths caused by infectious diseases, and according to the World Health Organisation (WHO, http://www.who.int/en/) about 13 million deaths (of all ages) were caused by infectious diseases in 1998. Even though the data was not that recent, I concluded that the first premise on health burden might not be completely accurate. However, ultimately that was not the reason I was looking for. Therefore, why is cancer so fascinating that attracts so much scientific and medical efforts?

Among the nonmedical community in the "developed" world, infectious diseases are not the problem they historically used to be, apart from some exceptions such as AIDS. We owe this to the introduction of hygiene measures and sanitation, which are possibly more significant factors than vaccines in controlling "everyday" infections. Nevertheless, there is no doubt in the medical community that mass vaccination is an effective way of achieving population immunity, essential for the eradication of infectious agents [3, 4]. It is worthy to mention that the overall population has in some cases forgotten the importance of mass vaccination for the most common infectious agents. This is a well-known phenomenon denominated "vaccine refusal" and has serious consequences that delay the eradication of infectious diseases [5]. As a recent example of this, there was an important measles virus outbreak in England in 2012. This outbreak with

serious consequences in a proportion of infected children was caused by "vaccine refusal" for the triple measles-mumps-rubella vaccine due to fears from unfounded links with autism [6–8]. Therefore, good-intentioned nonrational decisions made by following inaccurate perceptions have a real significant impact on the population's health status and the propagation of infectious diseases.

However, cancer is a different matter. There is not any doubt in my mind that anyone would vaccinate their children against cancer. Why is that so? Well, on one hand the longer that we live, the higher the chances of suffering from some type of cancer. Secondly, cancer will quickly kill the patient without much that modern medicine can do to prevent it. Thirdly, radiotherapy and chemotherapy cause severe secondary effects, and in many cases they will prolong life but not cure cancer.

So, this is from the point of view of the population. However, why is it so fascinating for scientists? Well, for me, the answer lies in the scientific challenge itself. Cancer arises from the complications caused by the uncontrolled growth of transformed cells (tumours). It would not be such a problem, as tumours can be removed by surgery. However, if left untreated, these cells will eventually colonise the organism through a process called metastasis. The establishment of secondary tumours from these cancer cell colonies significantly interferes with the physiological functions of the organism. And here is where the challenge lies. These cells come from self-tissues, and therefore, the immune system is largely tolerant to them. In the case of infectious diseases, viral and bacterial products are "foreign" or "non-self", so it is relatively easy for immune cells to detect them and get rid of the infectious agents. That is why the development of vaccines against infectious diseases is relatively straightforward in comparison to cancer vaccines. In the case of cancer, how can we alert the immune system against mutated "self-cells"? Does it really play a role in controlling cancer?

2. Tumour Antigenicity

At the beginning of the 20th century, Paul Ehrlich put forward the tumour immunosurveillance theory. He had already worked in the role of immune responses to control infections caused by microorganisms. He then applied the same observations to cancer. He proposed that cancer cells spontaneously arise in the organism and that immune responses could effectively eliminate them [9]. This same concept was later refined by Burnet [10]. The fast development of organic chemistry, biochemistry, and molecular biology (and nuclear physics!) that followed in the 20th century provided the tools to systematically study cancer and develop chemotherapeutic agents that could inhibit cancer cell growth [11]. For the first time, drugs could be developed that were effective to at least control (and in some cases cure in combination with surgery and radiotherapy) cancer. Therefore, biomedical research directed its efforts in the development of these new drugs. The study of anticancer immune responses steadily continued, but it never reached a therapeutic status as achieved by other "conventional" methods. The lack of success of cancer vaccines led to another misconception which still lingers within a relatively large proportion of the scientific community; immune responses did not play a significant role in controlling cancer, unlike "classical" antineoplastic strategies such as radiotherapy and chemotherapy. Interestingly, there is strong recent evidence that classical anticancer treatments heavily rely on the immune system for their effectiveness [12–20]. These treatments include radiotherapy and chemotherapy and depend on cellular stress responses [17]. These responses lead to enhanced antitumour activities through the activity of TLR agonists released from necrotic tumour cells and activation of the inflammasome in antigen presenting cells by released ATP [14, 15]. Interestingly, these conventional antineoplastic treatments lose their efficacy when immunocompromised mice are used in the experiments, or when human patients have deleterious mutations on TLR4 [14].

What determines whether cancer vaccines can become a success in human immunotherapy? Exactly the same as required for infectious diseases, cancer has to be immunogenic and activate cytotoxic T cell responses. Consequently, cancer cells have to possess immunogenic antigens susceptible of being targeted by vaccination.

Since Ehrlich's proposal, researchers have been looking for tumour-associated antigens (TAAs) that could be exploited for cancer immunotherapy. And even though early studies found some experimental evidence towards their existence (particularly from virus-induced tumours), the problem of the immunological tolerance always came back for the counterattack [21–26]. Many of these studies concentrated on immune responses against virus-induced tumours rather than endogenous cancer antigens [24, 27–29]. In fact, at the time there was an increasingly accumulating body of evidence supporting the viral aetiology of nearly all human cancers [30–32]. This resulted in a major (slightly misguided?) change of view for cancer therapy, as it was much easier to target foreign viral antigens expressed by tumour cells than mutated self-antigens [30, 33–35]. Now we know that only a number of human cancers are caused by viral infections. Nevertheless, the "viral aetiology" theory for human cancer could not explain the immune responses against chemically induced cancers that were also observed. However, in many instances these results could not be reproduced by other research groups [36]. Even so, these early studies provided evidence that immune responses could be raised against tumours of nonviral origin. Spontaneous tumour regressions were also sporadically observed in human patients, in some cases provoked after immunisation towards common pathogens, strongly supporting the existence of TAAs of nonviral origin [20, 37, 38].

A turning point came from the study of oncogenic viruses, especially from the Retroviridae family which led to a "shocking" discovery. These viruses induced cancer through the expression of oncogenes, which had their corresponding cellular variants [39–47]. These oncogenes included v-raf, c-my, c-rel, and k-ras among others. All these proteins were largely involved in the regulation of cell proliferation and survival. So after all, transforming oncoviruses had "hijacked" cellular oncogenes for their own advantage. But, in cancers of nonviral aetiology, are the corresponding cellular versions involved in carcinogenesis?

That turned out to be the case [48]. Cancerous cells accumulate a series of mutations leading to genetic instability, which results in protein expression changes, increased survival, and uncontrolled proliferation [47–52]. Many of the mutated proteins were transcription factors (c-myc), part of key signalling pathways (human Ras), cell cycle regulators (retinoblastoma protein, Rb), and antioncogenes (p53). As a result of uncontrolled proliferation and defects in DNA repair/apoptosis pathways, further mutations and chromosomal rearrangements appear. "Fortunately," as a direct consequence, these cells express a collection of mutated self-proteins that confers them with a degree of immunogenicity (quasi-antigens). In some cases, self-proteins can be aberrantly overexpressed in tumours, which would not normally be expressed in the corresponding healthy tissue. This acquired immunogenicity allows the immune system to identify and destroy them.

3. Identification of Human TAAs

Evidence for the existence of immunogenic autologous tumour antigens appeared about 50–60 years ago [35], and since then an increasingly growing collection of TAAs has been identified [53]. It is not the intention of this review to provide an extensive and detailed list of the TAAs that have been identified so far. However, it is worthy to mention the most widely used in human therapy and how they were discovered.

In the 1950s, tyrosinase overexpression in melanomas had already been observed [54, 55]. Tyrosinase is an oxidase involved in melanin production which is specifically expressed in melanocytes. Interestingly, it was shown to be immunogenic and tyrosinase-specific CD8 T cells could lyse melanoma cell lines [56, 57]. In 1994, CD4 T cells were demonstrated to recognise HLA-DR-specific tyrosinase peptide epitopes [58]. Tyrosinase-derived peptides have been used in vaccines against melanoma, alone or in combination with other TAAs [59, 60].

Carcinoembryonic antigens (CEA) were indirectly identified in the 1960s, by the detection of anti-CEA antibodies in human colon cancer patients [61, 62]. CEA is a cell adhesion glycoprotein of the immunoglobulin superfamily which is expressed during foetal development [63]. Interestingly, CEA is again reexpressed especially in colorectal cancer, although it can also be expressed in many other carcinomas [63–67]. This expression pattern made it a good candidate for immunotherapy. In 1995-1996, CEA-specific T cell responses were demonstrated, and a CEA-derived immunogenic T cell peptide was identified [68, 69]. From then on, several different CD8- and CD4-restricted peptide epitopes have been found and used in human immunotherapy [70–75]. Licensed CEA-based vaccines are extensively used in many variations of cancer immunotherapy [76]. There is strong evidence supporting the role of anti-CEA T cell responses in breaking immunological tolerance towards other tumour antigens.

Alpha-fetoprotein (AFP) was reported to be expressed in some tumours in the 1970s [77]. AFP is a globulin-like plasma protein present at high levels during foetal development, and it is elevated particularly in hepatic cancers [78, 79]. AFP is currently used as a tumour marker rather than an immunotherapy target, because it strongly inhibits T cell responses [80]. Nevertheless, immunogenic T cell epitopes have also been identified [81–85] that could be used as targets in cancer vaccines, alone or in combination with other TAAs [86, 87].

The cancer antigen 125 (CA-125), highly expressed in ovarian carcinomas, was discovered in 1981 by the specific reactivity of the murine OC125 monoclonal antibody towards ovarian carcinoma cell lines [88, 89]. CA-125 is a glycoprotein belonging to the mucin family, which is generally immunosuppressive and correlates with tumour progression. There is evidence that immune responses can be raised against CA-125 that could be therapeutically relevant [90–92].

In the 1980s, elevation of prostate-specific antigen (PSA) was observed in prostate hyperplasia and cancer [93, 94]. PSA is a peptidase expressed by epithelial cells of the prostate gland, and it has been classically used as a tumour maker although its reliability has been questioned. PSA has also been used in cancer immunotherapy as a prostate cancer vaccine [95–97], and immunogenic T cell epitopes have also been described for it [98–101].

The melanoma-associated antigens (MAGE antigens, cancer-testis antigens), also expressed by a wide variety of cancers [102, 103], were described in the early 1990s and shown to contain many immunogenic T cell peptide epitopes [104–110]. MAGE antigens are also big players in the development of cancer vaccines. They have been successfully applied in human immunotherapy protocols leading to melanoma regression, and their use can also break tolerance towards other TAAs such as gp100 [111].

Also in the 1990s, the immunogenic tyrosinase-related proteins 1 and 2 (TRP-1, TRP-2) were cloned. Both of them are frequently overexpressed in melanoma [112–114]. CD8 and CD4 T cell epitopes have also been identified for these TAAs, especially for the "highly immunogenic" TRP-2 protein [115–121]. TRP-1 is a TAA of interest in human therapy although not one of the most immunogenic melanoma-associated antigens [122, 123]. Interestingly, the use of selectively mutated TRP1 CD8 T cell epitopes can effectively break immunological tolerance and drive effective antimelanoma responses [120]. TRP-2-targeted immune responses can also lead to melanoma regression [124, 125].

The gp100 protein was identified in 1988 by its reactivity with a melanocyte lineage-specific monoclonal antibody used for diagnosis of human melanoma [126]. It was later cloned and demonstrated that human cytotoxic T cells could recognise immunogenic gp100 epitopes [117, 127, 128]. This particular TAA is extensively used in the development of melanoma vaccines [59].

In 1997, NY-ESO-1 (cancer-testis antigen 1B) was isolated from esophageal squamous cell carcinoma, and immune responses demonstrated in humans against it [129, 130]. NY-ESO-1 is also expressed by a wide range of cancers, including ovarian cancer, melanoma, sarcomas, neuroblastoma, leukaemias, and lymphomas, just to name a few. Several CD8 and CD4 T cell epitopes have been defined which are relevant to trigger anti-cancer immune responses [131–135].

NY-ESO-1 is possibly one of the best antigens for human immunotherapy due to its wide expression patterns and immunogenicity [136–138]. T cells modified to express NY-ESO-1-specific TCRs were shown to induce tumour regression in metastatic synovial cell sarcoma and melanoma [139].

MART-1/Melan A was cloned in 1994 from a HLA-A2+ melanoma cell line-derived cDNA library by transfection into target cells for lysis by established melanoma-specific cytotoxic T cells [140]. MART-1-specific TCRs were identified from human patients shortly after and shown to be specific to a HLA-A2-specific MART-1 peptide epitope [141]. Again, MART-1 is extensively used together with other TAAs for vaccination against melanoma [59, 60, 134]. In fact, there is evidence suggesting that the activities of MART-1-specific CD8 T cells are prominent in effective immunotherapy for melanoma [142]. Genetically engineered autologous CD8 T cells for the expression of MART-1-specific TCRs demonstrated their capacities to achieve melanoma regression and long-term cures in animal models and human patients [143–145].

Summarising, cancer cells are in fact immunogenic, and they are not immunologically silent. There is a wide range of common TAAs expressed in a range of cancers which raise both CD4 and CD8 T cells. In fact, there are numerous studies both in preclinical experimental models and in human clinical trials that report effective T cell responses. However, overall, the clinical success of cancer immunotherapy is rather low even though TAA-specific autologous T cells can be expanded. In fact, these T cells exert good cytotoxic activities *ex vivo* and can mediate strong tumour regression *in vivo*. But at the end, long-term cures are infrequent and tumours usually come back by escaping from the immune system using a variety of mechanisms. Some of these mechanisms involve the selection of cancer cell variants that lose TAA expression or decrease the expression of MHC molecules. In this review, the involvement of regulatory immune cells that expand in cancer and strongly inhibit anti-cancer immune responses will be discussed.

4. Concept and Application of Cancer Immunotherapy

As we have discussed above, cancer cells express many TAAs that in some cases appear in a wide range of cancers with relatively high frequency. However, the presence of potentially immunogenic TAAs does not directly answer the question on whether the immune system plays a central role in controlling cancer in physiological conditions, in other words, whether the immune system is competent in protecting the organism against cancer in the absence of external interventions. If the immune system would in fact be competent, then strategies to boost its natural anti-neoplastic activities could succeed for human therapy. How to prove whether the "cancer immunosurveillance" hypothesis has any basis?

Compelling evidence on the role of the immune system in keeping potential cancer cells at bay when they arise in the organism came in 2001, by the observation of cancer immunoediting [146]. Several research groups noticed that cancers growing in immunodeficient mice were by far more immunogenic than the same cancers grown in immunocompetent mice [20]. When T cell responses were raised against these tumours, their regression was apparent, but some cancer cells escape from the immune system. These escapees had lost immunogenicity, demonstrating that the immune system is exerting at all times a selective pressure. In the absence of immune responses, tumours are immunogenic. In the presence of competent immune responses, less immunogenic tumours are selected out of the immune pressure.

Nowadays, a four-stage process for oncogenesis is widely recognised by tumour immunologists: (1) transformation of cancerous cells, (2) raising of anti-cancer immune responses, (3) establishment of a cancer growth-immune attack equilibrium which exerts a selective pressure, and (4) immunological escape of poorly immunogenic cancer cells.

To succeed against cancer, it is necessary to strongly boost step 2, while reducing at maximum the establishment of cancer-immune system equilibrium. In a "natural" anti-cancer immune response, there is clearly amplification of TAA-specific effector T cells. However, this amplification is much smaller than that found in T cell responses against infectious agents. TAA-specific T cells are usually of low affinity or even anergic. An antitumour attack of a limited number of these effector T cells will shift the equilibrium balance towards selection of escaping cancer cells. For effective anti-cancer therapies, the amplification of a very large number of effector T cells is necessary to culminate in a "blitz" attack leaving no chance for immune escape (Figure 1). So, anti-tumour T cell responses need to reach a critical mass to be effective. We recently proposed this mechanism based on our experimental observations, the critical mass hypothesis of T cell responses [147–149] (Figure 1).

Therefore, the main objective of cancer immunotherapy is to strongly stimulate the immune system through a variety of procedures to boost the natural anti-tumour immunity or to implement novel antitumour properties to immune cells by, for example, genetic modification. Cancer immunotherapy groups a variety of techniques directed to increase the activities of T, B and dendritic cells (DCs) to raise strong anti-tumour immune responses. The manipulation of these cell types is carried out by conventional techniques such as the use of vaccine adjuvants together with TAAs, cytokine/chemokine therapies or by molecular/genetic techniques [148, 150–155]. Some successful examples of these procedures are the expression in T cells of TAA-specific T cell receptors (TCRs) and chimeric antigen receptors (CARs) directed towards tumour antigens [139, 145, 156, 157] or the administration of TAA-loaded DCs [158]. Nevertheless, for any successful cancer immunotherapy, it is imperative to achieve a strong, effective TAA presentation to effector cytotoxic cells, especially CD8 T cells [159].

Cancer immunotherapy depends on the enhancement of potent cytotoxic T cell (CTL) responses against TAAs. Antigens are processed into a series of antigenic peptides which are loaded into MHCs. These peptide-MHC complexes are exposed on the surface of both antigen presenting cells (APCs) and also normal and transformed cells. While antigen presentation by APCs will lead to the activation of effector

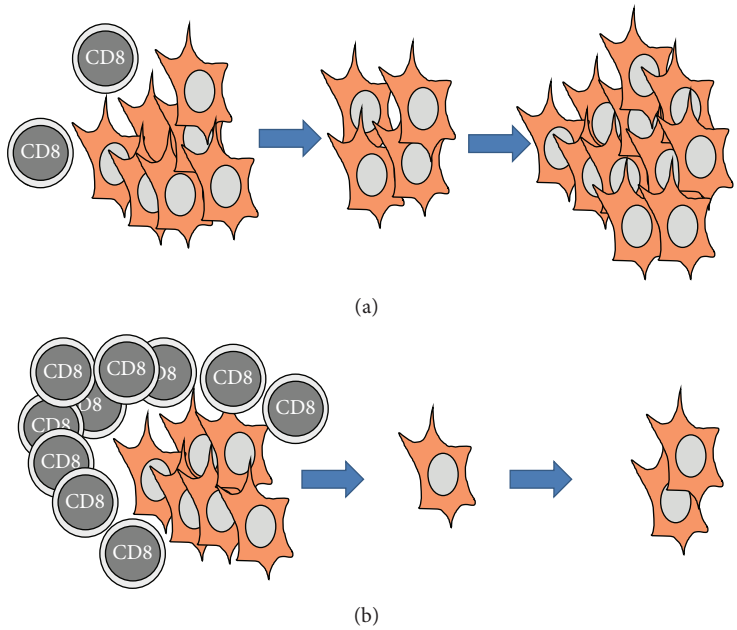

Figure 1: A critical mass of TAA-specific cytotoxic T cells is necessary to reduce immunological editing and cancer escape. (a) A tumour mass represented as a group of transformed cells (orange) is being attacked by a limited number of TAA-specific T cells (left). Immunological editing takes place as indicated above the arrow, due to limited and slow killing of TAA-expressing T cells leaving a significant number of poorly immunogenic cancer cells alive (centre). These cells eventually regrow through reduced immunogenicity and lack of CD8 T cell attack (right). (b) The same situation as in (a) with the difference that a large pool of TAA-specific cytotoxic CD8 T cells has expanded following immunotherapy (left). In this way, a larger number of cancer cells will be killed in the tumour mass, leaving no cells or a very reduced number of them alive. This situation will minimise immunological editing and cancer escape (centre). At the end, as turns out in many cancer immunotherapy treatments, the tumours usually come back. However, in comparison with the situation shown in (a), it will take longer to the tumour to regrow with an associated increase in survival.

T cells, their presentation on the surface of normal cells discriminates normal, healthy cells from infected, transformed cells. Briefly, antigen-specific T cells expanded by APCs will look for cells exposing their corresponding antigenic peptide-MHC complexes. Once they are found, T cells will exert their cytotoxic activity and eliminate infected/transformed cells.

T cells recognise their cognate antigenic peptides and activate by direct binding of their TCR to peptide-MHC complexes present on the surface of APCs (Figure 2). This recognition delivers a signal to T lymphocytes, which is insufficient to effectively activate them. Antigen-presenting APCs have to provide additional signals in the immunological synapse to the T cell [153]. These cosignals will regulate the degree of T cell activation and can be stimulatory or inhibitory [160]. A major costimulatory interaction takes place after the binding of CD80 on APCs with CD28 on T cells. However, an array of inhibitory interactions also occurs in the immunological synapse, such as binding of CD80 with CTLA4 or PD-L1 with PD1 [153, 161]. The integration between positive and negative costimulation will determine the degree of T cell activation [160] (Figure 2). In addition to these two signals, the specific cytokine profile produced during the DC-T cell engagement will drive T cell differentiation towards CD4 T helper (Th) subtypes [153]. These different responses can be proinflammatory, such as those driven by Th1, Th17, antibody responses controlled by Th2, or immune suppression as regulated by regulatory T cell differentiation.

While responses against infectious agents are usually strong and specific, as they rely upon the recognition of pathogen-derived molecules by APCs such as DCs, this is not the case with anti-cancer responses. Activation of TAA-specific T cells can be certainly challenging. Firstly, TAAs can be expressed at low concentrations, leading to suboptimal antigen presentation. In this situation, T cells that recognise antigen-presenting cells (in minute concentrations) differentiate towards Tregs [162, 163]. On the other hand, it is well known that T cells can be efficiently activated by the engagement of a low number of peptide-MHC complexes by a process called "serial TCR triggering" [148, 155]. It has to be taken into account that most p-MHC complexes bind TCRs with low affinities anyway, and co-stimulation plays the part of strengthening TCR-pMHC binding. However, TCR manipulation by genetic engineering can increase signal 1 leading to effective cancer immunotherapy in animal models and human clinical trials [164].

Engagement of DCs with pathogen-derived molecules causes DC maturation by upregulation of co-stimulatory signals, MHC molecules and secretion of pathogen-specific cytokine profiles [154]. Then, maturing DCs migrate to secondary lymphoid tissues and present pathogen-derived antigens to specific T cells. However, in the case of cancer, TAAs are frequently aberrantly overexpressed endogenous proteins or quasi-antigens. A major problem for anti-cancer immune responses is that the frequency of TAA-specific T

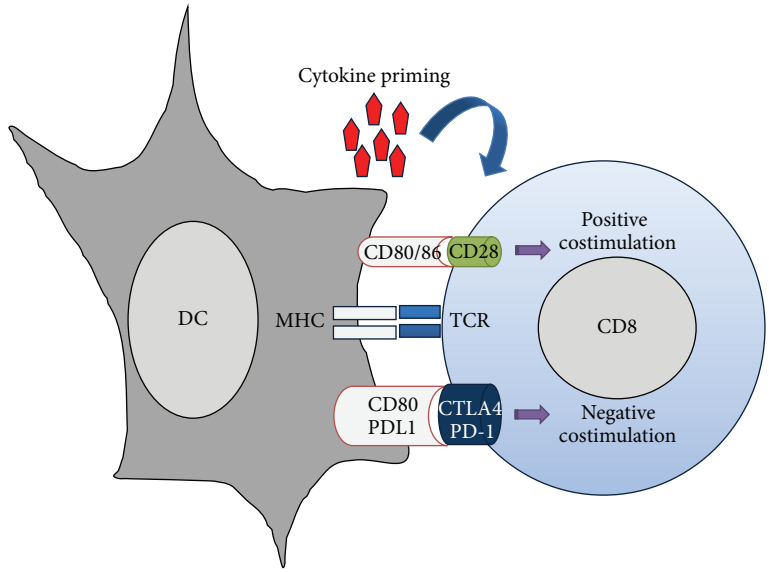

Figure 2: T cell activation by antigen presentation. Tumour antigen presentation to T cell by dendritic cells (DC, on the left) is schematically depicted in this picture. The antigenic peptide complexed to the MHC, as indicated within the DC on the left, is recognised by the TCR on the T cell surface, as indicated within the T cell on the right. To effectively activate TAA-specific T cells, strong costimulation is needed. Costimulation depends on the integration between activatory (positive, as indicated on top of the MHC-TCR interaction) and inhibitory (negative, as indicated below the MHC-TCR interaction) bindings between ligands on the DC cell with their corresponding receptors on the T cell. For effective T cell activation and acquisition of effector activities, a third signal is necessary, provided by cytokines present in the immunological synapse, as indicated on top.

cells is generally low, as they are removed by clonal deletion in the thymus. The circulating autoreactive T cells that escaped from clonal deletion possess low-affinity TCRs or had already differentiated into natural Tregs in the thymus [165].

5. Dendritic Cell-Based Cancer Immunotherapy

As already discussed throughout this review, there are many immunotherapeutic approaches for the treatment of cancer. Here, we will briefly focus on the use of DCs for cancer immunotherapy, since a significant research effort has been dedicated for the therapeutic targeting of DCs. Steinman and Cohn discovered conventional DCs in 1973 [166]. These cells were found to be the most potent activators of T cell responses [167] but also strong inhibitors when not appropriately activated [168, 169]. Dendritic cells comprise a heterogeneous group of cell types derived from haematopoietic precursors. They are frequently classified in two broad groups according to their lineage, myeloid (or conventional) DCs and plasmacytoid DCs [13]. A major breakthrough occurred about twenty years ago when conventional DCs were shown to efficiently differentiate *ex vivo* from mouse bone marrow using GM-CSF. Four years later, the corresponding DC production method from human monocytes was published, using a combination of GM-CSF and IL4 [170, 171]. The possibility of their *ex vivo* expansion significantly stimulated DC-based immunotherapy, due to the high reproducibility of these procedures and their ease of use. In addition, no previous specific knowledge on the particular antigenic peptide epitopes for a given antigen was required. DCs could efficiently take up any antigen and process it for antigen presentation (Figure 3). Or even the specific HLA genotype of the patient, as autologous DCs could be differentiated, loaded *ex vivo* with antigen and reintroduced into the patient. In addition, DC numbers were not a limiting factor any more. Around 50 million DCs can be reproducibly obtained from a single mouse's bone marrow in one week. From PBMCs, large numbers of DCs can still be obtained, although for human therapy the procedure is slightly more cumbersome as blood has to be taken in high quantities from patients [172]. These cultured DCs are immature, and this ensures that they can be modified and matured using the required stimuli for immunotherapy [154, 173, 174]. The efficient cell yields have allowed the systematic characterisation of a wide variety of immunostimulatory/inhibitory treatments (Figure 3). As one of the key problems of cancer immunotherapy is the effective activation of TAA-specific effector T cells, DC vaccination might be one of the best immunotherapeutic strategies. The enhancement of their potent antigen-presenting strategies might be sufficient to strongly activate low-affinity effector T cells and break the natural tolerance towards endogenous TAAs.

DCs present on their surface a wide collection of receptors which recognise a variety of pathogen-associated molecular patterns or inflammation-related cellular proteins. The binding of these molecules with their appropriate receptors triggers their phenotypic and function maturation, by the strong upregulation of MHC and costimulatory molecules of antigen presentation (Figure 2). The ability to grow *ex vivo* large quantities of DCs has allowed the detailed study of cellular and molecular mechanisms of antigen presentation to T cells [153].

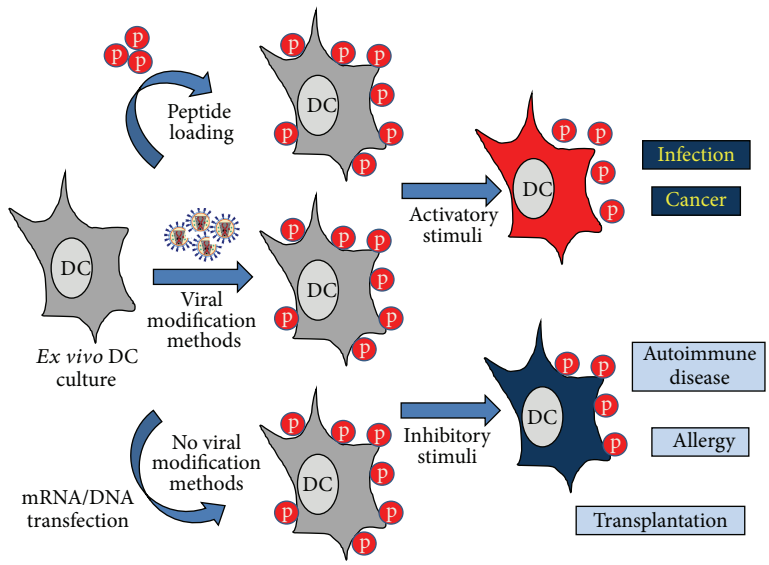

FIGURE 3: *Ex vivo* manipulation of DCs for immunotherapy. This figure schematically represents the manipulations performed with murine or human *ex vivo*-generated DCs for immunotherapy. DC cultures grown for about 5–10 days (left of the figure) are treated to present antigens of interest. This can be carried out by either direct peptide loading on DC cultures, as indicated by the top arrow, by genetic manipulation using viral vectors expressing the antigens of interest (central arrow), or by nonviral genetic manipulation using mRNA/DNA electroporation or transfection (bottom arrow). Any of these procedures will end up with DCs presenting the antigenic peptides in their surface associated to MHC molecules (represented as spheres labelled with a "p"). After that, the maturation stage and functional properties of the modified DCs can be manipulated by incubation with either stimulatory, as indicated by the top arrow on the right, or inhibitory stimuli, as indicated by the bottom arrow. These stimuli can be TLR agonists or antagonists, cytokines, chemokines, chemotherapy drugs, and even achieved by genetic manipulation. Pro-inflammatory DCs (DCs in orange) presenting antigenic peptides can be used for the treatment of infectious diseases and cancer, as indicated on the right by the text boxes. On the other hand, inhibitory (tolerogenic) DCs can be applied as immunosuppressive agents for the treatment of disorders and transplantation, as indicated by the text boxes.

Obviously, the large-scale DC production was quickly applied for immunotherapy and vaccination, particularly to achieve the strong antigen presentation that was required to stimulate effective anti-tumour immune responses. Immature cultured DCs can be incubated with antigens, which are phagocytosed, processed into antigenic peptides, and loaded onto MHC molecules (Figure 3). These antigens can also be TAAs, either as peptides or directly from tumour lysates [13]. Another advantage is that *ex vivo* differentiated immature DCs can be genetically modified with a variety of viral vectors including adenovirus, retrovirus, lentivirus, or poxvirus or even with nonviral methods such as mRNA electroporation [13, 154, 175, 176]. As their maturation state can also be controlled using appropriate adjuvants or even molecular activators/inhibitors [12, 134, 149, 176, 177], they are ideal for immunotherapy (Figure 3). Consequently, *ex vivo* generated DCs have been used to raise T cell responses to infectious agents [178–182] and in cancer immunotherapy [134, 149, 183–188]. They have also been used as tolerising agents for autoimmune disorders in experimental animal models and in human therapy [168, 169, 189–194] (Figure 3).

Most of the preclinical research in immunotherapy using *ex vivo* DCs has provided relevant data [13, 158, 195–197]. However, these results have not always been translated into the expected clinical success in human cancer immunotherapy [159, 198, 199]. Why is that so? To try to answer this question, our research group has been studying DC intracellular signalling pathways during the last 8 years to control DC functions.

6. Constitutive Activation of Intracellular Signalling Pathways to Boost DC Anticancer Activities

As commented before, the administration of foreign, pathogenic antigens on their own is not sufficient to raise effective immune responses. They need to be presented to T cells in a context that will lead to their activation and clonal expansion. This is achieved by inflammation driven by several stimuli including pathogen-derived molecules. These pathogen products will bind to receptors recognising pathogen molecular patterns such as toll-like receptors (TLRs) on the surface of DCs. As discussed above, DCs will then mature and present pathogen-derived antigens to specific T cells. That is exactly the strategy of using adjuvants in vaccination. Adjuvants can be TLR agonists or mediators of inflammation that in combination with the antigens of interest will boost antigen-specific B and T cell responses. The classical adjuvant alum was recently found to be a potent inflammasome activator [200, 201]. TLR agonists are being widely used to break immunological tolerance towards autologous TAAs and activate anti-cancer immune responses [202, 203].

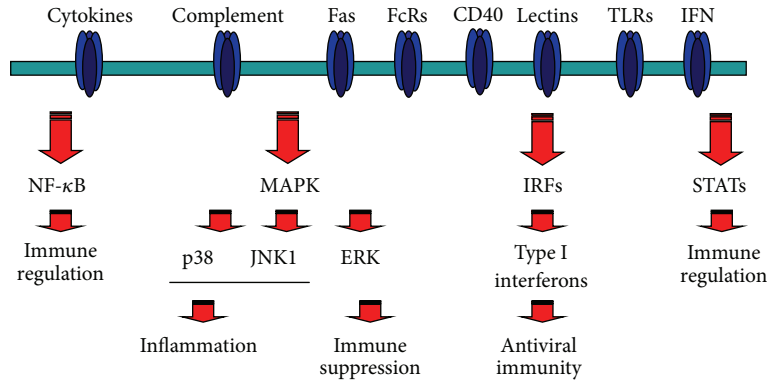

Figure 4: Intracellular signalling pathways controlling DC functions. A simplified scheme of the intracellular signalling pathways which control DC maturation and antigen presentation functions is indicated in the figure. On top, the DC plasma membrane containing a series of receptors which will recognise a wide variety of ligands. Some of these receptors are shown on top, such as Fas, immunoglobulin receptors (FcR), CD40, lectin receptors, toll-like receptors (TLR), and interferons (IFN). The binding of the corresponding ligands to their receptors will activate critical signalling pathways, as indicated below the membrane in the figure. Their involvement in several aspects of immune regulation is briefly indicated in the figure below each pathway.

TLR agonists and other adjuvants act by inducing DC maturation. We reasoned that instead of classical adjuvant formulations, we could directly modify DC antigen presenting functions by activating specific signalling pathways by genetic modification. When maturation stimuli as found in adjuvants trigger their corresponding receptors on the surface of DCs, an intricate network of intracellular signalling cascades is initiated. Simplifying, the four key main signalling pathways that activate following TLR and cytokine stimulation are the NF-κB, mitogen-activated protein kinases (MAPKs), interferon-regulatory factors (IRFs), and signal transducer and activator of transcription (STAT) pathways [12, 154, 204] (Figure 4). These signalling pathways regulate the expression levels of MHC and costimulatory molecules, as well as cytokine production. The administration of adjuvants will stimulate these pathways, but they will also trigger negative feedback mechanisms that will terminate their signalling [187, 205–211]. These inhibitory mechanisms ensure a control over excessive inflammation that protects the organism against autoreactive disease. However, in cancer immunotherapy, these mechanisms are detrimental for the breaking of immunological tolerance towards TAAs.

To circumvent this problem, we decided to express constitutively active mutants of some of these intracellular signalling pathways. For this purpose, we chose mutants that could be resistant to the activity of negative feedback mechanisms such as MAPK phosphatases. One of the key pathways regulating immunity and inflammation is the NF-κB, a transcription factor that translocates to the cell nucleus and transactivates a wide array of inflammation-related genes [13, 212]. Constitutive NF-κB activation in DCs was achieved by the overexpression of the NF-κB inducing kinase (NIK) using adenoviral vectors [176]. This approach strongly potentiated the antigen presentation activities of DCs to T cells by triggering their phenotypic maturation and production of proinflammatory cytokines and mediators. NIK-mediated immunostimulatory properties were superior to those of classical adjuvants. However, this strategy was tested using GFP as a model antigen and not for anti-cancer immunotherapy. A similar strategy was applied using lentiviral vectors (lentivectors) by the expression of Kaposi's sarcoma herpesvirus vFLIP protein. vFLIP is a constitutive activator of the NF-κB pathway, and its activities are associated to cell transformation and oncogenesis [213–215]. Interestingly, its expression in DCs using lentivectors resulted in their phenotypic maturation and production of proinflammatory cytokines, similarly to NIK overexpression using adenoviral vectors [216]. In this case, increase in survival was achieved in an experimental MHC II-negative thymoma model using OVA as a surrogate tumour antigen (EG7 cells) [178, 216]. Another strategy to achieve sustained NF-κB activation is to interfere with the negative feedback mechanisms. Recently, the ubiquitin ligase A20 has been found to be a major negative regulator of the NF-κB pathway [217, 218]. A20 plays a key regulatory role in innate TLR and cytokine dependent immune responses [219–221]. Consequently, siRNA silencing of A20 expression in DCs prolonged NF-κB and AP-1 activation [187]. A20-silenced DCs exhibited a higher secretion of IL10, IL6, and IL12. Interestingly, this strategy increased IL10 expression, a potent immunosuppressive cytokine. Therefore, Breckpot and cols simultaneously silenced A20 and IL10 expression, which in turn increased the antigen presenting capacities of human DCs and stimulated the expansion of MART-1-specific CD8 T cells.

TLR stimulation activates the three classes of MAPK pathways: p38, c-jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (ERK). MAPK p38 has been extensively linked in proinflammatory responses and described a key driver of DC phenotypical and functional maturation [182, 222–224]. Therefore, we tested the effects of its constitutive activation by lentivector expression of an MKK6 mutant [225] that cannot be inactivated by phosphatases. Interestingly, sustained p38 activation in murine bone-marrow-derived DCs (BM-DCs) significantly increased the expression of just a few costimulatory and adhesion molecules (CD80, CD40, and ICAM-I) [134]. To

our surprise, it did not increase expression of IL12 even though there was at the time experimental evidence using p38 inhibitors that it contributed to IL12 production [12, 224]. However, recent evidence is challenging this view [226], and its contribution might be indirect through NF-κB activation [212] and dependent of the source of DCs [227]. Direct vaccination with a lentivector coexpressing the MKK6 mutant and OVA strongly stimulated the expansion of OVA-specific CD4 and CD8 T cells. Sustained p38 activation in DCs also significantly enhanced CD8 T cell responses against NY-ESO-1 in a human HLA-A2 transgenic mouse model [134, 228]. MAPK p38-activated human DCs could also support the *ex vivo* proliferation of MART-1-specific CD8 T cells. Consequently, *ex vivo* p38-activated OVA-expressing DCs were used as a therapeutic cellular vaccine in the EG7 lymphoma model [134]. Interestingly, an increase in survival was observed although all mice subsequently developed lymphomas due to immunological escape. These results suggested that constitutive p38 activation was not strong enough yet, even though it enhanced the antigen presentation properties of modified DCs. However, tumours always came back even though they were expressing a strongly immunogenic xenoantigen (OVA).

Constitutive JNK1 activation was also tested by DC genetic modification using lentivectors [134]. JNK1 has also been linked to proinflammatory responses and regulation of DC survival [222, 229, 230]. The expression of a fusion protein between MKK7 (JNK upstream kinase) and JNK1 resulted in its constitutive activation and had only a minor effect on DC maturation [134, 231]. Even so, it clearly enhanced CD8 T cell proliferation after vaccination with a lentivector encoding MKK7-JNK1 and OVA. Nevertheless, this strategy was never tested in a tumour model.

Similarly, the expression of a constitutively active MEK1 mutant using lentivectors resulted in sustained ERK phosphorylation. ERK activation in murine BM-DCs and human monocyte-derived DCs (MoDCs) drove DCs towards a regulatory phenotype, suppressed antigen-specific T cell expansion, and expanded antigen-specific CD4 Foxp3 Tregs [134, 189]. Obviously, these DCs could be used to suppress autoimmune disorders such as inflammatory arthritis [189] but could not be used for anti-cancer immunotherapy. Therefore, we constructed a dominant negative MEK1 mutant by replacing the activatory threonine and serine residues by alanines in its activation loop (MEK1 AA). Now the expression of this MEK1 AA mutant in DCs inhibited ERK phosphorylation, which drove DCs to their phenotypical maturation and strongly enhanced their T cell antigen presenting properties [189]. Even so, and following our experience with the MAPK p38, we reasoned that ERK inhibition on its own might not be sufficient to achieve effective anti-cancer therapeutic activities. Therefore, we combined MEK1 AA expression with delivery of a PD-L1 targeted shRNA in murine DCs and used these genetically modified DCs as a cellular vaccine in an EG7 lymphoma model. PD-L1 is a member of the B7 costimulatory molecules, although it delivers an inhibitory signal to T cells through binding to PD-1. However, we had observed that PD-L1 silencing in antigen presenting DCs interfered with ligand-induced TCR downmodulation in CD8 T cells [148, 155]. This lack of TCR downmodulation hyperactivated T cells which started to proliferate much earlier and acquire their effector cytotoxic activities shortly after antigen presentation. However, in "physiological" conditions these T cells exponentially expand and down-modulate their TCR, which prevents an untimely attack until a "critical mass" of T cells has been reached [147, 148, 155, 161]. Therefore, just by PD-L1 silencing alone, the expanding T cells started to attack the tumour much earlier, before reaching their peak of proliferation. The net result was that cancer cells underwent immunoediting early, and the reduced T cell numbers favoured the selection of escaping T cells. However, with PD-L1 silencing in combination with the ERK inhibitor in DCs, these T cells could effectively control tumour growth [149]. By using this combination, up to 80% long-term survival was observed in the EG7 lymphoma mouse model [149]. Interestingly, constitutive activation of p38 together with PD-L1 silencing also achieved similar survival outcomes.

Following this strategy, we launched further experiments using endogenous TAAs instead of OVA. We observed that raising significant TAA-specific T cell responses using our PD-L1 silencing strategy is harder than what we had expected. As discussed above, there are many discrepancies between the successes observed in experimental cancer models and their corresponding strategies in human clinical practice. These discrepancies are principally due to the activity of potent immunological regulatory mechanisms that ensure immunological tolerance in physiological conditions. These mechanisms are truly immunological barriers for cancer immunotherapy.

7. Immunological Barriers for Cancer Immunotherapy

The immune system is capable of recognizing, controlling, and eliminating cancer cells, as they are certainly immunogenic [37, 232]. However, there are two key barriers for the immune system to overcome to raise effective antitumour responses. The first one is to break the natural immunological tolerance towards self-antigens, which stops the activation of TAA-specific cytotoxic T cells. The second one is tumour-induced immune suppression, which strongly inhibits any immunotherapy treatment used in clinical practice. In fact, this is a main factor contributing to the pathological manifestations of cancer. Patients with advanced cancer possess an immune system so compromised that it is unable to fight infections. These infections complicate their treatment and can quickly lead to their death. So, what causes tumour-induced immune suppression? There are several contributing mechanisms, but it is mainly caused by the expansion of potent immunosuppressive cells. From these, especial mention deserves regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs).

Thus, the two main goals of cancer immunotherapy are the achievement of strong activation of circulating autoreactive TAA-specific T cells and to counteract the activity of Tregs and MDSCs. As mentioned earlier in this review, activation of effector TAA-specific T cells occurs in cancer

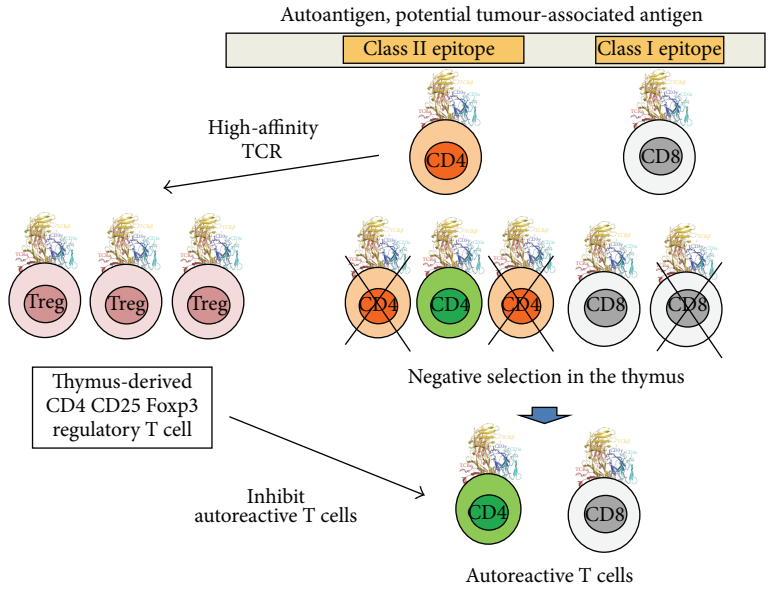

Figure 5: Negative selection of autoreactive T cells and natural Treg development. This figure schematically shows a very simplified version of negative selection that takes place in the thymus and removes auto-reactive T cells. On top, a cellular autoantigen expressed in the thymus is shown, containing both class I and class II epitopes as indicated. When developing T cells recognise these peptides, most of them will undergo negative selection as indicated below the represented antigen. These cells will die, and their differentiation will not continue (crossed-out cells). However, a small percentage of low-affinity autoreactive T cells survives negative selection. These can be both CD4 (in green) and CD8 T cells, as shown at the bottom of the figure. However, if CD4 T cells strongly recognise the autoantigen epitope, they can also differentiate into highly suppressive natural Tregs (left on the figure, Treg). When released out of the thymus, these nTregs will control the activity of autoreactive T cells, in case that these get activated "by mistake" during antigen presentation. This is possibly one of the major barriers for cancer immunotherapy, as many of these autoreactive T cells can be potential TAA-specific cytotoxic T cells.

patients, and it can be stimulated by enhancing antigen presentation at the level of DCs. However, the neutralisation of the profound systemic immune suppression in cancer patients with heavy tumour burden remains a challenge. Any immunotherapy treatment, including DC vaccination or the use of strong adjuvants to enhance antigen presentation, has to overcome the strongly immunosuppressive environment in cancer patients. Here lies one of the main factors for the discrepancy between *ex vivo* preclinical data and *in vivo* human cancer immunotherapy.

8. Regulatory T Cells: The "Re-Discovery"

In physiological conditions, millions of innocuous organisms and compounds are in direct contact with our organism, including bacteria, viruses, all types of "allergens" such as dust mites, pollen and man-made chemicals. During the early-middle 20th century, the medical community including scientists and researchers thought that the immune system was constantly fighting against all these organisms and antigens. Therefore, the immune system was considered a very active sentinel keeping potential threats at bay. This interpretation of immunity has turned out to be not that accurate. It seems that our immune system is kept in a type of "stand-by" state. More precisely, the immune system actively suppresses immune responses, and the net result is systemic tolerance towards most organisms and potential allergens. It would certainly be highly detrimental to raise uncontrolled immune responses towards, let us say, commensal bacteria in our gut. When a truly dangerous threat arises, the immune system is triggered to mount immediate protective responses, leading to medium- and long-term adaptive responses.

However, there was another puzzling property of the immune system. When antipathogen immune reactions take place, no autoimmune reactions appear, apparently. How can our organism differentiate "self" from "non-self"? Obviously, this is a critical question considering cancer immunity, as cancer could be considered as "self." Firstly, systemic central self-tolerance starts during the development of T cells. A high variety of antigen-binding regions are generated when TCRs are assembled, and these also include TCRs that can recognise autoantigens with high, medium, and low affinities. Fortunately, high-affinity autoreactive T cells are removed in the thymus by a process called clonal deletion [233] (Figure 5). This mechanism eliminates most of the autoreactive T cells, which certainly helps in the keeping of systemic self-tolerance. However, clonal deletion did not explain autoimmune disorders, a pathological situation in which B and T cells recognise certain autologous antigens. These autoreactive cells will attack self-tissues and amplify inflammation leading to the development of diseases such as rheumatoid arthritis, lupus, or ankylosing spondylitis [234]. Therefore, autoreactive T cells can in fact escape clonal deletion, although in physiological conditions they are

inactive. The answer to this riddle is that many high-affinity autoreactive T cells that survive clonal deletion differentiate into natural CD4 Foxp3 Tregs [165, 233] (Figure 5).

8.1. Natural Tregs. Natural Tregs (nTregs) largely contribute to the establishment of central tolerance. These T cells are intrinsically immunosuppressive and can inhibit the proinflammatory activities of a range of immune cells [165]. Many scientists might believe that Tregs have been recently discovered. It is true that research in Treg biology has exponentially increased relatively recently. However, Tregs are not a new finding by any means. There was ample evidence on their existence in the 1970s. In 1973, suppressor CD4 T cells originated in the thymus were described [235–237]. However, as the authors mention, they were basically very similar to their immunostimulatory T helper counterparts and difficult to separate from them [235, 238]. There was even evidence for inducible Tregs [239]. Even more suprisingly, these early studies published experimental designs and conclusions which were nearly identical to the most recent papers on Tregs. For example, there was compelling evidence in the 70s that cyclophosphamide could break immunological tolerance by counteracting T cell suppressor activities [240]. This observation was "re-discovered" in 2006, 32 years later from the original finding [241]. Even as early as 1974, suppressor cells that could correspond to either Tregs or MDSCs were described in spleens of tumour-bearing mice [242]. The recognition of the existence of MDSCs as a separate immunosuppressive lineage, which accumulate at large numbers in spleens from tumour-bearing mice, was less than 10 years ago [243]. Also in 1974 suppressor T cells were isolated from tolerised mice, and the authors demonstrated their capacities to suppress immune responses in a cell-to-cell dependent manner [244]. Similar recent studies are still clarifying these suppressive cell-to-cell mechanisms exerted by Tregs [245–247].

So, if there was so much evidence regarding the existence, how is it possible that all this research was forgotten and "re-discovered" around 2003 [248]? There was a surprising large body of evidence regarding suppressor T cells, but at the time there were not specific cell markers associated to them. We have to take into account that monoclonal production technology appeared in 1973 [249], and their use for phenotyping of immune cells was not regulated until 1982. Therefore, the phenotypic characterisation of Tregs during the late 70s and 80s just simply did not occur. This fact made it difficult to isolate, study, and manipulate them. It was not until the early 2000s when Sakaguchi and colleagues associated high expression levels of CD25 in CD4 T cells with natural Treg activity [248, 250]. CD25, the high-affinity IL2R chain, has been directly associated to Treg suppressor activities by sequestration of IL2 required for T cell survival and clonal expansion [251, 252]. In addition, IL2 signalling is required for Treg homeostasis [253].

A major breakthrough took place in 2003 when the Foxp3 transcription factor was shown to be required for Treg development [248]. Other markers and transcription factors have been associated to Tregs [254], but so far only Foxp3 remains the most reliable of them, particularly in mice. The strong association of high levels of CD25 and Foxp3 expression with regulatory T cells strongly relaunched research on Tregs.

8.2. Inducible Tregs. Nevertheless, nTregs and clonal deletion did not explain the acquired tolerance towards other autoantigens not expressed in the thymus and without mentioning a plethora of foreign antigens to which we constantly come in contact with. Theoretically speaking, immune responses should be constantly in place towards these antigens, although this is not by any means the case. The organism remains tolerant towards most antigens whether they are autoantigens or foreign/xenoantigens. Interestingly, once Foxp3 was described as a Treg marker, another set of Tregs that did not originate directly in the thymus was discovered. These Tregs also coexpressed CD25 and Foxp3 but differentiated in the periphery from naïve and antigen-experienced nonregulatory CD4 T cells [250]. This inducible Foxp3 Tregs (also called Th3 cells) differentiated during antigen presentation by tolerogenic DCs in the absence of inflammation [255]. Negative costimulation and TGF-β expression mediate inducible Treg differentiation during antigen presentation [189, 256–259]. Another set of inducible CD4+ IL10+ lacking Foxp3 expression cells can also be differentiated by antigen presentation in the presence of IL10, a potent immunosuppressive cytokine [260]. They are known as Tr1 and also play a key role in maintaining peripheral and mucosal tolerance [261].

8.3. Treg Cells in Cancer. Tregs are major players in keeping systemic immunological tolerance. Unfortunately for cancer immunotherapy, natural Tregs which recognise autoantigens differentiate in the thymus. And even though autoreactive T cells escape, their activities are controlled by nTregs targeting the same autoantigens. The elimination of Tregs using CD25-depleting antibody results in tumour regression, demonstrating a direct link between the inhibitory activities of Tregs and lack of anti-tumour immune responses [262]. In fact, using a systemic B cell lymphoma model expressing flu haemagglutinin as a surrogate tumour antigens, Zhou and cols demonstrated that tumours induce antigen-specific Tregs [263]. Even after vaccination, both effector antitumour cells and antigen-specific Tregs were expanded, but Tregs could control the antitumour cytotoxic cells. This is a key observation, as it demonstrates that tumours expand Tregs to escape from the T cell attack. Increased numbers of circulating and tumour-infiltrating Tregs were also observed in cancer patients [264, 265]. Therefore, even though tumours are certainly immunogenic, TAA-specific T cells are at the end rendered inactive by Treg activity [266–268]. Thus, Tregs strongly influence the equilibrium between tumour growth and antitumour immune responses towards cancer growth and immune escape.

There is evidence that tumours cause Treg differentiation by converting myeloid DCs into tolerogenic DCs [269]. In addition, tumour cells produce a range of cytokines and metabolic enzymes that stimulate the conversion to Tregs, and some of these also promote MDSC differentiation such as cyclooxygenase and prostaglandin [270].

Interestingly, MDSC infiltration in tumours recruits Tregs through chemokine secretion in a CCR5-dependent manner [271]. Therefore, there is a concerted action between MDSCs and Tregs that stimulate tumour progression. Tumours also produce key cytokines and growth factors that can convert Tregs in situ, or in the draining lymph node, such as the prototypical TGF-β and IL10.

Tregs inhibit immune responses by a wide range of mechanisms, such as infectious tolerance by amino-acid consumption [272, 273], expression of potent immunosuppressive cytokines such as TGF-β and IL10 [80, 260, 269], and by the delivery of negative signals to antigen presenting cells. Particularly important is this last point, usually mediated by CTLA4 expressed on the surface of Tregs [274]. Even though CTLA4 in Tregs has been extensively studied, the specific mechanisms by which it inhibits immune responses still remain a sort of a mystery [247]. Firstly, as it exhibits a higher affinity for CD80/CD86 than CD28, it has been proposed that CTLA4 competes with CD28 binding CD80/CD86 [275]. This completion would be responsible for the inhibition of APC antigen presentation functions [247, 275]. It could be possible that CTLA4-CD80/CD86 binding would deliver a negative signal to both T cells and APCs. Interestingly, there is strong evidence that Tregs physically remove CD80/CD86 molecules from the surface of antigen presenting cells by transendocytosis [246]. Then, CTLA4-CD80/CD86 complexes would be degraded, leaving APCs without costimulatory molecules. The result would be APCs with highly reduced antigen presenting capacities.

8.4. Tregs as a Target for Cancer Immunotherapy. The therapeutic elimination of Tregs or inhibition of their functions is a major goal for cancer immunotherapy to improve its therapeutic activities [276]. As discussed above, Tregs express CTLA4 and CD25, surface markers that can make them susceptible to antibody-targeted strategies. The combination of a CTLA4 blocking antibody with a CD25-depleting antibody in a B16 melanoma model resulted in effective immunotherapy [277]. The elimination of Tregs enhanced immune responses against endogenous TAAs such as TRP2. Likewise, the simultaneous blockade of CTLA4 and PD-1 signalling also strongly inhibits Treg function [278]. Preclinical and clinical data from systemic administration of CTLA4-blocking antibodies clearly show that targeting these immunosuppressive T cell pathways is an effective therapeutic procedure [279]. The humanised approved anti-CTLA4 antibody ipilimumab clearly boosts antitumour activities in human therapy, significantly prolonging survival in melanoma [280–282] and nonsmall cell lung carcinoma patients [283]. Interestingly, it has been demonstrated that the activity of the CTLA4 "blocking" antibody is in fact dependent on the specific Treg depletion within the tumour but not systemically. So, instead of blocking CTLA4 activities on Treg cells, it seems that this antibody physically removes Tregs via macrophages [284]. However, it might not be that effective in less immunogenic cancers [285]. Something to be taken into account is that these antibody-based immunostimulatory strategies exhibit important inflammatory cytotoxicity [281, 282, 285]. Nevertheless, it is likely that better outcomes will be expected in combination therapies [286].

Some DC immunostimulatory treatments can make them resistant to Treg suppressive activity. Genetic modification of DCs using mRNA electroporation expressing a constitutively active TLR4 ligand together with CD40L and CD70 can confer Treg resistance to T cells and inhibit their suppressive activity towards effector T cells [183]. Likewise, constitutive inhibition of ERK phosphorylation by the expression of the MEK1AA dominant negative mutant or TGF-β silencing in antigen presenting DCs can also strongly inhibit the *in vivo* expansion of inducible Tregs. [189]. Consequently, ERK inhibition synergistically enhanced the antitumour properties of PD-L1 silencing in DCs [149].

Recently, commonly used chemotherapeutic agents such as cyclophosphamide, fluorouracil, and oxaliplatin have been shown to inhibit the development of Tregs *ex vivo* and *in vivo* [240, 241]. These effects contribute to their antitumour capacities by stimulating anticancer immune responses. In fact, there is evidence that broad spectrum kinase inhibitors frequently used in chemotherapy have potent immunostimulatory capacities, such as sunitinib [287, 288]. Without them, it is likely that their direct actions against cancer cells [289, 290] would not be sufficient for their full efficacy. Inhibition of tyrosine kinases and MAPK ERK could have strong immunostimulatory activities by stimulating DC maturation and simultaneously inhibiting the expansion of Tregs and MDSCs [12, 288].

9. Myeloid-Derived Suppressor Cells in Cancer

While Tregs are certainly important immunosuppressive cells that enhance cancer progression, the central role of another suppressive cell type has come to light in recent years, the myeloid-derived suppressor cell (MDSC).

MDSCs include a heterogeneous myeloid cell population with common phenotypic and functional characteristics, which expand in highly inflammatory environments [291], infection, transplantation, and cancer [292]. In mice, they express high levels of CD11b+ and GR1, a granulocyte-specific marker. GR1 comprises two surface markers, namely, Ly6C and Ly6G, and on the basis of their relative expression, MDSCs are classified into monocytic ($Ly6C^{high}$ $Ly6G^{neg/low}$) and granulocytic ($Ly6C^{high}$ $Ly6G^{high}$). These murine populations have also their corresponding counterparts in humans [293]. In recent years, MDSCs have been recognised as the cell type largely responsible for tumour-induced immune suppression, causing the inefficacy of immunotherapeutic procedures in clinical practice. These cells have suddenly become a target of interest for researchers, medics, and pharmaceutical companies [294].

In cancer patients with heavy tumour burden, tumours produce cytokines and other molecules that direct myeloid differentiation towards MDSCs instead of monocytes, DCs, macrophages, and inflammatory granulocytes [295–297]. These MDSCs are systemically distributed and infiltrate the tumours where they inhibit antitumour T cell responses by antigen-specific and unspecific mechanisms [298–300].

The spreading of cancer cells from the primary tumour is in itself a key pathological feature of cancer. However, systemic immunosuppression hampers the immune system from eliminating cancer cells. The existence of myeloid cells that contributed to cancer progression has been known for several years. However, only recently MDSCs have been recognised as a specialised lineage of immunosuppressive cells which strongly expand in cancer [301, 302]. Although the recognition of MDSCs as a specific cell lineage has been controversial [243], all experimental evidence strongly supports their existence and role. Tumour-expanded MDSCs possess strong T cell inhibitory activities by antigen-specific and nonspecific mechanisms [292]. Antigen-specific suppressive mechanisms may include the induction of Tregs due to antigen presentation in the presence of immunosuppressive cytokines or induction of T cell anergy. T cell anergy encompasses a two-step process. Firstly, T cells undergo antigen presentation in the absence of significant positive costimulation. That might be the case of MDSCs, which are highly immature myeloid cells. These T cells expand, but with limited, if any, effector activities. Then, after a second antigen encounter such as antigen recognition in target cells, these T cells become unreactive (anergic). Antigen nonspecific mechanisms include amino-acid depletion by upregulation of amino-acid-consuming enzymes such as arginase-1 and iNOS expression that would also contribute to arginine depletion. Also, production of immunosuppressive cytokines such as IL10 and TGF-β by MDSCs overly inhibits the activity of immune cells [292].

10. MDSC Differentiation: A Possibility for Therapeutic Targeting?

MDSCs differentiate *in vivo* through the activity of a range of cytokines and other cancer cell products. The inflammatory environment also strongly contributes to their differentiation and acquisition of their suppressive activities. Granulocyte monocyte colony stimulating factor (GM-CSF) produced by cancer cells strongly contributes to MDSC differentiation [296, 298, 303, 304], which would explain why the use of GM-CSF in human cancer immunotherapy has mixed responses [305–311].

A proinflammatory environment driven either by cytokines or TLR ligands seems to strongly contribute to their differentiation. The combination of GM-CSF with the TLR4 agonist lipopolysaccharide (LPS) drives the differentiation of bone marrow myeloid precursors into MDSC-like cells [296]. These BM-derived MDSCs strongly inhibited T cell responses by a cell-to-cell contact mechanism and iNOS expression, respectively. Cancer cells produce a range of cytokines that not only differentiate MDSCs but also provide them with strong immunosuppressive capacities. The combination of GM-CSF with the proinflammatory cytokine IL6 strongly potentiated their suppressive activities [312, 313]. A range of other cytokines produced by primary tumours and cancer cell lines also contributed to MDSC differentiation although to a minor extent [313]. The presence of IL13 in combination with GM-CSF and M-CSF also results in particularly suppressive MDSCs [314], and these cells could inhibit experimental graft-versus-host disease through expression of arginase 1.

These observations pose a critical problem for cancer immunotherapy and the possibility of therapeutically targeting MDSC differentiation. A proinflammatory Th1/Th17 type of response is usually thought to be beneficial to activate anticancer cytotoxic cells [315, 316]. And this might be true to some extent. However, the same proinflammatory environment favours MDSC activation, which in physiological conditions regulates excessive inflammation. Here lies a major problem for an adequate design of cancer immunotherapy protocols. How to raise Th1/Th17 responses without significantly expanding MDSCs and Tregs.

Apart from myeloid-differentiation and proinflammatory cytokines, prostaglandin E2 (PGE2) and TGF-β can effectively drive MDSC differentiation, both of them found in abundance in tumour-derived exosomes [317, 318]. PGE2 in combination with GM-CSF, IL4, and LPS differentiates human MDSCs by a positive loop involving COX2 induction [319].

11. MDSC Targeting for Cancer Immunotherapy

MDSCs are a tougher therapeutic target in comparison to Tregs. There are Gr1-specific depleting antibodies, but in general terms, any myeloid-specific blocking/depleting molecule will possibly eliminate proinflammatory myeloid cells. These cells play a critical role in TAA antigen presentation. As discussed above, there are some chemotherapy drugs and metabolic compounds that inhibit the expansion of MDSCs and Tregs [294], possibly through inhibition of STAT3 signalling. In general terms, both inhibitory T and MDSC cells rely on STAT3 activation for their suppressive activities [288]. In fact, genetic modification of myeloid cells using STAT3-targeted silencing shRNA delivered by lentivector vaccination [320].

It is also highly likely that broad spectrum kinase inhibitors already in use in human oncology might shift the balance towards proinflammatory DC differentiation [12, 154]. However, from a scientific point of view, in preclinical studies, how could these MDSC counteracting drugs be identified without taking them to clinical trials? Currently, MDSCs are isolated directly from spleens or tumours from tumour-bearing mice, which renders relatively low numbers. These cell yields are insufficient for large screenings of candidate drugs. Isolated MDSCs fail to proliferate *ex vivo,* and their survival is severely compromised, which hampers their study and therapeutic applications [321]. Even though they can be differentiated *ex vivo* from bone marrow or mononuclear cells, the MDSC yields range from 20% to 40% in the best cases [314, 319]. Additionally, there are many differing protocols, which will possibly result in heterogeneous and nonrepresentative MDSC populations. Another key point to take into account is that MDSCs subtly differ depending on the cancer background in which they differentiate [321, 322]. This means that for each type of cancer, large numbers of MDSCs should be reproducibly obtained for large scale, high throughput drug testing. In the meantime, small scale studies

in mice or from limited human samples have to be performed to study the effects of antineoplastic treatments on MDSC differentiation and functions.

12. Conclusions

Does cancer immunotherapy have a "niche" in oncology? According to my opinion, it does. Tumours are certainly immunogenic, and TAA-specific T cells do in fact expand and infiltrate tumours. Even in some infrequent cases there is spontaneous tumour regression. However, a key point in cancer immunotherapy is the development of strong TAA-specific T cell responses, not only in quality but in quantity to prevent immunological escape as a result of tumour immune editing. One way to achieve this is to potentiate antigen presentation from the beginning, by manipulating the most potent antigen presenting cell, the DCs.

Many biomedical research groups including our own use murine or human *ex vivo* differentiated DCs to develop anticancer vaccines. These DCs are manipulated to process and present TAA antigen peptides on their MHCs, so that they can be used in antigen presentation assays to T cells to evaluate the therapeutic potential of a range of antineoplastic treatments. These *ex vivo* antigen presentation systems and *in vivo* tumour animal models certainly provide invaluable information to evaluate novel anticancer immunotherapy treatments [149]. However, there are major inconsistent results between preclinical drug testing and clinical trials in human patients. A reason for these discrepancies is that human cancer patients have been suffering the disease for some time. By the time that they go to their physician, tumours have likely exerted their immunosuppressive activities. Therefore, the question is, how can we counteract immunosuppressive cells in their organism?

Treg depletion and inhibition of their differentiation are becoming a reality, even though Treg-depleting treatments are highly toxic. However, new cancer immunotherapy agents have to be aimed at counteracting activity of MDSCs. There is a strong correlation between the success of conventional antineoplastic treatments with inhibition of MDSC and Treg expansion [288, 294, 302].

However, nowadays it is not yet possible to faithfully and efficiently replicate cancer-driven MDSC differentiation *ex vivo*. If a reproducible and efficient MDSC differentiation system could be set up *ex vivo*, similar to DC production protocols, antineoplasic treatments could be firstly assessed at preclinical assays. Immortalised MDSC cell lines could be used as substitutes of primary MDSCs [323]. However, these cell lines were obtained by retrovirus transduction to express the oncogenes v-myc and v-raf. It is highly likely that this will alter their behaviour towards antineoplastic treatments.

As a conclusion, tumours are more immunogenic than previously thought. However, the concerted action of several immunosuppressive cells keeps the immune system at bay. Researchers should be looking for a system to test anticancer treatments over Treg and MDSC functions *ex vivo*. While Tregs can be eliminated using depleting antibodies, MDSCs are more difficult to target. An *ex vivo* MDSC-T cell antigen presentation assay should conserve MDSC's proliferative capacities and replicate their behaviour as found within tumours [321].

These *ex vivo* strategies will uncover novel antineoplastic drugs at preclinical assays, which will save the precious economic and human resources required for human clinical trials.

Conflict of Interests

The author declares no conflict of interests.

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