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Master's project in Bioengineering and Biotechnology

ELUCIDATING THE MECHANISMS OF PACLITAXEL-LOADED-NANOPARTICLE-INDUCED IMMUNE RESPONSE

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ABSTRACT

Dendritic cells (DC) are the most potent antigen-presenting cells and play a key role in the induction of effective immune responses, suggesting a novel target for anti-tumor immunotherapy. Paclitaxel (PXL) is a widely used chemotherapeutic, initially characterized as a mitotic inhibitor, that has been shown to enhance maturation and function of DCs as well as up-regulate the production of inflammatory cytokines. Thus, modulation of DC function by the approved-for-human-use PXL could constitute a novel approach for reshaping immune responses against a tumor. For targeted delivery of PXL to DCs in the lymph nodes (LNs), where they are present in high concentrations and apt for antigen uptake, poly(propylene) sulfide core nanoparticles (NPs) developed by our laboratory have been used. PXL loaded-NP (PXL-NP) treatment induces massive immune cell infiltration into the draining LN 24h after intradermal injection of PXL-NPs in mice, suggesting that PXL-NP treatment induces a local and robust immune response (Thomas et al, in preparation). We hypothesized that this effect stems directly from the action of PXL-NPs on LN-resident DCs. We demonstrate that PXL-NPs are able to induce maturation and cytokine production in murine and human DCs *in vitro*, which may explain the *in vivo* observations of robust cell recruitment to the draining LN and generation of adaptive immune responses. Furthermore, the striking cell infiltration in the LN upon PXL-NP treatment was abrogated in complement protein 3 (C3) knockout mice *in vivo* (Thomas et al, in preparation). Interestingly, we show that the presence of C3 regulates IL-12p40 expression and, in turn, plays a crucial role in the activation of mature, functional DCs in response to PXL-NPs. Thus, the absence of functional DCs in case of C3 deficiency might account for the hindered infiltration. Together, this data suggests that PXL-NPs may effectively direct the immunomodulatory action of PXL to LN-resident DCs to alter immune responses.

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1. MOTIVATION

Cancer immunoengineering aims to better understand and manipulate the immune system to eliminate cancer by reshaping immune responses against tumor and prevent tumor growth. Many studies have been focused on the identification of potent immunostimulatory agents that could enhance immunogenicity while overcoming immune-suppressive mechanisms to achieve effective and long-lasting anti-tumor responses. Interestingly, it has been widely reported that many chemotherapeutic agents, when used in low doses, can activate dendritic cells (DCs). Such an agent is Paclitaxel (PXL), a successfully used chemotherapeutic that can be loaded efficiently into the Poly(propylene) sulfide core nanoparticles(NPs), developed in our laboratory, for targeted delivery to immune cells. It has been shown that PXL-loaded NPs induce massive immune cell infiltration (~4-fold increase in cell number) into the lymph nodes (LNs) 24 h after intradermal injection in mice, suggesting that PXL-NP treatment induces a local immune response (Thomas et al, in preparation). Furthermore, when applied to the tumor-draining LN via intradermal administration daily after tumor implantation, PXL-NP treatment dramatically reduced tumor growth and reshaped the immune milieu inside both the tumor-draining LN and tumor itself. The drastic reduction in tumor growth was coupled with increased maturation of tumor-draining LN CD11c+ cells, up-regulation of antigen-specific CD8+ T cells within the tumor and decrease in the frequency of TReg cells within both the tumor-draining LNs and tumor (Thomas et al, in preparation). We hypothesized that the above mentioned effects result from the immunomodulatory capacity of PXL-NPs to induce mature and functional DCs upon treatment. To investigate the validity of our hypothesis, we evaluated the capacity of PXL-NPs to enhance maturation and cytokine production of murine and human DCs *in vitro*. Additionally, it has been observed that the induced cell infiltration to the draining LN in response PXL-NPs treatment *in vivo* was impaired when treatment was applied in complement protein 3 (C3)- knockout mice (Thomas et al., in preparation). To elucidate the potential role of C3 in the immunoregulating capacity of PXL-NPs, we assessed the expression of costimulatory molecules as well as cytokine production in C3 knockout DCs *in*

vitro. We hope that these studies could enlighten the therapeutic role of approved-for-human-use PXL supporting its application in biomaterials-based schemes for immunomodulation.

2. INTRODUCTION

2.1. *The interface between innate and adaptive immunity*

To generate effective and durable immune responses, which would lead to pathogen eradication and host immunity, the action of both the innate and adaptive immune system is essential. The innate immune system acts as the first-line defense against pathogenic organisms or abnormal cell growth initiating a local and immediate response [1]. Efficient detection is mediated by several distinct components of innate immunity. These include the complement system, specialized receptors expressed on natural killer (NK) cells, intracellular sensors and most importantly, the toll-like receptor (TLR) family of proteins that are expressed on both myeloid and lymphoid cells [2]. The TLR family of pattern-recognition receptors (PRR) plays a crucial role as sensor of infection since it ensures innate system's specificity with different TLRs recognizing distinct types of structures [3]. All these components ensure the establishment of a prompt response which allows the development of the adaptive immune response. The recruitment of dendritic cells (DC) and T and/or B cell interactions in an antigen-specific way is required for complete eradication and generation of host immunologic memory. Therefore, the requirement for antigen presentation provides the link between innate and adaptive immunity. It is thus, conceivable that antigen-presenting cells (APC) bridge and coordinate innate and adaptive immune responses.

2.2. *Antigen-presenting cells (APCs)*

Since they frame the cooperation between innate and adaptive immunity, antigen-presenting cells (APCs) are one of the main targets in the modulation of the immune system. APCs play a key role in immunity due to their capacity to capture and process antigens leading to T cell activation [1, 4, 5]. Among the cells displaying these characteristics, the ones with the greatest efficiency are B cells, macrophages and dendritic cells, which are considered as "professional" antigen-presenting cells [5, 6]. Dendritic cells (DCs) are thought to be the most specialized, since they are inherently more efficient for antigen

presentation while they tend to assemble within the T cell areas of lymphoid organs [1, 4, 7, 8]. Additionally, DCs exhibit the ability to lead T cell responses by promoting T cell development or T cell tolerance [2, 9]. All the processes mediated by dendritic cells rely highly on their maturation [4, 9, 10]. Maturation is closely related to DCs function and it is displayed by structural as well as phenotypical changes (Figure 1) [1, 4, 9]. Phenotypically mature DCs express high levels of surface major histocompatibility class I (MHC I) and class II complexes (MHC II), determining the T cell response (signal 1), and costimulatory molecules, such as CD40, CD80, CD83, CD86, which regulate the ability of naïve T cells to expand (signal 2) [9]. Additionally, the expression

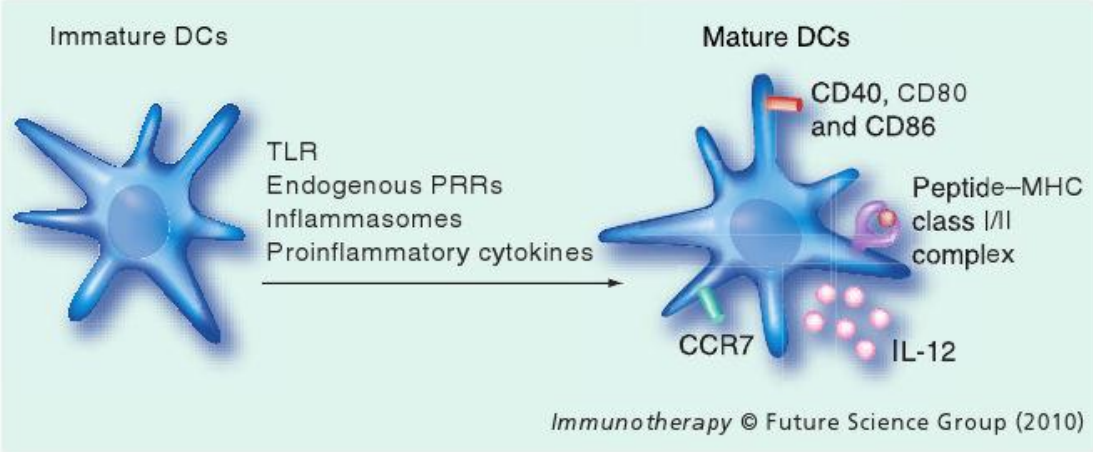


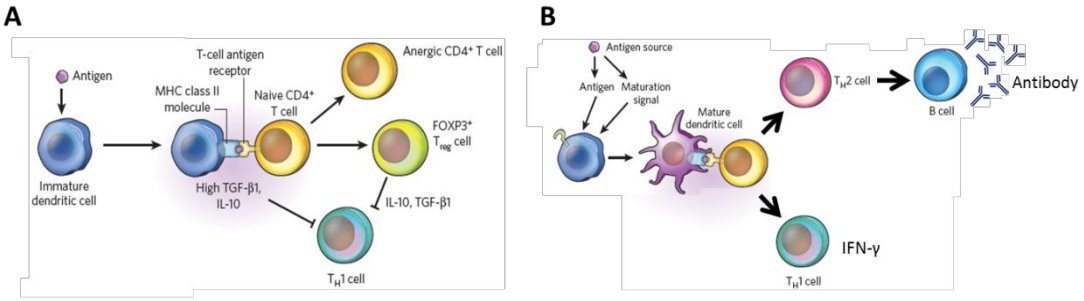
Figure 1. Dendritic cell functional states [1]. DCs are the most specialized antigen-presenting cells, being inherently more efficient for antigen presentation. All the processes mediated by dendritic cells rely highly on their maturation. Maturation is induced by the interaction through several receptors, such as TLRs or PRRs. Upon maturation, DCs express high levels of surface major histocompatibility class I (MHC I) and class II complexes (MHC II), up-regulate costimulatory molecules CD40, CD80 and CD86, as well as the production of inflammatory cytokines such as IL-12. CCR7 is also up-regulated, allowing the migration of DCs from the periphery to lymphoid tissues to determinine the nature of the ensuing immune response.

of CCR7, which controls the migration from the periphery to lymphoid organs, is up-regulated [1, 8]. Upon arrival in the secondary lymphoid organs, mature DCs secrete chemokines to recruit macrophages, natural killer (NK) cells, B-cells, and antigen-specific T cells as well as cytokines which determinine the nature of the ensuing immune response (signal 3) [1, 9, 11]. The state of maturation, the way the antigen was taken up, the local environment as well as the tissue in which activation took place determine the fate of the response.

Following antigen uptake, DCs load antigen-derived peptides onto MHC molecules for presentation to T cells [10]. The uptake and processing of the antigen by DCs depends on its origin. Endogenous antigens are degraded into peptides by the proteasome in the cytosol [1]. The released peptides are then transported by special transporters for antigen presentation (TAP) into the endoplasmic reticulum (ER) and loaded on MHC class I molecules. Exogenous antigens are processed in endosomes in which they are degraded into peptides followed by loading on MHC class II molecules[4]. Antigens presented on MHC class I molecules can activate CD8+ T cells, whereas antigens bound to MHC class II molecules can activate CD4+ T cells. Cross-presentation is a distinctive characteristic of DCs which allows them to present exogenous antigens on MHC class I molecules [1, 4].

The various types and levels of cytokines expressed by DCs shape the microenvironment determining the induced immune response [12]. IL-12 is one of the most important cytokines produced by DCs since it regulates T cell differentiation towards a Th1 phenotype [1, 9]. More specifically, IL-12 expression by dendritic cells induces IFN-γ production in naïve Th cells promoting Th1 responses [9]. The ability of IL-12 to strongly direct the response towards a Th1 phenotype is further demonstrated by the fact that transfection of DCs with the IL-12 encoding gene led to a shift from a Th2 to a Th1 response [13]. Furthermore, local IL-12 production enhances local inflammation and activates IFN-γ expression by NK cells or effector CD4 T cells [14]. At the same time, IL-12 has been shown not only to activate cytotoxic CD8 T cells [15] but most importantly, to be able to act as the necessary third signal for the generation of cytotoxic CD8 T cells responses from naïve CD8 T cells [16]. IL-10, IL-6 and other cytokines are also significantly expressed or induced by DCs with IL-6 inhibiting regulatory T cells [17], whereas TNF-a is induced upon maturation of DCs and interaction with T cells. IL-10 has been shown to block the expression of costimulatory molecules and IL-12 production preventing DCs from Th1 responses [18]. Similar studies have confirmed these findings showing that IL-10 deficient APCs are potent activators of Th1 response [19].

DCs in an immature state, expressing low levels of costimulatory molecules, reside in peripheral tissues, where they continuously sample antigens to present to T cells for the maintenance of self-tolerance [1, 9, 20]. In the presence of an exogenous or endogenous stimulus while taking up antigen, DCs begin to mature and drain to the nearest draining lymphatic vessels and then to the lymph node to initiate an adaptive immune response. To this extent, when immature DCs encounter an antigen in the presence of transforming growth factor-β1 and Interleukin-10 (IL-10), they induce tolerogenic responses (Figure 2) [5, 20]. On the other hand, when mature DCs encounter antigens in the presence of immune cytokines, they activate antigen-specific responses by instructing T cells. Stimulation of T cells depends on the strength and the duration of the interaction between DCs and T cells, which are crucially determined by the up-regulation of costimulatory molecules during DC maturation [1].



JA Hubbell, SN Thomas and MA Swartz, *Nature* 2009.

Figure 2. The state of DC maturation determines the fate of the response [20]. Dendritic cells in an immature state continously sample antigens in their environment. When immature DCs encounter an antigen in the presence of transforming growth factor-β1 and Interleukin-10 (IL-10), they present it, without costimulatory molecules to T cells, inducing tolerogenic responses (A). When DCs encounter an antigen in the presence of a maturation signal, they begin to mature and migrate into the nearest lymph node. There, they present processed antigenic peptides with maturation induced costimulatory molecules to T cells to initiate an antigen-specific immune response (B).

There are many different subtypes of dendritic cells, such as plasmacytoid dendritic cells (pDCs) expressing interferon-a (IFN-a), or myeloid dendritic cells secreting IL-12, as well as lymphoid dendritic cells and follicular dendritic cells, which do not express major histocompatibility complex (MHC) class II molecules [1, 7]. The DCs that we use for in vitro studies are generated from peripheral blood monocytes, hematopoietic progenitor cells or bone marrow which are differentiated into immature dendritic cells by using IL-4 and

granulocyte-macrophage colony-stimulating factor (GM-CSF) and then activated by lipopolysaccharide (LPS) or tumour-necrosis factor-a (TNF-a).

2.3. *Manipulating DCs for tumor immunotherapy*

Antigen presentation by DCs is a critical process in the induction of specific antitumor responses. Therefore, manipulation of DCs to enhance immune responses could be a powerful tool to achieve anti-tumor immunity. Recent studies have shown that certain chemotherapeutic drugs, used at low doses, may strengthen antitumor efficacy by inhibiting tumor angiogenesis [21] while supporting antitumor immunity by providing tumor antigens from dying tumor cells to antigen-presenting DCs [22]. Additionally, specific chemotherapeutic agents were found to directly up-regulate DC maturation and function in murine and human DCs without affecting cell viability as well as to enhance antigen presentation to T cells [23, 24]. There are several studies indicating that modulation of DC function by non-cytotoxic concentrations of chemotherapeutic drugs could constitute a novel approach for altering the tumor microenvironment to augment the efficacy of cancer vaccines. Such an antineoplastic-agent, paclitaxel, combined with the injection of DCs resulted in complete tumor regression compared to only partial eradication of the tumor in case of chemotherapy or DCs alone, in the murine fibrosarcoma model [25]. Zhong et al. have recently shown that pretreatment of tumor-bearing mice with paclitaxel *in vivo* enhanced the antitumor potential of dendritic cell vaccine administered intratumorally [26].

2.4. *The dual role of Paclitaxel*

Paclitaxel (PXL) has been successfully utilized in the treatment of breast, lung, ovarian, and other solid tumors. It was initially characterized as a mitotic inhibitor that provokes apoptosis by binding to microtubules [27, 28]. Paclitaxel binds on P-tubulin and stabilizes microtubules (alp tubulin polymers) against depolymerization. Because microtubule function requires a dynamic flux of polymerization/depolymerization, PXL interferes with the critical role of

microtubules during cell division and blocks cell progression through metaphase[29].

However, Paclitaxel has also been found to mimic the action of Toll-Like Receptor (TLR) agonists such as lipopolysaccharide (LPS), which is a TLR4 ligand, inducing the maturation of mouse macrophages and major histocompatibility complex (MHC) class II expression on mouse dendritic cells [30, 31]. Stimulation of macrophages with paclitaxel activates pathways involved in the elimination of microbes as well as the production of cytokines such as interleukin IL-1β, tumor necrosis factor TNF-α, IL-8 and IL-12 [31]. Recent studies have shown that Paclitaxel, in low non-cytotoxic concentrations, can enhance maturation and function of dendritic cells, without affecting cell viability [32], as well as up-regulate inflammatory cytokine production [24, 33]. Additionally, it has been demonstrated that Paclitaxel, acting via the above mentioned mechanisms, can enhance antigen-specific IFN-γ secreting CD8+T cell in vivo suggesting that administration of Paclitaxel with a tumor vaccine improves T cell priming leading to slower tumor growth [32]. Similar studies support the immunomodulatory activity of Paclitaxel showing its ability to up-regulate Th1 and Th2 immune responses pointing out its adjuvant property in vaccine development [34]. Zhong et al have shown that Paclitaxel pretreatment of tumors can enhance vaccine efficacy by altering the intratumoral microenvironment towards antitumor immunity [26]. Apart from reshaping immune responses, Paclitaxel can inhibit tumor escape in the treatment of 3LL bearing mice by eliminating Treg cells [35]. Many studies have been carried out on combined treatment of conventional chemotherapy with an intratumoral injection of syngeneic dendritic cells (DCs) as a potent cancer treatment strategy. Choi et al [25] have demonstrated that combined treatment of paclitaxel chemotherapy and the injection of DCs led to complete tumor regression, in contrast to only partial eradication of the tumors with chemotherapy or DCs alone in murine fibrosarcoma model. Kaneno et al [24] have recently reported that low-dose paclitaxel markedly up-regulates antitumor immune responses in mice bearing lung cancer and treated with DC vaccines.

These data strongly suggest that Paclitaxel could potentially have greater therapeutic application beyond cancer chemotherapy. The immunomodulatory activity of Paclitaxel has been attributed to signaling through TLR4 leading to the activation of transcription factor NF-κB. Nuclear factor kB (NF-kB) is an inducible eukaryotic transcription factor of the rel family of proteins which is important for the regulation of genes that participate in inflammatory, immune, and acute-phase responses. The induction of many cytokine genes, including TNF-a, IFN-b, IL-1β, IL-8 and IL-6 is regulated by NF-kB [36]. By means of targeted disruption of the rel family genes in mice, it has been demonstrated that the members of the rel family have distinct functions that cannot be fully compensated for by the other members. The targeted disruption of the RelB gene indicated a role for this transcription factor in maintaining the immune system of adult mice [37], whereas the targeted disruption of the p50 gene indicated its importance as a transcription factor responsible for both innate and adaptive immune responses[38]. Given the important impact of the rel family proteins to a functional immune system, Perera et al have studied the ability of paclitaxel to activate members of this family in murine macrophages. Their data demonstrated that macrophages treated with paclitaxel induced the translocation of NF-kB complexes from their cytoplasmic location to the nucleus. More specifically, the NF-kB subunits induced by paclitaxel were indistinguishable from those induced by LPS. Ding et al have also shown that paclitaxel rapidly activated NF-kB in mouse peritoneal macrophages [39].

2.5. Poly(propylene) sulfide core nanoparticles (NPs) as a platform for targeted delivery of PXL

Many studies have been focused to the delivery of stimulating agents directly to the endogenous dendritic cells in the draining lymph node after injection into the tissue interstitium. Macromolecules can enter lymphatics driven by the interstitial flow allowing immature DCs in the lymph node to sample self-molecules and pathogen-derived molecules present in the tissues [20]. Exploiting this mechanism to target DCs requires an adjusted control of the

particle's size since particles should be neither too big, to prevent entrapment in the interstitium, nor too small so that they cannot be absorbed in the blood. It has been previously shown that particles of ~20-25 nm in diameter were very efficiently delivered to lymph nodes.

One of the successfully used schemes for the synthesis of polymer nanoparticles in the 20-30nm range is emulsion polymerization [40]. In this technique, surfactant micelles are formed in an aqueous environment, and hydrophobic monomer is loaded within the micelle and polymerized. The size of the resultant surfactant-stabilized polymer nanoparticle can be regulated by the ratio of surfactant to monomer. The particles consist of a hydrophobic core within a hydrophilic corona (from the surfactant) which allows the conjugation of hydrophilic molecules such as antigens to its surface [20].

Since there is a clear need for release of the pay load (drug, stimulating agent) in response to the oxidative environment of inflammatory reactions, an oxidation-responsive material should be used for the design of nanoparticles. Poly-(propylene sulfide) (PPS) is a hydrophobic polymer, which is readily converted to hydrophilic poly-(sulfoxide) or poly(sulfone) by mild oxidizing agents. Thus, it could be an ideal matrix for hydrophobic drugs, such as paclitaxel. Apart from controlled release, sensitivity is also required as well as prevention of non-specific phagocytic uptake [41]. Poly(ethylene glycol) (PEG) coating accounts for escape from the cells of the mononuclear phagocyte system, as well as for specific cell targeting by decorating the particle's surface with cell-specific ligands. Pluronic F-127, a PEG block copolymer (PEG-bl-polypropylene glycol-bl-PEG) offers stability as well as the ability to conjugate bio-active groups on the surface of the nanoparticles. PPS NPs constitute a very potent carrier for the delivery of hydrophobic Paclitaxel to DCs, slowly degrading after injection in an oxidative environment and rapidly cleared from the body after release thanks to its low molecular weight.

2.6. *Targeting the lymph node*

Upon maturation and antigen uptake, DCs residing in the peripheral tissues migrate to the lymph nodes, where they interact with T and B cells [1]. The trafficking of DCs to lymph nodes is crucial for the execution of their functions. In the lymph nodes, dendritic cells are not only present in much higher concentration but there is also a significant fraction of them being phenotypically and functionally immature, meaning that they are prone to uptake of new antigens [42]. On the contrary, targeting DCs in peripheral tissues, where DCs are present in much lower concentrations, necessitates for travelling to the lymph node after antigen uptake [43] with the risk of premature antigen presentation [44]. Additionally, the physical continuous drainage of fluid and macromolecules of the lymphatic system from the interstitial space, creates small flows providing a delivery route directly to the lymph nodes [45]. Exploiting interstitial flow to target dendritic cells in the lymph node is a promising strategy for effective DC activation. Small size nanoparticles, such as the PPS NPs of around 20-25nm in diameter, can enter the lymphatics and reach the lymph nodes convected by this interstitial flow, as previously shown [45].

2.7. *The role of complement in adaptive immunity and its interaction with DCs*

The complement system consists of serum proteins, membrane-bound regulators and receptors for interactions with various molecules. Complement proteins are mostly synthesized in the liver and then, released into circulation however, some components are also expressed by macrophages [46]. The complement cascade can be activated through three different pathways, called the classical, lectin and alternative pathway, all converging at the third component of complement (C3). The classical pathway is activated by antibodies released after a humoral response, whereas the lectin pathway is triggered by the binding of pathogen-associated molecular patterns (PAMPs) by lectin proteins [46]. The alternative pathway has the distinct capacity of being continuously turned on due to the spontaneous activation of C3. The

activation of C3 is mediated by C3 convertases inducing the generation of effector molecules involved in various pathways. These pathways include the recruitment of inflammatory cells, microbial opsonization and phagocytosis as well as lysis of pathogens by the C5b–9 membrane attack complex [MAC]. Recognition of pathogens by the complement systems is mainly based on their deficiency in complement regulatory proteins that would inhibit complement activation [3]. Additionally, complement involves pattern recognition, similarly to TLRs, as complement elements such as C1q (properdin) and the mannose-binding lectin, can recognize conserved microbial structures and carbohydrate motifs.

Apart from its principal role in innate immunity, there is strong evidence that complement also participates in several pathways of adaptive immunity. It has been shown that complement is implicated in the regulation of T cells either by direct opsonization of foreign antigens by APCs or by altering cytokine secretion [46]. Furthermore, it has been recently demonstrated that human DCs are able to express several complement proteins such as C3, C5, C9, as well as C1q, C7, C8, and the complement receptors (CR)-3 and CR4 [12, 47]. Among the proteins of the complement system, C3 is the most abundant one and as mentioned above, its activation is involved in all the three pathways. C3 is cleaved by specific proteolytic enzymes, C3 convertases, generating the C3a, C3b, iC3b, C3dg cleavage fragments that control innate and adaptive immune responses through activation of specific complement receptors on various cell types including phagocytes, DCs and B cells. Interestingly, Reis et al. have revealed that complement C3 is critical for the expression of surface molecules in DCs with a considerable impact in the capacity of antigen-uptake and in turn, in T cell activation [12].

In the present study we tried to investigate the potential immunostimulating role of Paclitaxel-loaded PPS nanaparticles (PXL-NPs). Our interest was driven by the study of Thomas et al. (in preparation), who observed massive immune cell infiltration into the lymph nodes (LNs) 24 h after intradermal injection of PXL-NPs in mice, as well as dramatic reduction in tumor growth along with the up-regulation of tumor-draining LN CD11c+ cells and antigen-specific CD8+ T

cells within the tumor. We hypothesized that the above mentioned effects result from the immunomodulatory capacity of PXL-NPs to induce mature and functional DCs upon treatment. To investigate the validity of our hypothesis, we evaluated the capacity of PXL-NPs to enhance maturation and cytokine production of murine and human DCs *in vitro*. Additionally, the same study has demonstrated that the observed cell infiltration of PXL-NPs *in vivo* was impaired when treatment was applied in complement protein 3 (C3)-knockout mice (Susan et al, in preparation). To elucidate the potential role of C3 in the immunomodulatory role of PXL-NPs, we first tested C3 expression in PXL-NP-stimulated DCs and then we assessed the induction of functional mature DCs upon PXL-NP treatment in C3 knockout DCs *in vitro*. We hope that these studies could enlighten the therapeutic role of approved-for-human-use PXL supporting its application in biomaterials-based schemes for immunomodulation.

3. MATERIALS AND METHODS

3.1. Cells and mice

Bone marrow dendritic cells (BMDCs) were harvested from C57Bl/6 C3 knockout mice as described [48] and used at day 8 after isolation. Briefly, femurs and tibiae were removed and the surrounding muscle tissue was detached with scissors and rubbed with kleenex tissues. The intact bones were kept in RPMI 1640 media. Both ends were cut with scissors and the marrow was flushed out with RPMI 1640 using a syringe with a 25-gauge needle. Marrow was then passed through cell strainer (70μm Nylon, BD Biosciences) to remove small pieces of bone and debris. Cells were centrifuged and resuspended in complete RPMI 1640 medium supplemented with penicillin/streptomycin (PS, Life Technologies), 50 mM beta-mercaptoethanol (Sigma-Aldrich), 10% heat-inactivated fetal bovine serum (FBS) and sodium pyruvate, which was passed through a vacuum driven disposable filtration system (MILLIPORE Stericup Express PLUS 0.22μm, Millipore corporations, Massachusetts, USA) . Cells were seeded in 100 mm diameter bacteriological petri dishes (BD Biosciences, San Jose, CA, US) at $5x10^6$ cells per dish in 10ml of complete RPMI medium containing 100μl of recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF). At day 3 after isolation an additional 10 ml of complete medium containing rmGM-CSF were added to the plates. At day 6 after isolation half of the culture supernatant was collected, centrifuged, and the cell pellet was resuspended in 10 ml of fresh complete medium containing rmGM-CSF, and transferred back to the original plate. BMDCs on day 8 after isolation were transferred in 50ml tubes, centrifuged and resuspended in fresh complete medium. Cells were plated in 96-well plates (BD Biosciences, San Jose, CA, USA) at a density of 300.000-400.000 cells per well in 100μl. 80μl of fresh complete medium were added per well and plates were incubated at 37o C until the treatments were prepared. Treatments were added at a volume of 20μl. Cells were pulsed with the above mentioned treatments for 24h.

Human dendritic cells (huDCs) were prepared from peripheral blood mononuclear cells (PBMCs) of healthy donors as described earlier [49]. Briefly, blood was collected in EDTA-coated tubes and PBMCs were isolated after gradient separation on Histopaque. Centrifugation was performed at 1600g without break for 30 minutes and the upper layer of platelets was carefully removed. PBMCs were collected in Wash buffer (HBSS (Invitrogen, Basel, Switzerland), 2.5% heat-inactivated FBS (Life Technologies, San Diego, CA, USA), 1% HEPES, 1% EDTA), centrifuged for another 15min at 1200g and resuspended in Iscove's Modified Dulbecco's Media (IMDM) medium supplemented with 1% PSA, 10% FBS. Cells were counted and seeded in 6-well plates at a density of $1.5x10^6$ cells/well in 1ml of complete medium. After a 2-hour incubation at 37°C, non-adherent cells were removed by washing 3 times with PBS. Monocytes were cultured in 1ml of complete medium and recombinant human (rh) GM-CSF and rhIL-4 were added at final concentrations of 500U/ml. GM-CSF and IL-4 were added on day 2 and day 4 after isolation and cells were used on day 6. HuDCs on day 6 after isolation were transferred in 50ml tubes, centrifuged and resuspended in fresh complete medium. Cells were plated in 96-well plates (BD Biosciences, San Jose, CA, USA) at a density of 100.000-150.000 cells per well in 100µl. 80µl of fresh complete medium were added per well and plates were incubated at 37◦ C until the treatments were prepared. Treatments were added at a volume of 20µl. Cells were pulsed with the above mentioned treatments for 24h.

The THP1-Blue™ cells (Invivogen, Version # 09G15-MT, San Diego, CA, USA) that are derived from the human monocytic THP-1 cell line were also used. These THP-1 cells cells carry a stable transfection with a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by the transcription factors NF-κB and AP-1. With activation by a toll-like receptor (TLR) ligand, nuclear transcription factor κB becomes activated and induces expression of the downstream SEAP gene. SEAP is then detectable using QUANTI-Blue™, a medium that changes color in response to SEAP. Cells were maintained in 75 cm^2 tissue culture flasks (TPP, Switzerland) in 20ml of RPMI medium containing 10% FBS,

1% HEPEs (1M), 1% PS, 1% sodium pyruvate and 0.1% beta-mercaptoehtanol (50mM), supplemented with 100 µg/ml of Zeocin (Invivogen, Version # 09G15-MT, San Diego, CA, USA). Cell concentration was preserved at around $1x10^6$ cells/ml and cells were passed every 3 to 4 days. THP-I Blue cells were plated in a 48-well plate at about 700,000 cells/well in 500µl and treated with PXL-NPs for 1h to 24h. At several time points, 50µl of each well were transferred in a new plate; cells were centrifuged for 1min at 2000rpm and the supernatants were transferred in a flat-bottom transparent 96-well plate. QUANTI-Blue™ was prepared according to the manufacturer's instructions. 150µl of QUANTI-Blue™ were added per well and the plate was incubated for 1 to 8h at 37° C. SEAP levels were then, determined using a spectrophotometer at 620nm.

The RAW-Blue™ Cells (Invivogen, Catalog # raw-sp, Version # 11E30-MM, San Diego, CA, USA)), which are derived from RAW 264.7 macrophages, are a mouse macrophage reporter cell line that stably express a secreted embryonic alkaline phosphatase (SEAP) gene inducible by NF-kB and AP-1 transcription factors. In the presence of TLR agonists NF-kB and/or AP-1 are activated leading to the secretion of SEAP which is easily detectable and measurable when using QUANTI-Blue™, SEAP detection medium that turns purple/blue in the presence of SEAP. Cells were maintained in 75 cm^2 Falcon tissue culture flasks in DMEM medium supplemented with 10% FBS, 1% PS and Zeocin (1:250). Before stimulation, cells were removed from their flask with scraping and resuspended in fresh medium at $2x10^6$ cells/ml. Cells were plated in a 48-well plate at about 200,000 cells/well in 150µl and treatments were added in equal volume, adjusting with complete medium if necessary. After 24h, cells were centrifuged for 1min at 2000rpm and 20µl of the supernatants were transferred in a flat-bottom transparent 96-well plate. 180µl of QUANTI-Blue™, which was prepared according to the manufacturer's instructions, were added per well and the plate was incubated for 1 to 8h at 37° C. SEAP levels were then determined using a spectrophotometer at 620nm.

3.2. Isolation of splenocytes

Spleens were homogenized in RPMI-1640 containing penicillin/streptomycin and passed through a 70 µm filter. Red blood cells were lysed for 5 min at RT in ammonium chloride. Splenic cells were then resuspended in restimulation media (RPMI-1640 (Life Technologies) with 10% heat-inactivated FBS (Life Technologies), 50 µm beta-mercaptoethanol (Sigma–Aldrich), 1% penicillin-streptomycin (Life Technologies)), counted and plated at 10^7 cells per well in a 96-well plate.

3.3. Isolation of lymph node cells

Lymph nodes were extracted from C57/Bl6 mice and C3 knockout mice and maintained in RPMI supplemented with 1% PSA. Collagenase D (Roche, Rotkreuz, Switzerland) was added and the nodes were incubated at 37o C for 1h to digest the lymph node capsule. Lymph nodes were dispersed by gently mashing the tissue and then passed through a cell strainer (BD Biosciences). Medium was added up to 40 ml and lymphocytes were spun down at 1200rpm for 5min. Then, cells were washed twice in IMDM and resuspended in IMDM containing 10% FBS, 1% PS, at a concentration of 10^7 cells/ml.

3.4. Reagents and antibodies Chemicals

Cell culture grade media, serum, and antibiotics were from Invitrogen (Basel, Switzerland) unless otherwise noted. The following primary antibodies were used for Western blotting: Rat-anti-mouse C3b/iC3b/C3c (Hycult biotechnology, Canton, MA USA), Mouse-anti-human C3/C3b (Human mAb , Clone 755, Abcam plc, Cambridge, UK), anti-rat IgG-HRP, anti-human IgG HRP (Bio-Rad Laboratories Inc, Reinach, Switzerland). Antibodies for flow cytometry were purchased from eBioscience (San Diego, CA, USA) and BioLegend (San Diego, CA, USA). Cell viability was determined using Live/Dead dye (Invitrogen). Primers for RT-PCR were designed in the Beacon Designer 4 software (Biorad) and were bought Microsynth (Balgach, Switzerland). The C3 forward primer used was *TTGTCGGTGGTGGCAGTGTATC* and the revere *GGCTTCCTCGGGCTTCTTGG*. The CpG used was CpG-B 1826

and it was purchased from Microsynth (Balgach, Switzerland). The LPS used was ultra-pure LPS 0111 B4 (Invivogen). Paclitaxel (Cat.number P-9600, MW 853.91) was purchased from LC Laboratories (Woburn, MA USA).

3.5. Detection of cytokine production

Cell culture supernatants were harvested and stored frozen at −20°C. ELISAs for interleukin 12p40 (IL-12p40), IL-4, IL-10, IL-6, tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ) were performed on duplicate wells according to the manufacturer's instructions (eBiosciences.). Briefly, ELISA plates were coated with 100 µl/well of capture antibody in Coating Buffer (diluted as indicated by the manufacturer), sealed and incubated overnight at 4°C. Wells were aspirated and washed 5 times with Wash Buffer before blocked with 200 µl/well of 1X Assay Diluent for 1 hour at room temperature. Wells were aspirated and washed for 5 times and 2-fold serial dilutions of the standard were added to the appropriate wells to make the standard curve. 100µl/well of the supernatants were added to the appropriate wells. Supernatants from BMDCs were previously diluted 10x in PBS. Plates were sealed and incubated at room temperature for 2 hours. Wells were aspirated and after a total of 5 washes, 100 µl/well of detection antibody diluted in 1X Assay Diluent were added. Plates were sealed and incubated at room temperature for 1 hour. Wells were aspirated and washed for 5 times. 100 µl/well of Avidin-HRP diluted in 1X Assay Diluent was added and the plates were sealed incubated at room temperature for 30 minutes. After aspiration a new round of 7 washes was performed, followed by adding 100 µl/well of Substrate Solution to each well. The plates were incubated at room temperature for 15 minutes maximum before 50 µl of Stop Solution were added. Immediately after plates were read plate at 450 nm and the values of 570 nm were subtracted. The standard curve was built and the samples' data were normalized to it to calculate the cytokine production in ng/ml.

3.6. NP synthesis

Chemicals were reagent grade and purchased from Sigma–Aldrich (Saint Louis, MO, USA) unless otherwise noted. Pluronic-stabilized PPS-NPs were synthesized in a one pot reaction as described earlier [41]. Briefly, we used an inverse emulsion polymerization of propylene sulfide, with 5% (w/v) Pluronic F-127 as the surfactant, wich was dispersed in water under argon flow. Pluronic F-127 is a block copolymer of polyethylene glycol and polypropylene glycol terminated by a and ω hydroxyl groups. The solution was stirred at maximum speed for 30 minutes. Polymerization was initiated using an in-house synthesized four-arm thiol initiator which was previously blocked in the form of a tetrathioacetate in order to avoid the formation of disulfide bonds between free thiols. Remaining free sulfhydryl groups on the NP surface were irreversibly capped by reaction with the alkylating reagent iodoacetamide. The initiator was activated in situ by the addition of 0.5M sodium methoxide in methanol in a separate flask reacting for 15min. The initiator was then added to the monomer emulsion, and 15min later diaza[5.4.0]bicycloundec-7-ene(DBU) was added. The reaction was stirred under inert conditions for 24 hours at room temperature. The nanoparticles were then exposed to air to produce disulfide cross-linking.

Particles were dialyzed with milliQ water in a 100,000KDa cut-off cellulose ester membrane (Spectrum Laboratories, Rancho Dominguez, CA) at room temperature and over a stirring plate for 72 hours. MilliQ water was replenished every 8 to 12 hours. After dialysis, nanoparticles are collected, sterile filtered through a 0.22μm filter membrane and stored at 4°C.

Particles were diltuted in PBS (1:20) and characterized for size with dynamic light scattering in a Zetasizer nano-ZS (Malvern Instruments Ltd,Worcestershire, U.K.).

3.7. Loading Nanoparticles with Paclitaxel and Dialysis of the loaded Nanoparticles

Paclitaxel was dissolved at 5 mg/ml in tetrahydrofuran (THF). 20% volume percent of this solution was added to PPS-core OH NPs in aqueous solution and immediately mixed by inversion for 2 to 3 minutes. Nitrogen was used to blow off the solvent and then, the loaded PXL-NPs were spun at 16,000 g for 15 min. The supernatant was then transferred in a sterile tube and a fraction of the supernatant as well as the pellet were freeze dried to determine the efficiency and extent of PXL loading by gel permeation chromatography (GPC).

The PXL-NP supernatant was then transferred with a sterile syringe in a Dialysis Cassette (Thermo Scientific Slide-A-Lyzer Dialysis Cassette 10K MWCO) for the removal of extra THF. The loaded NPs were dialyzed overnight in milliQ water on a stirring plate. The following day the dialyzed PXL-NPs were withdrawn with a syringe, transferred in a sterile tube and maintained at 4° C.

3.8. PXL-NPs measurements

The GPC measurements of the freeze dried pellet and supernatant from the loaded PXL-NPs allowed us to calculate the efficiency of loading as well as the exact content of paclitaxel in our treatments. Knowing the retention time of paclitaxel, we integrated the peak formed at around 17min to find out the amount of drug in our sample. Based on a calibration curve, previously designed, we then translated the area into nanograms of Paclitaxel.

3.9. PXL-NPs treatments

Treatments were added at a volume of 20µl and prepared as follows:

Three different kind of treatments were used; Paclitaxel alone, marked in the figures as "PXL in THF", OH nanoparticles loaded with Paclitaxel ("PXL-NPs")

and OH nanoparticles loaded with Paclitaxel that were dialyzed overnight after loading ("Dialyzed PXL-NPs"). Paclitaxel was diluted in tetrahydrofuran at a concentration of 5mg/ml. For the "Paclitaxel in THF" treatments, serial dilutions of the above mentioned solution were performed with fresh complete medium. The same solution was then used to load the OH NPs as described above to prepare the "PXL-NPs" treatments at 1mg/ml. The dialyzed OH NPs have been prepared earlier from the same batch of OH NPs and loaded with the same PXL concentration. All the NPs loaded with PXL treatments were centrifuged and the supernatant was transferred in sterile tubes. The PXL-NPs treatments were obtained by serial dilutions of the highest concentration with plain OH NPs of the same batch as the one from which they were prepared.

Apart from the different drug treatments, LPS and/or CpG, as well as PBS were used as positive and negative controls respectively. Cells were pulsed with the above mentioned treatments for 24h.

3.10. Analysis of DC phenotype

After 24 hours of stimulation, control non-treated and paclitaxel-treated DCs were harvested and pellets were stained in order to analyze their phenotype by flow cytometry. Briefly, pellets were washed in HBSS containing 0.1% BSA(FACS buffer) and centrifuged at 2000rpm for 1 min. Supernatants were aspirated and LD violet diluted in HBSS was added to cells for 20 minutes. Cells were washed with FACS buffer and monoclonal antibodies against human HLA-DR, CD83, CD80, CD86, or mouse MHC-II, CD11c, CD86 and CD40, conjugated with FITC, PECy5, PECy5.5, AF647 or PE and appropriately diluted in FACS buffer were added to cells and incubated for 30 minute at 4°C in the dark. Cells are washed again and analyzed in the flow cytometer (CyAn Cyril ADPS analyser from Beckman Coulter). Data analysis was performed using the FlowJo software and the results were expressed as the percentage of positive cells or Mean Fluorescent Intensity (MFI).

3.11. Real time polymerase chain reaction (RT–PCR)

Total mRNA from harvested DC was extracted by using the RNAqueous®-Micro Kit following the procedure recommended by the manufacturer. Briefly, cell pellets were resuspended by vortexing vigorously in at least 100 µl Lysis Solution and one-half volume of 100% ethanol was added to the lysate and mixed thouroughly. The mixture was passed through a Micro Filter cartridge at a volume of 150µl at a time by centrifuging for 10 sec at maximum speed until all of the mixture has passed through the filter. The collection tube was emptied every time before proceeding to the next step. The filter was washed with two different washing solutions and flow-through was discarded after a total of 3 washes. Finally, the RNA was eluted into the elution tube with a 10 µl preheated at 75° C elution solution. DNase treatment was performed as recommended by the manufacturer in order to remove trace amounts of contaminating genomic DNA. mRNA was then transferred in fresh tubes and stored at -20° C. The concentration of the isolated mRNA samples was measured at Nanodrop.

cDNA synthesis was carried out using the iSCRIPT cDNA synthesis kit (BIORAD). 200ng of mRNA were diluted in water in a total volume of 15µl and 4µl of iScript mix plus 1µl of reverse transcriptase were added per sample in PCR tubes. The samples were incubated in the PCR machine for 45 minutes according to the manufacturer's instructions.

The synthesized cDNA was subjected to PCR amplification with specific primers using the iCycler iQ (Multicolor RT-PCR detection system, BIORAD). 96-well thermo-plates were used and 1µl of the synthesized cDNA was added to the mixture of the primers with the double stranded DNA binding dye SYBGreen. Quantification of the PCR products was obtained by normalizing to the expression of the house-keeping gene GAPDH.

3.12. Western blot

The expression of complement protein 3 (C3) in DCs was assessed using the Western blot technique. Cells were harvested after a 24h stimulation, washed in PBS and lysed with the RIPA lysing buffer (SIGMA-ALDRICH) supplemented

with EDTA proteases inhibitor (Roche). 20µl of the lysed pellets were mixed with beta-mercaptoethano,l diluted 10x in Laemmli buffer, at the same volume. Samples were heated at 98° C for 5min and then maintained at 4° C until loading. Equal amounts of protein (30 µl) were loaded for each sample and electrophoretically separated on a 12.5% SDS-PAGE gel, followed by transfer to a PVDF membrane. The membrane was blocked with a dilute solution of 0.5% non-fat dry milk (BioRad, Laboratories Inc, Reinach, Switzerland) and 0.1% bovine serum Albumin (BSA) in Tris-Buffered Saline (TBS) with 0.05% Tween-20 (TBS/T).

C3 protein was detected using specific Rat anti-mouse primary antibodies (C3b/iC3b/C3c) diluted 1:100 in TBS/T, and Mouse anti-human primary antibodies (C3/C3b) diluted 1:250 in TBS/T for BMDCs and huDCs respectively by incubating the membrane for 1h at room temperature or overnight at 4°C. Membrane was washed 5 times for 5min and Anti-rat IgG HRP (1: 10.000 dilution) and Anti-mouse IgG HRP (1: 5000 dilution) respectively (Bio-Rad Laboratories Inc, Reinach, Switzerland), were used as the secondary antibodies. The membrane was then treated with chemiluminescence reagents (SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific)) for 5min and the bands were visualized on a Kodak film exposed to the membrane to detect chemiluminescence signals. After detection of C3 expression, membranes were washed and stripped for 15min with Western Blot stripping buffer (Thermo Fisher Scientific). After blocking as mentioned above, the membrane was incubated with anti-actin antibodies to ensure that the detected differences of C3 expression were not due to errors in loading or transfer.

4. RESULTS

4.1. Paclitaxel-loaded NPs induce murine DC maturation and up-regulation of costimulatory molecules without affecting cell viability in vitro

In order to determine whether DC maturation is the mechanism that induces the massive immune cell infiltration that was observed into draining lymph nodes 24h post intradermal injection with PXL-NPs, we decided to assess the ability of PXL-NPs to promote DC maturation and expression of costimulatory molecules in murine DCs *in vitro*. Immature DCs were generated from the bone marrow hematopoietic precursors in cultures supplemented with mGM-CSF as described in *Materials and Methods*. Medium was replenished on day 3 and day 6 after isolation. Paclitaxel-loaded Nanoparticles (PXL-NPs) that have been dialyzed overnight, and Paclitaxel diluted in THF (PXL in THF), as described in *Materials and Methods*, were added to mature DCs on day 8 after isolation in various drug concentrations indicated in Figure 3. Treatment with PBS, CpG and LPS served as controls. After 24h DCs were harvested and analyzed by flow cytometry (Figure 3A, 3B, 3D). Representative flow cytometry histograms with the event count on the y-axis and the fluorescence intensity of CD40/CD86 on the x-axis depict the significant up-regulation in CD40 and CD86 expression for the highest Paclitaxel concentration PXL-NP treatment compared to the zero one in immature murine DCs. The shift in fluorescence intensity between LPS and PBS ensures the validity of the experiment. Additionally, the results were expressed as the percentage of MHC class II positive and CD40 positive (Figure 3B) or CD86 positive (Figure 3B) cells within the CD11c positive population for increasing concentration of the PXL-NP treatment. Obviously, PXL-NP treatment shifted the immature DCs to a more mature phenotype with the curves reflecting a dose-dependent effect of the treatment in the expression of costimulatory molecules CD40 and CD86. More specifically, there is an 88% increase in the percentage CD86 positive cells between the highest and the lowest

Paclitaxel concentration treatment while CD40 percentage is 6 times more when cells are treated with the highest concentration of PXL-NPs. Remarkably, cell viability is not significantly affected by the PXL-NP treatment (Figure 3D) indicating that dose-dependent maturation of DCs upon PXL-NP treatment is not an effect of cell death (Figure 3D). On the contrary, the remarkable up-regulation of costimulatory molecules, also displayed in the flow cytometry histograms, when DCs are treated with the drug alone is disputable due to the noticeable increase in cell death with increasing concentration (Figure 3D). Obviously, PXL treatment of DCs led to a mature phenotype however, we cannot directly compare the effect of PXL-NPs and PXL alone due to the significant decrease in cell viability. The concentration of NPs was maintained constant in all PXL-NP treatments. The results presented in Figure 3 are from one representative experiment but they were reproduced in 6 independent experiments. Therefore, these data demonstrate that PXL-NPs do induce DC maturation and up-regulate the expression of costimulatory molecules on murine DCs in a dose-dependent way.

4.2. Paclitaxel-loaded NPs increase IL-12p40 cytokine production in DCs without affecting cell viability in vitro

Next, we wanted to investigate whether the phenotypically mature DCs after PXL-NP treatment do also present functional characteristics of APCs. IL-12p40 is an immune cytokine which is required for successful T cell activation [50]. Together with the expression of costimulatory molecules and MHC class II peptides on the surface of APCs, it plays a key role in T cell regulation. After 24h stimulation of murine DCs with PXL-NPs, as mentioned above, cell supernatants were collected and evaluated by ELISA for IL-12p40 cytokine production. Results of a representative experiment, which were reproduced 3 times, are shown in Figure 3C. In this figure, the IL-12p40 cytokine production in ng/ml is presented revealing that PXL-NPs strongly induce IL-12p40 in a dose-dependent pattern, in agreement with the DC maturation data. The higher PXL concentration of PXL-NPs increased 10 times the IL-12p40 production compared to the zero one while the same treatment induced a 33-fold increase compared to untreated cells (PBS).

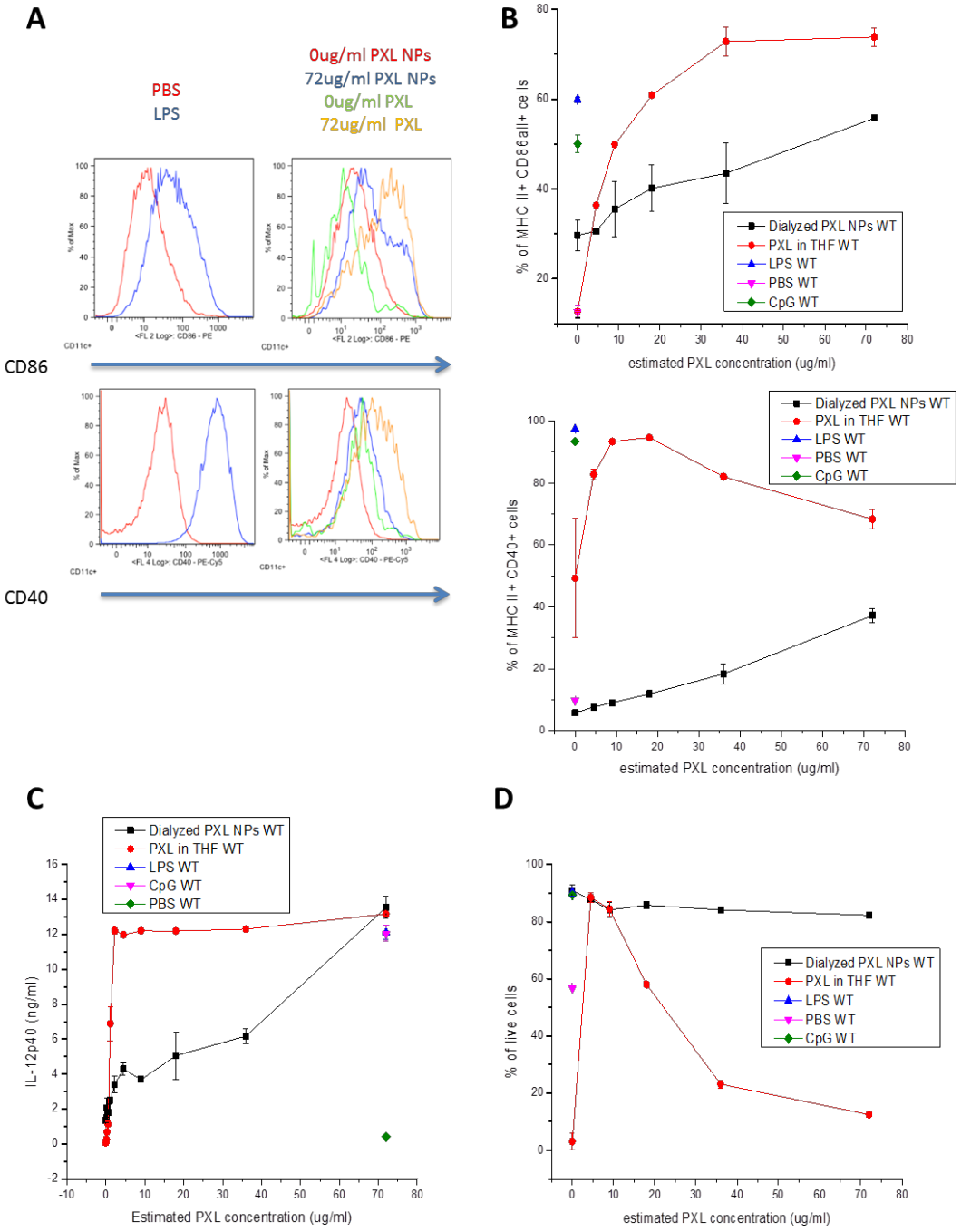


Figure 3. Paclitaxel-loaded NPs induce murine DC maturation and cytokine production without affecting cell viability in vitro. Immature DCs were generated from the bone marrow hematopoietic precursors in cultures supplemented with GM-CSF as described in *Materials and Methods.* PXL-NPs and PXL diluted in THF were added to mature DCs on day 8 after isolation in indicated concentrations. The concentration of NPs was maintained constant in all treatments. Treatment with PBS and LPS served as controls. After 24h DCs were harvested and analyzed by flow cytometry (A, B, D). Cell supernatants were collected and evaluated by ELISA for IL-12p40 cytokine production (C). Results of a representative experiment are shown. Representative flow cytometry histograms (A) and results assessing the co-expression of CD11c, MHC-II and CD40 (B) or CD86(B) indicate dose-dependency in murine DCs maturation after 24 h PXL-NP treatment without affecting cell viability (D). (C) Dose-dependent IL-12p40 cytokine production in ng/ml with 24 h treatment of murine DCs with PXL-NPs and PXL in THF. The remarkable maturation and cytokine production upon PXL treatment is disputable due to its negative effect in cell viability (D).(D) Cell viability after 24h treatment of murine DCs with PXL-NPs and PXL in THF.

The IL-12p40 production for the treatment of highest concentration even outreached the one induced from the LPS control. As previously mentioned, cell viability does not change significantly which means that the observed dose-dependent cytokine production does not result from reduced number of cells (Figure 3D).

Adversely, cell viability is significantly impaired when DCs are treated with the PXL alone implying that the evidently higher IL-12p40 production might be an artifact. These data demonstrate that PXL-NPs strongly induce IL-12 expression in non-activated DCs with the production increasing with increasing PXL concentration. Thus, PXL-NPs do promote DC maturation and cytokine production suggesting that they have dose-dependent immunomodulating activity.

4.3. Paclitaxel-loaded NPs induce human DC maturation and NF-κB activation in vitro

The immunomodulating activity of PXL-NPs in murine DCs raised the question of whether they can also alter phenotypic maturation and function of human DCs *in vitro*. DCs were generated from monocytes isolated from PBMC of healthy volunteers by culturing monocytes in complete medium supplemented with GM-CSF and IL-4, as described in *Materials and Methods*. Medium was replenished on day 2 and day 4 after isolation. PXL-NPs were loaded with PXL, as described in *Materials and Methods*, and added to mature DCs on day 6 after isolation in various concentrations, starting from 77μg/ml and doing serial dilutions with NPs. Treatments of PXL diluted in THF were also added at the same concentrations. Treatment with PBS and LPS served as controls. After 24h DCs were harvested and analyzed by flow cytometry (Figure 4A, 4B). Representative flow cytometry histograms are shown in Figure 4A, with the event count on the y-axis and the fluorescence intensity of CD80 on the x-axis, indicating increase in CD80 expression after treatment with the highest PXL concentration PXL-NPs for 24h compared to the lowest one in non-activated human DCs. The histograms for the positive and negative control, LPS and PBS respectively, certainly confirm the validity of the experiment. Apart from the shift in fluorescence intensity, the results

were also expressed as the percentage of HLA-DR positive and CD80 positive (Figure 4B) cells with increasing concentration of PXL-NP treatment. The curve obtained displays the co-expression of HLA-DR and CD80 costimulatory molecule and suggests, similarly to murine DCs, dose-dependent up-regulation of DC maturation.

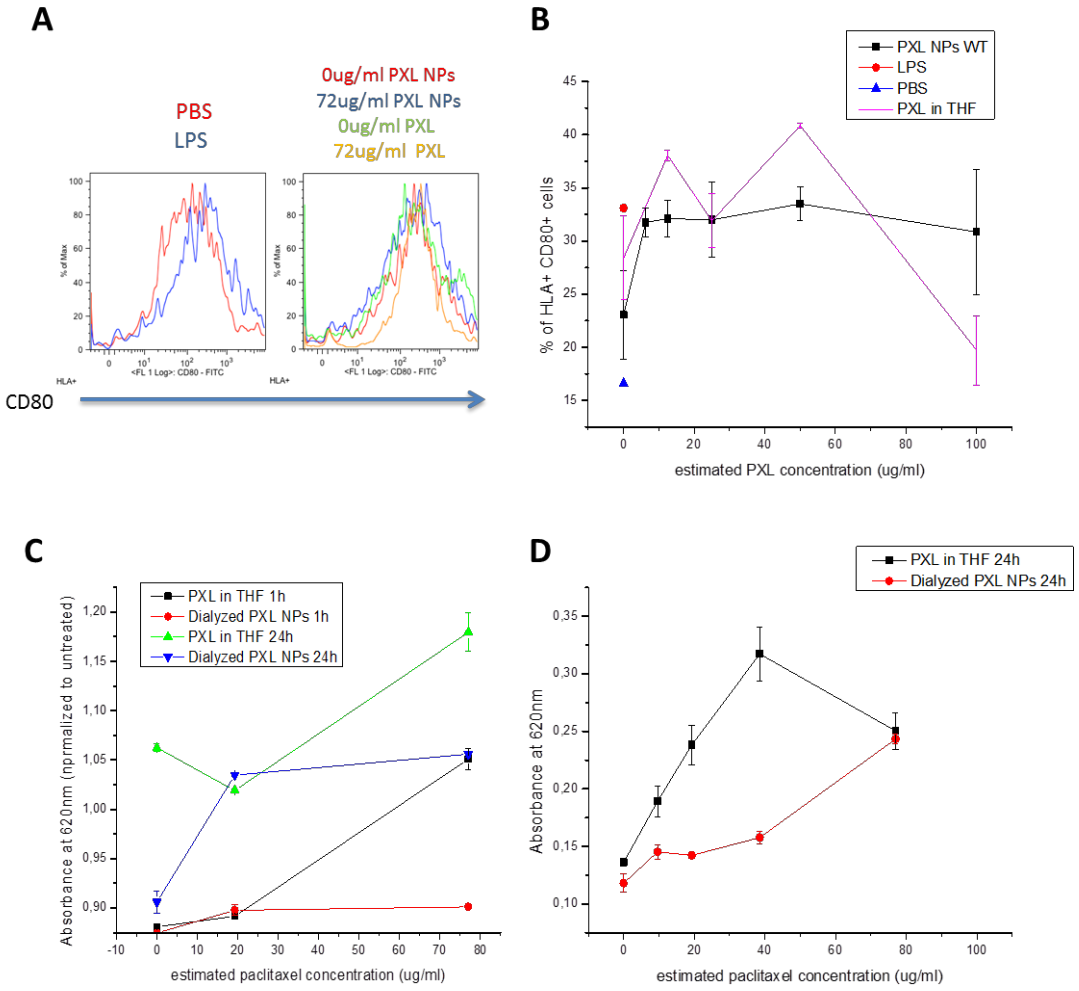


Figure 4. Paclitaxel-loaded NPs induce human DC maturation and NF-κB activation in vitro. DCs were generated from monocytes isolated from PBMC of healthy volunteers by culturing monocytes in complete medium supplemented with GM-CSF and IL-4 as described in *Materials and Methods*. PXL-NPs and PXL diluted in THF were added to mature DCs on day 6 after isolation in indicated concentrations. The concentration of NPs was maintained constant in all treatments. Treatment with PBS and LPS served as controls. After 24h DCs were harvested and analyzed by flow cytometry. THP-I Blue cells and RAW-Blue cells were treated with PXL-NPs and PXL in THF in indicated concentrations or PBS for 1h to 24h and 24h respectively. Cell supernatants were collected and evaluated for NF-κB activation by incubating with Quanti Blue at 37 ◦C for 5h. The secretion of SEAP was detected using a spectrophotometer at 620nm as described in *Materials and Methods* reflecting dose dependent NF-κB activation (C, D). The different responses upon PXL-NP and PXL treatment reveal greater and faster activation by the drug alone. Data of a representative experiment are shown. Representative flow cytometry histograms (A) and results assessing the co-expression of HLA-DR and CD80 (B) indicate dose-dependent maturation on human DCs after 24 h PXL-NP treatment. (C) Relative NF-κB activation compared to PBS treated cells as measured by AP development of THP I-Blue cell culture supernatants after 1h and 24 h PXL-NP or PXL treatment. (D) NF-κB activation as measured by AP development of RAW-Blue cell culture lysates after 24 h PXL-NP or PXL treatment.

Notably, PXL-NPs at 50μg/ml increased the percentage of phenotypically mature cells by 45% compared to PXL-NPs at 0μg/ml while it up-regulated co-expression twice relatively to untreated cells (PBS). DCs treated with the drug alone did not correspond in the same dose-dependent pattern (Figure 4B) even though up-regulation of CD80 costimulatory molecule was detected. These are data from one representative experiment and reproduction of the results did not always lead to the same expression levels, however, the dose-dependency effect was mostly apparent. Viability was not affected by PXL-NP treatment (data not shown). Altogether, these results imply that PXL-NPs were able to induce maturation of human DCs *in vitro*.

In order to assess then, the activating potential of PXL-NPs we decided to evaluate the activation of the NF-κB transcription factor in human cells. NF-κB is an inducible transcription factor with a diverse regulatory role, including the activation of immune cytokines. Previous studies have shown that NF-κB is implicated in IL-12p40 expression in macrophages [51]. Additionally, Paclitaxel has been shown to induce NF-κB translocation to the nucleus in murine macrophages [52]. Thus, we decided to investigate the activation of NF-κB in response to PXL-NPs in two distinct human cell lines, the human macrophages RAW-Blue cells and the human monocytic THP-I Blue Cells. Both types of cells carry a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by NF-κB. THP-I Blue cells and RAW-Blue cells were treated with PXL-NPs and PXL in THF in various concentrations, PBS and LPS for 1h to 24h and 24h respectively. Cell supernatants were collected and evaluated for NF-κB activation by incubating with Quanti Blue at 37 $^{\circ}$C for 5h, as described in the *Materials and Methods*. The secretion of SEAP was detected using a spectrophotometer and the absorbance at 620nm is shown in Figure 4C, 4D revealing dose dependent NF-κB activation with increasing concentration of PXL-NPs. NF-κB activation of human monocyte cells is displayed relatively to untreated cells (PBS) in Figure 4C after 1h and after 24h of treatment whereas the raw data are shown for the human macrophages. PXL-NPs at the highest concentration up-regulated above 16% the induction of NF-κB compared to the lowest concentration in human monocytes while they doubled the expression in

human macrophages. Consequently, PXL-NPs could activate, in agreement with the reported action of Paclitaxel, the translocation of NF-κB to the nucleus indicating their potential immunomodulating role in human cells as well. However, it seems that the drug alone can stimulate NF-κB activation more rapidly and efficiently. A possible explanation for that is that PXL directly interacts with TLR4 leading to NF-κB stimulation whereas the PXL-NPs have to be uptaken by the cells before the drug is released.

4.4. Limited cytokine production from murine splenocytes and lymphocytes treated with PXL-NPs ex vivo

Having demonstrated that PXL-NPs promote cytokine production in DCs *in vitro* we wanted to determine whether PXL-NPs are able to stimulate expression of cytokines in other cell populations and whether the increase in IL-12p40 expression after PXL treatment is an effect reserved to DC populations strengthening their antigen presenting capacity. Spleens from C57BL/6 mice were dispersed and cells were filtered followed by several washes. RBCs were lysed, as described in *Material and Methods*, and splenocytes were finally resuspended in complete medium. Lymph nodes from C57BL/6 mice were incubated with collagenase for capsule digestion and passed through cell strainer followed by several washes to get finally resuspended in complete medium, as described in *Materials and Methods*. Splenocytes and lymphocytes were plated and stimulated with PXL-NPs in various concentrations for 24h. Treatment with PBS and LPS served as controls. Cell supernatants were collected and levels of IL-12p40 production were determined by ELISA. Very low levels of IL-12p40 were detected as shown in Figure 5 more than 2 orders of magnitude lower compared to the IL-12p40 production by pure DC populations. More specifically, the highest PXL-NPs treatment induces only a 67% increase in IL-12p40 expression compared to untreated cells (PBS) in splenocytes (Figure 5A) whereas it is doubled for lymphocytes (Figure 5B). However, the dose-dependent trend that was observed in DCs is still noticeable in the lymphocyte population in which various concentrations were tested. That indicates that expression could be low due to the low percentage of DCs in the splenocyte and lymphocyte

populations and consequently, that the IL-12p40 induction by PXL-NPs is DC-specific reflecting the immunomodulating role of PXL-NPs.

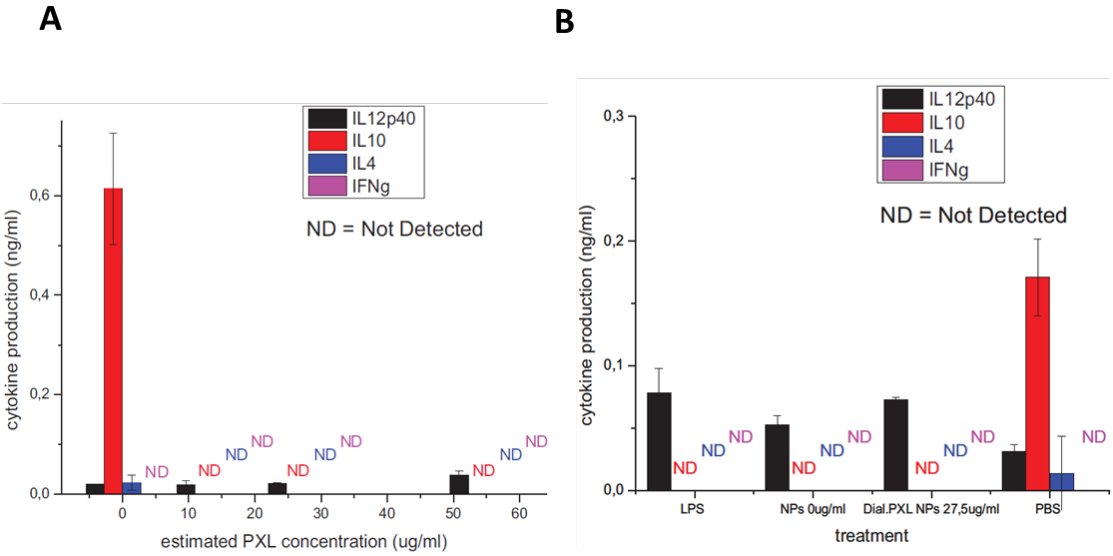


Figure 5. Limited cytokine production from murine splenocytes and lymphocytes treated with Paclitaxel-loaded NPs *ex vivo*. Splenocytes and lymphocytes from C57BL/6 mice were placed in culture and stimulated with PXL-NPs in indicated concentrations for 24h. The concentration of NPs was maintained constant in all treatments. Treatment with PBS and LPS served as controls. Cell supernatants were collected and levels of IL-12p40, IL-10, IL-4 and IFNg production were determined by ELISA. Low levels of IL-12p40 were detected, most probably as a result of the low percentage of DCs in the splenocyte and lymphocyte populations. IFNg was not detected at all while IL-4 is produced at very low levels in the absence of PXL-NPs or LPS stimulation. IL-10 production is evidently impaired in response to PXL-NPs both for lymphocytes and splenocytes, similarly to LPS (data not shown for splenocytes). Undetected cytokine levels are indicated by ND. (A) IL-12p40, IL-10, IL-4 and IFNg cytokine production (ng/ml) with 24 h treatment of murine splenocytes with PXL-NPs as measured by ELISA. (B) IL-12p40 , IL-10, IL-4 and IFNg cytokine production (ng/ml) with 24 h treatment of murine lymphocytes with PXL-NPs as measured by ELISA.

Next, we were interested to evaluate the expression of IFN-γ, IL-4 and IL-10 in the PXL-NP-treated splenocytes and lymphocytes. As shown in Figure 5, IFN-γ was not detected at all either in splenocytes (Figure 5A) or in lymphocytes (Figure 5B). IFN-γ is mostly expressed by natural killer (NK) cells as well as cytotoxic T and effector T cells in case of antigen specific responses. APCs are thought to regulate IFN-γ by producing IL-12 which promotes its expression [53]. In this context, the absence of IFN-γ could have been expected since stimulation was limited to 24h. IL-4 is detected at very low levels, only for the 0μg/ml PXL-NPs treatment in splenocytes (Figure 5A) and only for the PBS treatment in lymphocytes (Figure 5B). IL-4 is considered to favor Th2 responses by down-regulating IFN-γ and IL-12 expression. However, it has been also shown that IL-4 can instruct DCs to promote Th1 cell differentiation [54]. It is very likely that IL-4 is barely detected due to the short duration of stimulation.

4.5. PXL-NPs down-regulate IL-10 expression in splenocytes and lymphocytes

IL-10 is an anti-inflammatory cytokine inhibiting synthesis of IFN-γ and TNF-α. It has been shown that IL-10 blocks the expression of costimulatory molecules and IL-12 production preventing DCs from Th1 responses [55]. We detected a substantial expression of IL-10 for the 0μg/ml PXL-NPs treatment in splenocytes (Figure 5A) and for the PBS treatment in lymphocytes (Figure 5B) which was remarkably impaired in the presence of PXL-NPs or LPS. Despite the deviation observed between the duplicates, IL-10 production is high enough not to be doubted. The remarkable deficiency of IL-10 when cells are treated with PXL-NPs, in agreement with the dose-dependent increase in IL-12 expression, could suggest that PXL-NPs can down-regulate IL-10 expression allowing DCs to mature.

4.6. Paclitaxel and Paclitaxel-NPs induce dose dependent C3 expression in human and murine DCs

The massive immune cell infiltration that has been observed in the draining lymph nodes of mice 24h after the PXL-NP injection has been also shown to be dependent on the complement protein 3 (C3). C3 knockout and control mice were injected with PXL-NPs and the popliteal nodes were analyzed 24h post injection (Thomas et al., in preparation). The data demonstrated that in C3 knockout mice there was no increase in the amount of CD11c positive cells whereas an outstanding increase was detected in control wild type mice, as mentioned above. This interesting effect of C3 raised questions about its role in regulating PXL-NPs immunomodulating capacity. To elucidate the mechanism by which C3 deficiency, in C3 knockout mice, can impair the immune cell infiltration induced by PXL-NPs in control mice, we were first interested in finding out whether PXL-NPs can promote C3 expression by murine and human DCs. PXL-NPs and PXL (diluted in THF) were added to murine and human DCs respectively in various concentrations for 24h. Treatment with PBS and LPS served as controls (data not shown). Lysates of murine and human DCs were generated and analyzed for the presence of

C3b/iC3b (Figure 6A) and C3/C3b (Figure 6B) respectively as described in *Materials and Methods*. As illustrated in Figure 6A, there is a dose-dependent C3 complement expression in murine DCs in response to PXL-NPs. More specifically, the bands detected for the PXL-NPs from 12.5 to 100μg/ml (apart from the 25 μg/ml) demonstrate definitely more abundant C3 expression in comparison to 6.25μg/ml and to 3.12μg/ml for which expression is lower. For all the lower concentration treatments the protein is slightly identified.

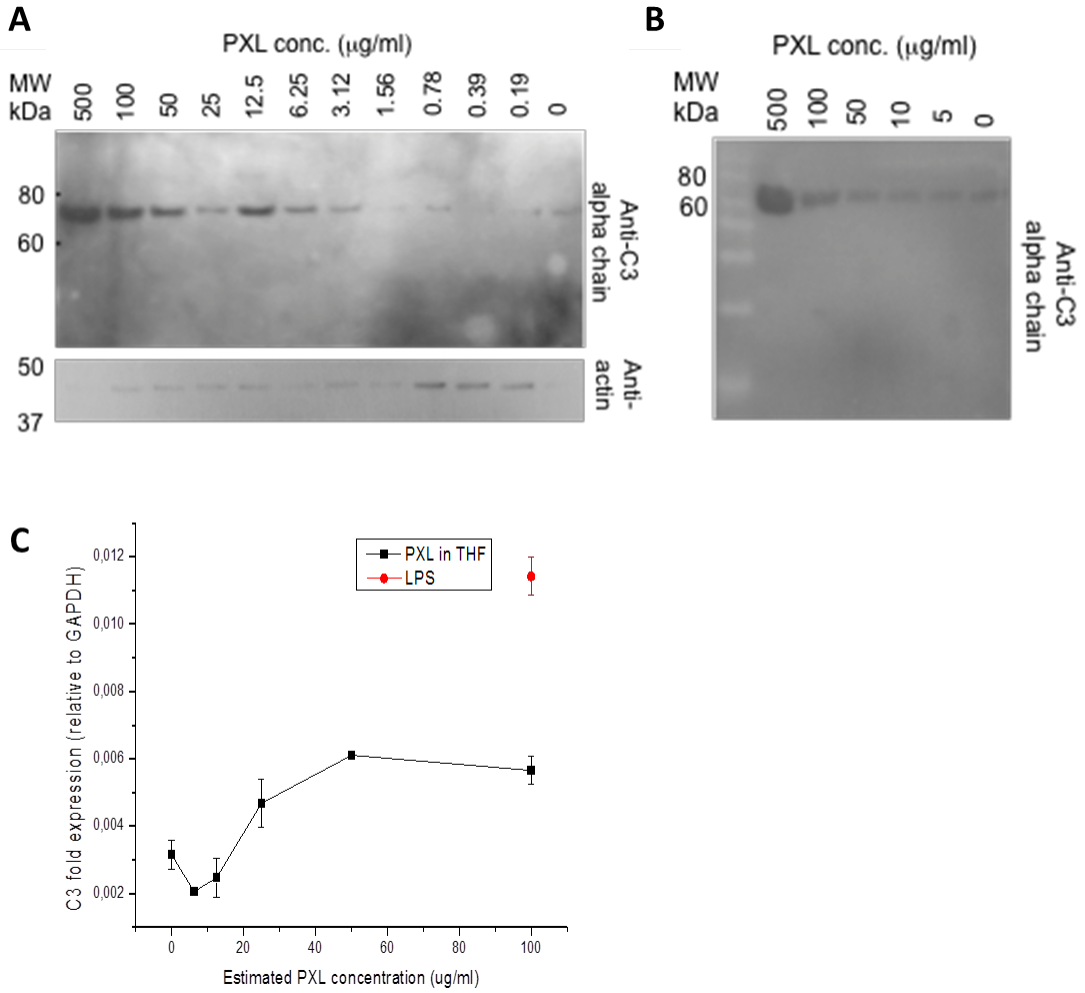


Figure 6. Paclitaxel and Paclitaxel-loaded NPs induce dose dependent C3 expression in murine and human DCs. Immature murine DCs were generated from the bone marrow hematopoietic precursors in cultures supplemented with GM-CSF as described in *Materials and Methods.* PXL-NPs were added to mature DCs on day 8 after isolation in indicated concentrations for 24h. Human DCs were generated from monocytes isolated from PBMC of healthy volunteers by culturing monocytes in complete medium supplemented with GM-CSF and IL-4 as described in *Materials and Methods*. PXL diluted in THF was added to mature DCs on day 6 after isolation in indicated concentrations for 24h. Treatment with PBS and LPS served as controls (data not shown). Lysates of murine and human DCs were generated, subjected to SDS and immunoblotted for C3b/iC3b (A) and C3/C3b (B) respectively as described in *Materials and Methods*. After chemiluminescent detection the membrane of murine DCs was re-blotted after removal of the first antibody with actin as a loading control (A). Gene expression of C3 by human DCs was analyzed by RT-PCR. Total cellular RNA was generated, reverse transcribed and detected as described in *Materials and Methods.* Results are presented relatively to GAPDH housekeeping gene that was used as a control. (A-B) Dose-dependent expression of C3 by murine (A) and human DCs (B) with 24 h PXL-NP and PXL treatment. (C) RT-PCR of C3 expression by human DCs with 24 h PXL treatment suggesting dose-dependent C3 mRNA levels in response to PXL treatment

The same effect was observed in human DCs treated with PXL, as shown in Figure 6B. Here, C3 expression seems to be much more abundant for the highest PXL concentration. After chemiluminescent detection the membrane of murine DCs was re-blotted, following the removal of the first antibody, with anti-actin as a loading control. Even though the loading might slightly differ between the lanes, it is evident that the greater expression for the higher concentrations is not due to unequal loading, since it appears to be less for these lanes. Additionally, to verify the detection of C3 protein, gene expression of C3 by human DCs was assessed by RT-PCR. Total cellular RNA was generated, reverse transcribed and detected as described in *Materials and Methods*. Results are presented relatively to GAPDH housekeeping gene that was used as a control. As illustrated in Figure 6C, C3 gene expression is dose-dependent in agreement with the Western blot analysis. These results, together with the *in vivo* data, strengthen the hypothesis that C3 is implicated in the mechanism of immunomodulating action of PXL-NPs and bring up the next question; whether C3 secretion induces DC maturation or whether it is an effect of it.

4.7. C3 affects maturation and IL-12p40 production in murine DCs in response to PXL-NPs

To delineate further the potential role of C3 in the mediation of PXL-NP-induced maturation and cytokine production in DCs, DC cultures where generated from the bone marrow hematopoietic precursors of C3 knockout and control C57/BL6 mice. DCs were treated on day 8 after isolation with PXL-NPs, dialyzed overnight, in various concentrations for 24h. The same PXL-NP treatments were added to C3 knockout and wild type (WT) DCs. The concentration of NPs remained constant in all treatments. Treatment with PBS and LPS served as controls. After 24h DCs were harvested and analyzed by flow cytometry (Figure 7A, 7B, 7D) to detect any phenotypic differences. Surprisingly, DCs from C3 knockout mice do mature, as illustrated in Figure 7A and 7B. The histograms of fluorescence intensity of CD40/CD86 (Figure 7A) reflecting the up-regulation of costimulatory molecules substantially co-align for C3 knockout and wild type DCs.

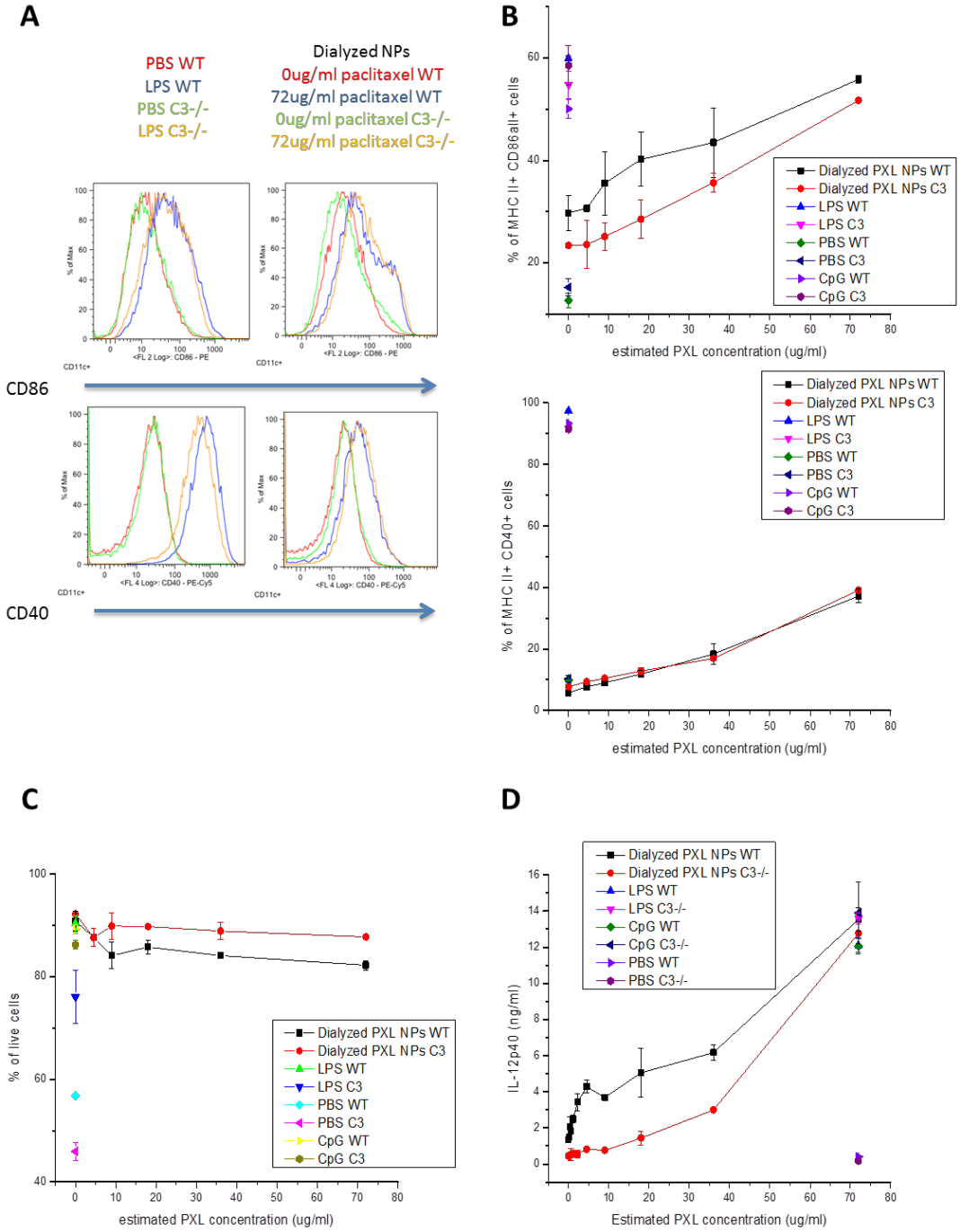


Figure 7. C3 affects maturation and IL-12p40 production in murine DCs in response to PXL-NPs. Bone marrow hematopoietic precursors were isolated from C3 knockout and control C57/BL6 mice and treated with PXL-NPs in indicated concentrations on day 8 after isolation for 24h. The concentration of NPs was maintained constant in all treatments. Treatment with PBS and LPS served as controls. After 24h DCs were harvested and analyzed by flow cytometry (A, B, D) to detect any phenotypic differences. Cell supernatants were collected and evaluated by ELISA for IL-12p40 cytokine production (C). The levels of IL-12p40 production by DCs from control and C3 knockout mice after PXL-NPs treatment were compared. Results of a representative experiment are shown. DCs from C3 knockout mice mature (A-B) but appear more sensitive in cytokine production (C) in response to PXL-NPs. Representative flow cytometry histograms (A) and results assessing the co-expression of CD11c, MHC-II and CD40 (B) or CD86(B) indicate no difference in maturation in murine DCs of control and C3 knockout mice after 24 h PXL-NP treatment (C) IL-12p40 cytokine production (ng/ml) is slightly impaired in DCs from C3 knockout mice. (D) Cell viability, no significant difference between DCs from control and C3 knockout mice.

The shift in fluorescence intensity for LPS and PBS is also similar in both types of mice, with the CD40 intensity being a bit higher for the control. The results assessing the co-expression of CD11c, MHC-II and CD40 (Figure 7B) or CD86 (Figure 7B) clearly demonstrate no significant difference in maturation between murine DCs in control WT and C3 knockout mice after 24 h PXL-NP treatment. Precisely, C3 knockout and control DCs present the same dose-dependent trend in the expression of costimulatory molecules with increasing PXL-NP concentration; the CD86 co-expression curve is slightly higher for the wild type control while the CD40 curves are totally aligned. The percentage of CD11c, MHC-II and CD40 positive cells in response to 35.1μg/ml PXL-NPs is 3.8 times higher in comparison with untreated cells (PBS) both for C3 knockout and wild type DCs, and 3.4 and 4.4 times higher for the CD86 positive cells respectively. However, when the cell supernatants were examined by ELISA for IL-12p40 cytokine production, the IL-12p40 levels seem to slightly differentiate. The curve of IL-12p40 production in response to increasing PXL-NP concentration, as shown in Figure 7C, turns out to be shifted upwards for the wild type DCs indicating greater expression. Indeed, plotting the IL-12p40 expression per number of live cells (data not shown) reveals that production is undoubtedly decreased in C3 knockout DCs compared to the wild type ones. The differences in IL-12p40 expression are more intensively pronounced for the lower PXL-NP concentrations with the wild type cells expressing 3 to 5 times more IL-12p40, while they tend to converge for the highest PXL-NP concentration. These data strongly suggest that even though wild type and C3 knockout DCs demonstrate similar capacity to mature and express costimulatory molecules, they do not exhibit the same functional characteristics. At the same time, it becomes evident that C3 regulates IL-12 expression and, in turn, plays a principal role in the activation of mature, functional, murine DCs in response to PXL-NPs.

4.8. C3 affects cytokine production from murine splenocytes and lymphocytes treated with PXL-NPs ex vivo

Having revealed the slight impairment in IL-12p40 production in C3 knockout DCs motivated us to elucidate the effect of C3 deficiency in cytokine production in different cell populations. Splenocytes and lymphocytes from C3 knockout and control C57BL/6 mice were stimulated with PXL-NPs in several concentrations for 24h.

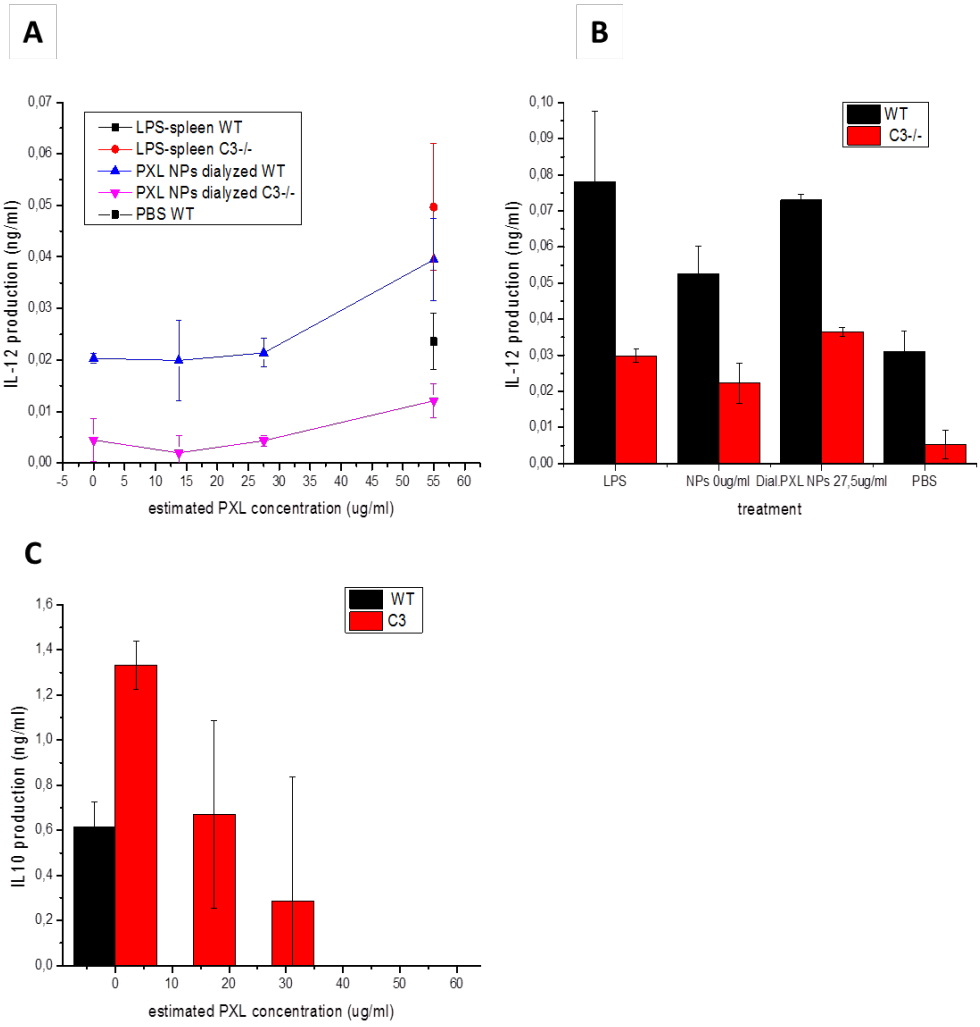


Figure 8. C3 affects cytokine production from murine splenocytes and lymphocytes treated with Paclitaxel-loaded NPs *ex vivo*. Splenocytes and lymphocytes from C3 knockout and control C57BL/6 mice were placed in culture and stimulated with PXL-NPs in indicated concentrations for 24h. The concentration of NPs was maintained constant in all treatments. Treatment with PBS and LPS served as controls. Cell supernatants were collected and levels of IL-12p40 (A,B) and IL-10(C) were determined by ELISA. IL-12p40 production decreased in splenocytes (A) and lymphocytes (B) from C3 knockout mice compared to control whereas C3 impairment upregulated IL-10 production (C) which appears to decrease with increasing PXL concentration. (A) IL-12p40 production (ng/ml) with 24 h treatment of murine C3 knockout and control splenocytes with PXL-NPs. (B) IL-12p40 cytokine production (ng/ml) with 24 h treatment of murine C3 knockout and control lymphocytes with PXL-NPs. (C) IL-10 production (ng/ml) with 24 h treatment of murine C3 knockout and control splenocytes with PXL-NPs.

Treatment with PBS and LPS served as controls. The concentration of NPs was maintained constant for all treatments. Cell supernatants were collected and levels of IL-12p40 (Figure 8A, 8B) and IL-10 (Figure 8C) were determined by ELISA. As expected, IL-12p40 production decreased in splenocytes (Figure 8A) and lymphocytes (Figure 8B) from C3 knockout mice compared to wild type ones. In accordance with our previous suggestion, the detected IL-12p40 expression stems primarily from DCs which constitute a small percentage of these cell populations. As shown in Figure 8A, there is barely any production of IL-12p40 in C3 knockout splenocytes.

Interestingly, C3 impairment seems to outstandingly up-regulate IL-10 production, as depicted in Figure 8C. The IL-10 expression is nearly doubled in the absence of C3 for the 0μg/ml treatment. Remarkably, IL-10 also appears to decay with increasing PXL concentration in C3 knockout DCs. These data support our previous observation that PXL-NPs have the ability to down-regulate IL-10 expression and further strengthen our hypothesis that this effect allows for DC maturation. The slight impairment of IL-12p40 in the absence of C3, which still presents a dose-dependent trend though, coincides with the decreasing expression of IL-10 to point out the immunomodulating action of PXL-NPs as well as the important mediating role of C3.

5. DISCUSSION

Dendritic cells (DC) patrol peripheral tissues for antigen uptake, express co-stimulatory molecules upon maturation, drain to the nearest lymph node and secrete cytokines to initiate adaptive immune responses by instructing T cells. Apart from lymphocyte activation, DCs can also induce T cell tolerance by continuously sampling self-antigens to prevent autoimmune responses. Antigen capture and presentation by DCs are critical processes in the induction of antigen specific responses, linking innate with adaptive immunity. Thus, DCs are a powerful tool to manipulate the immune system in order to achieve anti-tumor immunity. Paclitaxel (PXL), an FDA-approved chemotherapeutic, has been shown to stimulate DC maturation and antigen presentation as well as to induce secretion of inflammatory cytokines without affecting cell viability [23, 32, 56]. Poly(propylene) sulfide core nanoparticles (NPs), recently developed by our laboratory, were used for targeted delivery of PXL to immune cells. It has been recently shown that PXL-loaded NPs induce massive immune cell infiltration (~four-fold increase in cell number) into draining lymph nodes (LNs) 24 h after intradermal injection in mice, suggesting that PXL-NP treatment induces a local immune response (Thomas et al, in preparation). It is possible that this effect is induced by potential immune responses following maturation of DCs upon PXL-NP treatment. To pursue this possibility, we assessed the ability of PXL-NPs to promote DC maturation and expression of costimulatory molecules in murine DCs *in vitro*.

In the present study, we demonstrate that PXL-NPs induce murine DC maturation and up-regulation of costimulatory molecules CD40 and CD86 without affecting cell viability *in vitro*. We managed to show that PXL loaded in PPS-NPs retains its capacity of inducing maturation and MHC II expression in murine DCs, as reported earlier [23, 30, 31, 57]. Apart from the phenotypic maturation after PXL-NP treatment, functional characteristics of APCs were also promoted. We evaluated the expression of IL-12p40 by PXL-NP-treated DCs to reveal that PXL-NPs strongly increase IL-12 production. These results are consistent with the previously demonstrated PXL- induced IL-12 production in

DCs [23, 31, 33, 58]. IL-12 is one of the most important cytokines produced by DCs since it regulates T cell differentiation towards a Th1 phenotype [1, 9]. Importantly, IL-12 belongs to the so-called signal 3 factors [59], which are required for complete activation of naïve T cells for the generation of cytotoxic CD8+T cells responses from naïve CD8+T cells, and it is particularly effective as such [16]. Taken together, these data suggest that PXL-NPs, similarly to PXL, can directly enhance DC maturation and function and point out that they could be used for the up-regulation of functional activity of tumor and LN-residing DCs. Considering this, one may argue that DC maturation upon PXL-NP treatment could be the mechanism for the striking *in vivo* observed cell infiltration (Thomas et al., in preparation). If our hypothesis is true, it may further explain the increased maturation of tumor-draining LN CD11c+ cells as well as the increased antigen specific CD8+T cells in the tumor after intradermal injection to the tumor draining lymph node of tumor-bearing mice, which was also observed in vivo (Thomas et al., in preparation). Nevertheless, what makes these data really interesting and novel, compared to the already reported immunomodulatory action of the drug alone, is the competitive advantage of the delivery platform. Obviously, loading PXL in NPs allows for targeted delivery to the tumor draining lymph-node. In the lymph nodes, dendritic cells are not only present in much higher concentration but there is also a significant fraction of them being phenotypically and functionally immature, meaning that they are prone to uptake of new antigens [42]. On the contrary, targeting DCs in peripheral tissues, where DCs are present in much lower concentrations, necessitates for travelling to the lymph node after antigen uptake [43] with the risk of premature antigen presentation [44]. Additionally, PXL-NPs can be delivered to the lymph node without any special targeting thanks to the biophysical mechanism of interstitial flow. The small size of the PXL-NPs allows them to get convected by interstitial flow through the interstitial matrix, enter the lymphatic system and travel to lymph nodes [45]. The advantage of tumor-draining lymph node is also shown by Susan et al. (in preparation) who demonstrated that reduced tumor growth required delivery to the tumor-draining LN since application of PXL-NPs to non-tumor-draining LNs failed to slow tumor growth. Collectively, these data suggest that the approved-for-

human-use PXL can have greater therapeutic application than chemotherapy and that it could be reappropriated for use in biomaterials-based schemes for immunomodulation.

The next question raised was whether the immunomodulating activity of PXL-NPs can also alter phenotypic maturation and function of human DCs *in vitro*. Kaneno et al. have demonstrated that PXL is able to up-regulate maturation of human DCs *in vitro* as well as to increase their ability to stimulate T cell proliferation without inducing DC apoptosis [24]. Here, we show that human DCs treated with PXL-NPs express, similarly to murine DCs, dose-dependent increase in the co-expression of HLA-DR and CD80 costimulatory molecule. These results suggest that PXL-NPs were able, correspondingly to the reported PXL action, to induce maturation of human DCs *in vitro* and further confirm the above-manifested potential immunotherapeutic role of PXL-NPs delivered to LN-residing DCs, not just in mice but, in humans as well.

The immunomodulatory activity of PXL has been attributed to signaling through TLR4 leading to activation of the transcription factor NF-κB. Perera et al. have exhibited the translocation of NF-κB complexes to the nucleus in macrophages treated with paclitaxel, with the NF-κB subunits induced being identical to those induced by LPS [52]. Ding et al have also shown that paclitaxel rapidly activated NF-κB in mouse peritoneal macrophages [39]. Additionally, NF-κB has been shown to play a critical role for the induction of IL-12p40 in macrophages [60]. Therefore, the activation of NF-κB in response to PXL-NPs was examined in two different human cell lines, the human macrophages RAW-Blue cells and the human monocytic THP-I Blue Cells. Our data revealed dose dependent NF-κB activation with increasing concentration of PXL-NPs displaying, again, their potential immunomodulating role in human cells. The activation of NF-κB by PXL-NPs is very important since, apart from IL-12, it also regulates the induction of other cytokine genes, including TNF-a, IFN-b, IL-1β, IL-8 and IL-6 [36].However, the mechanism by which PXL-NPs stimulate NF-κB activation is not clear. Since PXL is loaded in the NPs and it is supposed to get released upon uptake, we suggest that released PXL might interact with the intracellular part of TLR-4.

Having demonstrated that PXL-NPs promote cytokine production in DCs *in vitro,* we investigated whether PXL-NPs are also able to stimulate expression of cytokines, such as Il-12, IFN-γ, IL-4 and IL-10, in other cell populations. We detected limited levels of IL-12p40 both in lymphocyte and splenocyte populations compared to IL-12p40 production by pure DCs. That may suggest that the IL-12p40 induction by PXL-NPs could be DC-specific reflecting the immunomodulating role of PXL-NPs via DC activation.

IL-4 is an immunomodulatory cytokine with diverse roles, known to be expressed in Th2 lymphocytes, mast cells and eosinophils [61]. IL-4 was initially considered as Th2 cytokine since it can initiate Th2 cell differentiation. However, IL-4 also directs DCs towards Th1 cell differentiation up-regulating IL-12 production with the requirement of IL-10 production by DCs [62]. The mechanism of this effect has been shown to be the IL-4 mediated inhibition of IL-10 production by DCs. IFN-γ expression was initially considered to be restricted to lymphoid cells. However, many studies have shown production and intracellular expression of IFN-γ by IL-12 activated macrophages and dendritic cells, as summarized by Frucht et al [63]. We did not detect any IFN-γ or IL-4 (apart from very low level in untreated cells) secretion neither in splenocyte nor in lymphocyte populations upon treatment with PXL-NPs for 24h *ex vivo*. We estimate that limited production of cytokines might be due to the short duration of stimulation.

IL-10 is an anti-inflammatory cytokine which is expressed in various cell types, including monocytes, mast cells, T regulatory cells, and tumor cells, and has been shown to participate in tumor escape from immune surveillance [64]. Several studies have shown that IL-10 blocks the expression of costimulatory molecules and IL-12 production preventing DCs from Th1 responses [55]. Corinti et al. have demonstrated that endogenous IL-10 down-regulates the antigen presenting functions of DCs and that DCs deficient in IL-10 undergo spontaneous maturation in vitro [18]. The same study has shown that IL-10 neutralization increased IL-12 and TNF-a production; however, it decreased IL-10 secretion, indicating that autocrine IL-10 is also involved in a positive

feedback mechanism for its own production. Similar studies have confirmed these findings showing that IL-10 deficient APCs are potent activators of Th1 response revealing that the suppressive immunoregulatory influence of IL-10 is manifested at the level of APCs [19]. In terms of PXL treatment, Mullins et al. have observed that tumor cell-derived supernatant, containing substantial quantities of IL-10, suppressed paclitaxel's capacity to induce IL-12 *ex vivo* [65]. Interestingly, upon PXL-NP treatment of splenocyte and lymphocyte populations *ex vivo*, we detected a substantial expression of IL-10 for the 0µg/ml PXL-NPs treatment in splenocytes and for the PBS treatment in lymphocytes which was remarkably impaired in the presence of PXL-NPs. The striking deficiency of IL-10 when cells are treated with PXL-NPs, together with the dose-dependent increase in IL-12 expression, could suggest that PXL-NPs can down-regulate IL-10 expression allowing DCs to mature towards the induction of Th1 response. At the same time, the IL-10 impairment in response to PXL-NPs could also confirm the induction of DC maturation, which is known to be coupled with decrease in IL-10.

The massive immune cell infiltration, which has been observed in the draining lymph nodes of mice 24h after the PXL-NPs injection, has also turned out to be dependent on complement protein C3 (Thomas et al., in preparation). The outstanding increase in the population of CD11c positive cells was abrogated in C3 knockout mice compared to the one detected in control mice, as mentioned earlier. This interesting C3-dependency raised questions about the role of complement in the regulation of PXL-NP immunostimulating capacity. To elucidate the mechanism by which C3 can impair the immune cell infiltration induced by PXL-NPs in control mice, we first evaluated the potential C3 expression by murine and human DCs upon PXL-NP treatment. It has been shown previously that human DCs can produce several complement proteins, such as C3, C5, C9, Factor I, Factor H and others at levels similar to macrophages as well as to express the complement receptors CR-3 and CR-4 [66]. For some of these proteins, such as C3, mRNA levels were increased after LPS stimulation. Most importantly, they revealed that the expression of complement mRNA does not result from DC maturation but appears to be mainly dependent on the stimuli. Nevertheless, they identified no significant

correlation between mRNA expression and protein secretion, showing, for example, no significant differences in C3 secretion upon LPS-induced DC maturation. We partly confirmed these findings by demonstrating dose-dependent C3 expression in the mRNA level in human DCs treated with PXL. However, we also detected C3 expression in the protein level following the same dose-dependent trend, which points out that PXL is among the stimuli that do induce C3 expression. Markedly, we also observed dose-dependent C3 complement expression in murine DCs in response to PXL-NPs, indicating that PXL-NPs seem to retain the ability of PXL for C3 induction. The local C3 production of complement proteins in the DC microenvironment could enhance significantly the strength of immune responses, particularly in the lymphoid tissues where circulating complement is not readily available [66]. Therefore, we have shown that PXL-NP treated murine DCs induce dose-dependent C3 expression enhancing the locally induced immune responses.

Taken these results, together with the *in vivo* data, it is evident that C3 is implicated in the mechanism of immunomodulating action of PXL-NPs in DCs. To further elucidate the potential C3 involvement in the PXL-NP induced maturation and cytokine production in murine DCs, we assessed maturation as well as IL-12p40 production by wild type and C3 knockout DCs treated with PXL-NPs *in vitro*. Surprisingly, PXL-NPs appeared to up-regulate CD86 costimulatory molecules in C3 knockout DCs, even though at slightly lower levels compared to WT DCs, while CD40 expression seemed not to be affected. Previous studies have shown that differentiation of human monocytes in the absence of C3 impaired CD86 but not CD40 expression, while it did not modulate complement receptors CD1 and CD18 expression [12]. Maturation upon LPS challenge led to increase in CD86 expression but in lower levels than in the presence of C3 and did not affect CD80 and CD40 [12]. We could hypothesize that PXL-NPs and C3 promote CD86 up-regulation through different pathways. In that case, C3 production by PXL-NPs in wild type, but not in C3 knockout mice, could lead to greater up-regulation of CD86. Importantly, IL-12p40 production, normalized to the number of live cells, revealed that production is undoubtedly decreased in DCs from C3 knockout mice compared to the wild type ones. The differences in IL-12p40 expression are more intensively pronounced for the lower PXL-NPs concentrations while

they tend to converge for the highest PXL-NPs concentration implying that the effect could be even dose-dependent. These data strongly suggest that even though wild type and C3 knockout DCs demonstrate similar capacity to mature and express costimulatory molecules, they do not exhibit the same functional characteristics. This is in agreement with the study of Reis et al. who observed a decrease in IL-12p40/p70 in the absence of C3 during DC differentiation which was only partly compensated after LPS-induced maturation in human DCs [12, 66]. Our data indicate that C3 deficiency affects IL-12p40 production in murine DCs in response to PXL-NPs. Thus, one may suggest that PXL-NPs, along with their capacity to induce the expression of C3, may be a powerful tool for the generation of mature, functional DCs and in turn, for the development of adaptive immune responses.

Next, we looked into the effect of C3 impairment in cytokine production in distinct cell populations. Upon treatment of splenocytes and lymphocytes from C3 knockout and wild type mice with PXL-NPs for 24h *ex vivo* we detected, as expected, a prominent decline in IL-12p40 production decreased in both splenocytes and lymphocytes from C3 knockout mice compared to control wild type ones. As mentioned earlier, we suggest that the IL-12p40 expression detected is primarily provided by DCs which constitute a small percentage of these cell populations. More specifically, in C3 knockout splenocytes there is nearly no production of IL-12p40. In lymphocytes we detected a greater expression of IL-12 which is though, significantly impaired in case of C3 deficiency. Our controls (LPS) confirm the above mentioned study of Reis et al. who have observed that in the absence of C3, maturation and thus, IL-12p40 production by DCs is impaired, as well as that LPS stimulation is not able to counterbalance the low expression resulting from the lack of C3 during DC differentiation in human DCs [12]. As we observed before, C3 plays a critical role in IL-12p40 expression. Interestingly, C3 impairment exhibits a striking up-regulation in IL-10 production in splenocytes. It is noteworthy that IL-10 also appears to decay with increasing PXL concentration in C3 knockout splenocytes. These data strongly support our previous observation that PXL-NPs might be able to down-regulate IL-10 expression and further strengthen our hypothesis that this effect allows for DC maturation. The slightly ascending trend in IL-12p40 production with increasing

concentration complies completely with the descending trend in the expression of IL-10 in C3 knockout splenocytes and evidently points out the important role of C3 in the immunomodulating mechanism of PXL-NPs.

6. CONCLUSION

Collectively, our data demonstrate that PXL-NPs directly enhance DC maturation and cytokine production *in vitro,* suggesting that DC maturation upon PXL-NP treatment could be the mechanism for the *in vivo* observed robust cell recruitment to the draining LN and generation of adaptive immune responses (Thomas et al., in preparation). Furthermore, we reveal that the presence of C3 regulates IL-12p40 expression and, in turn, plays a crucial role in the activation of mature, functional DCs in response to PXL-NPs. This indicates that the absence of functional DCs in case of C3 deficiency may account for the abrogated cell infiltration in the LN upon PXL-NP treatment in C3 knockout mice *in vivo* (Thomas et al., in preparation). Together, this data suggests that PXL-NPs may effectively direct the immunomodulatory action of PXL via targeted delivery to LN-resident DCs to alter immune responses and thus, they represent a powerful approach for immunomodulation.

7. FUTURE PROSPECTS

The present study proves that DC maturation could be a potential mechanism for the cell-infiltration-effect observed *in vivo* upon intradermal injection of PXL-NPs in mice. However, many questions are raised at the same time; how do PXL-NPs induce maturation and cytokine production? What is the role of C3 in this mechanism? Does C3 directly affect the PXL-NP-induced functional activity of DCs or is it a synergistic effect?

A potential mechanism for the induced maturation and cytokine production in DCs upon PXL-NP treatment is NF-κB activation through TLR4 signaling, similarly to the reported action of the drug. We suggested that released PXL may interact with the intracellular part of TLR4 inducing NF-κB pathways. Studies in TLR4 deficient mice could further elucidate the mechanism of action of PXL-NP. Perera et al. [33, 52, 58, 67] have shown that apart from TLR4 deficiency, CD14 deficiency also decreased NF-κB activation in macrophages upon PXL treatment at low concentration. They also demonstrated decreased NF-κB activation and impaired IL-12 production in CD11b/CD18 (also known as complement receptor 3, CR3)-deficient macrophages in response to PXL. Moreover, Kawasaki et al. did not manage to demonstrate binding of Taxol to either TLR4 or to its obligatory cofactor, MD-2 even though the cytoplasmic region of the TLR4 was essential for Taxol-induced NF-κB activation [68]. Taken all these data together, Perera et al. attempted to describe the formation of multimeric receptor complexes stimulating complex signaling pathways for the activation of genes in response to PXL. They suggested that the role of CD11b/CD18 might be analogous to that of CD14, where CD11b/CD18 functions to bind Taxol (via CD18 as reported earlier [69]) whereas associated TLR4 molecules function as signal transducing receptors. Therefore, there is evidence introducing the implication of complement receptors and TLRs (with a potential cross-talk) in the immunomodulating action of PXL with the concentration playing a crucial role. It would be interesting to verify whether and how that would impact the immunostimulating action of PXL-NPs.

Furthermore, even though we demonstrated that C3 deficiency down-regulates IL-12p40 production in DCs upon PXL-NP treatment we do not know whether this effect is due to impairment of the PXL-NP mechanism in the absence of C3 or whether C3 acts synergistically, but through different pathways, with PXL-NPs for the up-regulation of IL-12p40 expression. It has been reported that DCs differentiated in the absence of C3 lead to decrease in IL-12p40, which is only partly compensated by LPS treatment [12]. The same study has shown that differentiation of DCs in increasing C3 concentration revealed dose-dependent up-regulation of HLA-DR and costimulatory molecules CD80 and CD86 but it had no effect on CD40. Thus, it seems that C3, on its own, can strongly affect maturation and cytokine production in DCs. Consequently, it might be possible that the up-regulated C3 expression in DCs upon PXL-NP treatment significantly enhances the autocrine C3 expression in DCs creating a positive-feedback loop that, eventually, remarkably up-regulates the expression of costimulatory molecules and IL-12p40.

Another important aspect of C3, which has not been examined in the present study, is its role in the regulation of interferon-inducible protein (IP)-10 (also known as CXCL10). IP-10 is an IFN-γ inducible chemokine with distinct biological effects, such as stimulation of NK cells, T-cell migration, regulation of T-cell, up-regulation of adhesion molecules and inhibition of angiogenesis [70]. It has been also shown to enhance the ability of DCs to chemoattract and stimulate CD8+ T lymphocytes [71]. Interestingly, Jha et al. have demonstrated that in case of complement depletion, IP-10 is barely detectable both at the protein as well as at the mRNA protein level [72]. Taken these, along with the reported action of induced IP-10 expression by PXL in macrophages [33], they could suggest a potential mechanism for the immunomodulatory action of PXL. It would be very interesting to investigate whether PXL-NPs also do induce IP-10 production. If this hypothesis is true, that would also be a very good explanation for the observed impairement of PXL-NP immunomodulatory activity in C3 knockout mice *in vivo* upon PXL treatment.

All the above suggest that there is a strong association between PXL, C3 expression, IL-12 as well as IP-10 production and the observed *in vivo* effects upon PXL-NP treatment. Further studies could enlighten the therapeutic role of PXL supporting its application in biomaterials-based schemes for immunomodulation.

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