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OPTIMIZATION OF DENDRITIC CELL BASED IMMUNOTHERAPIES

OTTIMIZZAZIONE DELLE IMMUNOTERAPIE BASATE SU CELLULE DENDRITICHE

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To Giulia,
for always believing in me

To my family,
for their constant and doubtless support

To Eleonora, Marianna and Filippo
for driving me along this path with their teachings

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SUMMARY

Dendritic cells (DC) are professional antigen presenting cells that continuously sample the environment; capture and process antigens; and transmit gathered information to T cells (Steinman & Banchereau 2007; Geissmann et al. 2010; Steinman & Cohn 1973). In presence of danger signals, DC become activated and trigger an inflammatory response against processed antigens, otherwise they remain in an immature state that lead to immune tolerance (Steinman 2003). Similarly, DC can interact and activate both B cells and NK (Batista & Harwood 2009; Steinman & Banchereau 2007). Given their pivotal role in shaping the immune system, DC are considered among the most promising cell-based immunotherapeutic approach in cancer setting aiming at activating an immune response against tumor associated antigens (TAA) (Steinman & Banchereau 2007). Several strategies have been developed to directly target DC *in vivo* by conjugating TAA with antibodies specific for DC receptor. Similarly more recently initial results have been collected on the administration of *ex vivo* activated circulating DC loaded with TAA (Wimmers et al. 2014). However, by far the most explored approach is vaccination with *ex vivo* generated monocyte-derived DC (Palucka & Banchereau 2013). Over twenty years ago, in fact, it was discovered that monocytes cultured with GM-CSF and IL-4 differentiate into DC and that upon maturation these cells were capable of activating T cells against specific antigens (Sallusto & Lanzavecchia 1994). Since then many other strategies to differentiate monocyte into DC have been developed, each one generating DC with a different phenotype, but none has conquest a general *consensus* on which one is endowed with ideal phenotype and should be used clinically (Kalinski et al. 2009).

Despite the large number of studies and clinical trials on *ex vivo*-generated DC vaccines, clinical results so far obtained were disappointing. Even though these vaccines proved to be safe and capable of inducing immune and clinical response in patient with melanoma, prostate carcinoma, glioma, and renal cell carcinoma, the overall response rate was usually below 15% (Datta et al. 2014). Many reasons have been hypothesized for such low response rates, among which the generation of DC with suboptimal potency is considered the most relevant. It's not known yet how to generate the most potent DC; furthermore, differences in clinical setting, study design, sources of antigens, and route of administration make it almost impossible to compare results from previously conducted trials in order to clearly delineate the shared determinants of in vivo efficacy of DC-based vaccines.

Differently from previous attempts to optimize DC by modifying differentiation and/or maturation procedures, this project explores the possibility to identify factors affecting DC potency/efficacy *in vivo* in order to gain knowledge of molecular determinants essential for specific DC types

and that can thus be used for quality assessment of manufactured DC. Therefore, this project aimed at identifying factors affecting DC consistency and candidate molecular biomarkers of consistency, potency and efficacy of GMP manufactured DC.

In the first part of this project, we therefore analyzed factors affecting DC consistency as well as genes and proteins mostly affected by these factors. We analyzed a specific type of DC that is being tested clinically that are DC differentiated by GM-CSF and IL-4 and matured with LPS and IFN-gamma (LIg-DC). We showed that even when highly standardized procedures are used to generate LIg-DC, manufacturing, intra-donor and inter-donor related factors may affect DC phenotype with the last one being the most relevant (Castiello et al. 2013). Interestingly, these three factors mainly affected expression level of different genes and, while intra-donor variability diminished during differentiation (probably because of strong differentiation signals), inter-donor variability increased upon differentiation/maturation. Additionally, we observed that, while most of the well-known and usually tested DC markers (e.g., CD80, CD86, CD83, HLA-DR) did not show any differences in expression among LIg-DC generated at different times from different donors, the expression of several genes and the levels of several key secreted cytokines and chemokines showed significant variability among LIg-DC products. In particular, among top variable genes, many are likely to be functional important for LIg-DC and their expression correlated with the levels of inflammatory IL-12 as well as other key chemokines, such as MDC, MIG and CXCL10.

Then, in order to analyze whether such variability can be responsible of functional differences *in vivo*, we characterized from a molecular as well as immunophenotypic point of view LIg-DC vaccines administered to stage D0 prostate cancer patients. We observed a strong correlation between DC phenotype and development of clinical and immunological response in patient after vaccination. In particular, we identified a 303-gene signature made up of several well-known tolerogenic DC factors, such as CD14 and IL-10, that was capable to discriminate DC of patients that later showed clinical/immunological response from the ones of non-responders. The differential expression of CD14 and IL-10 was confirmed at the proteomic level and we also observed that MCP-1 and MDC protein levels correlated with the expression of the tolerogenic gene signature. Even though IL-10 secretion levels were able to predict strong immunological responses, it was only by combining CD14, IL-10, MCP-1 and MDC protein measures that it was possible to obtain an index able to replace the tolerogenic gene expression signature in its ability to discriminate both clinical and strong immunological responses.

In the final part of the project, we explored whether monocyte-derived DC differentiated in presence of GM-CSF and interferon-alpha (IFNa-DC) show patterns of variability similar to LIg-DC and whether biomarkers of efficacy are shared with LIg-DC. The DC used in this study were also manufactured to sustain a phase I clinical study aiming at activating an immune response in advanced melanoma patients. Even IFNa-DC were showing pretty invariable expression levels of major histocompatibility complex class I and class II molecules, as well as co-stimulatory receptors CD80 and CD11c marker. However, we did observe high variation in the level of expression of CD86, CD40, CD83 and CD1a among IFNa-DC made from different patients. At gene expression level, instead, we did not observe the existence of the tolerogenic signature we detected in LIg-DC, but even in these cells immune response genes showed high level of variability, therefore pointing to functional differences in DC vaccines. Also proteomic analysis suggested that lot-to-lot variability shown by IFNa-DC affects different cytokines and chemokines compared to LIg-DC.

Altogether, this project developed a methodological framework for the identification of biologically-relevant quality control markers of DC by combining genomic and proteomic analysis. When applied to clinical DC, such approach was able to identify genes and proteins that correlated with clinical and immunological response and that can therefore be used as efficacy biomarkers of LIg-DC. However, as highlighted from the analysis of IFNa-DC, such newer markers are specific for DC used. On a broader range, these results strongly support the need for in-depth analysis of DC for the identification of newer quality assessment markers and factors essential for DC activity *in vivo*. Once identified, these markers can be used for the advancement of DC immunotherapies and to foster their implementation in clinic.

RIASSUNTO

Le cellule dendritiche (DC) sono cellule specializzate nella presentazione degli antigeni che analizzano costantemente il microambiente in cui si trovano, catturano e processano antigeni, e in base a questi ultimi regolano l'attività dei linfociti T (Steinman & Banchereau 2007; Geissmann et al. 2010; Steinman & Cohn 1973). Solo in presenza di segnali di pericolo, infatti, le DC si attivano e innescano una risposta infiammatoria contro gli antigeni che hanno processato; alternativamente queste cellule rimangono in uno stato immaturo di solito legato al sostenimento della tolleranza immunologica (Steinman 2003). Inoltre, le DC possono interagire e attivare anche i linfociti B e le cellule NK (Batista & Harwood 2009; Steinman & Banchereau 2007). Proprio alla luce del loro ruolo centrale nel dirigere il sistema immunitario, le DC sono considerate la base di uno degli approcci più promettenti tra le immunoterapie cellulari in campo oncologico, il cui obiettivo è di attivare una risposta immunitaria contro antigeni tumorali (TAA) (Steinman & Banchereau 2007). Diverse strategie sono state sviluppate per dirigere TAA sulle DC direttamente *in vivo*, coniugando gli antigeni con anticorpi specifici per recettori delle DC. Altri gruppi invece stanno analizzando l'uso di DC circolanti che possono essere isolate dal sangue e che vengono brevemente attivate e caricate di antigeni *ex vivo* prima di essere somministrate al paziente. Tuttavia, l'approccio di gran lunga più studiato è la vaccinazione con DC ottenute *ex vivo* dal differenziamento di monociti circolanti (Palucka & Banchereau 2013). Da oltre vent'anni, infatti, è noto che i monociti differenziano in DC se messi in coltura con GM-CSF e IL-4 e che queste DC - previa maturazione - sono in grado di attivare i linfociti T contro specifici antigeni (Sallusto & Lanzavecchia 1994). Da allora molte altre strategie per il differenziamento e la maturazione delle DC sono state sviluppate, ognuno capace di generare DC con un peculiare fenotipo. Ciò nonostante nessuna di queste strategie ha conquistato il consenso generale per essere la più indicata per un uso clinico (Kalinski et al. 2009).

Nonostante il gran numero di studi e trial clinici con DC, i risultati clinici finora ottenuti con questo approccio sono stati insoddisfacenti. Infatti pur mostrando di essere in grado, in alcuni pazienti, di attivare una risposta immunitaria e clinica contro diversi tumori, la percentuale di risposta osservata è stata mediamente inferiore al 15% (Datta et al. 2014). Diverse cause sono state ipotizzate per spiegare questi insuccessi, e tra queste la generazione di DC con funzionalità subottimali è considerata la più importante. È ancora in discussione, infatti, come generare DC che inducano una spiccata attività infiammatoria una volta inoculate nei pazienti, e l'eterogeneità degli studi finora condotti non permette di comparare i diversi

risultati e determinare esaustivamente le caratteristiche necessarie per l'efficacia di queste cellule *in vivo*.
Diversamente dai precedenti tentativi di ottimizzare le DC modificando le strategie di differenziamento/maturazione, questo progetto esplora la possibilità di identificare i fattori che condizionano la funzionalità *in vivo* delle DC con l'obiettivo di identificare gli elementi molecolari essenziali per l'attività delle DC e che quindi possono essere usati per i controlli di qualità delle DC prodotte. Quindi, questo progetto mira a identificare i fattori che influiscono sul fenotipo finale delle DC e i biomarcatori molecolari candidati per valutare la consistenza, la potenza e predire l'efficacia di DC prodotte per studi clinici.
Nella prima parte del progetto, abbiamo analizzato quali fattori influenzano la consistenza delle DC, e i geni e le proteine che ne sono principalmente condizionati. Per questo studio abbiamo utilizzato un tipo specifico di DC che è attualmente in fase di sperimentazione clinica, ovvero di DC differenziate in presenza di GM-CSF e IL-4 e maturate con LPS e Interferon-gamma (LIg-DC). I risultati ottenuti mostrano come anche quando protocolli altamente standardizzati vengono utilizzati per generare le LIg-DC, differenze intra-donatore, inter-donatore, e di processamento delle cellule condizionano il fenotipo delle DC generate e, tra questi, le differenze inter-donatore sono le più rilevanti (Castiello et al. 2013). Questi tre fattori, inoltre, condizionano i livelli di espressione di geni differenti e mentre la variabilità intra-donatore diminuisce durante il differenziamento dei monociti in DC quella inter-donatore risulta più elevata nelle DC rispetto ai monociti di partenza. In aggiunta, abbiamo osservato che sebbene i livelli di espressione dei principali marcatori delle DC si mantengano costanti in modo indipendente dal donatore(es. CD80, CD86, CD83, HLA-DR), l'espressione di numerosi geni così come i livelli di secrezione di citochine e chemochine importanti per la funzionalità delle DC mostrino un'elevata variabilità tra le LIg-DC prodotte da diversi donatori. In particolare, molti dei geni tra i più variabili sono stati descritti come importanti per la funzionalità delle DC e la loro espressione correla con i livelli di secrezioni della citochina infiammatoria IL-12 e delle chemochine MDC, MIG e IP10, tutte essenziali per l'interazione con le altre cellule del sistema immunitario.
Poi, al fine di comprendere se tale variabilità potesse essere responsabile di una diversa funzionalità *in vivo*, abbiamo analizzato LIg-DC usate per vaccinare pazienti con carcinoma prostatico in stadio D0. Dai risultati è emersa una forte correlazione tra il fenotipo delle DC e lo sviluppo di una risposta clinica e immunologica nei pazienti dopo vaccinazione. In particolare, abbiamo identificato 303 geni – in gran parte già descritti come tipici di DC tollerogeniche come CD14 e IL-10– capaci di discriminare le DC somministrate ai pazienti che successivamente avrebbero risposto alla

vaccinazione da quelle di pazienti "non responders". La diversa espressione di CD14 e IL-10 è stata anche confermata a livello proteico, insieme a quella di MDC e MCP-1. Tuttavia sebbene i livelli di espressione di IL-10 fossero sufficienti a discriminare DC capaci di indurre forti risposte immunologiche, è stato solo combinando le quattro proteine (CD14, IL-10, MDC, MCP-1) che siamo stati in grado di ottenere una capacità predittiva paragonabile a quella dei 303 geni.
Al fine di stabilire l'applicabilità di questi marcatori di efficacia *in vivo* anche per altre DC, nella parte finale del progetto abbiamo poi cercato di comprendere se anche nelle DC differenziate in presenza di GM-CSF e interferon-alpha (IFNa-DC) fosse possibile osservare gli stessi schemi di variabilità osservati nelle LIg-DC. Anche in questo studio abbiamo analizzato DC che erano state prodotte per uno studio clinico di fase I, stavolta mirato a vaccinare pazienti con melanoma metastatico avanzato. Anche le IFNa-DC hanno mostrato un pressoché invariabile livello di espressione di alcuni marcatori ben noti delle DC, quali CD90, CD11c e i complessi maggiori di istocompatibilità di classe 1 e 2, anche se livelli di variabilità elevati sono stati registrati nell'espressione di CD86, CD40, CD83 e CD1a. A livello genico, invece, pur non osservando la presenza dell'impronta tollerogenica osservata nelle LIg-DC, anche nelle IFNa-DC è stata rilevata un'elevata variabilità in molti geni della risposta immunitaria, suggerendo anche qui l'esistenza di potenziali differenze funzionali tra le diverse DC analizzate. Anche a livello proteico, poi, l'analisi dei livelli di secrezione di citochine e chemochine ha messo in luce come le IFNa-DC siano caratterizzato da una diversa variabilità rispetto alle LIg-DC.
In generale, questo progetto ha sviluppato un approccio metodologico per l'identificazione di nuovi controlli di qualità delle DC, basato sulla combinazione di analisi genomiche e proteomiche. Quando tale approccio è stato utilizzato su DC usate in clinica, esso ha permesso di identificare geni e proteine che correlavano con lo sviluppo di una risposta clinica e immunologica e che quindi possono essere usati come biomarcatori di efficacia delle LIg-DC. Tuttavia, come evidenziato dall'analisi delle IFNa-DC, questi biomarcatori non sono condivisi universalmente da tutte le DC. Aldilà dell'aver identificato nuovi biomarcatori delle LIg-DC, questi risultati evidenziano la necessità di analisi approfondite delle DC per l'identificazione di nuovi marcatori e fattori essenziali per l'attività delle DC *in vivo*. Una volta identificati, questi nuovi marcatori possono essere utilizzati per controlli di qualità più affidabili e quindi facilitare l'implementazione clinica di queste cellule.

1. BACKGROUND

1.1 Dendritic Cells and their role in shaping immune response

Dendritic cells (DC) are bone marrow-derived cells that are present in all tissues in order to sample the environment and transmit gathered information to the adaptive immune system (Steinman & Banchereau 2007; Geissmann et al. 2010; Steinman & Cohn 1973). To initiate an immune response DC present the captured antigen, which is in the form of peptide–major histocompatibility complex (MHC) molecule complexes, to naive T cells in lymphoid tissues (Steinman & Banchereau 2007).

Normally, DC in peripheral tissues are immature. These immature DC have the ability to efficiently capture antigens; they can express low levels of co-stimulatory molecules; and have a limited capacity for secreting cytokines. Non-activated (immature) DC can present self-antigens to T cells (Steinman 2003), which leads to immune tolerance either through T cell deletion or through the differentiation of regulatory or suppressor T cells. However, DC promptly respond to environmental signals and differentiate into mature DC that can efficiently launch immune responses. Maturation is associated with the down-regulation of antigen-capture activity, the increased expression of surface MHC class II molecules and co-stimulatory molecules, the ability to secrete cytokines, as well as the acquisition of CCR7, which allows migration of the DC into the draining lymph node (Trombetta & Mellman 2005). However, DC maturation alone does not result in a unique DC phenotype. In fact, depending on the type of maturation signal, DC acquire distinct phenotypes that are distinct in both expression of co-stimulatory/co-inhibitory molecules as well as type and amount of secreted cytokines and chemokines.

Depending on the interaction with DC, naive CD4+ T cells and CD8+ T cells can differentiate into antigen-specific effector T cells with different functions. In fact, CD4+ T cells can become T helper 1 (TH1) cells, TH2 cells, TH17 cells or T follicular helper (TFH) cells that help B cells to differentiate into antibody-secreting cells, as well as regulatory T (TReg) cells that down-regulate the functions of other lymphocytes. Naive CD8+ T cells can give rise to effector cytotoxic T lymphocytes (CTLs). The type of T cell response — for example, CD4+ helper T cells or CD8+ CTLs — is at least partly linked to the subset of DC that presents the antigen (Banchereau & Steinman 1998). DC can also interact with cells of the innate immune system, including natural killer (NK) cells, phagocytes and mast cells (Steinman & Banchereau 2007; Banchereau & Steinman 1998).

DC also have an important role in controlling humoral immunity. They do so both directly by interacting with B cells and indirectly by inducing the

expansion and differentiation of CD4+ helper T cells (Batista & Harwood 2009). These key properties of DC, which allow the activation of both arms of the adaptive immune system (that is, cellular and humoral) and which launch the immune response, render DC the central candidates for antigen delivery and therapeutic vaccination against cancer.

1.2 Dendritic Cells subsets: localization and function

Both mice and humans have two major subsets of DC: myeloid DC (mDC; also known as conventional DC and classical DC) and plasmacytoid DC (pDC). Different subsets of human DC in the blood can be distinguished by the differential expression of three cell-surface molecules: CD303 (also known as BDCA2 and CLEC4C), CD1C (also known as BDCA1) and CD141 (also known as BDCA3 and thrombomodulin) (Dzionek et al. 2000). CD303+ pDCs represent a front line of anti-viral immunity owing to their ability to secrete large amounts of IFNα in response to virus encounters (Siegal et al. 1999). Their presynthesized stores of MHC class I molecules may allow a rapid initial CD8+ T cell response to viral infections. pDC-derived IFNα may also promote the immunogenic maturation of other subsets of DCs, thus helping to activate novel T cell clones. Human CD141+ DCs share with mouse CD8+ DCs the high capacity to capture exogenous antigens for presentation on MHC class I molecules (known as cross-presentation). CD141+ DCs express XCR1, which is the receptor for the chemokine XCL1 (also known as lymphotactin) that is produced by NK cells and activated CD8+ T cells (Bachem et al. 2010). Thus, mouse CD8+ DCs and human CD141+ DCs are equipped for the generation of CD8+ T cell-mediated immune responses. The unique functions of CD1C+ DCs also continue to be analyzed.

The human skin hosts two main subsets of mDC: epidermal Langerhans cells and dermal interstitial DC (dermal DC) (Valladeau & Saeland 2005). The dermal DC can be further subdivided into CD1a+ DC and CD14+ DC. Human CD14+ DC can directly help activated B cells, as well as induce naive T cells to differentiate into cells with the properties of TFH cells. CD14+ DC may thus be specialized for the development of humoral responses (Ueno et al. 2010). Langerhans cells are more efficient in cross-presenting peptides from protein antigens to CD8+ T cells and can prime the differentiation of CD8+ T cells into effector CTLs.

The development and homeostasis of tissue-resident DC subsets in steady state conditions (that is, when there is no infection or activation of the immune system) is dependent on the activation of the receptor tyrosine kinase FLT3 and of the macrophage colony-stimulating factor 1 receptor (M-CSFR; also known as CSF1R). However, inflammatory processes, such as those initiated by microbial invasion, substantially alter the populations of

DC subsets. The origin of DC that are recruited to sites of inflammation is still under investigation, although it is clear that monocytes can give rise to inflammatory DC in vivo (Cheong et al. 2010).

1.3 Monocyte-derived Dendritic Cells: pathways of differentiation and maturation

Over twenty years ago, Sallusto and Lanzavecchia described for the first time that immature DC can be generated culturing monocytes *in vitro* in presence of granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) (Sallusto & Lanzavecchia 1994). These cells (IL4-DC) showed typical dendritic morphology, expressing high levels of major histocompatibility complex (MHC) class I and class II molecules, CD1 family, and other co-stimulatory molecules. Even functionally, these cells proved to be highly stimulatory in mixed leukocyte reaction (MLR) and were also capable of triggering naive T cells. Since then, many different protocols have been developed to differentiate monocytes into DC, each one leading to DC with a unique phenotype.

Given their functional similarity, both IL-13 and IL-7 can substitute IL-4, even though signaling in different ways. In fact, IL-13DC are similar to IL-4DC both phenotypically and functionally, whereas IL-7DC express CD21, the complement receptor type 2. Even functionally, these cells proved to be more effective than IL-4DCs in eliciting proliferative responses of CD4 and CD8 T cells, and stronger T cell cytotoxicity (Takahashi et al. 1997).

Originally described for its anti-viral activity, type I interferon (IFN) exerts important effects on the immune system, including promotion of cellular and humoral responses, by virtue of its adjuvant effects on antigen-presenting cells (Belardelli 1995). As high amounts of IFN-α can be physiologically produced in response to infectious agents and inflammatory stimuli, this cytokine may be among the factors signaling danger to circulating monocytes, thus enabling them to rapidly differentiate into DC. In line with this hypothesis, it has been previously demonstrated that highly active partially mature DC can be generated from monocytes after a single step of 3-day culture with IFN-α and GM-CSF (IFNa-DC) (Santini et al. 2000). IFNa-DC proved to be more effective than immature DC generated in the presence of GM-CSF and IL-4 in inducing a Th-1 type of immune response and CD8+ T cell responses against defined antigens in different models (Lapenta et al. 2006; Santodonato et al. 2003). Although antigen uptake and endosomal-processing capabilities were similar for IFNa-DC and IL-4DC, and both DC types efficiently cross-presented soluble antigens to the specific CD8+ T cell clone, IFNa-DC were superior in cross-presenting low amounts of viral antigens. This property correlated with enhanced potential to express the specific subunits of the IL-23 and IL-27 cytokines.

Depending on the specific cytokine combination, blood monocytes can also give rise to Langerhans cells. In this respect, it has been reported that blood monocytes differentiated into LCs by replacing IL-4 with IL-15 (Mohamadzadeh et al. 2001). This cytokine cocktail gives rise to CD1a+, HLA-DR+, CD14− DCs, a proportion of which express LC markers, such as E-cadherin, Langerin and CCR6. Accordingly, IL-15DC, but not IL-4DC, migrated in response to CCL20. However, IL-15DCs cannot be qualified as genuine LCs because, despite Langerin expression, they do not express Birbeck granules (Mohamadzadeh et al. 2001).

Independently of the differentiation protocol used, DC can be matured by different *stimuli*. Mimicking signal microenvironment sensed by DC *in vivo*, DC have been matured *in vitro* using either inflammatory or tolerogenic compounds. Tolerogenic DC can be generated by maturing DC with IL-10 alone or in combination with IL-6, dexamethasone, or TGF-beta1 (Torres-Aguilar et al. 2010). Alternatively, tolerogenic DC have been generated by co-culturing DC with immunosuppressive cells, such as T regulatory cells and mesenchymal stromal cells (Pulendran et al. 2010). However, many more signals can be used to generate DC with inflammatory phenotype. Maturation of DC can be achieved by triggering Toll-like receptors (TLR) or through inflammatory cytokines. Triggering of TLRs on DC is thought to be critical for their functional maturation to immunogenic DC and the priming of naïve T cells in response to infection, and therefore coupling innate and adaptive immunity. Because of its bacterial origin and its predominant role as a pathogen associated pattern (PAP), LPS is recognized by TLR4 and represents a prototypical model of DC maturation. LPS-DC are endowed with strong chemotactic and immune activating properties (Castiello et al. 2011). Polyinosinic:polycytidylic acid (Poly I:C) is another of the most studied maturation signals in DC. Poly I:C is structurally similar to double-stranded RNA, which is present in some viruses, and is recognized by TLR3. Similarly to LPS, also Poly I:C induces maturation of DC with a strong immunogenic phenotype (Möller et al. 2008). Type I and II IFN, Tumor Necrosis Factor alpha (TNFa), IL-6 and IL-1b are all inflammatory cytokines that are capable to induce DC maturation either alone or in combination (Castiello et al. 2011). Differently from PAP, these cytokine do not trigger TLR, but activate different downstream signaling pathways, therefore leading to DC with different phenotypes.

1.4 Dendritic Cell Based Immunotherapies

Given their pivotal role in activating an antigen specific immune response, DC have been considered among the ideal cell-based immunotherapies. Over the last twenty years, many studies focused on three different DC-based

immunotherapies: *in* vivo targeting of DC, *ex vivo* activation of circulating DC and *ex vivo* generation of monocyte-derived DC.

Pioneering studies from Steinman and Nussenzweig demonstrated the feasibility of targeting antigens to DC *in vivo* by coupling the desired antigen to an antibody recognizing a DC receptor (Bonifaz et al., 2002; Hawiger et al., 2001; Soares et al., 2007a). Importantly, in the absence of adjuvants, (e.g., targeting antigens to DEC205+ DC) in vivo induces antigen-specific tolerance, which can be used as treatment against autoimmune diseases such as type I diabetes (Steinman, 2012). Administration of these complex vaccines with DC-activators such as TLR3, TLR7-8, or CD40 agonists enables the maturation of DC and thus the establishment of immunity rather than tolerance (Steinman, 2012). The induced immunity was shown to be protective in a number of diseases including various infections (malaria, HIV) and cancer (Steinman, 2012; Tacken and Figdor, 2011), but moving in vivo DC-targeting to human trials requires considerable work yet because activator to use, DC subset to target, and specificity of targeting *in vivo* have still to be optimized (Datta et al. 2014).

Due to the low occurrence of naturally circulating DC in blood, conclusive clinical evidence on their usability for immunotherapy is lacking. Only recently, thanks to technological advancement in cell separation techniques, some encouraging results have been achieved using either pDC and mDC. In both cases, circulating DC subsets are selected by magnetic separation kits and cultured with activating cytokines for short period of time (i.e., overnight). Then, cells are loaded with tumor associated antigens and injected back to patient. Interestingly, one clinical trial selecting/activating pDC in melanoma patients showed induction of cancer-specific immune response in 7 of 15 treated patients and similar encouraging results were obtained selecting CD1c+ mDC (Wimmers et al. 2014).

Thanks to the accessibility to high amount of monocytes, injection of *ex vivo* generated monocyte-derived DC has been under the spotlight as the most promising and ready to implementation immunotherapy. Since the discovery of monocyte differentiation into DC over 300 clinical trials have been performed, but the heterogeneity of cells used, sources of antigen, dose and site of injection makes impossible to clearly summarize results so far obtained. By far the majority of the studies involved conventional IL-4DC used immature or matured by different maturing compound or cocktails loaded with tumor associated antigens (either single peptides, whole proteins or tumor lysates). To date, none of these study showed conclusive results of efficacy of such approach, even though both clinical and immunological responses have been observed (Engell-Noerregaard 2009). Furthermore, more recently some studies were published utilizing DC injected intra-tumorally after chemotherapy or radiotherapy, therefore aiming at an *in situ*

loading of antigens released by therapy induced cell death (Yu et al. 2003; Kolstad et al. 2014; Tanaka et al. 2005). These studies showed preliminary promising results, but larger studies have to be performed to assess efficacy of this other approach. Altogether, even though a large body of research and clinical experimentation has been performed, monocyte-derived DC immunotherapies have still to be optimized and many parameters to be more deeply analyzed.

1.5 Open questions in immunotherapy based on monocyte-derived Dendritic Cells

Even though a large number of clinical trials has be conducted utilizing *ex vivo*-generated DC vaccines, several controversies linger. First, the optimal DC phenotype, and the differentiation/maturation protocol utilized therein remains contentious. In particular, given the complexity of DC biology and the wide array of molecular players essential for T cell activation, a consensus on optimal DC phenotype and the procedure to obtain it is still lacking (Castiello et al. 2011). Among molecular markers of ideal DC, it is increasingly recognized that abundant production of IL-12p70 during DC maturation *ex vivo*, as well as "burst" secretion during DC-activated Th interaction *in vivo* (via CD40-CD40L in lymphoid organs), are critical for the induction of CTL responses and Th1-polarized immunity (Strioga et al. 2013). In addition to IL-12p70 elaboration, other desirable functions of immunogenic DC include non-exhaustive capacity, expression of chemokines enhancing tumor microenvironment infiltration of T effector cells (e.g., CXCL9/10), low IL-10 secretion following restimulation with CD40L, and enhanced migratory ability to lymph nodes. Several cytokine cocktails have been proposed to achieve optimal DC characteristics, but other factors have also to be finely tuned, such as time of stimulation and cytokine concentrations. Also, most of the studies focused only on how to optimize DC in order to have the best activation of T cells; however there is increasing evidence that also DC interaction with B cells and NK is important for effective activation of immune system, but the ideal DC phenotypes for these interactions are mostly unexplored (Bray et al. 2011; Qi et al. 2006). Additionally, it has to be considered that the ideal DC phenotype may differ depending on tumor setting, type of antigen used, site of injection, and patient immune status, thus opening the door to multiple "ideal DC phenotypes", each one for a specific setting/use.

Second, the ideal strategy for DC antigen-loading is not universally agreed upon. The most common approach has been loading with tumor-associated peptides or whole recombinant tumor proteins (Palucka & Banchereau 2012). Other modalities of DC loading (engineered fusion proteins, autologous/allogeneic tumor cells, tumor cell-lysate, DC-tumor hybrids, and

DNA- or mRNA-transfected) have also emerged (Strioga et al. 2013). Even though clinical studies are still evaluating all of these strategies, the use of tumor lysate is thought to be the most promising one given the wide antigenic repertoire the DC can process and thus present. As already mentioned, another strategy is also emerging pointing to *in situ* loading of intra-tumorally injected DC, usually following chemo/radiotherapy (Kolstad et al. 2014; Tong et al. 2001; Akutsu et al. 2007; Finkelstein et al. 2012). Similarly to tumor lysate, this approach allows a wide antigenic repertoire, and compared to tumor lysate has the advantage of being more simple (given that tumor lysate has not to be processed *ex vivo*), but has the drawback that DC have to overcome tolerogenic tumor microenvironment in order to activate immune system.

Third, the optimal route for DC administration remains controversial. Historically, with intradermal/subcutaneous injection techniques, DC trafficking to regional lymph nodes was considered critically important to their function. Indeed, maturation cocktails (e.g., PGE2-containing) were designed to optimize trafficking ability (Strioga et al. 2013). However, depending on differentiation/maturation strategy and DC maturation status at time of injection, alternative routes might be more effective. Ultrasound-guided intranodal injection, which co-localizes DC1-derived IL-12p70 "burst" with the anatomic site of T-cell sensitization, has emerged as a feasible solution (Bedrosian et al. 2003) ideal in case of DC impaired migration ability or advanced maturation.

Fourth, *ex vivo*-generated DC vaccines, like all other cell therapies, is challenging because of their considerable lot-to-lot and patient-specific variability that in most cases has yet to be sufficiently quantified and characterized (Stroncek et al. 2010). In fact, while each cell therapy lot has to be tested for identity, purity and potency among other tests, feasibility issues dictate these tests to be focused on a handful of factors and, therefore, they cannot assure an exhaustive characterization of each lot. Identity testing aims to ensure the manufactured cells show a defined phenotype, purity testing evaluates the absence/low level of cell contaminants. Potency testing, instead, assesses one biologically-relevant activity of the product and therefore is more controversial because can be tested with relatively-easy and low-informative assays (such as the secretion of IL-12 or phagocytosis ability) or lengthy and complex high-informative ones (such as the ability to activate tumor specific immune response in animal models). An accurate characterization of DC should ideally assay all the factors affecting their *in vivo* biological functions: antigen processing and presentation, expression of co-stimulatory signals, absence or reduced expression of co-inhibitory signals, lymph node migration, and secretion of activating cytokines and chemokines. These are all essential features of potent DC and should be

thoroughly tested. Since it is impossible to routinely evaluate each product for every cell function using cellular assays, the identification of reliable biomarkers of identity, consistency and potency of cell therapies is highly encouraged by regulatory agencies beginning in the earliest phases of clinical development of the cellular product (Hinz et al. 2006; Vatsan et al. 2013).

1.6 Novel strategies for generation of monocyte-derived Dendritic Cells

As previously stated, a wide array of strategies to generate monocyte-derived DC has been developed and tested, but no general consensus exists on which one leads to ideal DC. In the next two sections, two novel strategies that represent promising candidates and that were used and analyzed for the PhD research project are discussed more in details.

1.6.1 LPS/IFN-gamma mature Dendritic Cells

Maturation of conventional IL-4DC with clinical grade LPS and IFN-gamma generated DC with strong immunostimulatory activity (LIg-DC) (Dohnal et al. 2009; Felzmann et al. 2005; Hüttner et al. 2005; Vopenkova et al. 2012). In fact, it has been shown that LIg-DC exhibit fully mature phenotype, the highest IL-12p70 production and stimulate T cell proliferation as well as their specific cytotoxic activity (Vopenkova et al. 2012). However, it has been observed that IFN-gamma and high-IL-12p70 production reduced migratory ability of DC, and thus LIg-DC do not migrate well. In particular, donor-dependent differences were observed in DC migratory capacity, suggesting that DC migration does not depend only on the maturation strategy used, but also on individual characteristics of the donor. Also, it was noted that timing of maturation play a critical role for this kind of cells. In fact, while only two hours were sufficient to induce maturation and potent immunostimulatory ability, cultivation of DC in the presence of both maturing agent for longer than 24 hours generated DC that were unable to release IL-12p70 and were less effective in triggering anti-tumor immunity(Hüttner et al. 2005).

Two clinical studies using LIg-DC have been published. In the first one, 22 pediatric cancer patients were vaccinated with LIg-DC pulsed with tumor cell lysate and keyhole limphet hemocyanin (KLH, as positive control). Following immunization, the majority of patients responded positively to KLH in a delayed-type hypersensitivity (DTH) test. In addition, three of six intra-nodally treated patients responded to the tumor Ag in the DTH test(Dohnal et al. 2007). In the second study, twenty-seven subjects with HER-2/neu over-expressing ductal carcinoma *in situ* of the breast were enrolled in a neoadjuvant immunization trial of LIg-DC pulsed with six HER-2/neu promiscuous MHC class II-binding peptides, plus two additional HLA-A2.1 class I-binding peptides. Interestingly, sensitization of Th cells to at least 1 class II peptide was observed in 22 of 25 evaluable subjects, while

eleven of 13 HLA-A2.1 subjects were successfully sensitized to class I peptides (Koski et al. 2012).

1.6.2 IFN-alpha Dendritic Cells

As already mentioned culture of monocyte with GM-CSF and IFN-alpha generate DC showing a semi-mature phenotype and endowed with potent functional activities (IFNa-DC) (Santini et al. 2000; Farkas et al. 2008; Paquette et al. 1998; Santini et al. 2009). In fact, these cells produce mostly T-helper-1 (Th-1) cytokines and chemokines, express toll-like receptors (TLRs) 1 to 8, show migratory response to chemokines, and are capable of stimulating Th-1 polarized immune responses after injection into severe combined immunodeficient mice reconstituted with human peripheral blood leukocytes(Santini et al. 2009; Farkas et al. 2008). Of interest, in a variety of in vitro and in vivo preclinical models, IFNa-DC proved to be superior with respect to IL-4DC in inducing potentially protective immune responses . Notably, IFNa-DC exert a direct cytotoxic effect on tumor cells (Santini et al. 2000), are capable to take up apoptotic cells through the scavenger receptor Lectin-like oxidized-LDL receptor-1 (LOX-1) (Parlato et al. 2010) and cross-present their antigens to CD8+ T cells, thus leading to an efficient cross-priming of these cells(Santodonato et al. 2003; Tosi et al. 2004; Lapenta et al. 2006). In addition, IFNa-DC are capable of expanding both Th1 and Th17 responses as a result of the production of cytokines such as IL-23 and IL-12(Santini et al. 2011). Remarkably, IFNa-DC do not require TLR triggering to induce antigen specific cytotoxic T lymphocytes and to stimulate allogeneic CD4+ T cells (Bracci et al. 2008). Interestingly, it was also shown that IFNa-DC can mediate TRAIL-dependent cytotoxicity of tumor cells and that even human CD11c(+) blood DCs express TRAIL after stimulation with IFN-alpha, thus acquiring the ability to kill TRAIL-sensitive tumor cell targets (Fanger et al. 1999; Santini et al. 2000; Servet et al. 2002). Of special note, a cell population resembling IFNa-DC was identified in immune cell infiltrates in Molluscum contagiosum virus-induced cutaneous lesions undergoing spontaneous regression (Vermi et al. 2011). Accordingly, a type I IFN signature associated with pDC infiltration was demonstrated in both keratinocytes and inflammatory cells. All these features make IFNa-DC highly promising new candidates for the development of more effective DC-based strategies of cancer immunotherapy (Farkas & Kemény 2011; Bracci et al. 2013).

2. AIM

Dendritic cells (DC) play a key role in the activation of immune system by presenting antigens to T cells and, by so, generating an antigen-specific immune response (Ueno et al. 2010). For this reason, several attempts have been done so far in order to develop effective immunotherapeutic approaches that consist of ex vivo generated fully-functional DC to be infused in patients in order to induce an antigen specific T cell expansion (Palucka & Banchereau 2012). DC can be generated ex vivo by culturing monocytes in presence of differentiating cytokines (such as GM-CSF, IL4, IL15 and IFNa) to obtain immature DC and maturing them with single agents or cocktails of agents (such as TNFa, LPS, IFNg, CD40L, IL6, IL1) (Kalinski et al. 2009). Once generated, DC are usually pulsed with tumor antigens and injected into patients (usually intranodally or intradermally) or directly injected intratumorally aiming at in situ antigen loadings usually after chemo/radiotherapy. Clinical results, mainly from studies in cancer patients, clearly showed the feasibility and efficacy of this approach even if the overall response rate is below the 15% (Engell-Noerregaard 2009). Several possible reasons have been hypothesized to justify such low response rate, among which the suboptimal generation of DC able to activate an anti-inflammatory Th1-polarized T cell response is considered the main bottleneck, even if there is no general consensus on how to improve DC function and which factors are responsible for the discrepancies between preclinical and clinical results (Castiello et al. 2011). Furthermore, cell based immunotherapies would strongly benefit of new markers for quality control assessment. Since many more factors are responsible for the function and effectiveness of cellular therapies than those of drugs and other biological products, an in depth evaluation of the characteristics of all newly developed cellular therapies is needed (Stroncek et al. 2010).

Differently from previous attempts to optimize DC by modifying differentiation and/or maturation procedures, this project explores the possibility to identify factors affecting DC potency/efficacy *in vivo* in order to gain knowledge of molecular determinants essential for DC function and that can thus be used for quality assessment of manufactured DC. Therefore, this project aimed at identifying factors affecting DC consistency and candidate molecular biomarkers of consistency, potency and efficacy of GMP manufactured DC. The project initially focused on a preclinical setting in order to evaluate feasibility of this approach by analyzing factors affecting DC consistency by combining genomic and proteomic approaches. Successively, given that the only reliable indicators of DC potency/efficacy derive from results in humans, the project focused on analyzing DC from two

clinical trials. The two trials utilized two different DC (in one case DC were differentiated with GM-CSF and IL-4 and matured with LPS and IFN-g; in the other one DC were only differentiated in presence of GM-CSF and IFN-a), therefore allowing the analyze whether factors affecting DC potency/efficacy are shared among different DC or are unique to each type of DC.

3. IDENTIFICATION OF FACTORS AFFECTING REPRODUCIBILITY OF LIg-DC AND CANDIDATE BIOMARKERS OF LIg-DC CONSISTENCY AND VARIABILITY

3.1 Introduction

Monocyte-derived dendritic cells (DC) have been used in vaccine trials and represent one of the most promising approaches in inducing a targeted immune response(Palucka & Banchereau 2012). However, despite of twenty years of research and clinical experimentation in different settings, response rate are still pretty low, ranging from 5% to 15%(Engell-Noerregaard 2009). Reasons behind these results have to be identified in the complexity of DC biology as well as the lack of knowledge on best antigens, adjuvants and route of administration(Kalinski et al. 2009). In particular, DC function depends strongly on several factors, such as the differentiation process, maturation stimulus and duration of the manufacturing processing. In facts, monocytes can be differentiated using different cytokines leading to DC showing differences both on phenotype and functional activity. Since the initial discovery that monocyte can differentiate into DC when cultured in presence of GM-CSF and IL-4 (Sallusto & Lanzavecchia 1994), it has been shown that simple culture in presence of only GM-CSF leads to tolerogenic DC(Conti & Gessani 2008), whereas differentiation in presence of GM-CSF and IL-15 leads to DC more similar to Langherans cells(Dubsky et al. 2007) and that replacing IL-4 with IFN-alpha leads to DC with a semi-mature phenotype and potent co-stimulatory activity (Santini et al. 2000; Santodonato et al. 2003; Santini et al. 2009). Also the length of differentiation has a role in DC activity: several groups have shown that reducing the length of DC differentiation from the classical 7 days to 3-4 days leads to more potent DC(Dauer et al. 2003). Lastly, even maturation stimulus has a central role in shaping DC function, but even if there are over a dozen of different maturation cocktails no consensus exists on which signal leads to more potent DC(Castiello et al. 2011). Therefore, all of these factors drive DCs to develop a specific qualitative and quantitative immune activation, ranging from strong pro-inflammatory Th1 response to regulatory T cell induction. Even though several elements are known to affect the function of monocyte-derived DCs, the best methods for manufacturing DCs and for characterizing key DC functions are yet to be defined.

On the other hand, quality control is becoming a critical part of cellular therapy(Stroncek et al. 2010). Major aspects of ensuring product consistency and quality involve process control, adhering to standardized procedures, using GMP grade reagents, training staff and validating instruments and

equipment. However, the final product characterization is the main critical aspect of ensuring product consistency, especially for assuring that every production lot exceeds determined minimum standards. DC product characterization is performed at each step of the manufacturing process and at lot-release. Final products are evaluated for identity, sterility, purity, consistency, stability and potency; the latter being a quantitative measure of a product-specific biological activity that is linked to a relevant biological property. Feasibility issues dictate that actual product characterization must be a balance of what should and what can be tested (e.g., time needed for functional assays, lack of animal models). However, since many more factors are responsible for the function and effectiveness of DC-based therapies than those of other drugs, an in depth evaluation of the characteristics of newly developed cellular therapies is extremely desirable and needed for the identification of markers that are able to easily reveal characteristics relevant to important biological functions.

Additionally, even if high level of variation in DC phenotype has been observed even when using the same procedure of differentiation, factors behind such variability have never been analyzed(Stroncek et al. 2010).The understanding of these factor may thus play an essential role in the implementation of DC immunotherapies in clinical practice by defining molecular biomarkers of DC with increased potency, thus driving the development of newer protocols and procedure for DC-based immunotherapies. In fact, a prerequisite for the implementation of DC immunotherapies, as well as other cell based therapies, is the ability to consistently produce high quality products. Therefore, the project started analyzing what are the main factors affecting consistency of DC generated under GMP and whether such variability may hinder functional differences relevant to DC biology.

To dissect factors affecting DC consistency, the project focused on understanding how much manufacturing, intra-donor and inter-donor related causes have a role in DC variability. To test these factors we evaluated DC generated at different times from aliquots of the same monocyte collection (manufacturing related variability), DC generated from monocytes deriving from leukapheresis collected at different times from the same donors (intra-donor related variability), and DC generated from monocytes of different donors (inter-donor related variability). In addition, starting monocytes and intermediate DC products (i.e., immature DC) were included in the study to make possible the selection of candidate molecular markers of DC consistency and variability. In order to have a wide DC characterization we combined standard flow cytometry of known DC surface markers with gene expression profiling and protein secretion profiling of a broad panel of cytokines and chemokines.

Regarding the type of DC to analyze in this study, DC differentiated in presence of IL-4 and GM-CSF for 3 days and matured with LPS and IFN-g (LIg-DC) were selected because of their promising Th1-polarization phenotype and their use in multiple clinical trials at National Institute of Health(Shin et al. 2008; Jin et al. 2010).

3.2 Results

3.2.1 Role of manufacturing, intra-donor and inter-donor variability in consistency of LIg-DC

3.2.1.1 Study design and LIg-DC production. When reviewing the overall manufacturing process, we realized that many factors could affect LIg-DC. Among these factors we decided to evaluate the effects of manufacturing variability, inter-donor variability and within-donor time-dependent fluctuations in the starting material (intra-donor variation) on the features of the final DC product. Manufacturing-related variability was tested by generating 5 DC preparations on different days using 5 aliquots from the same starting material. Inter-donor variation was assessed by studying DC derived from 9 different healthy donors. Intra-donor variation was assessed by preparing DC starting from 5 monocytes preparations derived from as many apheresis products from the same donor. Although it could be argued that the evaluation of a larger number of samples should lead to a more robust model for the description of manufactured products' characteristics, the conditions that we defined are sufficient for the early-stage progressive assay implementation.

LIg-DC were manufactured according to standard GMP procedures established to support phase I/II vaccine trials at the NCI, NIH Bethesda, Maryland (NCI-09-C-0139, NCI-08-C-0051 and NCI-07-C-0206) and the clinical DC product release criteria were used to evaluate the quality of each experimentally manufactured product. All LIg-DC passed release criteria by showing cell viability >70%, CD83 expression >80%. Sterility tests were not performed given that cells were not use as clinical products.

3.2.1.2 Variability of LIg-DC expression of surface markers. More than 95% of the cells in the final product were CD80+, CD86+, CD83+, CD209+, HLA-DR+, CD40+, CD54+, CD123+, CD11c+ as individually assessed by flow cytometry (see Table 3.1). Other markers were showing more variable levels of expression

When assessed for variability among the three tested conditions (i.e., manufacturing, intra-donor and inter-donor related variability), the majority of surface markers did not show any appreciable variation among different

DC. Levels of variations were only observed for CD14, CCR7 and CD54 expressionlevels. While CD14 and CCR7 were expressed only by a proportion of DC, CD54 was expressed by all DC but at a very different intensity. In particular, manufacturing- and inter-donor-related variability did affect both CD14 and CCR7 expression levels (Figure 3.1), while intra-donor showed lower level of variation. Instead, even though >90% of the LIg-DC were CD54+, we observed high variability in all three conditions tested when the signal intensity was evaluated (Figure 3.2).

3.2.1.3 Variability of LIg-DC at gene expression level. Next, instead f focusing on few markers, gene expression profiles were examined by using Agilent Microarray technology. However, in order to appraise the confounding effect due to the variability of gene arrays, replicate samples were tested to estimate the within assay variability (shown by replicate samples of RNA amplified, labeled and hybridized on the same day) and the between assay variability (replicate samples of RNA prepared and hybridized on different days). The Coefficient of Variation (CV) was calculated for the samples used for assessing assay variability. The within assay and between assay samples showed CV median values of 6.1% and 14.4%, respectively (data not shown). These values are consistent with those obtained by the MicroArray Quality Control project for intra-site repeatability and reproducibility and indicated sufficient reliability for clinical and regulatory purposes(Shi et al. 2006). Then, we calculated the Intra-class Correlation Coefficient (ICC) for each possible source of variability that could impact the final product (assay, manufacturing, intra-donor and inter-donor). As expected, the within and between assay ICCs were much greater than manufacturing, intra-donor and inter-donor ICCs indicating that assay variability had a low confounding effect in our experimental approach (Figure 3.3), thus reconfirming the role of high-throughput gene expression analysis for the assessment of manufactured cell products. Interestingly, manufacturing, intra-donor and inter-donor factors all affected the final product. Although inter-donor samples show the lowest ICC value (0.925), it should be noted that manufacturing (0.948) and intra-donor variation (0.947) also played a significant role in final product consistency. This was also demonstrated by unsupervised hierarchical clustering and similarity matrix analysis of the samples based on the entire dataset (Figure 3.4). However, to place results into context and correctly assess the biological value of the obtained ICCs we also evaluated the ICC of an artificial class made up of a mixed cell populations that are known to show functional and molecular differences (i.e., immature DC andLIg-DC). We selected immature DC and mDC from 5 donors, and obtained an ICC value of 0.867. The ICC for the mixed immature and LIg-DC population was, as expected, less than the ICC

associated with LIg-DCs. However, since the ICC for mixed cells was similar to the inter-donor ICCs, these results indicate that LIg-DCs from different donors show a degree of variability that likely reflects functional differences.

Table 3.1 Expression level of LIg-DC surface markers

	% of positive cells											
Sample type	**CD86**	**CD83**	**CD14**	**CD209**	**CCR7**	**CD40**	**HLA-DR**	**CD123**	**CD11c**	**CD80**	**CD154**	**CD54**
manufacturing 1	98	NA	27	95	3	97	99	63	98	93	0	99
manufacturing 2	100	100	8	82	70	100	95	100	97	96	11	100
manufacturing 3	100	100	9	72	62	100	93	100	92	89	10	100
manufacturing 4	100	100	9	86	63	100	100	100	96	92	6	100
manufacturing 5	100	100	7	83	43	100	100	100	98	96	10	100
intra-donor 1	100	100	8	82	70	100	95	100	97	96	11	100
intra-donor 2	100	100	8	81	83	99	95	100	94	98	13	100
intra-donor 3	100	99	8	80	81	99	94	99	94	97	10	99
intra-donor 4	100	99	11	83	57	100	97	97	98	94	10	100
intra-donor 5	100	99	3	68	83	98	89	99	86	97	4	98
inter-donor 1	100	100	7	88	92	99	98	100	81	99	NA	99
inter-donor 2	99	100	0	61	91	93	89	100	65	84	NA	95
inter-donor 3	100	100	21	86	58	100	100	100	98	92	8	100
inter-donor 4	100	99	19	90	61	100	99	100	98	98	11	100
inter-donor 5	100	100	15	68	37	99	100	100	96	95	12	100
inter-donor 6	100	100	19	76	55	100	100	100	98	99	10	100
inter-donor 7	99	NA	30	93	2	99	100	46	99	50	1	84
inter-donor 8	99	NA	26	94	1	99	98	56	99	97	0	99
inter-donor 9	100	100	7	83	43	100	100	100	98	96	10	100

NA: Not available

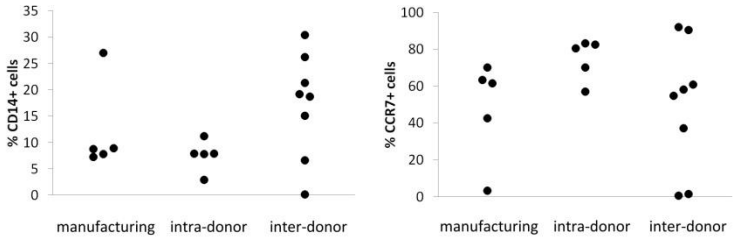

Figure 3.1 Variability of CD14 and CCR7 surface expression levels. On the left, percentages of CD14+ cells measured on LIg-DC evaluating manufacturing-related variability (n=5), intra-donor-related variability (n=5) and inter-donor-related variability (n=9).On the left, percentages of CCR7+ cells measured on LIg-DC as above.

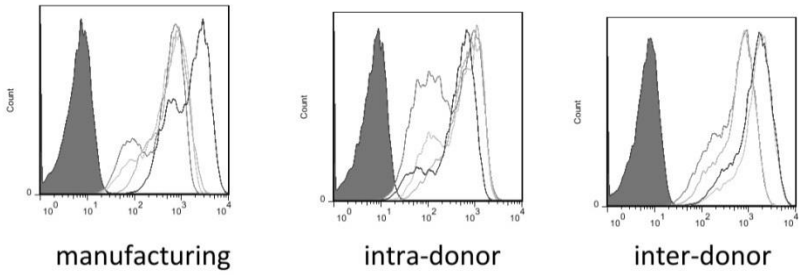

Figure 3.2 Variability of CD54 surface expression levels. Histograms of CD54 expression levels on LIg-DC evaluating manufacturing-related variability (n=5), intra-donor-related variability (n=5) and inter-donor-related variability (n=9). Histogram of isotype labeled cells is shown with a grey surface

3.2.1.4 Origin of LIg-DC variability. In order to determine if the observed LIg-DC variability was due to differences already present in the cellular starting material (i.e., the monocytes), or due to variability introduced during the manufacturing process, we evaluated the ICC values of the source material, monocytes, both for intra- and inter-donor variability. We assessed the variability of monocytes at the very beginning of the manufacture process (i.e., thawing and washing of the monocytes). As shown in Figure 3.5, the early steps of monocyte manufacturing had a lower impact on variability (ICC=0.955). Interestingly, we observed a lower value for intra-donor variability in the starting monocytes (ICC=0.938) than in the final product, mDCs, suggesting that *in vitro* culture decreases initial differences; whereas the inter-donor variability increased in LIg-DCs (ICC of monocytes was 0.939), indicating that differences due to genetic make-up increased during manufacturing.

3.2.1.5Role of sources of variation on single-gene expression levels. After characterizing the degree of consistency of the final cell product related to intra-donor, inter-donor and manufacturing factors using the entire gene expression data set, we focused on single gene variability in order to understand whether these three factors affect variability in expression of the same specific genes (i.e., whether the three factors might affect the same or different pathways/functions of the final cell product). To address this point, we calculated the assay-adjusted manufacturing, intra-donor and inter-donor variances for each gene (see Methods). We then ranked the genes according to variability and selected the most variable genes for each factor (one

percent or 344 genes per factor for a total of 877 genes due to some overlap among the three sets). As depicted on Figure 3.6, the three factors mainly affected different genes. However, it is important to note that 138 genes were present among the most variable genes of more than one factor (hypergeometric p-value < 10^{-10}). This result indicated that even if the three factors affect mostly different genes, a subset of genes showed a strong susceptibility to more than one factor.
Next, we checked whether the variability shown by these genes was already present in the starting monocytes. Monocyte manufacturing, intra-donor and inter-donor variances were calculated for each of the 877 genes. The analysis did not show any pre-existing differences in the monocytes (Figure 3.6c, d).

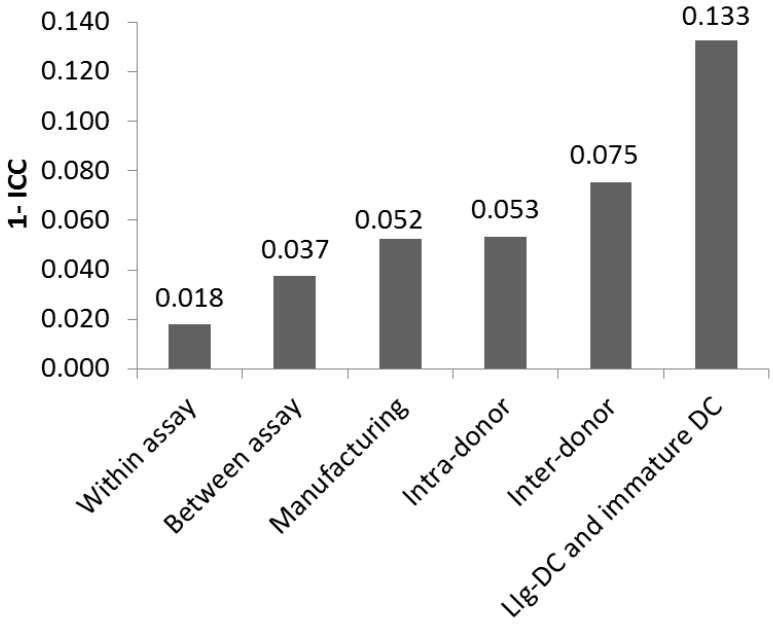

Figure 3.3 Intra-class Correlation Coefficient of gene expression profiles. Bar plot showing the 1-intra-class correlation coefficient calculated LIg-DC assessing the different sources of variability. “Within assay” and “Between assay” were included to evaluate the microarray-related confounding effect, whereas the “LIg-DC and immature DC” group was included to show the ICC value of a class showing high variability and well-known functional differences

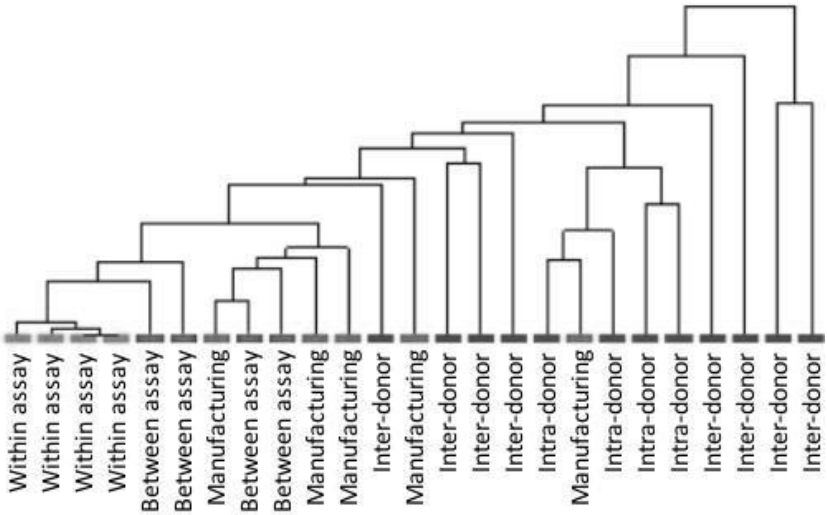

Figure 3.4 Unsupervised hierarchical clustering of LIg-DC gene expression profiles. Whole dataset was used to cluster LIg-DC samples. Pearson correlation was used to calculate distances.

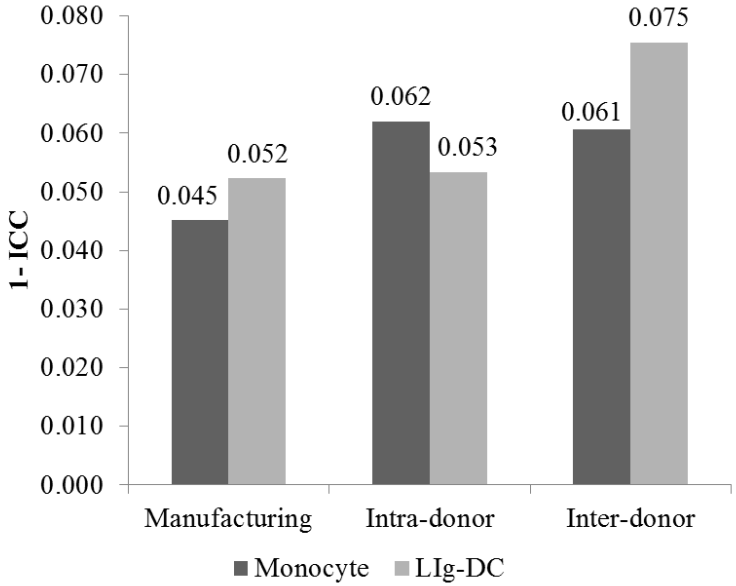

Figure 3.5 Comparison of Intra-class Correlation Coefficient of LIg-DC and monocytes. Bar plot showing the 1-intra-class correlation coefficient calculated onLIg-DC and starting monocytes assessing the different sources of variability

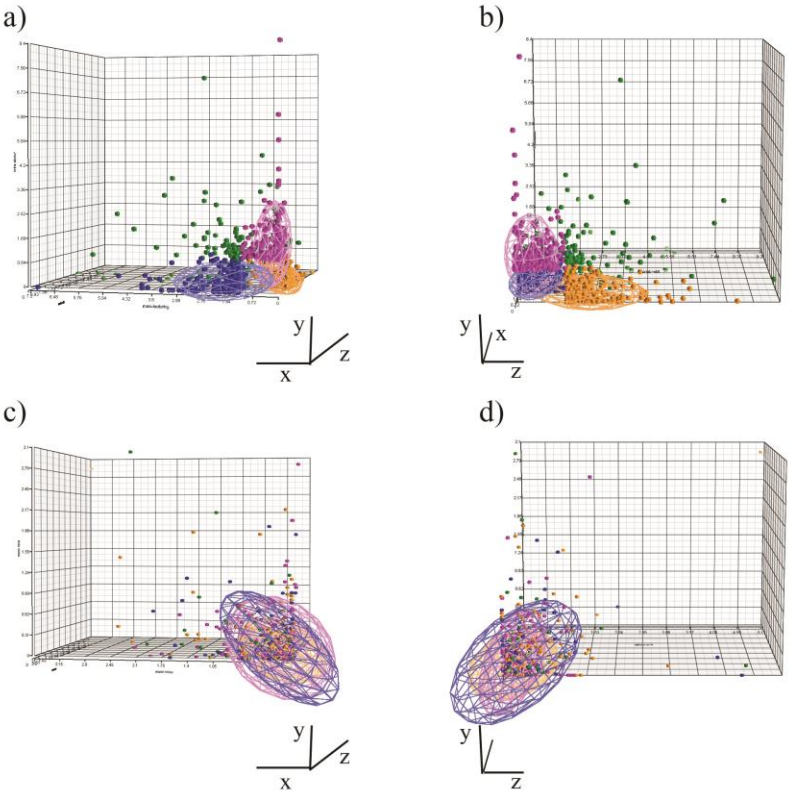

Figure 3.6 Genes with the greatest assay-adjusted manufacturing, intra-donor and inter-donor variability. (a) (b) Three-dimensional plots of the 877 genes whose expression was most variable in the DC gene expression data set in at least one factor. Each genes is represented according to its assay adjusted variances in the DC dataset: manufacturing related variability (x-axis), intra-donor related variability (z-axis) and inter-donor related variability (y-axis). Genes whose expression was most variable in more than one factor are represented in green. Genes most variable in LIg-DC manufacturing,intra-donor and inter-donor samples are shown in blue, purple, and orange, respectively. For each factor, ellipsoids are depicted to include 2 standard deviations from the mean value of each of the three factors. Each panel shows a different perspective.(c) (d) Three-dimensional plots of the assay adjusted variances of the same 877 genes in monocytes.

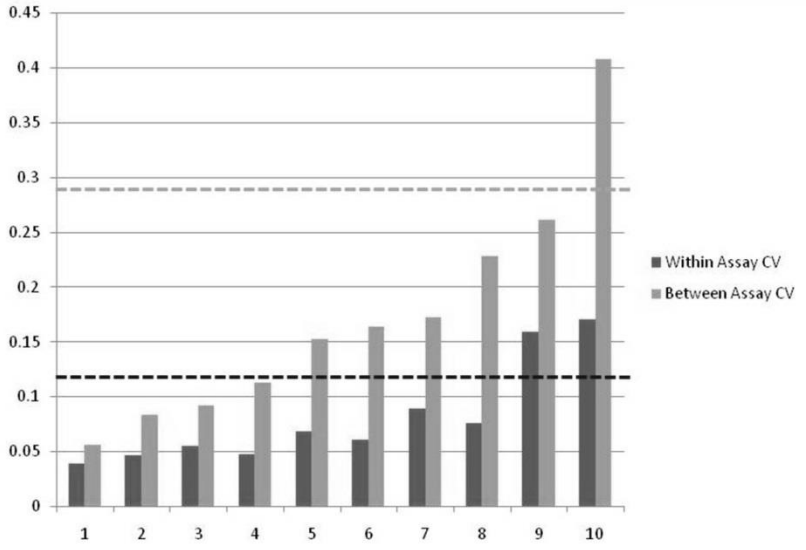

Figure 3.7CVs of the 323 genes grouped in deciles according to assay variance. Dark and light grey dotted lines indicate the threshold of 2*median CV of Within assay and Between assay samples, respectively

3.2.2 Identification of candidate markers for quality assurance of LIg-DC

3.2.2.1 Selection of quality assurance gene markers. After having defined global consistency/variability of LIg-DCs at both the whole transcriptome-level and single-gene level, we focused on candidate markers for quality assurance and quality control assessment. Ideal markers of cellular therapies must be precise and reliable, while detecting essential and distinctive features of the final product. Since DC maturation stimuli have a strong impact on the function of these cells and their gene expression profile(Castiello et al. 2011), reliable maturation-related markers are ideal candidates for assessing the identity and consistency and possibly the stability and potency of DC products at lot release. For this reason, the most critical markers of manufactured monocyte-derived mature DCs are those that indicate that maturation has progressed beyond the starting and intermediate material and thus their expression ensures the completeness of the manufacturing process. Therefore, we applied highly stringent statistical filters to our dataset: only probes that were induced in LIg-DCs versus both the starting monocytes (9 samples/class) and immature DCs (5 samples/class) with a *p*-value lower than 0.001, a false discovery rate lower than 0.005 and a fold-change greater than 5 were selected. A total of 323 probes passed the defined criteria. Then, even though as a whole the gene expression assay was found to be reliable, we evaluated whether for each gene the assay showed high repeatability. We estimated the median CV for each decile of these 323 genes according to assay variance and filtered out the tenth decile because both the within and between median CV exceeded by more than 2-fold the median CV of the whole gene list (Figure 3.7). The remaining 291 genes were studied further. A similarity matrix based on gene expression levels in mDCs clearly showed the existence of several gene correlation networks that might reflect different functional potentials of the manufactured product (Figure 3.8). To better define the characteristics of these genes, for each one we evaluated an index of variability (IV) calculated as the sum of the adjusted manufacturing, intra-donor and inter-donor variances of the gene (see Method)(Figure. 3.9).

3.2.2.2 Identification of candidate markers of consistency of LIg-DC. Considering the specificity of the 291 genes on the manufactured product, we hypothesized that those genes showing the least variability as assessed by the IV in our highly controlled manufacturing process would be the best consistency and identity markers. To select genes showing the lowest variability among all the products (i.e., having a similar level of expression independently of manufacturing, intra- and inter-donor variability), we selected the first decile of the 291 genes according to the IV index.

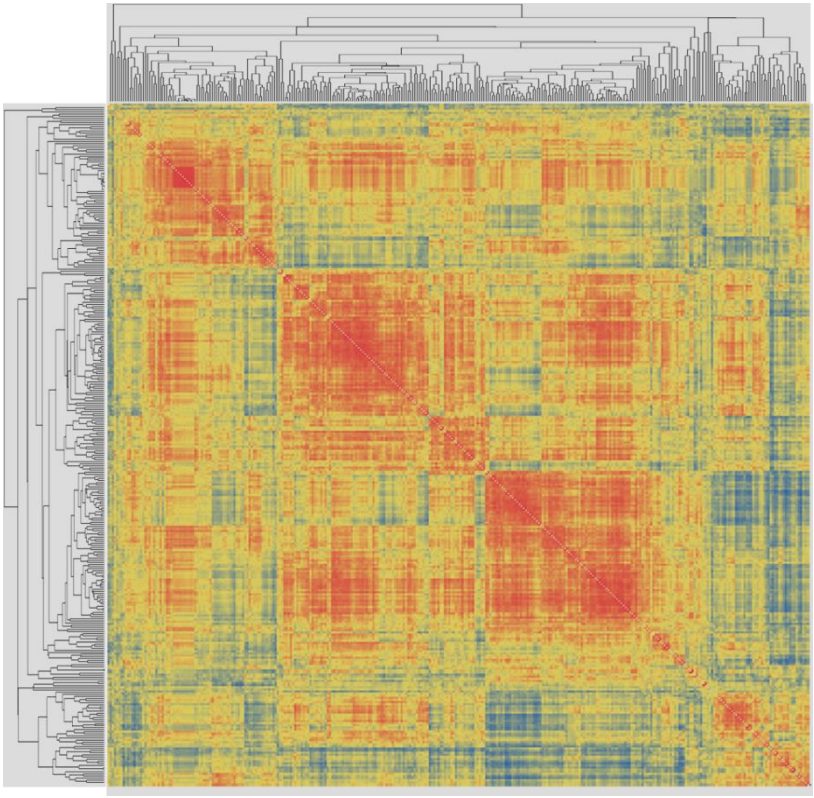

Figure 3.8 Existence of multiple gene networks among the 291 quality assurance candidate marker genes. Similarity matrix of the 291 genes induced reproducibly in LIg-DC compared to both monocytes and immature DC with p-value < 0.001, FDR < 0.005 and fold change > 5. Pearson correlation values were calculated based upon mDCs gene expression levels. The genes are sorted according to unsupervised clustering in order to reveal gene correlation networks in the LIg-DC

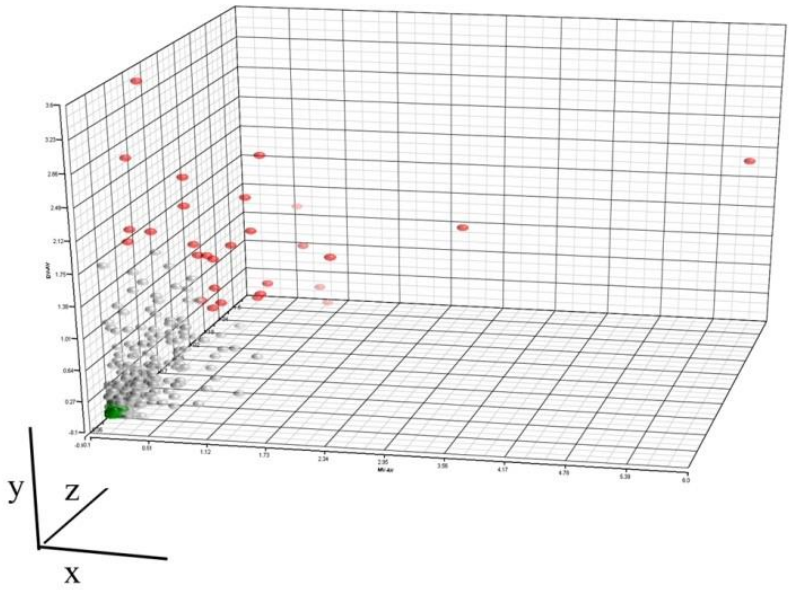

Figure 3.9 Manufacturing, intra-donor and inter-donor related variability of the 291 candidate marker genes. The 291 genes are plotted three-dimensionally according to the assay adjusted variances: manufacturing related variability (x-axis), intra-individual related variability (z-axis) and inter-individual related variability (y-axis). Genes included in the first decile according to the Index of Variability are represented in green, genes in the tenth decile in red and the others in grey.

Interestingly, most of these 29 potential marker genes have already been described as being induced in DCs by IFNγ or LPS, the maturation agents used in this study, such as AIM2, FEM1C, APOL1, NUB1, MAZ, DRAM1, AK4; or induced by both agents, like IFI27, WARS, PSME2 and ICAM1 (CD54). All of these potential markers encode for proteins belonging to inflammation or immune-related functional groups indicating a phenotype of the manufactured mDCs that could sustain a Th1 response once administered *in vivo*.

3.2.2.3 Identification of candidate markers of variability/potency of LIg-DC. By using the described experimental setting and computational approach we also selected genes that may be good markers of variability and possibly useful markers for stability and potency of LIg-DC products by simply focusing on the tenth decile of the 291 genes according to the IV index. Interestingly, CD80, CCL1, CCRL1, CD70 were among these genes. CD80 is a costimulatory protein essential for T cell activation. CCL1 is a chemokine that attracts several immune cells by interacting with CCR8(Gombert et al. 2005). CCRL1 binds the chemokines CCL19, CCL21, and CCL25 all of which play a fundamental role in lymphnode homing of DCs(Comerford et al. 2006). CD70 has been reported to play a critical role in the immunogenicity of CD40-independent, CD4+ T cell-dependent CD8+ T cell response(Van Deusen et al. 2010). Of particular note is the observation that although most of the other highly variable mDC induced genes that encode for proteins that have not been reported to play a key role in DC function, the expression of most of these genes clearly correlate (positively or negatively) with the level of mDC secretion of 14 functionally important cytokines (Figure 3.10). This feature makes these genes possible candidates as surrogate markers of the secretion of LIg-DC key cytokines and indirectly LIg-DC phenotype and function.

3.2.2.4 Characteristics of candidate markers are conserved in an independent clinical LIg-DC product dataset. To assess the robustness of our findings, we tested the identified potential quality assurance marker genes in a different dataset obtained from the transcriptional profiling of 80 LIg-DC samples manufactured for the clinical trial NCI-09-C-0139. The autologous LIg-DC products were manufactured from 14 patients and for each patient a median of 6 different products were manufactured and administered. An aliquot from each of these products was saved and tested. For these 80 products, IV was calculated for the 291 identified as potential markers for quality assurance, based on the same principles used for the analysis of the initial LIg-DC samples (see Methods). As clearly depicted in Figure 3.11, the features previously observed were confirmed in the clinical data set,

suggesting the potential relevance of the 29 most and least variable markers for quality assurance analysis.

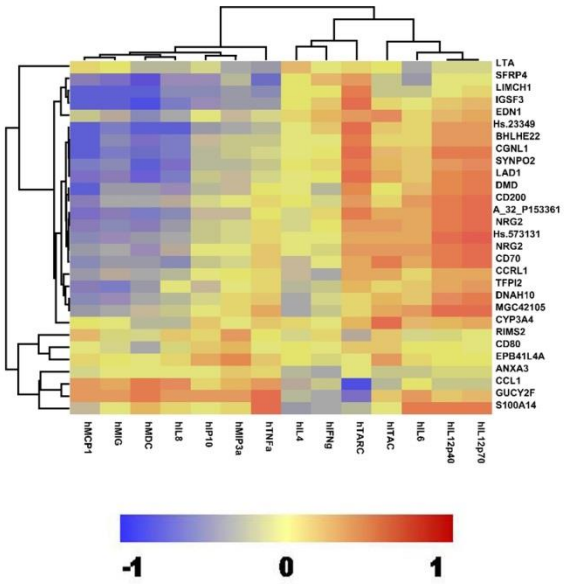

Figure 3.10 Expression levels of candidate markers of variability/potency of LIg-DC correlate with secretion levels of key cytokines. Pearson correlations between the level of expression of genes in the tenth decile of the index of variability and the concentrations of selected cytokines measured in the culture media. Both genes and cytokines are ordered according to unsupervised hierarchical clustering

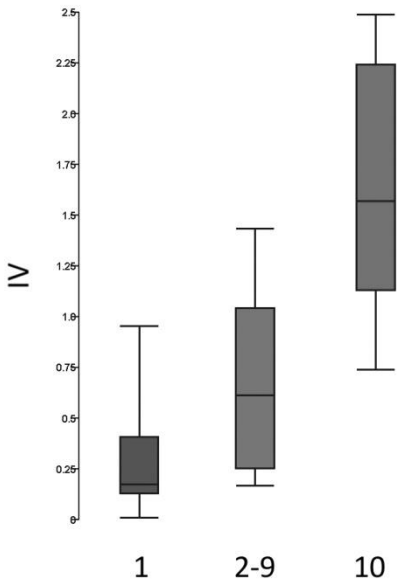

Figure 3.11Analysis of the variability of expression of the 291 candidate LIg-DC markers in a clinical dataset. The expression of 291 candidate genes were measured in a clinical dataset made up of 80 different LIg-DC products (14 patients, between 2 and 8 products were manufactured for each patient). The Index of Variability was calculated for each gene. The genes were grouped in deciles according to their Index of Variability calculated in the initial LIg-DC dataset. The boxes indicate the 25% and 75% percentiles, and whiskers indicate the 10% and 90% percentiles.

3.3 Discussion

Although preclinical and early clinical studies of DC-based therapies have been highly promising, several issues have hindered their translation into clinic. In particular, quality control of cellular products, an essential step to assess identity, consistency, stability and potency, has been one of the major stumbling blocks for the scale up and out of these therapies. Without high quality markers, it is difficult to establish and implement manufacture processes for moving products from phase I/II studies to phase III clinical trials and licensure. Testing cellular therapy products using the same methods and standards applied to conventional drugs is not possible for several reasons including their biological complexity, short shelf life, the timing of complex assays and the difficulty associated with implementing an effective

assay. The identification of the factors that affect cell therapy product consistency is essential for the discovery of markers reflecting relevant changes in the final cell product (Hinz et al. 2006). In this study, analyzing LIg-DC we developed a method for characterizing factors affecting product consistency that may be useful for identity, consistency, stability and potency testing. Also, we proved the importance of in-depth testing of DC vaccines by gene expression as a tool to identify phenotypic differences among different DC preparations in order to select candidate markers for advanced quality assurance of DC.

Our main goal was to define factors affecting LIg-DC consistency and identify candidate gene markers to use for quality control. Considering the complexity of events related to the mode of action of DC; microarray technology provides an effective tool for large scale gene expression profiling of cells and tissue, allowing the simultaneous measurement of thousands of genes and therefore capturing a snap shot of all possible molecular markers associated with cellular function both expected (known) and unexpected (unknown). Traditional analytical assays, such as flow cytometry immunophenotyping and ELISA, have a lower power in discovering such global signatures, given their a priori selection of a limited number of factors to be tested. Other major advantages offered by gene expression microarray techniques are the small number of cells needed, often a limiting factor when cells are manufactured for autologous use, and the potential to use either fresh or cryopreserved material. Major disadvantages are the still relatively high cost of high through-put technologies and time needed to complete the assays, precluding their use for in process and lot release testing.

Using global gene expression profiling, we were able to characterize the magnitude of variability introduced into LIg-DC by intra-donor and inter-donor differences and by manufacturing and determined how these three major factors affected DC consistency. Each of these factors provides useful information related to cell manufacturing. Low manufacturing consistency suggests that the manufacturing process includes critical steps that need additional optimization. Low intra-donor consistency indicates the existence of differences in the starting material and more comprehensive testing of the starting material should be considered. Low inter-donor consistency suggests that the genetic makeup of the cell donors affects the final products and supports the search for genetic factors contributing to the consistency of the final products. Our analysis of LIg-DC suggests that manufacturing and intra-donor variability affected the final products less than inter-donor factors. Such information is essential for correctly evaluating the existence of correlations between DC properties and clinical or immunological results derived from clinical studies using cells manufactured with the same

protocol. Monocyte phenotype may be used to predict DC phenotype. Others have also shown that monocyte-derived DC from healthy donors differ from those derived from patients with colorectal cancer, non-small-cell-lung-cancer (Kvistborg et al. 2009), systemic lupus erythematosis(Decker et al. 2006), Chagas disease (Cuellar et al. 2008)and allergies (van den Heuvel et al. 1998). Although this phenomenon has been known for more than 10 years, a complete understanding of the reasons for these differences is lacking. Here we showed that at least part of the variability in the final products could be traced back on monocytes strengthening the hypothesis that final DC potentials can be predicted by studying the phenotype of monocytes. However, our data indicates that two confounding factors should be considered: on one hand that even under highly standardized procedures manufacturing may introduce variation in final product consistency, while on the other hand that intra-donor variability observed in monocytes could diminish during processing. While our observations are based on the analysis of cells obtained from healthy subjects, it has to be noted that greater differences in starting material and final products are possible when our approach is applied to clinical samples from heavily pretreated cancer patients.

We found the consistency of expression of donor genes is affected differently by manufacturing, intra-donor and inter-donor variables. Although we observed a statistical significant number of overlapping genes among the most variable genes for each factor, this represented a relatively small subset and each factor mainly perturbed a different set of genes, indicating that functions of the final LIg-DC products are affected dissimilarly by manufacturing, intra-donor and inter-donor variability. However, in order to define the specific functions that may be affected by each factor, further studies are needed to construct models of manufacturing and intra-donor variability based on more than one single donor. Such an approach may be able to unravel the degree of variability that could normally be expected for each specific important cell function and consequently to set parameters for determining when the final production quality is low and it would be worthwhile repeating the entire collection and manufacturing process to produce a more potent DC.

By applying highly stringent statistical filters to the gene expression data to select markers induced in the final product, LIg-DC, but not in an intermediate, immature DC, or in the starting monocytes, we identified potential markers for final product identity, consistency and potency testing according to their index of variability. Although LIg-DC identity can be assured by the analysis of the expression of classical DC markers, such as CD80, CD86, CD83, using flow cytometry this analysis is of limited usefulness considering that these markers are expressed by DC having

different phenotypes. This observation suggests that new and more specific DC markers are needed to better assess quality of the final cellular product. In particular, since cells have more than a single critical function and since multiple markers may be required to assess some functions, it is likely that a panel of markers is needed to quality control assessment. We focused on genes specifically induced during maturation by comparing LIg-DC with both monocytes and immature DC. The genes strongly and reproducibly differentially expressed in LIg-DC were further categorized according to their IV.

Among the donor genes that are highly expressed in LIg-DCs, those whose expression showed the least variability should be good markers for identity testing since they are effected least by donor and manufacturing factors. Most of the 29 potential markers for identify testing were DC genes already known to be induced by IFN-gamma and LPS. All of the proteins in this group encoded inflammation or immune-related genes. One of these genes is already being used as a quality control marker, CD54. The protein encoded by ICAM1 (CD54) is a ligand for the leukocyte integrin complex CD11a/CD18 (LFA-1) that strengthens immune cross-talk (Carrasco et al. 2004)and, because it indicates antigen presenting cell (APC) activation, its protein expression has been selected for potency testing of the APC based vaccine Provenge (Sipuleucel-T) – the only cellular immunotherapy approved by the FDA for clinical use (Sheikh & Jones 2008). While the genes we identified may be good candidate markers, their usefulness must be tested by other comparability studies and functional testing.

Among the 29 highly variable genes were some factors that are likely to be functional important for LIg-DC. Furthermore, the expression of many of the 29 highly variable genes correlated with the levels of several cytokines and chemokines in the LIg-DC supernatant, In particular, the secretion of IL-12 is considered essential for the induction of a desirable Th1 immune activation (Trinchieri 2003). Similarly, the induction of chemokines capable of attracting Th1 cells (e.g., MDC, MIG and IP10) is considered critical for DC effectiveness for cancer immunotherapy (Lebre et al. 2005). This feature suggests that these genes may reflect mDCs function and might be potential markers of LIg-DC consistency and potency.

In conclusion, although specific studies will be needed to clearly define newer biomarkers, the approach described proves the feasibility of gene-expression-profile based characterization to address essential information on the nature of the sources and factors affecting the consistency of cellular based immunotherapies. Moreover, by studying the level of variability of a selected group of highly induced genes, new candidate markers can be detected for the assessment of identity, stability comparability and possibly potency. Although other gene-expression characteristics (e.g., the kurtosis

and the skew of expression levels) might reveal features impacting the function of single products, these features can only be truly detected by correlation with in vivo evidence.

4. IDENTIFICATION OF CANDIDATE BIOMARKERS OF LIg-DC EFFICACY IN PROSTATE CARCINOMA PATIENTS

4.1 Introduction

Dendritic cells (DC) are potent antigen presenting cells that are able to activate both innate and adaptive arms of the immune system (Ueno et al. 2010). For this reason DC-based vaccines represent a promising immunotherapeutic approach in several clinical settings. In fact, since the discovery of monocyte differentiation into DC (Sallusto & Lanzavecchia 1994), over 300 clinical trials have been conducted which have proven the feasibility and safety of DC vaccines (Castiello et al. 2011).However, despite extensive preclinical and clinical studies, very few clinical trials have demonstrated the desired clinical efficacy. For the majority of trials, the overall response rates have been well below 20%. Many reasons have been hypothesized for such low response rates, among which the generation of DC with suboptimal potency is considered the most relevant. It's not known yet how to generate the most potent DC; furthermore, differences in clinical setting, study design, sources of antigens, and route of administration make it almost impossible to compare results from previously conducted trials in order to clearly delineate the shared determinants of in vivo efficacy of DC-based vaccines.

Compared to drug therapy, cell therapies are more challenging because of their considerable lot-to-lot and patient-specific variability that in most cases has yet to be sufficiently quantified and characterized (Stroncek et al. 2010). In fact, while each cell therapy lot has to be tested for identity, consistency and potency among other tests, feasibility issues dictate these tests to be focused on a handful of factors and, therefore, they cannot assure an exhaustive characterization of each lot. An accurate characterization of DC should ideally assay all the factors affecting their in vivo biological functions: antigen processing and presentation, expression of co-stimulatory signals, absence or reduced expression of co-inhibitory signals, lymph node migration, and secretion of activating cytokines and chemokines. These are all essential features of potent DC and should be thoroughly tested. Since it is impossible to routinely evaluate each product for every cell function using cellular assays, the identification of reliable biomarkers of identity, consistency and potency of cell therapies is highly encouraged by regulatory agencies beginning in the earliest phases of clinical development of the cellular product (Hinz et al. 2006; Vatsan et al. 2013).

Many factors are known to play a key role in DC-induced activation of the immune system. Secretion of interleukin-12 (IL-12) is considered the most

important driving factor for Th1 inflammatory T cell activation. Surface expression of CCR7 is necessary for DC migration into lymph nodes and expression of co-stimulatory molecules (i.e., CD80 and CD86) is essential for the activation of T cells (Kalinski et al. 2009). On the other hand, several detrimental factors have also been identified. Secretion of IL-10 is considered a hallmark of tolerogenic activities exerted by DC (Vieira et al. 2000; Kalinski et al. 2009). Similarly, the maintenance of immature/monocytic factors (e.g., CD14) are also known to be characteristic of tolerogenic DC (Chitta et al. 2008; Torres-Aguilar et al. 2010).Even though these and many other molecular factors have been characterized thoroughly for their role in DC function and many of them have been used to discriminate among DC produced using different differentiation/maturation procedures, it has rarely been determined how these factors are differentially expressed among DC manufactured using the same differentiation/maturation procedure and whether such difference has a functional relevance.

In the previous part of this project, we have shown that even when highly standardized procedures are used to generate monocyte-derived LIg-DC, manufacturing, intra-donor and inter-donor related factors may affect DC phenotype (Castiello et al. 2013). In particular, we observed that while most of the well-known and usually tested DC markers (e.g., CD80, CD86, CD83, HLA-DR) did not show any differences in expression among LIg-DC generated at different times from different donors, the expression of several genes and the levels of several key secreted cytokines and chemokines showed significant variability among LIg-DC products. However, whether such lot-to-lot variability affects the identity, potency and/or efficacy of LIg-DC-based vaccines used in human clinical trials has yet to be determined.

NCI-09-C-0139 (NCT00908258) is a randomized, prospective, pilot study of vaccination with a mixture of wild type (TARP27-35) and epitope-enhanced (TARP29-37-9V) T cell receptor gamma chain alternative reading frame protein (TARP) peptides in HLA-A*0201 patients with stage D0 prostate cancer (no evidence of visceral or bone metastasis with persistently elevated or rising PSA levels i.e. biochemical progression) and at increased risk for disease progression based on PSA doubling time (PSADT) (Wood et al., 2014 Submitted to Science Translational Medicine). TARP is a tumor-associated antigen expressed in over 90% of prostate and 50% of breast carcinomas (Epel et al. 2008). The study compared two vaccination regimens: in one TARP peptides were admixed with Montanide ISA 51 VG plus Sargramostim to generate a peptide emulsion administered by deep subcutaneous injection; in the other, TARP peptide-pulsed autologous LIg-DC were administered intradermally. TARP vaccines were administered every three weeks at weeks 3, 6, 9, 12, and 15 as part of the primary vaccination series, with a conditional sixth booster at week 36 dependent on

documented immunologic and/or clinical responses at week 24. The original 48 week study design was amended and extended to subsequently allow seventh and/or eighth booster doses of vaccine at weeks 48 and 96 after initial immunologic and clinical activity of TARP vaccination was documented.
In the current study we analyzed 114 peptide-pulsed LIg-DC preparations manufactured to vaccinate 18 patients randomized to the autologous TARP peptide-pulsed DC arm in order to understand which factors are affected by lot-to-lot variability in clinical GMP manufactured DC and whether such variability has an impact on DC identity, potency and efficacy. By analyzing DC surface marker expression, gene expression profiles, protein secretion profiles and culture data, we observed the existence of a tolerogenic DC signature that was negatively correlated with the development of clinical and immunological response.

4.2 Results

4.2.1 LIg-DC induced clinical and immunological response in prostate carcinoma patients. Each LIg-DC vaccine was manufactured starting from one aliquot of autologous cryopreserved monocytes cultured for 3 days with GM-CSF and IL-4. On day 2, Keyhole Limpet Hemocyanin (KLH) was added to the culture as a control antigen. On day 3 cells were matured for additional 24 hourswith LPS and IFN-gamma and then pulsed for 2 hours with WT and EE peptides.
13 out of 16 evaluable patients were considered to have achieved clinical response (decrease in slope log PSA) at week 48 (two additional patients completed the treatments but their week 48 clinical responses were not included as a result of the data analysis cut-off date for the dataset).The development of TARP-specific immune response (assessed by IFN-gamma ELISPOT) was observed in 10 out of 18 evaluable patients (ELISPOT was performed on patient samples taken at baseline and weeks 12, 18 and 24 after vaccination). Immune activation against control antigen KLH was observed in the majority of patients (15 out of 16 subjects in whom KLH reactivity was assessed). Clinical (change in slope log PSA) and immunological responses were assessed both qualitatively (i.e., in term of Responder or Non responder)and quantitatively (Table 4.1). Interestingly, slope log PSA responses were observed almost independently of TARP-specific T cell responses, however, a strong immunological response (defined as a median ELISPOT reading greater than 500) was observed only in patients with a notable decrease in slope (log PSA) (i.e. equivalent to a lengthening in PSADT and considered to be a stronger clinical response).

Table 4.1. Clinical and immunological patient responses

ID	Week 24 clinical response	ΔlogPSA 24week-0	Week 48 clinical response	ΔlogPSA 48week-0	Immuno-logical response	Median ELISPOT count[b]	Immuno-logical response to KLH
201	Yes	-0.172	Yes	-0.093	No	0	No
202	Yes	-0.017	Yes	-0.011	Yes	210	Yes
203	No	0.144	Off Study		Yes	30	Yes
204	No	0.039	No	0.066	No	50	Yes
205	Yes	-0.148	Yes	-0.178	Yes	1180	Yes
206	No	0.036	No	0.03	Yes	100	Yes
207	Yes	-0.002	Yes	-0.008	No	0	Yes
208	Yes	-0.054	Yes	-0.026	No	0	Yes
209	Yes	-0.018	Yes	-0.119	No	0	ND
210	Yes	-0.052	Yes	-0.038	No	0	Yes
211	Yes	-0.087	Yes	-0.113	Yes	710	Yes
212	Yes	-0.002	Yes	-0.041	No	0	ND
214	Yes	-0.028	Yes	-0.049	Yes	4480	Yes
215	Yes	-0.103	Yes	-0.066	Yes	4370	Yes
216	Yes	-0.013	Yes	-0.083	Yes	230	Yes
217	Yes	-0.028	Yes	-0.008	No	0	Yes
218	Yes	-0.025	/	/	Yes	50	Yes
220	Yes	-0.07	/	/	Yes	90	Yes

[*]Absence or presence of a clinical response was defined as having negative difference in the slope log PSA at either 24 or 48 weeks minus the pre-treatment slope log PSA.
[a]Intensity of clinical response was calculated as the difference in slope of PSA trend over time observed at time of analysis compared pretreatment value (e.g., log PSA slope at week 24 –log PSA slope before treatment)
[b]Intensity of immunological response was calculated as the median # of spots observed through 7d *in vitro* stimulation ELISPOT against wild type 27-35, epitope enhanced 29-37-9V and wild type 29-37 TARP peptides tested at week 12, 18 and 24
/ Not Available
ND: not done

4.2.2 LIg-DC showed high level of variability on CD14 and CCR7 surface expression. Phenotypically all lots of DC products were positive for CD80, CD83, CD86, CD123. CD11c CD38, CD54, HLA-DR (all > 95%) by flow cytometry (Figure 4.1). The markers showing significant degrees of variability among DC products were CD14 (ranging from 14% to 90% CD14+) and CCR7 (ranging from 5% to 90%). This variability was dependent on both manufacturing and inter-patient factors, but only for CD14 the inter-patient variability was substantially greater than manufacturing variability (Figure 4.2). Interestingly, when we analyzed DC for differential expression among those from patients that achieved a decreasing slope log PSA clinical response (RespDC)versus those from patients that did not (NonRespDC), we observed a trend with RespDC expressing higher levels of CCR7 and lower levels of CD14 compared to NonRespDC (not statistically significant).To analyze how CCR7 or CD14 levels were able to discriminate RespDC vs NonRespDC we used receiver operating characteristic (ROC) analysis. The underlying assumption of ROC analysis is that a variable under study (e.g. % of CCR7+ DC) is used to discriminate between two mutually exclusive states (i.e., RespDC vs NonRespDC). For these analyses, ROC curve represents an easy visualization tool because it illustrates the performance of the variable under study by plotting specificity vs sensitivity of the test for each possible cut-off; and the area under the curve (AUC) summarizes the overall ROC curve and can be considered as a summary statistic of its ability to classify cases correctly. A perfect test would have an AUC of 100%; a worthless test would have an AUC of 50%. According to an arbitrary guideline AUC values may be classified as follows: 90%–100%, excellent; 80%–90%, good; 70%–80%, fair; 60%–70%, poor; 50%–60%, fail (Hanley & McNeil 1982).

When qualitative clinical responses were evaluated by ROC curves both factors led to an Area Under the Curve (AUC)of 76.3% based on percent of CD14+ cells and of 69.6% based on percent of CCR7+ cells (Figure 4.3).

In addition to phenotypic expression of surface markers we also analyzed cell culture data and noticed a great variability in final product viabilityand DC yield (i.e., the percentage of initial cells that were recovered at the end of DC manufacture), respectively ranging between 37% and 91% and between 6% and 48%. For these factors the sources of variability were also traced back to both manufacturing and inter-patient differences (Figure 4.2). A non-random distribution was also observed for these factors between RespDC and NonRespDC, but with a much lower relevance (AUC based onDC viability was 60.8% and the AUC based on DC yield was 61%)(Figure 4.3).All together these data indicate that lot-to-lot variability can be observed in clinical DC products and that inter-patient variability might be responsible forphenotypic differences among RespDC and NonRespDC.

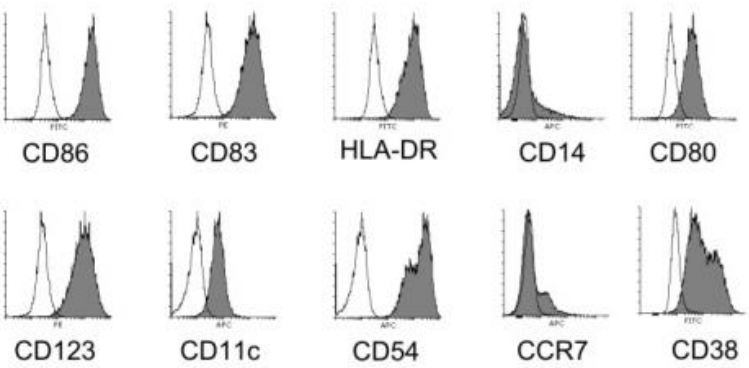

Figure 4.1 Flow Cytometry Analysis of LIg-DC. Histograms of the expression of surface markers CD86, CD83, HLA-DR, CD14, CD80, CD123, CD11c, CD54, CCR7, CD38 of a representative DC product;

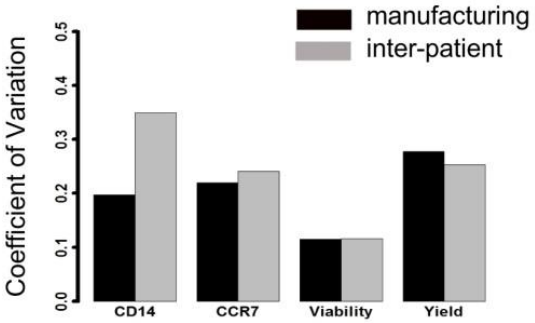

Figure 4.2 Coefficients of Variation(CV) of % of CD14+, % CCR7+, % of viable cells and final DC Yields CV were calculated for manufacturing (black bars) and inter-patient variability (light grey bars) among all manufactured DC. Manufacturing related CV was calculated as the average CV registered among all the DC generated from each patient, whereas inter-patient CV was calculated on patients averaged values;

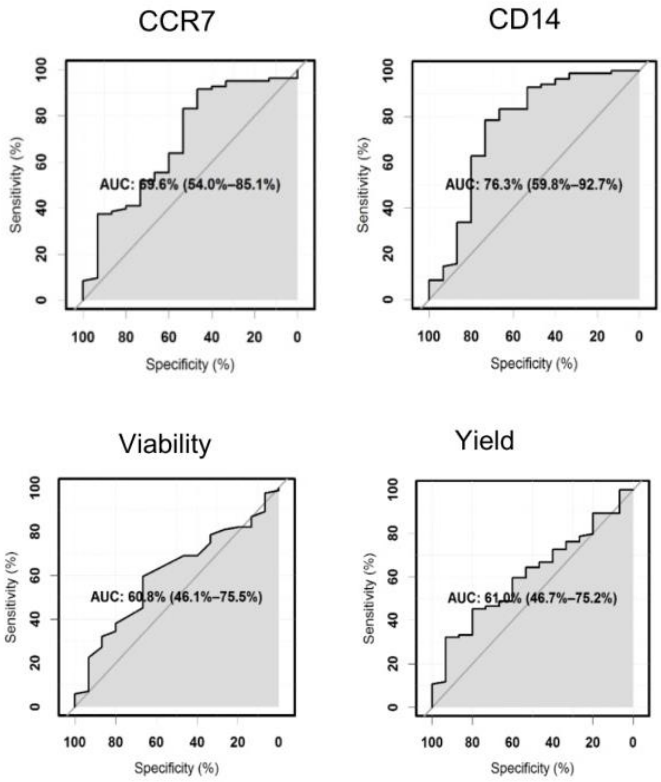

Figure 4.3 ROC curves of CD14, CCR7, viability and yield on clinical response. ROC curves of showing the power of % of CD14+, % CCR7+, % of viable cells, and final DC Yields to discriminate among RespDC and NonRespDC. In a ROC curve plot, the "true positive" diagnosis rate (sensitivity) is plotted against the "false positive" diagnosis rate (1-specificity) for a test with a binary outcome. The AUC summarizes the discrimination of the test, i.e., its ability to classify cases correctly. A perfect test would have an AUC of 100%; a worthless test would have an AUC of 50%. AUC values may be classified as follows: 90%–100%, excellent; 80%–90%, good; 70%–80%, fair; 60%–70%, poor; 50%–60%, fail(Hanley & McNeil 1982)

4.2.3 DC transcriptomes clustered according to patient. Next, we analyzed gene expression profiles of 99 DC vaccine products derived from the 18 patients who received at least 5 vaccines using microarray technology. Unsupervised hierarchical clustering analysis grouped the DC products according to patient (Figure 4.4), confirming the prominent role of inter-patient variability in affecting DC lot-to-lot variability shown in our previous

report (Castiello et al. 2013). In addition, the node analysis of the unsupervised hierarchical clustering did not show the existence of separated subclusters but rather indicated that the DC products were spread on continuum levels of variability as indicated by the fact that except for a few outliers samples, the vast majority of DC showed similar inter-patient distances. Similar observations were obtained using principal component analysis (PCA) of the whole dataset and through Davies-Bouldin Index testing on partitioning the dataset into defined numbers of groups (not shown). All together these analyses suggested that clinical DC products show inter-patient differences that cannot be easily grouped into well-defined phenotypes. In particular, in both clustering and PCA analysis RespDC were not separated from NonRespDC, pointing to the fact that differences between RespDC and NonRespDC, if they exist, are hidden by inter-patient variability.

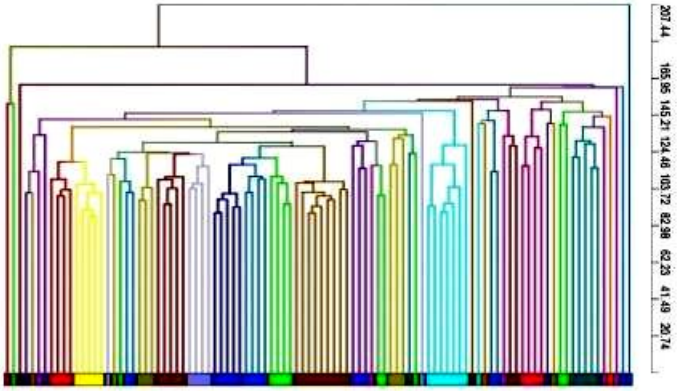

Figure 4.4 Unsupervised cluster of gene expression data. DC products (n=99) were analyzed by gene expression profiling using Agilent microarrays. Unsupervised hierarchical clustering of the DC based on the whole dataset (35753 genes). Branches are colored according to patient

4.2.4 Class comparison failed showing differences between RespDC and NonRespDC. After observing that unsupervised analysis were not able to separate RespDC from NonRespDC, we tested whether statistical analysis was able to identify gene expression signatures that distinguish the two groups. A direct class comparison of RespDC versus NonRespDC revealed the presence of only 55 statistically differentially expressed genes(p-value <

0.001). Such a low number of significant genes (false discovery rate was equal to 65%) pointed to the fact that RespDC and NonRespDC do not represent two strictly different classes. In our opinion, these results could be the consequence of three different possibilities: i) RespDC and NonRespDC do not have any differences, ii) given the heterogeneity in the magnitude of responses, only DC with strong clinical responses should be compared to NonRespDC, or iii) responses could be the consequence of multiple factors and therefore DC cannot be simply grouped into classes based on response, but have to be characterized for their inter-patient variability first. Regarding the first hypothesis, surface marker expressions and culture data clearly pointed to the existence of some difference between RespDC and NonRespDC, therefore we decided to test the other two hypothesis.
In order to evaluate the second hypothesis, we decided to tailor our analysis using more restrictive clinical and immunological response criteria, but even in this case we did not observe a significant number of genes differentially expressed among DC from patients showing a strong clinical and/or immunological response compared to NonRespDC. For example, when we used only immunological response to discriminate DC only 3 genes were differentially expressed with a p-value < 0.001.
Altogether, and in line with unsupervised clustering analysis, these data suggested that to delineate differences between RespDC and NonRespDC more complex models must be implemented in order to unbiasedly analyze inter-patient variability.

4.2.5 Weighted Gene Coexpression Analysis revealed the presence of 8 modules in DC. To characterize the inter-patient variability without any a priori assumption, we applied to our dataset the weighted gene coexpression analysis (WGCNA)in order to identify modules of genes that are coexpressed (i.e., whose expression changes similarly among different samples) and thereforeshould bestrongly representative of inter-patientvariability (12).WGCNA revealed the existence of 8 modules that were differentially expressed among the DC in our dataset(Figure 4.5).Modules were labeled numerically in decreasing order (i.e., Module 1 being the one made of the highest number of genes). To dissect the characteristics of the eight modules and define whether the modules reflect manufacture-related variability or inter-patient variability, we calculated the manufacturing and inter-patient standard deviations for each module. As shown in Figure 4.6 while module 1 and 8 clearly showed a low level of inter-patient variability, the other modules showed a much higher degree of inter-patient variability indicating that differences in the expression levels of these modules exist among patient DC(Figure 4.6 and 4.7). Interestingly, modules 2, 3 and 7 showed somewhat low manufacturing related variability, suggesting that levels of expression of

these modules are mainly affected by manufacturing unrelated factors (Figure 4.6). On the other hand, modules 4, 5 and 6 were characterized by manufacturing-related variability levels comparable to inter-patient variability, indicating that genes belonging to these modules were more susceptible to manufacture-related variability.

4.2.6 Low expression of module 2 genes correlated with clinical and strong immunological responses. We then analyzed modules for their differential expression among RespDC and NonRespDC. Notably,only module 2 showed a statistically significant correlation with clinical response (r = 0.5278, p-value = 0.035, Figure 4.8), therefore suggesting that expression level of genes belonging to module 2 may play a role in clinical response. In particular, when we analyzed the expression of module 2 among different patient DC we observed that while up-regulation of module 2 led to mixed clinical responses, down-regulation of module 2 was strongly associated with clinical and immunological responses (Chi-square p-value = 0.008829, Figure 4.9). Next we evaluated module 2 expression for its predictive value for clinical response and through a ROC curve we obtained an AUC of 85.5% (Figure 4.10). However, when tested as a predictor of strong immunological response, module 2 led to an almost perfect prediction with an AUC of 97.9%. All together these data indicate that lower expression of module 2was correlated with more potent DC vaccines that resulted in strong immunological and clinical responses, even though clinical responses were observed even in patients that received DC expressing high levels of module 2.

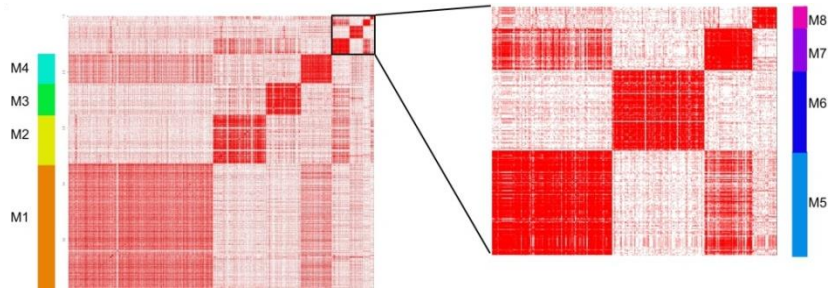

Figure 4.5 WGCNA modules in LIg-DC. Similarity matrix analysis of the 1864 genes belonging to the 8 modules identified by WGCNA. On the right, magnification of the top-right corner of the matrix to show less abundant modules. Similarity matrix is on a white-to-red gradient, where white represents a correlation equal to 0, whereas red is 1

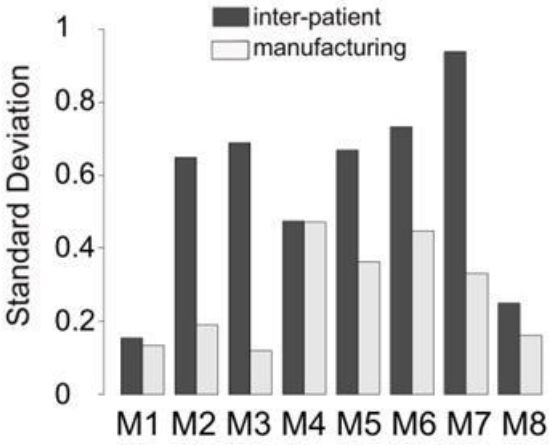

Figure 4.6 Manufacturing and inter-patient variability of the expression levels of the 8 modules in clinical DC. For each module, the standard deviation of module expression is shown for both inter-patient (black bars) and manufacturing variability (grey bars). Manufacturing variability was calculated as the average standard deviation registered among all the DC generated from each patient, whereas inter-patient variability was calculated on patients averaged values

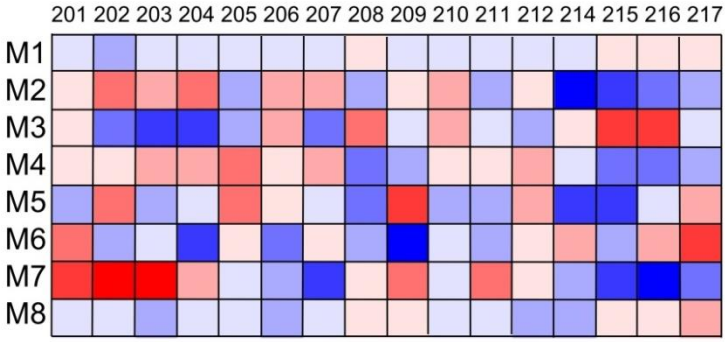

Figure 4.7 Average expression levels of the 8 identified modules in the DC of each patient. The heatmap is shown on a Blue-White-Red Gradient, where blue represents an expression level below the average, white is an average expression level and red represents an expression above the average

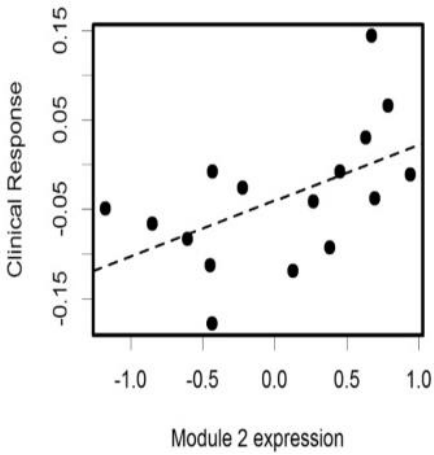

Figure 4.8 Module 2 expression correlated with clinical response. Plot showing the correlation of module 2 expression in DC and the quantitative measure of clinical response at week 48 (measured as the decrease in slope (log PSA) over time)

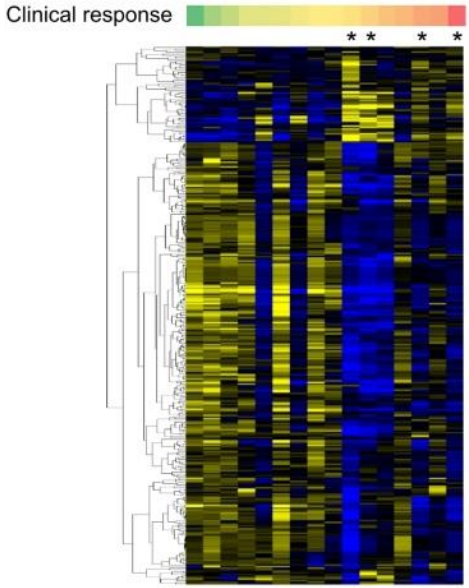

Figure 4.9 Heatmap of the patient-averaged expression level of the genes belonging to module 2. Each column represents the average value observed among the DC manufactured from the same patient. Columns are ordered according to the quantitative measure of clinical response at week 48 with non-responders on left shown by the color bar on the top of the heatmap (green: no response, yellow: mild response, red: strong response). * indicates DC of patients showing strong immunological response (median ELISPOT count >500). The top gene cluster of the heatmap shows genes more expressed in Resp-DC whereas the lower cluster shows genes more expressed in NonResp-DC.

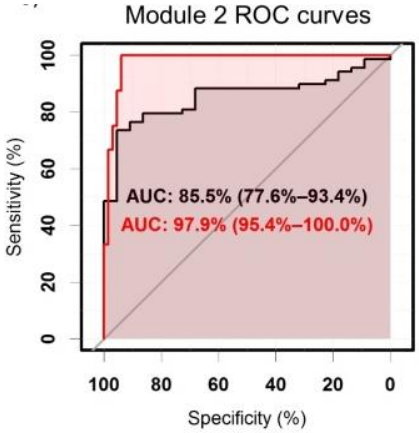

Figure 4.10 ROC curves of module 2 expression and clinical and immunological response. ROC curves showing the ability of module 2 expression on DC to discriminate among clinical and non-clinical responders in black and strong immunological responders vs non-strong immunological responders in red

4.2.7 Module 2 was a tolerogenic DC module. To characterize module 2 genes we performed a gene ontology (GO) analysis and among the most over-represented "biological process" GO families we observed: immune response, chemotaxis, and endocytosis (not shown). In particular, network analysis revealed that the dominant module 2 factors were CD14, IL-10, thrombospondin, estrogen receptor 1, Insulin-like growth factor-binding protein 4, and hepatocyte growth factor (HGF). Most of these genes are known factors in driving immune tolerance and specifically the first two are widely described as the major markers of tolerogenic DC (Krispin et al. 2006; Pulendran et al. 2010; Torres-Aguilar et al. 2010; Rutella et al. 2006; Gregori et al. 2010). To better understand whether module 2 represents a module of tolerogenic DC, we performed a meta-analysis of all the publicly available tolerogenic DC gene expression studies and looked at how genes belonging to module 2 behaved in these other datasets of tolerogenic DC. In total, we found 8 gene expression datasets describing in vitro generated tolerogenic DC that could be used for the analysis. In these studies, tolerogenic DC were generated according to different protocols using IL-10 alone or in combination with other cytokines, mesenchymal stromal cells, T regulatory cells, or adhesion protein disruption. In 5 of the 8 analyzed datasets, we observed a statistically significance concordance of module 2

overexpression in tolerogenic DC (Table 4.2), strengthening the link between the expression of module 2genesandtolerogenic DC.

Table 4.2 Meta-analysis of tolerogenic DC dataset

Dataset # (Source)	Type of DC	Ref	p-value
GSM468775 (NCBI GEO)	IL-10/IL-6 mDCs vs mDCs	(Torres-Aguilar et al. 2010)	3.56E-06
GSE23371 (NCBI GEO)	IL10/dexamethason DC vs LPS DC	(Jansen et al. 2011)	7.34E-06
GSE18921 (NCBI GEO)	IL-10/IL6 DC vs standard DC	(Torres-Aguilar et al. 2010)	7.52E-06
MTAB-286 (EMBL-EBI)	DC grown in presence of MSC vs normal DC	(Aldinucci et al. 2010)	7.34E-05
GSE18921 (NCBI GEO)	IL-10 DC vs standard DC	(Torres-Aguilar et al. 2010)	0.005962
GSE7387 (NCBI GEO)	Induced-regulatory T cell treated-and untreated-DC from patients with ITP	(Zhang et al. 2009)	>0.05
GSE9241 (NCBI GEO)	E-cadherin-stimulated DCs vs bacteria activated DCs	(Jiang et al. 2007)	>0.05
GSE18921 (NCBI GEO)	IL-10/TGFb1 DC vs standard DC	(Torres-Aguilar et al. 2010)	>0.05

Ref: Publication reference
p-value: p-value of the overlap of tolerogenic genes in the dataset with genes in module 2

4.2.8 Low IL-10 concentrations correlated with strong immunological response. Next, we analyzed media supernatants obtained from the last 6 h of DC culture (see methods) in order to characterize the cytokine/chemokine secretion profiles of DC immediately before they were injected. We tested 93 supernatants (90 of which corresponding to the same DC we tested by gene expression) by ELISA for the presence of 11 proteins: IFN-gamma, IL-10,IL-12p70, IL-6, IP10 (CXCL10), MCP1 (CCL2), MIG (CXCL9), TNF-alpha, I-TAC (CXCL11), MDC(CCL22), and TGF-beta1. Interestingly, we observed high levels of both manufacturing and inter-patient related variability for most of the tested proteins, with coefficients of variation ranging between 0.27 and 0.67 for manufacturing-related variability and between 0.33 and 1.34 for inter-patient related variability (Figure 4.11). When tested for their predictive value of clinical response none of the proteins showed an AUC greater than 80% indicating that single cytokine concentrations in supernatants may not be good predictors of DC efficacy. However, when we tested protein concentrations for their predictive value of strong

immunological response (similarly to what was observed with module 2 genes) we observed an impressive predictive value for IL-10 with an AUC of 95.8% (Figure 4.12). In particular and as expected, minimal levels of IL-10 were detected in the supernatants of those DC that led to a strong immunological response compared to the ones that did not. A similar result was obtained when we tested the IL-12/IL-10 ratio. In this case, we observed an AUC of 94.3%, with the highest IL-12/IL-10 ratios leading to strong immunological responses.

4.2.9 Module 2 expression correlated with CD14, IL-10, MDC and MCP-1 secretion. Given that it is not possible to routinely test DC by gene expression profiling, we analyzed how the expression of module 2 genes correlated with the other analyzed factors that can be tested more easily assayed. As expected, we observed a statistically significant correlation between module 2 expression levels and percentages of CD14+ DC assessed by flow cytometry ($r = 0.71$, p-value < 0.0001) and IL-10 secretion levels ($r = 0.604$, p-value < 0.001), confirming at a proteomic level, the observations made on gene expression profiles. Also, module 2 expression correlated positively with secreted concentrations of MCP-1 (CCL2)($r = 0.537$, p-value < 0.0001) and negatively with level of MDC (CCL22)($r = -0.534$, p-value < 0.0001). Given that none of these proteins was able to replace module 2 for its predictive value as single factor, we evaluated whether by combining all four proteins we were able to obtain a better correlation with module 2. We, therefore, calculated for each DC that we analyzed by gene expression, CD14 expression by flow and supernatant analysis by ELISA (n= 89) a CD14/IL10/MCP1/MDC index(made by adding up DC ranks of the expression level of the 4 proteins, see methods for details)and observed that it strongly correlated with module 2 expression ($r = 0.867$, p-value < 0.0001, Figure 4.13). Next we tested its predictive value for both clinical and strong immunological responses and we obtained AUC of 88.7% and 97.2%, respectively(Figure 4.14). All together, these data suggest that the analysis of CD14 expression by flow cytometry combined with IL-10, MCP-1 and MDC cytokine concentrations was able to discriminate between RespDC and NonRespDC.

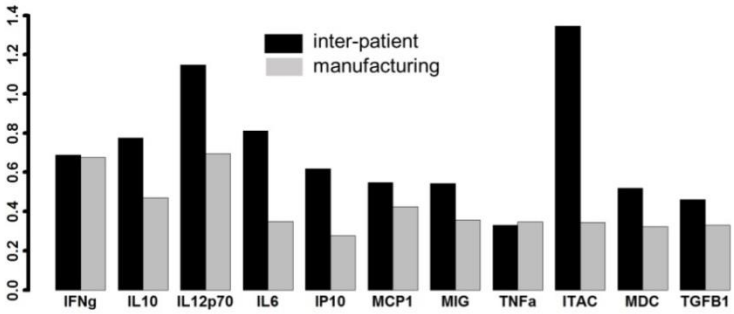

Figure 4.11 Variation in cytokine/chemokine secretion levels. Coefficients of Variation (CV) of supernatant concentrations of indicated cytokines/chemokines calculated for manufacturing (black bars) and inter-patient variability (light grey bars) among 93 manufactured DC. Manufacturing related CV was calculated as the average CV registered among all the DC generated from each patient, whereas inter-patient CV was calculated on patient-averaged values

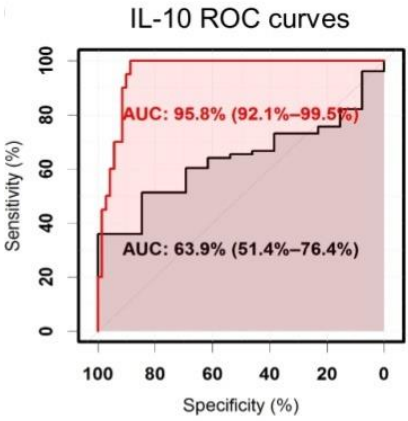

Figure 4.12 ROC curves of IL-10 on clinical and immunological response. ROC curves showing the ability of IL-10 concentrations measured on DC supernatants to discriminate among clinical and non-clinical responders in black and strong immunological responders vs not-strong immunological responders in red

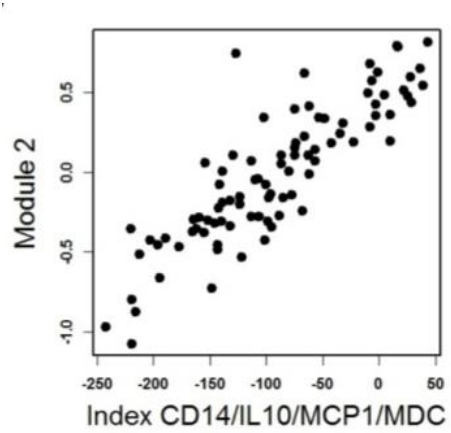

Figure 4.13 CD14+/IL-10/MCP-1/MDC index correlated with module 2. Plot showing the correlation of module 2 expression with the CD14+/IL-10/MCP-1/MDC index

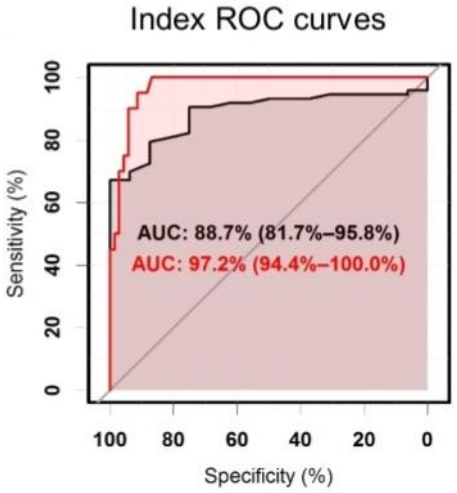

Figure 4.14 ROC curves the CD14+/IL-10/MCP-1/MDC index on clinical and immunological response. ROC curves showing the ability of CD14+/IL-10/MCP-1/MDC index to discriminate among clinical responders and non-responders in black and strong immunological responders vs not-strong immunological responders in red.

4.2.10 Module 2 genes are co-expressed also in other DC. Lastly, we wanted to check whether co-expression of module 2 genes is shared among DC differentiated by different protocols or is unique of LIg-DC. Therefore, we performed a metanalysis on publicly available gene expression dataset on monocyte-derived DC. We selected only human dataset that were made of at least 8 samples, with more than 3 biological replicates per condition, that were testing at least one maturation agent for more than six hours, and that had sufficient level of details about experimental design. A total of six dataset were selected covering a broad array of IL-4-DC and IFNa-DC both immature as well as matured by several stimuli. Gene expression data were generated on three different platforms (i.e., Agilent, Illumina, Affymetrix). Therefore data were normalized each according to standard procedures for the specific platform used. Module 2 genes were selected, and co-expression patterns analyzed by hierarchical clustering. In three out of six dataset module 2 genes were mostly co-expressed, whereas in the remaining dataset co-expression was not observed (Table 4.3 and Figure 4.15). No clear factors affecting the presence/absence of co-expression of module 2 genes were identified.

Table 4.3 Meta-analysis of matured monocyte-derived DC for co-expression of module 2 genes

Series #	**Series Title**	**# of samples**	**Co-expression?**
GSE39745	Human monocyte-derived dendritic cells treated with U0126 or SB203580	35	Yes
GSE26438	Recombinant human lactoferrin activates human dendritic cells via Toll-like receptors-2 and -4	12	Yes
GSE47621	Interferon-gamma critically determines dendritic cell function	8	Yes
GSE44719	IFNa and IL4 DCs stimulated with microbial components for 6hr	77	No
GSE44721	IL4 DCs and monocytes stimulated by 13 human vaccines and LPS for 6hr	128	No
GSE44720	IFNa DCs and IL4 DCs exposed to H1N1, heat killed S. aureus, or heat killed S. enterica (HKSE) for 1h, 2h, 6h, 12h, or 24h	120	No

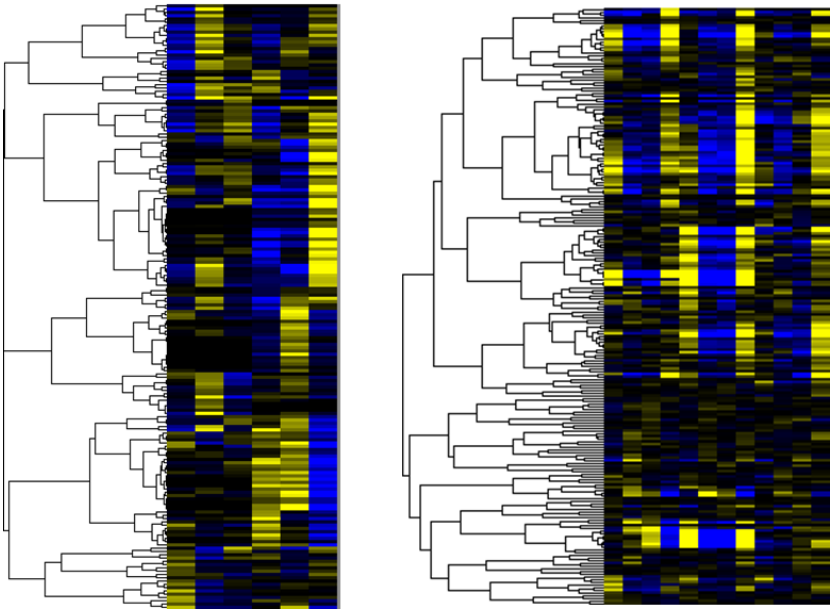

Figure 4.15 Co-expression of module 2 genes in DC. Heatmaps of module 2 genes in two different dataset. On the left, representative heatmap of dataset in which module 2 genes are not co-expressed, on the right representative heatmap of dataset in which module 2 genes are co-expressed.

4.3 Discussion

DC-based cell therapies represent a promising approach to activate immune responses against tumors even though the vast majority of clinical trials have failed to show efficacy for such approach. Several reasons for such disappointing results have been identified: suboptimal generation and delivery of DC, inappropriate selection of immunogenic tumor associated antigens, systemic inactivation of the immune system in advanced tumors and the ability of the established tumor microenvironment to inhibit T cell function. These factors have all been widely described and analyzed as possible justification of poor clinical trial result outcomes (Castiello et al. 2011; Whiteside 2006; Whiteside 2013; Vasaturo et al. 2013; Hargadon 2013). It has also been recently recognized that response evaluation of immunotherapies, especially cell-based therapies, should be based on different criteria compared to standard chemotherapy drugs and treatments, implying that the previous reports should be careful reevaluated (Hinz et al. 2006).

In this study, we focused our analysis on the DC products administered to stage D0 prostate cancer patients, by eliminating issues related to systemic tumor burden and a local immune-tolerizing microenvironment, and observed a strong correlation between DC phenotype and slope log PSA responses (a well-established surrogate for clinical outcomes in the stage D0 population) and immunological responses. In particular, we identified a gene signature made up of several well-known tolerogenic DC factors such as CD14 and IL-10that was able to discriminate RespDc from NonRespDC. The differential expression of CD14 and IL-10 was confirmed at the proteomic level and observed that MCP-1 and MDC protein levels correlated with the expression level of the tolerogenic gene expression signature. Even though IL-10 secretion levels were able to predict strong immunological responses, it was only by combining CD14, IL-10, MCP-1 and MDC measures that it was possible to obtain an index able to replace the tolerogenic gene expression signature in its ability to discriminate both clinical and strong immunological responses.

Lot-to-lot variability is a critical issue for DC-based immunotherapies. Our data revealed a correlation between the phenotype of DC used as vaccines and the induction of clinical and immunological responses. Even though functional analyses are needed to support a causative role of the identified phenotype, our observations further strengthen the need for extensive characterization of cellular products used in preclinical and early phase clinical trial in order to identify manufacturing and inter-patient related issues that may hamper identity, consistency and potency of final DC products. In a previous report, we described a framework for preclinical analysis of cell therapies for the identification of factors affecting consistency of cell products (Castiello et al. 2013), but only by using accepted surrogates for clinical outcomes such as slope log PSA used in this study was it possible to correctly determine which factors play a role in product efficacy. The consistency of DC-based products is critical considering that many reports have highlighted how DC generated from patient monocytes show phenotypic differences compared to those manufactured from healthy donor monocytes (van den Heuvel et al. 1998; Kvistborg et al. 2009; Cuellar et al. 2008; Decker et al. 2006). Therefore, lot-to-lot variability should be carefully characterized for each cell product in the early phases of product development to determine which factors should be analyzed routinely to control and manage product consistency.

The generation of potent DC capable of inducing a strong anti-tumor immune response is highly sought after, but a consensus concerning optimal DC generation protocols is still lacking.The changes in slope log PSA and TARP-specific immunogenicityfollowing therapeutic vaccination observed in the current study were encouraging and highly statistically significant. Clinical

responses were observed in 15 of 18 evaluable patients at 24 weeks and 13 out of 16 evaluable patients at 48 weeks, whereas immunological responses were detected in 10 out of 16 evaluable patients. However and more interestingly, our results suggest that even among DC products manufactured following identical standard operating procedures it is possible to identify more potent DC. The detrimental role of tolerogenic signals on DC function, such as expression of CD14 and secretion of IL-10 has been widely discussed in literature (Kalinski et al. 2009; Torres-Aguilar et al. 2010; Gregori et al. 2010),but the direct involvement of these signals in clinical DC products has not yet been described. DC generated with our protocol and expressing low levels of tolerogenic genes, as determined by scoring low on our CD14/IL-10/MCP-1/MDC index, strongly correlated with the induction of strong immunological and clinical responses. How to more consistently manufacture DC with such a potent phenotype is under investigation in our laboratory, but the possibility of predicting which patients are more likely to benefit from vaccination is already an appealing scenario that will be tested further in forthcoming clinical trials at our institution.

Response to DC-based vaccine depends on several factors. In the current study, by analyzing DC administered to patients with relatively low tumor burdens i.e. micrometastatic disease since the only evidence of disease is PSA biochemical progression, we were able to more directly link DC phenotype with clinical and immune responses. However, it's reasonable to expect that in more complex clinical settings, additional factors related to DC phenotype as well as unrelated factors (e.g., overall patient immune system status following multiple chemotherapies, tumor phenotype and tumor burden) should also be considered. Therefore, complex data modeling should be developed that is able to extract precious information on DC phenotype and identify factors that correlate with clinical and immunologic outcomes.

In this study, when we used standard statistical tools for the analysis of gene expression data (i.e., t-test based class comparison) we were not able to observe statistical differences among RespDC and NonRespDC. It was only when using a novel unsupervised method for the selection of gene modules which were co-expressed across the dataset (i.e., WGCNA) we were able to identify the tolerogenic gene signature. In fact, some of RespDC did express similar levels of the tolerogenic signature of NonRespDC. What is the mechanism behind the ability of these DC to induce clinical responses in the absence of a conventional immunological response will be tested in future clinical trials, but considering the multiplicity of effects DC can exert (Steinman & Banchereau 2007), it is possible that these DC worked by activating immune cells other than T cells (Bray et al. 2011). However, further investigations are needed to explore such hypotheses, including

studies that involve expanded patient immunomonitoring and/or systems immunology.

5. CHARACTERIZATION OF IFNa-DC USED IN METASTATIC MELANOMA PATIENTS AND THEIR LOT-TO-LOT VARIABILITY

5.1 Background

Dendritic cells (DC) are professional antigen presenting cells that are able to activate both innate and adaptive arms of the immune system (Ueno et al. 2010). Given their pivotal role in shaping the immune system, DC-based vaccines represent a promising immunotherapeutic approach in several clinical settings. In particular, over 300 clinical trials have been conducted in cancer setting which have proven the feasibility and safety of DC vaccines (Castiello et al. 2011). However, so far clinical trials have not demonstrated the desired clinical efficacy and in most of the cases stopped in very early phases. In fact, for the majority of trials, the overall response rates have been well below 20%. Many reasons have been hypothesized for such low response rates, among which the generation of DC with suboptimal activity *in vivo* is considered the most relevant, even though it's not known yet how to generate the most potent DC. Also, it has to be noted that differences in clinical setting, study design, sources of antigens, and route of administration make it almost impossible to compare results from previously conducted trials in order to clearly delineate the shared determinants of in vivo efficacy of DC-based vaccines.

DC-based therapies, similarly to other cell therapies, face an additional issue for their implementation into clinics: their considerable lot-to-lot and patient-specific variability. Extensive characterization is extremely costly, time-demanding, and hypothetically endless, given the fact that differently from standard drugs, cells cannot be completely characterized (Stroncek et al. 2010). Therefore, the identification of reliable biomarkers of identity, consistency and potency of cell therapies is highly encouraged by regulatory agencies beginning in the earliest phases of clinical development of the cellular product (Hinz et al. 2006; Vatsan et al. 2013).

In the previous part of this project, we have shown that even when highly standardized procedures are used to generate monocyte-derived LIg-DC, manufacturing, intra-donor and inter-donor related factors may affect DC phenotype (Castiello et al. 2013). In particular, we observed that while most of the well-known and usually tested DC markers (e.g., CD80, CD86, CD83, HLA-DR) did not show any differences in expression among LIg-DC generated at different times from different donors, the expression of several genes and the levels of several key secreted cytokines and chemokines showed significant variability among LIg-DC products. Next, we analyzed 114 peptide-pulsed LIg-DC preparations manufactured to vaccinate 18

patients with autologous TARP peptide-pulsed DC in order to characterize whether lot-to-lot variability in clinical GMP manufactured DC has an impact on DC identity, potency and efficacy. By analyzing DC surface marker expression, gene expression profiles, protein secretion profiles and culture data, we observed the existence of a tolerogenic DC signature that was negatively correlated with the development of clinical and immunological response. Then, by analyzing publicly available gene expression datasets, we observed that such tolerogenic signature was shared also in other DC differentiation protocols, while other datasets were not showing the co-expression of such a signature.

In the current part we explored whether DC differentiated in presence of GM-CSF and interferon-alpha (IFNa-DC) show patterns of variability similar to LIg-DC and whether biomarkers of efficacy are shared with LIg-DC. IFNa-DC represent a relatively new DC showing a semi-mature phenotype and endowed with potent functional activities (Santini et al. 2000; Farkas et al. 2008; Paquette et al. 1998; Santini et al. 2009). In fact, these cells produce mostly T-helper-1 (Th-1) cytokines and chemokines, express toll-like receptors (TLRs) 1 to 8, show migratory response to chemokines, and are capable of stimulating Th-1 polarized immune responses after injection into severe combined immunodeficient mice reconstituted with human peripheral blood leukocytes(Santini et al. 2009; Farkas et al. 2008). Notably, IFNa-DC exert a direct cytotoxic effect on tumor cells (Santini et al. 2000), are capable to take up apoptotic cells through the scavenger receptor Lectin-like oxidized-LDL receptor-1 (LOX-1) (Parlato et al. 2010) and cross-present their antigens to CD8+ T cells, thus leading to an efficient cross-priming of these cells(Santodonato et al. 2003; Tosi et al. 2004; Lapenta et al. 2006). In addition, IFNa-DC are capable of expanding both Th1 and Th17 responses as a result of the production of cytokines such as IL-23 and IL-12(Santini et al. 2011). Remarkably, IFN-DC do not require TLR triggering to induce antigen specific cytotoxic T lymphocytes and to stimulate allogeneic CD4+ T cells(Bracci et al. 2008). All these features make IFNa-DC highly promising new candidates for the development of more effective DC-based strategies of cancer immunotherapy(Farkas & Kemény 2011; Bracci et al. 2013).

Here, we characterized IFNa-DC manufactured to sustain a phase I clinical trial for advanced melanoma patients. Differently from most of the DC-based immunotherapies, in this case IFNa-DC were injected intratumorally one day after dacarbazine aiming at an *in situ* loading of tumor antigens relesead by cancer cell death induced by the chemotherapeutic agent. By analyzing DC surface marker expression, gene expression profiles, protein secretion profiles and culture data, we observed a different pattern of variability compared to LIg-DC and that in this setting biomarkers of efficacy might be

mostly related to phagocytic activity coupled to secretion of high level of chemokines.

5.2 Results

5.2.1 IFNa-DC showed variability in final viability, phagocytosis and surface expression of key markers. Monocyte-derived IFNa-DC generated from 5 patients were characterized according to release criteria for cell viability, cell counts, cell phenotype and antigen uptake on cryopreserved aliquots. As shown in Figure 5.1, IFNa-DC showed some lot-to-lot variability in all factors analyzed. In fact, viability ranged between 73% and 91% (release threshold 70%); cell recovery was in the range of 55-90% (release threshold 50%); and phagocytic activity averaged in the range of 40-50% with IFNa-DC of patient 3 showing a much higher level (ranging approximately at 70%) (release threshold 30%).

Phenotypic analysis showed that IFNa-DC from all patients displayed significant and barely invariable expression of class I and class II molecules, co-stimulatory receptors CD80 and CD11c marker. IFNa-DC also retained CD14 expression at the same level among different patients, in line with their semi-mature phenotype. CD86, CD40, CD83 and CD1a, instead were expressed at different levels among the IFNa-DC made from different patients.

5.2.2 Gene expression profiling revealed changes occurring upon differentiation from monocytes. Then, to more deeply characterize IFNa-DC and monitor molecular changes occurring during the manufacturing process, we also analyzed gene expression profiles of starting monocytes, of DC at the end of the culture before cryopreservation, and of one aliquot of the thawed DC product from each of the five patients for which DC were manufactured. As shown in Figure 5.2, unsupervised clustering of the whole dataset clearly separated monocytes from DC samples, showing that a huge change occurred during differentiation of monocytes into IFNa-DC. Also, DC samples clustered according to patient, implying that changes occurring along cryopreservation and subsequent thawing of the cells are little compared to inter-patient differences and insignificant when considering changes occurring during DC differentiation, which was the main focus of our microarray analysis.

Therefore, to better characterize molecular pathways affected during IFNa-DC differentiation, we performed a paired class comparison between monocytes and IFNa-DC and observed that 5,725 genes were differentially expressed with a p-value < 0.001 (false discovery rate < 0.01) (Figure 5.3).

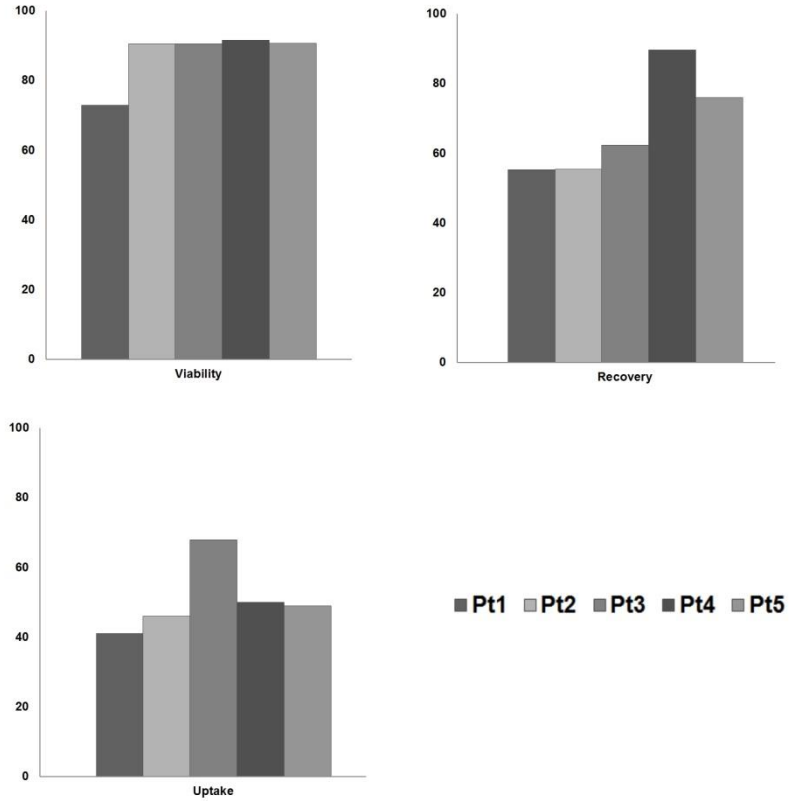

Figure 5.1 Viability, Post-thaw recovery and antigen uptake ability of IFNa-DC. IFNa-DC were analyzed after thawing for cell viability by trypan blue, cell recovery was assessed as the number of living cells after thaw divided by the number before cryopreservation. Antigen uptake assay was set as described in Appendix to assess phagocytic activity of IFNa-DC and % of FITC-OVA positive cells is shown.

As expected, among top up-regulated genes there were many well-known IFN-alpha-induced ones, such as ISG15, MX1, IFI27 and IFIT1. Interestingly, several chemokines, such as chemokine (C-C motif) ligand 13 (CCL13), CCL17 and CCL19, were all strongly up-regulated showing fold changes above 100, suggesting a strong chemotactic potential of IFNa-DC towards T cells and other immune cells. Then, to classify genes induced by IFN-DC differentiation, we performed gene ontology (GO) analysis on genes up-regulated in IFNa-DC compared to monocytes, focusing on mostly modulated genes (fold change >3) (Figure 5.3C).

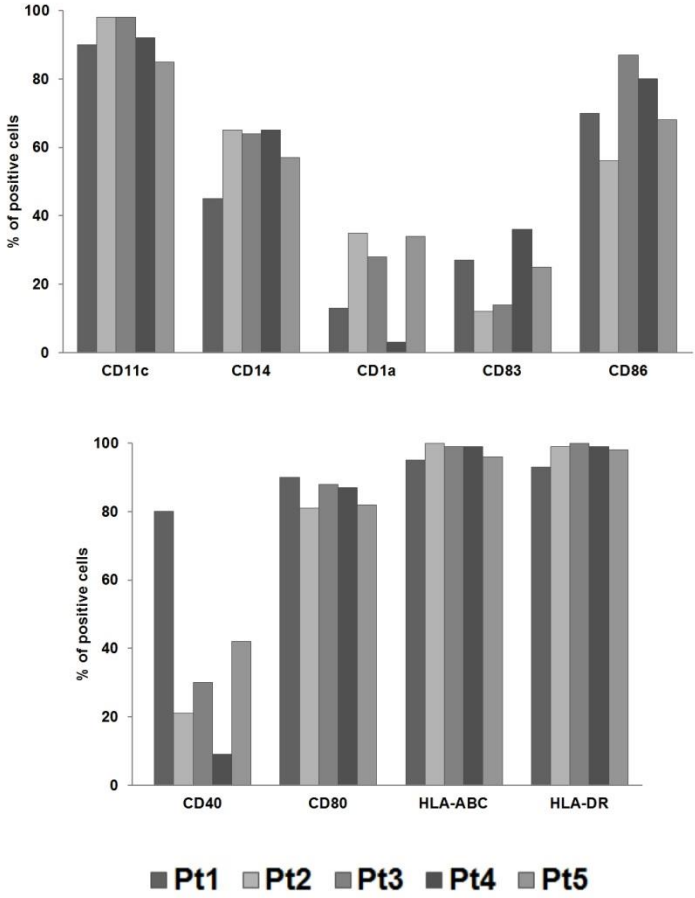

Figure 5.2 Expression of surface markers by IFNa-DC. Percentages of cells expressing the indicated surface markers as assessed by flow cytometry after subtraction of signal from isotype labeled cells.

Most over-represented families were immune related with a strong up-regulation of genes belonging to "antigen processing and presentation" and "response to virus". Considered the relevance of these GO families, we looked at exactly which genes were in our analysis falling into these families. Up-regulated "antigen processing and presentation" genes were mainly class II HLA genes and CD1 genes (a, b, c, and e), highlighting the well-

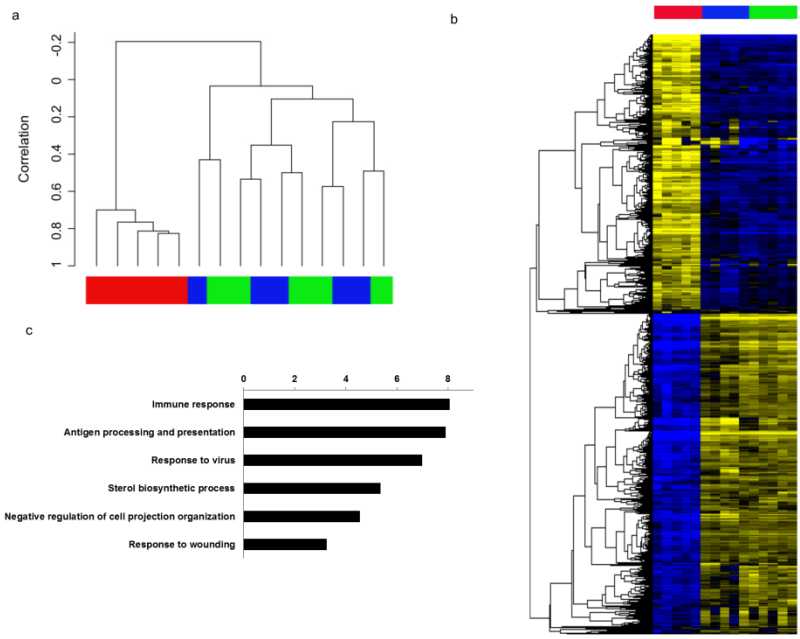

Figure 5.3 Gene expression analysis of IFNa-DC. A. Unsupervised Hierarchical Clustering of samples using the whole dataset. Monocyte, IFNa-DC and prior to cryopreservation DC are shown by red, green and blue bars, respectively; B) Heatmap of the average corrected expression levels of the 5725 genes differentially expressed between IFNa-DC and monocytes with a p-value < 0.001. Genes are in rows and samples in columns. Monocyte, IFNa-DC and prior to cryopreservation DC are shown by red, green and blue bars, respectively; C) Gene Ontology Analysis of up-regulated genes in IFNa-DC vs monocytes (p-value<0.001 and ratio >3). The plot show for each GO "biological function" term the enrichment among genes up-regulated in IFNa-DC expressed as –log10(p-value). Enrichment p-values were calculated through hypergeometric test. Statistical significance threshold for hypergeometric test was set to 0.05 (i.e., -log10(p-value) > 1.3 were statistically significant).

documented ability of IFNa-DC to strongly process and present antigens. Altogether, these data indicated that strong molecular changes are induced upon monocyte differentiation into IFNa-DC and that GMP-manufactured IFNa-DC were empowered, at least at gene expression level, with strong chemotactic and antigen processing and presentation abilities.

5.2.3 Module 2 genes were not co-expressed in IFNa-DC. Next, we analyzed whether genes previously identified as co-expressed in LIg-DC that were correlating with the induction of clinical and immunological response in prostate carcinoma patients (i.e., module 2 genes) resulted co-expressed also among IFNa-DC. Therefore, given the different platform we selected only annotated genes belonging to the tolerogenic signature and found 142 genes. As shown in Figure 5.4a, IFNa-DC did not co-expressed module 2 genes as can be observed by different expression of the genes within a sample (i.e., to be co-expressed all genes should show an expression level above or below average within the same sample). Therefore, this analysis pointed to the absence of a clear tolerogenic signature among DC made from different patients according to this protocol and suggested that inter-individual variability in IFNa-DC may be different from the one observed in LIg-DC. Therefore, to better characterize inter-individual IFNa-DC variability we selected top ventile showing the highest variability among IFNa-DC generated from the 5 patients (2127 genes, Figure 5.4b) and analyzed through gene ontology (GO) their biological relevance. Interestingly, the most over-represent GO family was "Immune Response" (p-value = 2.13×10^{-39}). In particular, we found highly variable many cytokines and chemokines (CCL1, CCL2, CCL3, CCL4, CCL5, CCL8, CCL13, CCL14, CCL18, CCL19, CCL20, CXCL1, CXCL2, CXCL3, CXCL9, CXCL10, CXCL11); CD1 family genes (CD1a-e); co-stimulatory/co-inhibitory molecules CD83, CD274, CD276; interleukins (IL1A, IL6, IL8, IL10, IL27, IL32) and many leukocyte immunoglobulin-like receptor (LILRA3, LILRA5, LILRA6, LILRB1, LILRB3, LILRB4, LILRB5). Altogether these results highlight how even if inter-individual patterns of variability in IFNa-DC are different from the ones observed in LIg-DC, even for these cells most affected genes play a key role in DC biology and therefore may hinder functional differences in IFNa-DC generated from different individuals.

5.2.4 IFNa-DC showed a different pattern of variability in cytokine secretion. Next, we wanted to characterize cytokine secretion profile of IFNa-DC. Thus, we analyzed culture media supernatants by ELISA for concentration levels of IL-10, IL-12p70, CCL3, CXCL10, CXCL9, and CCL22. Interestingly, both IL-10 and IL-12 levels were always below lower detection limit. However, we detected high concentrations of CXCL10, CXCL9, and CCL22 (usually above 1000 pg/ml), whereas CCL3 was secreted at much lower levels (ranging between 0.7 and 11 pg/ml). As expected, secretion levels of IFNa-DC were completely different to the ones of LIg-DC, given the wide difference existing between these two types of DC.
Then, we analyzed whether at least variability patterns in cytokine secretion ability of IFNa-DC were similar to LIg-DC, but even in this case we

observed that inter-individual variation affect cytokines secretion with a different pattern compared to LIg-DC (Figure 5.5). Therefore, in line with gene expression results, even at proteomic level IFNa-DC show inter-individual differences that are not comparable to the ones observed in LIg-DC and thus strengthening the hypothesis that different DC are characterized by not-universal patterns of variability.

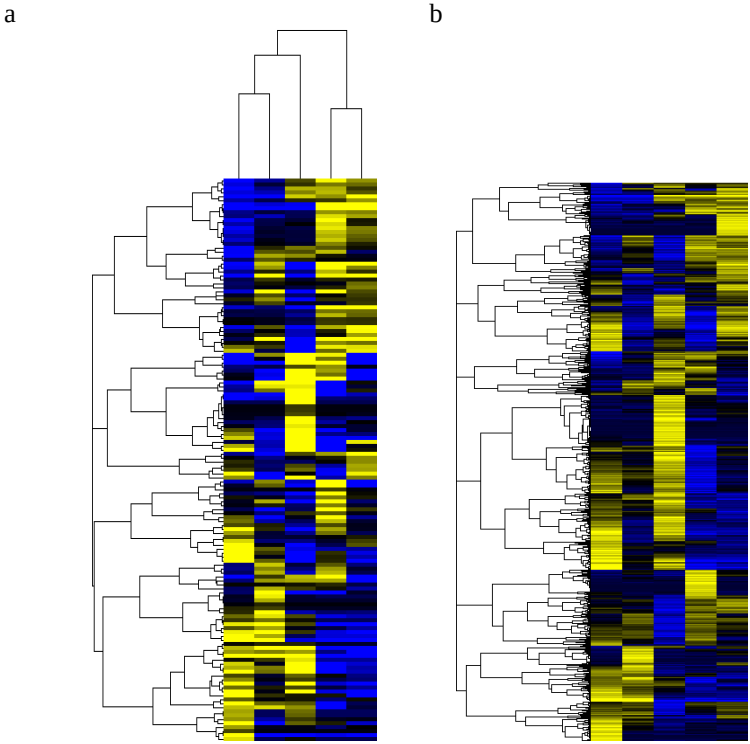

Figure 5.4 Inter-individual variability of IFNa-DC. a) Heatmap showing the absence of co-expression of module 2 genes in IFNa-DC. The 142 genes that were found in this dataset are in rows and the 5 samples in columns. b) Heatmap showing the top ventile genes in IFNa-DC showing the highest inter-individual variability. The 2127 genes are in rows and the 5 samples in columns.

5.3 Discussion

The possibility to activate immune response against tumor through DC-based immunotherapies remains extremely attractive despite the results so far observed in early phases clinical trials. Many causes have been identified for the failure of previous studies, but many issues are still on debate. Suboptimal generation and delivery of DC, inappropriate selection of immunogenic tumor associated antigens, systemic inactivation of the immune system in advanced tumors and the ability of the established tumor microenvironment to inhibit T cell function are all considered grounding factors for the success DC-based immunotherapy (Castiello et al. 2011; Whiteside 2006; Whiteside 2013; Vasaturo et al. 2013; Hargadon 2013). It has also been recently recognized that different criteria should be used when evaluating immunotherapies because standard criteria may be less meaningful or even fallacious conclusions (Hinz et al. 2006).

In this study, we analyzed IFNa-DC manufactured for a clinical trial for advanced melanoma patients. Even though the paucity of treated patient did not allow the identification of molecular markers associated with increased potency/efficacy in this setting, we were able to characterize molecular changes occurring upon their differentiation from monocytes and gene expression patterns. In particular, we observed a different pattern of inter-individual variability from the one previously observed in LIg-DC. In fact, IFNa-DC did not show a clear tolerogenic signature, but even for IFNa-DC it was possible to observe that inter-individual variation affects relevant genes and proteins and that therefore can affect their ability in vivo to activate immune response.

Lot-to-lot variability is a critical issue for DC-based immunotherapies. In the previous parts of this study, we described a framework for preclinical analysis of cell therapies for the identification of factors affecting consistency of cell products (Castiello et al. 2013) and described a correlation between the phenotype of LIg-DC used as vaccines and the induction of clinical and immunological responses. Here, by analyzing IFNa-DC used in a clinical trial we strengthened the importance of in-depth characterization of cellular products for the identification of manufacturing and inter-patient related issues that may hamper identity, consistency and potency of final DC products. The consistency of DC-based products is a critical issue considering that many reports have highlighted how DC generated from patient monocytes show phenotypic differences compared to those manufactured from monocytes of healthy donors (van den Heuvel et al. 1998; Kvistborg et al. 2009; Cuellar et al. 2008; Decker et al. 2006). Therefore, lot-to-lot variability should be carefully characterized for each cell product in the early phases of product development to determine which factors should be analyzed routinely to control and manage product consistency.

The generation of potent DC capable of inducing a strong anti-tumor immune response is highly sought after, but a consensus concerning optimal DC generation protocols is still lacking. IFNa-DC have shown very promising results in vitro and in animal models, but had not been tested in human. Here, we showed the analysis of the IFNa-DC used for the first time clinically in advanced melanoma patients. Even though the results cannot be considered conclusive given the paucity of vaccinated patients, we observed disease stabilization and the induction of immune response against melanoma antigens in two of the five treated patients. Similarly to previous studies (Santini et al. 2009), IFNa-DC used in the trial showed a semi-mature phenotype characterized by immature features (such as the expression of CD14 and an high phagocytic activity) mixed with maturing ones (such as high secretion of cytokines and chemokines, increased surface expression of MHC and co-stimulatory molecules). In particular, these features may be especially crucial in the setting of intra-tumoral injection as occurred in this trial. High phagocytic activity is in fact essential for a proper antigen uptake *in situ*; in a similar way chemotactic activity and partial mature features may have played a favorable phenotype. However, the low number of treated patients did not allow us to perform any analysis aimed at identifying IFNa-DC molecular markers associated with clinical results in patients.

Even though with different patterns also in IFNa-DC inter-individual variability affects key genes/proteins/functions. In fact we observed among top variable genes key cytokines and chemokines such as CCL2, CCL3, CCL5, CXCL9, CXCL10, CXCL11; CD1 family genes (CD1a-e); co-stimulatory/co-inhibitory molecules CD83, CD274, CD276; interleukins (IL1A, IL6, IL8, IL10, IL27, IL32) and many leukocyte immunoglobulin-like receptor (LILRA3, LILRA5, LILRA6, LILRB1, LILRB3, LILRB4, LILRB5). How variability affects also protein level and eventually function has to be carefully study for each identified gene, but we did observe high level of variation also at the protein level for CXCL10/IP10 and CXCL9/MIG as revealed by ELISA on culture media supernatants.

In conclusion, our study strongly suggests that pattern of variability are different among different DC preparations and that an in-depth characterization of DC vaccines may strongly benefit the identification of key factors and candidate biomarkers of DC identity, consistency and potency. Also it highlighted the potent phenotype shown by IFNa-DC, strengthening the rationale that these DC represent the ideal candidate in the setting of intratumoral injection aimed at in situ antigen loading.

6. CONCLUSION

Dendritic cells (DC) play a key role in the activation of immune system by presenting antigens to T cells and, by so, generating an antigen-specific immune response (Ueno et al. 2010). For this reason, several attempts have been done so far in order to develop effective immunotherapeutic approaches that consist of ex vivo generated fully-functional DC to be infused in patients in order to induce an antigen specific T cell expansion (Palucka & Banchereau 2012). Many methods have been developed to generate DC from monocytes. In fact, DC can be generated ex vivo by culturing monocytes in presence of differentiating cytokines (such as GM-CSF, IL4, IL15 and IFNa) to obtain immature DC. Usually these cells are then matured with single agents or cocktails of agents (such as TNFa, LPS, IFNg, CD40L, IL6, IL1)(Kalinski et al. 2009). Once generated, DC are usually pulsed with tumor antigens and injected into patients (usually intranodally or intradermally). More recently a newer approach has been developed that is based on directly injection of unloaded DC intratumorally aiming at in situ antigen loadings usually after chemo/radiotherapy. This latter approach even if less studied and tested, is surging as an attractive option given promising results shown in early phase clinical trials (Tanaka et al. 2005; Finkelstein et al. 2012; Kolstad et al. 2014) .

Clinical results, mainly from studies in cancer patients, clearly showed the feasibility and efficacy of this approach even if the overall response rate is below the 15% (Engell-Noerregaard 2009). Several possible reasons have been hypothesized to justify such low response rate, among which the suboptimal generation of DC able to activate an anti-inflammatory Th1-polarized T cell response is considered the main bottleneck, even if there is no general consensus on how to improve DC function and which factors are responsible for the discrepancies between preclinical and clinical results (Castiello et al. 2011). Furthermore, cell based immunotherapies would strongly benefit of new markers for quality control assessment. Since many more factors are responsible for the function and effectiveness of cellular therapies than those of drugs and other biological products, an in depth evaluation of the characteristics of all newly developed cellular therapies is extremely desirable and needed(Stroncek et al. 2010).

Differently from previous attempts to optimize DC by modifying differentiation and/or maturation procedures, this project explored the possibility to identify factors affecting DC potency/efficacy *in vivo* in order to gain knowledge of molecular determinants essential for DC function and that can thus be used for quality assessment of manufactured DC. Therefore,

this project aimed at identifying factors affecting DC consistency and candidate molecular biomarkers of consistency, potency and efficacy of GMP manufactured DC.

In the first part, the project focused on a preclinical setting in order to evaluate feasibility of this approach by analyzing factors affecting DC consistency by combining genomic and proteomic approaches. Using global gene expression profiling, we were able to characterize the magnitude of variability introduced into LIg-DC by intra-donor and inter-donor differences and by manufacturing and determined how these three major factors affected DC consistency. Our analysis of LIg-DC suggested that manufacturing and intra-donor variability affected the final products less than inter-donor factors. Interestingly, we also observed that part of the variability in the final products could be traced back on monocytes, therefore strengthening the hypothesis that final DC potentials can be predicted by studying the phenotype of monocytes.

Next we focused on the identification of candidate markers for quality assessment of DC and by using restrictive filters, we selected 29 genes that were showing the highest variability among DC. Interestingly many of these genes play a key role in DC biology and therefore their expression level may affect how LIg-DC will behave once injected in humans. To strengthen this observation we noted that the expression of many of the 29 highly variable genes correlated with the levels of several cytokines and chemokines in the LIg-DC supernatant, such as IL-12, whose secretion is considered essential for the induction of a desirable Th1 immune activation (Trinchieri 2003), and the Th1-cell-attracting chemokines MDC, MIG and IP10 (Lebre et al. 2005). This feature suggests that these genes reflect LIg-DC function and might be potential markers of LIg-DC consistency and potency.

Given that the only reliable indicators of DC potency/efficacy derive from results in human, in the second part of the project we focused on the analysis of DC from a phase I/II clinical trial for prostate carcinoma patients. Interestingly, from the analysis of over 100 DC vaccines used to sustain the trial we discovered that DC given to patients that developed a strong immunological and clinical response under-expressed a gene signature made of over 300 genes. This signature was made of many well-known tolerogenic DC genes, such as CD14 and IL-10, and a meta-analysis with other publicly available dataset of tolerogenic DC revealed a statistically significant overlap between the signature identified and other tolerogenic signatures. The detrimental role of tolerogenic signals on DC function, such as expression of CD14 and secretion of IL-10 has been widely discussed in literature (Kalinski et al. 2009; Torres-Aguilar et al. 2010; Gregori et al. 2010), but the direct involvement of these signals in clinical DC products has not yet been described. Lastly, we were able to show that such a signature could be

replaced by the analysis of 4 proteins: CD14, IL-10, MDC and MCP-1. Overall, we identified new molecular markers, both a gene and protein level, that can be used as advanced quality control biomarkers of LIg-DC.

Lastly, to conclude the project, we analyzed whether factors affecting DC potency/efficacy in the setting previously analyzed were shared among different DC or differed among DC generated with different protocols. Therefore, we started with a meta-analysis of gene expression data of DC generated by different protocols and observed that only in some DC we were able to observe the co-expression of the tolerogenic signature. Thus, we analyzed more in detail DC generated in presence of IFNa that were manufactured for a phase I clinical trial in advanced melanoma. As expected from previous studies (Santini et al. 2009), these DC showed a completely different phenotype, with some immature features (e.g., retained CD14 expression and high phagocytic activity) mixed with mature ones (e.g., increased expression and secretion of activating signals). However, even though these DC were not showing co-expression of tolerogenic genes, the analysis of genes affected by inter-individual variability revealed that also IFNa-DC can strongly differ in many immune genes whose expression is essential for their function in vivo. This observation was also confirmed at proteomic level, where variability in secretion level of key cytokines strongly differed from LIg-DC one.

Altogether, this project developed a methodological framework for the identification of biologically-relevant quality control markers of DC by combining genomic and proteomic analysis. When applied to clinical DC, such approach was able to identify genes and proteins that correlated with clinical and immunological response and that can therefore be used as efficacy biomarkers of LIg-DC. However, as highlighted from the analysis of different DC, such newer markers are specific for DC used. On a broader range, these results strongly support the need for in-depth analysis of DC for the identification of newer quality assessment markers and factors essential for DC activity *in vivo*. Once identified, these markers can be used for the advancement of DC immunotherapies and foster their implementation in clinic.

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APPENDIX

Materials and Methods

Mature and Immature LIg-DC Cell Manufacturing Process

LIg-DC were manufactured according to a standard procedure established in the Cell Processing Section (CPS), Department of Transfusion Medicine (DTM), Clinical Center (CC), NIH, Bethesda, Maryland, USA. Briefly, peripheral blood mononuclear cell (PBMC) concentrates were collected by apheresis using an Amicus Separator (Baxter Healthcare Corp., Fenwal Division, Deerfield, IL) from healthy donors or prostate cancer patients in the DTM. All donors and patients signed an informed consent approved by a NIH Institutional Review Board. Monocytes were enriched directly from the leukapheresis products by elutriation using the Elutra (Gambro BCT Lakewood, CO) automatic mode according to the manufacturer's recommendations and cryopreserved in aliquots of $100x10^6$ or 300 $x10^6$ cells each. Immature DC and LIg-DC were manufactured from single monocyte aliquots after assessing post thaw viability and purity; in all cases both were greater than 80%. DC were manufactured in our cGMP facility by operators trained on this specific procedure whose competency was assessed periodically according to internal policies. At the time of culture initiation the cells were resuspended in RPMI-1640 media, containing 10% single donor AB heat inactivated plasma, 10 mcg/ml gentamicin, GM-CSF (Leukine Sargramostin, 2000 IU/ml, Genzyme, Cambridge, MA, USA) and IL-4 (USP grade recombinant human IL-4, 2000 IU/ml, CellGenix, Gmbh, Freiburg, Germany) at a final concentration of $1.5x10^6$/mL in T162 or T225 flask (Corning Incorporated Life Sciences, Lowell, MA, USA). The flasks were incubated at 37^oC in 5% CO_2. On day 2, fresh cytokines were added to the culture at the same concentrations. The culture was terminated on day 3 and immature DCs harvested or maintained for 24 hours after adding the maturation cocktail. The maturation cocktail contained lipopolysaccharide (LPS) (30 ng/ml, CTEP, NIH Frederick, MD) and interferon gamma (IFN-γ) (Actimmune Interferon gamma-1b, 1000 IU/ml, Intermune, Brisbane, CA, USA). Healthy donor LIg-DC were analyzed after harvesting whereas patient LIg-DC were additionally processed as follows. After two washes LIg-DC were also re-suspended in infusion media made of Plasma-Lyte A and 10% autologous heat-inactivated plasma. LIg-DC were then pulsed with wild type 27-35 and epitope-enhanced 29-37-9V TARP peptides (NeoMPS, Inc., San Diego, CA) at 37oC in 5% CO2. After pulsing, LIg-DC were combined and tested for recovery, viability, purity, sterility, mycoplasma absence, endotoxin concentration and expression of surface markers by flow

cytometry (see below). Release criteria for both healthy donor and patient lots were defined based on CD83 expression by flow cytometry and trypan blue viability set as equal or greater to 70% and 60% respectively. If the cells met all the release criteria, then 20 million viable DC were used for vaccination and were administered intradermally to patients. The remaining cells were centrifuged, the supernatant was used for cytokine profile analysis (see below) and excess DC were used for RNA extraction.

IFNa-DC Cell Manufacturing Process

Leukapheresis was performed by a Fresenius Com-Tech blood cell separator (Fresenius Kabi, Friedberg, Germany) using the White Blood Cell Set (P1YA) for the collection of mononuclear cell (MNC) products. Monocytes enrichment from aphaeresis was performed according to Elutra® Cell Separation System Monocytes Enrichment Protocol. The monocyte enriched fraction was analyzed for cell viability and cell counts and purity were assessed by flow cytometry using CD14 mAb associated with the pan leukocyte CD45 mAb (all from BD Biosciences, San Jose, CA). When the purity of monocytes was less than 60%, an additional step of separation, by centrifugation on an isosmotic medium with a density of 1.077 g/ml as a Lymphoprep™ (Axis-Shield, Oslo, Norway), was performed. The enriched monocytes were cultured for three days in bags (Afc/American Fluoroseal Corporation, Gaithersburg, MD) at the concentration of 2x106 cell/ml in CellGenix DC medium (CellGenix GmbH, Freiburg, Germany) containing GM-CSF (600 IU/ml) (Leukine sargramostim, Bayer Healthcare Pharmaceuticals, Seattle, WA) and IFN-α (10,000 IU/ml) (Merck Sharp & Dohme Limited, Hoddesdon, UK).

IFNa-DC were then harvested, counted and re-suspended in freezing medium, prepared by mixing 9 volumes of 5% Human Serum Albumin (HSA) (Baxter S.p.A., Rome, Italy) + 1 volume of DMSO (WAK-Chemie Medical GmbH, Steinbach, Germany), at the final concentration of 1-2x107 cells/ml. Aliquots of 0.5 ml cell suspension were transferred to 2 ml cryo-vials, that were deep-frozen under decreasing controlled temperature conditions and stored in liquid nitrogen vapor phase.

Cell count, viability and recovery were evaluated by using trypan blue staining, counted into at least two large different squares of the Neubauer chamber. The viability was calculated as viable Cell Densityx100/total Cell Density. The recovery was evaluated as a ratio between the number of thawed viable IFNa-DC over the number of frozen viable IFNa-DC. Sterility was determined by Direct Inoculation technique and endotoxin status was evaluated by the LAL test.

Flow Cytometric analysis of LIg-DC

Analysis of expression of surface markers was performed using fluorescent labeled antibodies (Abs) and flow cytometry. The purity of the elutriated monocytes was assessed by flow cytometry using CD33-PE, CD15-FITC, CD3/CD19/CD56-APC and CD45-APC-Cy7 (Becton Dickinson, Mountain View, CA, USA) and isotype controls (Becton Dickinson). The analysis of healthy donor LIg-DC was undertaken after harvest on Day 4. This included a panel consisted of CD86-FITC, CD83-PE, CD14-APC, CD209-FITC, CCR7-PE, CD40-APC, HLA-DR-FITC, CD123-PE, CD11c-APC, CD80-FITC, CD154-PE, CD54-APC, CD16-FITC, CCR7-PE, and CD1a-APC. Instead, patient LIg-DC were analyzed after pulsing on Day 4. The analysis included the standard "DC panel" adopted in our institution as lot release for mature DC products and other investigational markers. The panel consisted of CD86-FITC, CD83-PE, CD14-APC, HLA-DR-FITC, CD123-PE, CD11c-APC, CD80-FITC, CD54-APC, CCR7-APC, and CD38-FITC (Becton Dickinson). Flow cytometry acquisition and analysis were performed with FACScanto flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ USA) according to CPS standard operating procedures. Spectral overlaps were electronically compensated using single color controls. Quality controls were run before each session according to internal quality control policy.

Flow Cytometric analysis of IFNa-DC

Immunophenotype of IFNa-DC was analyzed by flow cytometry using a panel of antibodies including HLA-ABC, HLA-DR, CD45, CD11c, CD1a, CD86, CD83, CD80, CD40 and CD14 (all from BD Biosciences, San Jose, CA). The capability of IFNa-DC to phagocytize antigens was verified by flow cytometry using OVA conjugated with fluorescein (OVA-FITC) (Molecular Probes, Inc., Eugene, OR). Flow cytometry was carried out with a FACSCanto flow cytometer and the data were analyzed using the FACSDiva software (BD Bioscience, San Jose, CA). IFNa-DC release criteria were: cell viability >70%, cell recovery >50%, antigen uptake >30%, CD80+ >80%, CD86+ >50%, CD83+ >10%, HLA-DR+> 80%, HLA-ABC+ >80%, CD14+ <65%, CD14 MFI <1000.

Gene Expression Profiling of LIg-DC

Total RNA was extracted from the unused fraction of DC using a miRNeasy kit (Qiagen, Valencia, CA, USA). Universal Human Reference RNA (Stratagene, Santa Clara, CA, USA) was used as reference. Test samples and reference RNA were amplified and labeled using an Agilent kit according to the manufacturer's instructions and hybridized on Agilent Chip (Whole Human genome, 4X44k, Agilent Technologies, Santa Clara, CA, USA). The

arrays were scanned using an Agilent Microarray Scanner and images analyzed using Agilent Feature Extraction Software 9.5.1.1. The resulting data were uploaded onto mAdb Gateway (http://madb.nci.nih.gov), the Agilent-normalized processed signals retrieved and analyzed with BRB Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). The processed data set was subjected to filtration based on signal intensity, quality and presence across the data set.

Gene expression profiling of IFNa-DC

Total RNA was isolated from at least 5 million cells using RNeasy kit (Qiagen, Valencia, CA, USA) for both monocytes and IFNa-DC before and after cryopreservation/thawing. After passing quality control assessment of integrity of purity analyzed with ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany), RNA was amplified and labeled using Agilent kit according to manufacturer's instructions and hybridized on Agilent Chip (SurePrint G3 Human GE 8x60K Microarray) at 65 °C for 17 hours. At the end of the hybridization, chips were washed following manufacturer's instructions and scanned on SureScan Microarray Scanner (Agilent) and images analyzed using Agilent Feature Extraction Software. Data were then analyzed with BRB Array Tools. The processed data set was subjected to filtration based on signal intensity, spot quality and presence across the data set.

Protein Analysis of LIg-DC

Supernatants of DC-conditioned infusion media were collected and properly stored. The levels of indicated soluble factors were further assessed on a customized antibody-based platform (Aushon, Boston, MA, USA) consisting of a multiplex array with different monoclonal antibodies spotted per well in standard 96-well plates. A sandwich enzyme-linked immunosorbent assay technique was used to generate signals via chemiluminescent substrate. Light corresponding to each spot in the array was captured by imaging entire plates with a commercially available cooled charge-coupled device camera. Data were reduced using image analysis software (Aushon Proteome Arrays, Boston, MA, USA) that calculates exact values (pg/mL) based on standard curves. Prior to further analysis, protein concentrations were normalized according to the number of DC.

Protein Analysis of IFNa-DC

Supernatants of DC-conditioned infusion media were collected and properly stored. The levels of indicated soluble factors were further assessed on plate by Bio-Plex® Multiplex System, Bio-Rad Laboratories, Inc. (Hercules, CA).

Assay was performed by a custom kit for simultaneous detection according to a capture sandwich immunoassay format. Briefly, the capture antibody-coupled beads are first incubated with antigen standards or samples for a specific time. The plate is then washed to remove unbound materials, followed by incubation with biotinylated detection antibodies. After washing away the unbound biotinylated antibodies, the beads are incubated with a reporter streptavidin-phycoerythrin conjugate (SA-PE). Following removal of excess SA-PE, the beads are passed through the array reader, which measures the fluorescence of the bound SA-PE.

Data Analysis

Intraclass correlation coefficient was calculated for each class of samples to compare the variability of each group of samples (within assay, between assay, manufacture, intra-individual and inter-individual) as described in Korn 2004 (Korn et al. 2004). Briefly, using a component of variance model:

$$Y_{ij} = g_i + e_{ij}$$

where Y_{ij} is the log expression ratio for the i^{th} spot and j^{th} replicate, the intraclass correlation can be calculated as

ICC = $\hat{\sigma}_g^2/\left(\hat{\sigma}_g^2 + \hat{\sigma}_e^2\right)$

where $\hat{\sigma}_e^2$ is the error variance component and $\hat{\sigma}_g^2$ is the between-gene variance component. The error variance component ($\hat{\sigma}_e^2$) is estimated by

$$\hat{\sigma}_e^2 = \sum_{i=1}^{n_g}\sum_{j=1}^{n_a}\left(Y_{ij} - \overline{Y}_{i.}\right)^2 / \left[n_g(n_a - 1)\right]$$

Where n_a is the number of arrays of the class in exam, n_g the number of genes and $\overline{Y}_{i.} = \sum_{j=1}^{n_a} Y_{ij}/n_a$. The between-gene variance component is estimated by

$$\hat{\sigma}_g^2 = \sum_{i=1}^{n_g} \frac{\left(\overline{Y}_{i.} - \overline{Y}_{..}\right)^2}{\left(n_g - 1\right)} - \hat{\sigma}_e^2/n_a$$

Where $\overline{Y}_{..} = \sum_{i=1}^{n_g}\sum_{j=1}^{n_a} Y_{ij}/\left(n_g n_a\right)$

The index of variability was calculated as the sum of the variances evaluated for three factors affecting the final level of gene expression: manufacturing, intra-individual and inter-individual. In detail, manufacturing variance for the i^{th} gene ($\sigma^2_{i,manufacturing}$) was calculated as the variance of the i^{th} gene among 5 DC products manufactured on 5 different days starting from cryopreserved monocytes deriving from the same apheresis product. Intra-individual variance for the i^{th} gene ($\sigma^2_{i,intra-individual}$) was calculated as the

variance of the i^{th} gene among the 5 DC products manufactured starting from monocytes of the same donor collected by 5 different apheresis procedures. Inter-individual variance for the i^{th} gene ($\sigma^2_{i,inter-individual}$) was calculated as the variance of the i^{th} gene among 9 DC products derived from 9 different donors. For the clinical data set, manufacturing variability was calculated as the average of all the manufacturing variances measured for each donor/apheresis product. Similarly, intra-individual variance was calculated as the average of all the measured intra-individual variances where average values were used when more than one DC product was generated starting from the same apheresis material. Inter-individual variance was calculated as the variance among the patient-averaged values.
Considering that each gene shows a different assay-related repeatability, assay adjusted variances of the three factors were used by subtracting assay variance to the manufacturing, intra-individual and inter-individual variances. In conclusion, the index of variability fot the i^{th} gene was calculated as:

$$IV_i = \left(\sigma^2_{i,manufacturing} - \sigma^2_{i,assay}\right) + \left(\sigma^2_{i,intra-individual} - \sigma^2_{i,assay}\right) + \left(\sigma^2_{i,inter-individual} - \sigma^2_{i,assay}\right)$$

Receiver operating characteristic (ROC) curves were generated using the R package "pROC" (Robin et al. 2011). The area under the curve (AUC) was used as a measure of the performance of a classifier and confidence intervals were computed with Delong's method. Clinical responses as assessed by changes in slope log PSA (mathematically equivalent to an inverse calculated PSADT) at weeks 24 and 48 or strong immunological responses (defined as a TARP-specific ELISPOT count > 500) were used to classify DC. Clinical Response at week 24 was used for patient #203 that went off study during the trial.
Unsupervised hierarchical clustering and Principal Component Analysis (PCA) of the whole dataset were run with Partek Genomic Suite (Partek, St. Louis, MO, U.S.A.). Davies-Bouldin Index was calculated with Partek to identify the number of clusters between 2 and 20 that better separates samples in subgroups. Class comparisons to identify genes differentially expressed between DC were performed with BRB ArrayTools with a p-value threshold of 0.001. In order to control false discoveries, the False Discovery Rate (FDR) was calculated for each analysis as the ratio of the expected number of false discoveries divided by the number of discoveries as described by Sorić (Sorić 1989).

Weighted Gene Co-expression Network Analysis (WGCNA) was performed using the R package "WGCNA" (Langfelder & Horvath 2008). The analysis

was applied only on the most variable quartile (9112 genes) as suggested by package instructions. To apply more stringent criteria in module definition we applied a modification to standard protocol. The data set was split in two and WGCNA was then performed in both data sets. Only genes assigned to the same module in both analyses were considered as forming a module and used in subsequent analysis.

Gene Ontology was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/) (Huang et al. 2009) and Network analysis was performed using QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, CA, USA, www.qiagen.com/ingenuity). Clustering of genes was performed with Cluster (Eisen et al. 1998) and results were visualized with Java Treeview (Saldanha 2004). For the meta-analysis of tolerogenic DC, all publicly available tolerogenic DC data sets with clear sample information were selected from GEO and EMBL-EBI database. P-values were calculated with Fisher's exact test. For the meta-analysis of different DC protocols, GEO dataset containing monocyte derived DC with at least 10 samples in total and at least 3 replicates for condition were included in case sufficient info about experimental condition were included.

CD14/IL10/MCP1/MDC index was calculated as follows: each DC was ranked according to % of CD14+ cells and concentrations levels of IL-10, MCP-1 and MDC measured in supernatants in decreasing order (i.e., rank 1 to the highest expression DC). Then, taking into account that MDC levels negatively correlated with module 2, whereas CD14, IL-10 and MCP-1 levels positively correlated with module 2, the index was calculated as: MDC rank – (CD14 rank + IL-10 rank + MCP-1 rank). In this way, high scoring DC showed low expression of MDC and high expression of CD14, IL-10 and MCP-1.