HUMAN ANTIBODIES TO DENDRITIC CELLS
GENERATION, ANALYSIS AND USE IN VACCINATION

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HUMANE ANTILICHAMEN TEGEN DENDRITISCHE CELLEN

Ontwikkeling, analyse en gebruik in vaccinatie

(met een samenvatting in het Nederlands)

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When you take a flower in your hand and really look at it, it's your world for the moment.

Georgia O'Keeffe

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The life cycle of dendritic cells

Dendritic cells (DCs), first described in 1973 by Steinman and Cohn [1], are now widely recognized as professional antigen presenting cells (APCs) that play a pivotal role in directing the immune response. The ability of DCs to process and present a broad variety of antigens, to which an organism is exposed, is unmatched.

The functioning of the immune system is based on two distinct types of responses, the innate and the adaptive immune response. The innate immune response, mediated by a variety of cell types including natural killer (NK) cells and macrophages, forms the first line of defense towards infections and is regarded as non-specific. The adaptive immune response depends on the activity of antigen-specific effector T and B cells and is notable for its exquisite specificity for the inciting antigen and the generation of memory for antigens. DCs are innate immune cells and acutely activated when a pathogen invades the body, but they also play a decisive and instructive role in the adaptive immune response that arises [2]. Given that the induction of productive T cell responses depends upon activation of DCs, it follows that the DC serves as a pivotal interface bridging the innate and adaptive immune system.

Generated in large numbers in the bone marrow every day, precursor DCs circulate in the blood stream and migrate into tissues, where they reside as sentinels in an immature state, the prototype being Langerhans cells in the epidermis. Immature DCs have a high phagocytic and endocytic capacity and constantly sample their environment for antigens at potential sites of pathogen entry.

Upon encounter of an antigen and the exposure to ‘danger’ signals (pathogens, tissue damage and local inflammation) immature DCs migrate to the lymphoid organs (Fig. 1). During this migration, which is regulated by altered expression of chemokine receptors, DCs undergo the maturation process that is characterized by down-regulation of the capacity to capture antigen and up-regulation of antigen processing and presentation, and of expression of co-stimulatory molecules. In the secondary lymphoid organs, mature DCs present antigens captured in the periphery to resting or naive T cells, inducing an adaptive immune response. After delivery of their pathogen-related information, the DCs, no longer necessary for the acute response to antigen, presumably undergo apoptotic cell death and are eliminated.

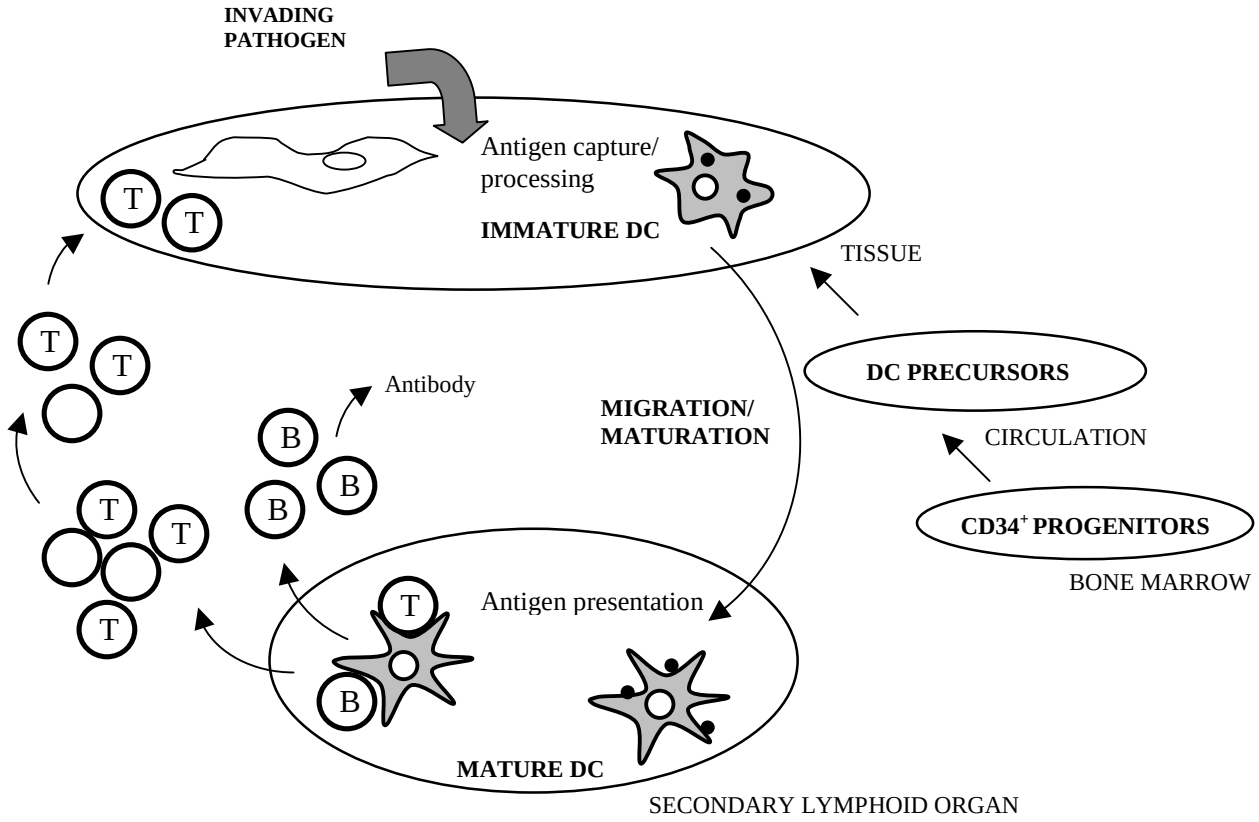

Figure 1 The life cycle of DCs. DCs, originating from bone marrow-derived hematopoietic $CD34^+$ progenitors, enter the circulation. Precursor DCs migrate into tissues all over the body, where they reside as immature DCs. Invading pathogens are captured by immature DCs, followed by antigen processing. The immature DCs mature and migrate to secondary lymphoid organs where they present antigens to T cells and initiate an immune response. The activated T cells migrate to the site of inflammation. B cells become activated after contact with T cells and DCs, migrate to various areas where they mature into plasma cells, secreting antibodies that can neutralize the invading pathogen.

Identification of DCs

DCs received their name on account of their distinctive cell shape [1]. Morphological characteristics, however, are insufficient as a discriminatory factor to distinguish DCs from other cell types. The identification of DCs relies on a combination of morphological, phenotypical and functional criteria. This includes the presence and absence of combinations of cell surface markers and the capacity to take up, process and present antigens. The relative scarcity of molecules that are uniquely expressed by DCs has hampered unraveling the ontogeny and lineage relationships of different DC subsets. The search for novel molecules, not only using monoclonal antibodies but also by differential display technology, continues and has resulted in several new molecules that are DC-specific, such as the intracellular maturation marker DC-LAMP [3], the chemokine DC-CK1 [4], and the C-type lectin DC-SIGN [5], or expressed relatively selectively by DCs, such as CMRF44 [6], CMRF 56 [7] and DC-STAMP [8].

HETEROGENEITY OF DCS

Generation of DCs

Over the past few years, many different cell culture systems using various combinations of cytokines and diverse starting populations to generate DCs have been described. In general, DCs are generated *in vitro* from hematopoietic $CD34^+$ progenitor cells or from peripheral blood monocytes. In cultures of $CD34^+$ progenitor cells, two different myeloid pathways as well as a putative lymphoid pathway of DC differentiation have been identified depending on the cytokine environment [9-11]. In the presence of GM-CSF and TNFα, the myeloid pathways lead to the generation of either Langerhans cells-like DCs or DCs with a dermal DC phenotype. The Langerhans cells-like DCs express CD1a, Birbeck granules and langerin, and DCs with a dermal DC phenotype are characterized by the expression of CD1a, CD68, CD2 and factor XIIIa [9]. A $CD34^+CD45RA^+CD10^+$ progenitor cell population derived from bone marrow has been described, that develops, in the presence of a mixture of cytokines, into T, B, NK cells and DCs, but not myeloid cells [11]. In addition, a $CD34^+$ $CD45RA^-$ progenitor has been shown to develop into a putative lymphoid, plasmacytoid DC in the presence of Fms-like tyrosine kinase receptor 3 ligand (Flt3L) [12].

In cultures of monocytes stimulated with GM-CSF and IL-4, immature myeloid DCs are generated. These immature DCs are characterized by high antigen capture and processing capacity and the induction of CD1a [13;14]. When cultured with M-CSF, the immature DCs differentiate to the macrophage phenotype, demonstrating the plasticity of these cells. Activation signals such as TNFα, LPS or CD40 ligand, induce the maturation of these immature DCs, indicated by the upregulation of adhesion and co-stimulatory molecules, the induction of CD83 expression and extremely efficient presentation of antigens. Once they have matured, DCs become resistant to modulation.

In vivo, the choice of whether a monocyte becomes a DC or a macrophage may in part be influenced by the endothelium. In a culture system involving endothelial cells grown on a collagen matrix, a subset of monocytes that have reverse transmigrated, display a DC phenotype, while the monocyte-derived cells that remain in the subendothelial layer become macrophages [15]. This model mimics the trafficking of DC precursors from blood to tissues through endothelium and the subsequent reverse transmigration mimics their movement from tissues to lymphatic vessels. Since this system is free of any exogenous cytokine, the relevance of monocytes as DC precursors is investigated in a more physiological context.

Additionally, CD2 expression by monocytes might be a property of DC precursors, as $CD2^+$ monocytes acquire a DC phenotype when cultured in the absence of cytokines, while $CD2^-$ monocytes remain $CD14^+$ and become macrophages under the same conditions [16].

Overall, the exact physiological differentiation pathway that generates DCs *in vivo* remains to be established [17]. However, a very recent study provides evidence for a DC-committed precursor in the mouse, which has the capacity to generate all the different DC subsets as found in mouse lymphoid organs, pointing toward an independent, common DC differentiation pathway [18].

Heterogeneity of DCs in vivo

Morphologically and phenotypically distinct DC subsets may be identified at many different anatomical sites and within a single tissue [19]. These differences probably may not only reflect different stages of maturation but also diverse origin of DC subsets. In humans, two subsets of DCs were originally identified in the peripheral blood based on the differential expression of CD11c [20]. Both subsets have in common the expression of CD4, MHC class II molecules, and the absence of lineage-specific markers such as CD14 (monocytes), CD3 (T cells), CD19, CD20 (B cells), CD56 (NK cells) and CD66b (granulocytes).

The $CD11c^-$ DCs express high levels of CD123 (interleukin (IL)-3Rα) [21] and display characteristic plasmacytoid morphology [22]. A lymphoid origin has been proposed for these so-called plasmacytoid DCs. Plasmacytoid DCs express pre-T cell receptor α chain transcripts [23] and development of plasmacytoid DCs, T and B cells, but not myeloid DCs is blocked by ectopic expression of inhibitor of DNA binding (Id)2 and Id3 inhibiting transcriptional activation of many basic helix-loop-helix-transcription factors [24]. When cultured with IL-3 or CD40L [22] or viruses [25;26], plasmacytoid DCs develop into mature DCs. Plasmacytoid DCs are identical to the natural type 1 interferon (IFN) producing cells that secrete high amounts of type 1 IFN (IFN α/β) in response to viruses [27]. DCs with a plasmacytoid phenotype are localized around high endothelial venules and in T cell areas within lymphoid organs such as tonsil [22], spleen [28] and thymus [29;30].

The $CD11c^+$ DCs express myeloid markers and require GM-CSF for survival in culture and are thus thought to be of myeloid origin [31]. $CD11c^+$ DCs constitute a heterogeneous subset with a variable differentiation capacity. Under different culture conditions, $CD11^+$ DCs develop in either $CD1a^+$ DCs or $CD14^+$ macrophages [20]. More recently, it was suggest that a distinctive $CD11c^+$ $CD1a^+$ DC subset in blood represent Langerhans cell precursors [32].

Whether $CD11c^+$ DCs in peripheral blood represent a reservoir that constitutively repopulates the pool of tissue DCs or are circulating while constantly sensing the endothelium followed by recruitment at inflammation sites is not known [33].

A panel of four recently described antibodies (BDCA-1-4) might help to further characterize DC subsets in human peripheral blood [34]. Using these antibodies, three phenotypically distinct subsets have been identified. BDCA-2 and BDCA-4 are uniquely expressed on plasmacytoid DCs, whereas BDCA-3 is only expressed on a small $CD11c^+$ $CD123^-$ DC subset and, in addition, on monocytes. BDCA-1 was identified as CD1c and is expressed, in addition to B cells, on the $CD11c^+$ $CD123^-$ DC subset.

In vivo, interstitial DCs have an immature phenotype and are found in almost all organs. It is thought that these immature interstitial DCs are precursors of the mature interdigitating DCs found in the T cell areas of secondary lymphoid organs. Another view is that Langerhans cells, immature DCs found in skin and other epithelia [35] are the precursors of interdigitating cells, whereas interstitial DCs move into germinal centers as germinal center DCs (GCDCs) [19;36]. The identification of distinct DC subsets at different anatomical localizations has lead to idea that distinct DC subsets may have different specialized functions [37].

DCS AND THE ADAPTIVE IMMUNE RESPONSE

Antigen uptake by DCs

Three pathways of antigen uptake have been described in DCs: phagocytosis, macropinocytosis and receptor-mediated endocytosis. Receptors that mediate endocytosis include C-type lectins (DEC-205 [38], mannose receptor [39], DC-SIGN [5]), Fc receptors that allow uptake of antigen opsonised with antibody [40;41], and Toll-like receptors (TLR) that recognize microbial products [42]. DCs also can very efficiently take up dying cells via CD36 and $\alpha_v\beta_5$ integrins [43;44]. The captured antigens enter the endocytic pathway of antigen presentation of DCs and are targeted to MHC class II compartments, followed by loading on MHC class II molecules. Subsequently, the MHC class II-peptide complexes are translocated to the cell surface.

Endogenous MHC class I pathway

For a long time, it was thought that exogenous antigens could not access the antigen presentation pathway that loads peptides onto MHC class I molecules of APC. For MHC class I-peptide complexes to form, only peptides derived from newly synthesized, endogenous proteins in the cytoplasm, were thought to be processed via the proteasome and transported into the endoplasmic reticulum via transporters for antigen processing. However, it has been shown that DCs efficiently present exogenous proteins that have gained access to the cytosol and are therefore presented on MHC class I molecules, a phenomenon called cross-presentation [45-47]. The nature of the antigen is critical for cross-presentation as soluble proteins are poorly presented, whereas antigens introduced via receptor-mediated endocytosis and phagocytosis are presented very efficiently. Heat shock proteins, released from dying cells, can chaperon peptides into the MHC class I pathway [48].

T cell response

Adaptive immunity is controlled by the generation of MHC-restricted effector T cells and the production of cytokines. T cells recognize peptides bound to MHC molecules by their T cell receptor (TCR). In addition, T cells express CD4 or CD8 coreceptors. $CD8^+$ T cells detect MHC class I-peptide complexes and differentiate into cytotoxic T effector cells (CTL) that directly kill cells infected with intracellular pathogens such as viruses. $CD4^+$ T cells detect MHC class II-peptide complexes and perform mainly helper functions for CTLs or B cells, and are thus called T helper (T_H) cells. These differentiated T cells develop from a common pool of naive precursor T cells. In secondary lymphoid organs, DCs prime naive $CD4^+$ T cells by displaying MHC class II-peptide complexes, which are recognized by the TCR (signal 1:antigen-specific stimulation), in combination with an interaction of co-stimulatory molecules and their ligands on T cells (signal 2: co-stimulation). This physical DC-T cell interaction is referred to as the 'immunological synapse' [49].

Activation of $CD4^+$ T cells induces T_H development and accordingly the recruitment of non-antigen specific effectors including NK cells, eosinophils and macrophages, resulting in diverse immune responses. The capacity of DCs to induce stimulatory T cells is dependent on the state of DC maturation. In the absence of inflammatory stimuli, immature, resting DCs are relatively poor stimulators of the proliferation of resting T cells and can presumably induce tolerance.

T helper 1 vs. T helper 2

Immune responses to different pathogens are associated with different types of effector responses directed by polarization of T_H cells into either a T_H type 1 (T_H1) response or a T_H type 2 (T_H2) response. The cytokines produced by these T_H subsets have a definitive influence on the outcome of the cellular and humoral immune response. In general, T_H1 cells secrete primarily IFNγ and promote cell-mediated immunity against intracellular pathogens.

In contrast, extracellular pathogens induce the development of T_H2 cells, producing IL-4, IL-5, IL-10 and IL-13, which are instrumental for B cell activation in a humoral immune response. Evidence has accumulated that in addition to providing signal 1 and signal 2, DCs produce cytokines,such as IL-12, IL-18, IL-4 or IL-10, in response to different stimulating tissue or pathogen-related factors, which are able to polarize the emerging T cell response (signal 3: polarization) [50].

DC -B cell interactions

DCs can also directly activate naïve and memory B cells. DCs express BAFF, a member of the TNF superfamily, which plays an important role as co-stimulator of B cell proliferation and function [51]. CD40-stimulated DCs also secrete IL-12 and other factors that, together with IL-2, may signal naive B cells during initiation of the immune response. GCDCs present in the germinal center induce differentiation into plasma cells secreting high levels of IgM in the presence of IL-2 [52]. DCs can also produce factors that can specifically regulate naive and memory B cell migration [53] of relevance within secondary lymphoid organs.

FUNCTIONAL HETEROGENEITY OF DC SUBSETS

Functionally, DCs represent a heterogeneous cell population. DCs exist in different maturational states, and exhibit functional and phenotypic differences. Functional heterogeneity is reflected by the observation that distinct DC subsets polarize T_H cell responses, i.e. elicit IFNγ producing T_H1 cells or IL-4 producing T_H2 cells. In humans, mature monocyte-derived DCs, termed DC1, were shown to generate a T_H1 response by producing the T_H1-polarizing cytokine IL-12 and induce a strong CTL response [54]. Especially CD40 ligand induced mature monocyte-derived DCs produce high levels of IL-12 [55]. Immature monocyte-derived DCs induce IL-10 producing regulatory T cells [56;57]. The

proposed human DC2 lineage comprises the plasmacytoid DCs, which induce a strict T_H2 response by a deficiency in IL-12-producing capacity [54], although that this was not observed in another study [25].

The observation that murine Peyers patch $CD11c^+$ DCs elicit T_H2 responses whereas $CD11c^+$ DC from spleen induce T_H1 responses [58] indicate that DC subsets isolated from discrete tissue microenvironments differently affect T_H responses. Immature DCs are also susceptible to pathogen-derived products or pathogen-induced nonspecific tissue factors that modulate the capacity of immature DCs to polarize naïve T cells into T_H1 or T_H2 cells [50;59]. Prostaglandin E2, IL-10 or glucocorticoids induce a T_H2-inducing DC subtype while the addition of IFNγ during DC maturation results in high IL-12 producing DCs that promote a T_H1 response [50;60;61]. Thus, functional specialization of DCs can be related to maturation stage and subset. Functional plasticity of DC subsets is demonstrated by tissue derived-factors and pathogen-related molecules that modulate DC responses and thus signal 3.

DCS IN INNATE IMMUNITY

The Toll-like receptors

In addition to their role in adaptive responses, DCs play a critical role in innate immunity. Many of the 'danger' signals that influence surveillance DC activity are mediated via or modified by the innate immune system. These signals may come from molecules such as LPS, IFN α and γ, and IL-1β and directly from invading pathogens.

Viruses and bacteria are identified by DCs by the presence of pathogen-associated molecular patterns (PAMPs) that trigger pattern recognition receptors (PPRs) expressed on DCs. Toll-like receptors represent a newly recognized family of vertebrate PPRs. To date, ten TLRs (TLRs 1-10) have been reported [42]. Signaling through TLRs strongly activates DCs to upregulate co-stimulatory molecules CD80 and CD86 and to produce cytokines [62]. The involvement of TLRs in maturation provides an attractive mechanism by which DCs link innate to adaptive immunity.

Each TLR appears to recognize a distinct microbial product: human TLR2 recognizes an array of molecules including bacterial lipoproteins [42], whereas TLR4 recognizes lipopolysaccharide (LPS) [63;64], TLR5 recognizes bacterial flagellin [65], and DNA containing unmethylated CpG motifs, which are found in greater abundance in microbial DNA than in

mammalian DNA signal trough TLR9 [66]. In addition, extension of the repertoire of recognition modules may be achieved by TLR heterodimerization, for example TLR2 is able to form functional pairs with TLR1 or TLR6 [67].

Plasmacytoid DCs strongly express TLR7 and 9, whereas $CD11c^+$ blood DCs preferentially express TLR 1, 2 and 3 [68]. In addition, monocytes express TLR 1,2, 4, 5 and 8. Since distinct DC subsets express distinct sets of TLRs, they may recognize different microbial products, in turn produce different sets of chemokines involved in trafficking of DCs, and cytokines, leading to polarization of the immune response. This suggests a limited plasticity in DC function.

DCs and NK cells

NK cells and NK T cells are effector cells of the innate immune system that are able to kill a variety of cells spontaneously, including tumor and virus-infected cells and produce important cytokines, particularly IFNγ. DCs can activate NK and NK T cells, by direct cell-cell interactions or indirectly e.g. by the production of IFNα or IL-12. This leads to enhanced anti-viral and anti-tumor activity of these cells [69;70]. In turn, NK cells are capable of enhancing DC maturation [71].

DCS IN CANCER IMMUNOTHERAPY

Cancer vaccines

Anti-tumor vaccination has a defined goal: to induce the specific recognition of tumor-associated antigens (TAAs) by cells of the immune system with the aim of selectively eliminating tumor cells. DCs have acquired a central role in vaccination approaches for the treatment of cancer because DCs are uniquely potent in inducing a CTL response against exogenous antigens. In addition, DCs are capable of generating and maintaining immunological memory [72]. Encouraging results in initial vaccination studies using DCs presenting tumor antigens and the identification of a growing number of tumor antigens have accelerated the development of DC-based vaccine approaches to human cancer [73-76]. The development of protocols to generate sufficient numbers of human DC *ex vivo* from either hematopoietic $CD34^+$ progenitor cells or peripheral blood monocytes for clinical application have led to an increase in clinical vaccination trials using DCs *ex vivo* loaded with tumor antigens.

The capacity of DCs to engage both arms of the human T cell response is crucial in light of evidence that resistance to experimental tumors is enhanced when both CTLs and T_H cells are generated [77-79]. Results of clinical trials suggest that DC-based vaccines are safe and well tolerated. Encouraging clinical data are demonstrated, at least for some tumors, confirming the promise of DC-based immunotherapy[80-82].

Different strategies to delivers antigens to DC

Different strategies are employed to get a TAA delivered to DCs so that an effective immune response is elicited (Table 1). *Ex vivo* loading of MHC class I molecules on DCs with peptides derived from known tumor-associated antigens (TAA) such as MAGE-1 [83] and MAGE-3 [84] or Melan-A/MART [75;85;86] has been most commonly used. Because of MHC restriction, this approach is limited to patients that express a defined specific HLA haplotype. Moreover, using MHC class I restricted peptides ignores the role of MHC class II restricted T_H cells in initiation and sustaining an immune response [78]. Foremost, immunization with defined tumor antigens is limited to cancers where effective TAA have been identified. In addition, the use of vaccines consisting of single TAA bears the risk of leading to the generation of antigen-loss escape mutants.

Using unfractionated tumor material or tumor lysates to pulse DCs *ex vivo*, tumor types for which no TAA have been identified can be treated. The presence of multiple tumor antigens in this approach results in a broad variety of epitopes presented via both the MHC class I and II pathways, minimizing the risk of escape mutants. The disadvantage of this approach lies in the availability of sufficient amounts of tumor tissue from patients. Moreover, the complete lack of control on the nature of the antigens that are being present by the DCs may cause induction of undesired autoimmune responses directed against self antigens [87]. Another appraoch to improve the diversity of tumor-derived peptides presented by DCs, is the uptake of dead or dying tumor cells by DCs, which also leads to cross-presentation [45]. However pulsing of apoptotic bodies may require necrotic material for optimal antigen presentation [88].

Ex vivo transfection of DCs with tumor RNA also appears to lead to the presentation of a wide array of tumor peptides [89]. DCs can be transduced with RNA coding for a specific TAA or the entire RNA extracted from tumor cells. The RNA can be used directly of after amplification from small amount of starting material from a few tumor cells in a biopsy. In this RNA approach, the lack of control over the nature of antigens that are being presented by the DC forms a possible limitation.

Naked plasmid DNA has been used extensively in the development of tumor vaccines [90;91]. A further possibility is the expression of genes encoding a TAA in DCs by means of appropriate viral vectors. Among the viral vectors, retroviral, adenoviral, and vaccinia vectors have been widely used to transduce either monocyte-derived or $CD34^+$-cell derived DCs. The advantage of using retroviral vectors is that retroviral transduced DCs should be able to constitutively express and process TAA to produce a long-term antigen presentation *in vivo* [92]. However, their low efficiency of transduction limits the clinical use of retroviruses. In contrast, adenoviral vectors transduce monocyte-derived or $CD34^+$-cell derived DCs with a high efficiency but the preexisting natural immunity against adenoviral vectors may pose a major hurdle [93].Yet another strategy to deliver antigens to DCs exploits the *ex vivo* somatic fusion of tumor cells and DCs. These heterohybrids have already been explored to vaccinate cancer patients in clinical trials and have shown some promising therapeutic effect [94;95].

Table 1 Delivery of antigens into DCs in DC-based vaccines

Defined TAA
Synthetic or eluted peptides
Transfection with cDNA or RNA encoding TAA
Viral vectors
Whole tumor vaccine
Tumor lysates
Dying cells
Tumor RNA
DC-tumor cell fusions
Exosomes

Another strategy to involves the usage of exosomes. Exosomes are small vesicles either released from tumor cells and presented by DCs [96] or released by DCs that have processed tumor cells [97] and contain MHC class I and II products, co-stimulatory molecules and other cell-derived products. Exosomes can potentially be developed into a standard form of TAA for *in vivo* or *ex vivo* loading and stimulation of DCs.

Source of DCs

The majority of clinical studies to date have been carried out with *ex vivo* generated monocyte-derived DCs [91]. Purified peripheral blood monocytes are cultured with GM-CSF and IL-4 that require additional maturation stimuli such as TNFα or monocyte-conditioned medium to increase their stimulatory capacity [98;99]. Alternatively, DCs can be generated form purified $CD34^+$ progenitor cells using appropriate cytokine combinations. Sources of $CD34^+$ cells include bone marrow, cord blood, and G-CSF mobilized peripheral blood. Although the

high antigen capture and processing capacity of immature DCs makes them suitable for uptake of exogenous antigens such as RNA, proteins or dead tumor cells, recent reports demonstrate that immature DC can induce tolerance to antigen used for vaccination [56;57;100].

However, other reports show that in murine models, mature monocyte-derived DCs were unable to elicit an anti-tumor response while immature monocyte-derived DCs elicit therapeutic effects [101;102]. Perhaps mature DCs have lost migrating capacity necessary to reach the appropriate secondary lymphoid organs. In general it is unclear to what extent DCs *ex vivo* cultured and loaded with TAA are able to migrate to relevant lymphoid organs in humans. Parameters to define effective versus ineffective antigen presenting cells for use in clinical trials and appropriate co-stimulatory and activation molecules to expose DCs in order to optimize DC function *in vivo* are addressed in current studies [103]. Also the route of administration may effect the quality of the response [104]. Several parameters, such as the dose of DCs, the route and the frequency of injection are also examined in ongoing clinical trials to optimize immune responses in order to maximize clinical efficacy against cancer [105].

The future of DC-based cancer vaccines

Cellular vaccines, such as the DC-based approach, are expensive, time consuming, and complex, and may not be simply implemented as a standard treatment of cancer patients. A perhaps more realistic strategy may be the induction of large numbers of DCs through the use of growth factors *in vivo.* For example, the administration of Flt3L in humans results in a reversible accumulation of functionally active DCs. Both the $CD11c^+$ and $CD11c^-$ DC subsets are expanded: the increase in circulating $CD11c^+$ DCs adds up to a mean of 48-fold, and in $CD11c^-$ DCs to a mean of 13-fold [106;107].

Direct *in vivo* targeting of TAA to receptors expressed on DCs may abolish the need for the tedious generation and loading of DCs *ex vivo* [108]. Antigen targeting using monoclonal antibodies may provide an elegant way for receptor-mediated targeted delivery of antigens to DCs [109;110]. Early studies, using an anti-MHC class II monoclonal antibody in a rat model, has validated this idea [111;112]. Targeting to a monocytic cell line by means of a fusion protein consisting of a antibody directed against FcγRI (CD64) and the prostate specific antigen PSA, results in the induction of presentation of PSA antigens on MHC class I molecules *in vitro* [113].

In a murine model, selective delivery of antigens was achieved *in vivo* by means of a fusion protein between an antibody against the DEC-205 (CD205), an endocytic receptor,

and HEL peptide resulting in presentation on MHC class II molecules [100]. However, in this study tolerance was induced due to lack of addition stimuli to mature DCs. In addition, adjuvants such as CpG oligonucleotides may be valuable for *in vivo* maturation of DCs, while they also prolong the survival of DCs [114;115]. Targeting direct to DCs *in vivo* by means of an antibody as vector could result in presentation of antigens on both MHC class I and II molecules leading to a broad immune response involving both T_H cells and CTLs. The specificity of the antibody permits selective targeting of the desired DC subset.

SCOPE OF THIS THESIS

The goal of the research described in this thesis was to identify human antibodies that bind to DCs and to explore whether these antibodies could be used to further delineate DC subsets and to evaluate their potential as targeting vehicles for the *in vivo* delivery of tumor antigens to DC. Human antibodies do not appear to be immunogenic, making human antibodies the most desirable antibody format for clinical application. DCs play a pivotal role in the induction of immune responses, explaining why they have received much attention in the design of new cancer vaccination strategies. Current DC-based vaccine approaches mostly rely on the *ex vivo* generation of DCs from precursor cells, which are subsequently loaded with tumor antigens and re-infused into patients to evoke an anti-tumor response. An alternative approach is provided by human antibodies as vectors for *in vivo* targeted delivery of tumor antigens to DCs.

In the search for suitable cell surface molecules expressed by DCs in peripheral blood and tonsil, we employed a phage antibody display approach to generate phage antibodies binding to cell surface molecules [116;117]. We performed selections using a phage antibody display library in combination with flow cytometry on freshly isolated DCs found in human peripheral blood (*Chapter 2A*) and human tonsil (*Chapter 2B*). We obtained several phage antibodies with desirable characteristics, recognizing both DCs and their precursors the monocytes, which have been used as tools in further delineation of DC subsets.

These phage antibodies were converted to fully human monoclonal antibodies (huMabs) of the IgG4 isotype. Furthermore, different DC subsets have been described based on phenotype, function and anatomical localization. The diversity of DC subsets and their potential role in polarization of the immune response, prompted us to determine in detail the

reactivity patterns of our huMabs on *in vivo* DC subsets found at different anatomical localizations and *in vitro* generated DCs (*Chapter 3*).

In light of the potential application of our huMabs as tools to target antigens to DCs, we analyzed whether engagement of the molecules recognized by the huMabs exerted functional effects (*Chapter 4*).

In a first step toward *in vivo* targeting of DCs in cancer immunotherapy, we explored whether antibody-mediated targeting of tumor antigens to DCs using a huMab as vector could provide a novel antigen delivery strategy. This strategy involves the construction of an eukaryotic expression vector encoding a huMab, genetically fused to a tumor antigen. We established proof of principle of this approach in an *in vitro* setting (*Chapter 5*).

In a cell-based approach to cancer vaccination, we explored whether these lipid-tagged scFvs incorporated in tumor cells could induce receptor-mediated endocytosis by monocytes (*Chapter 6*). Therefore, the phage antibodies were converted to lipid-tagged scFv antibody fragments.

At the end of this thesis, a summarizing discussion is provided in which our findings are discussed and the main perspectives for future studies emanating from the research described in this thesis are presented (*Chapter 7*).

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CHAPTER 2A

PHAGE ANTIBODIES AGAINST HUMAN DENDRITIC CELL SUBPOPULATIONS OBTAINED BY FLOW CYTOMETRY-BASED SELECTION ON FRESHLY ISOLATED CELLS

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ABSTRACT

In one application of phage display technology, large libraries of antibody fragments displayed on phage particles are used to select antibodies that bind to molecules expressed on the surface of eukaryotic cells. The advantage of this method is that antibodies can be selected against antigens in their native configuration, without the need to purify or express the antigen as a recombinant protein. Moreover, this approach may be used to search for novel membrane molecules expressed by subpopulations of cells that are difficult to address by conventional methods, e.g. small numbers of cells present in heterogeneous mixtures. It has been shown that the isolation of cell-bound phages is compatible with immunofluorescence staining and flow cytometric identification and sorting of cells based on multiparameter analysis. Here, we have employed a semi-synthetic phage display library of human single chain Fv antibody fragments in combination with flow cytometry to isolate antibodies against rare populations of precursor and mature dendritic cells present in human peripheral blood. Dendritic cells are a phenotypically heterogeneous population of professional antigen presenting cells of bone marrow origin with complex and only partly understood developmental relationships and functions. We have isolated phage antibodies against subpopulations of blood DCs and analyzed the distribution of the target antigens. The results show that these phage antibodies are useful tools to further dissect relationships and function of DCs in healthy and diseased tissues.

INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells that play a central role in the initiation of the immune response. DCs are well equipped to take up antigens from their environment, to process antigens that have entered the endocytic pathway and to present them as immunostimulatory peptides to T lymphocytes in the context of major histocompatibility (MHC) antigens. In addition to their role in stimulating primary T cells, DCs also have a direct effect on the growth and immunoglobulin secretion of B lymphocytes [1].

DCs are derived from bone marrow stem cells and are found in small numbers in most lymphoid and non-lymphoid tissues [2]. There appear to be several developmental pathways that lead to the generation of DCs [3]. To this date, no cell surface markers that exclusively identify DC subpopulations or lineages have been found. The identification of DCs relies on a combination of morphological, phenotypic and functional criteria, including the presence

and absence of combinations of cell surface markers and the capacity to take up, process and present antigens.

In the bone marrow, the small subset of $CD34^+$ hematopoietic progenitor cells contains the progenitors for at least two discrete DC populations: the epidermal Langerhans cells and the dermal or interstitial type DCs [4;5]. In blood, the minute numbers of cells with DC characteristics belong to at least two different phenotypically and functionally distinct subpopulations. One population is defined as $CD33^{dim}CD14^-CD16^-$ [6;7], and is also referred to as the $CD11c^-$ DC population that lacks lineage-specific markers (Lin^-) [8] and the $Lin^-HLA\text{-}DR^+$ cells that express high levels of the interleukin-3α receptor [9]. The $CD33^{dim}CD14^-CD16^-$ DCs represents a precursor population that efficiently takes up and processes antigens yet it fails to efficiently present peptides to T cells. The blood $CD33^{dim}CD14^-CD16^-$ DCs phenotypically resemble a recently described DC precursor population in T cell areas of lymphoid tissues [10]. The second blood DC population has been defined as $CD33^+CD14^-CD16^-$, whereas DC populations with similar characteristics have been identified as Lin^-CD11c^+. This population also expresses the CMRF44 marker [11;12], yet lacks CD83 [13]. The $CD33^+CD14^-CD16^-$ cells are considered more mature as they have an increased accessory function compared to the $CD33^{dim}CD14^-CD16^-$ DC precursors [7]. A phenotypically similar population has been found in germinal centers of lymphoid tissue [14].

Recent studies have shown that large numbers of cells with DC-like properties can be generated in vitro from $CD34^+$ bone marrow and cord blood cells and blood monocytes by stimulation with cytokines such as granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL) 4, and tumor necrosis factor α (TNF-α) [15-17]. Most of our current understanding of the developmental relationships between DC subpopulations has been based on results obtained with these culture systems [3]; the relevance for *in vivo* DC relationships and differentiation pathways largely remains to be established.

Monoclonal antibodies represent an important tool in delineating developmental relationships between cells and in unraveling functional properties of cell surface molecules. In search for unique surface markers expressed by DCs in peripheral blood and other tissues, we have employed a phage antibody display approach to generate recombinant antibody fragments binding to cell surface molecules. We have previously shown that semi-synthetic libraries of recombinant human single chain (sc) Fv antibodies expressed on phage particles, in combination with flow cytometry may be used to isolate phage antibodies against subpopulations of cells present in heterogeneous mixtures [18;19]. This selection procedure is

rapid and independent of the immunogenicity of the target antigen, a limitation associated with conventional hybridoma technology. Monoclonal phage antibodies (MoPhabs) isolated by this method are directed against antigens in their native configuration, unaltered by purification or antigen immobilization procedures that may introduce neo-epitopes. This approach was shown to be applicable to very rare populations of cells such as hematopoietic stem cells that occur in the bone marrow mononuclear cell fraction at a frequency of $1/10^4$-$1/10^5$ cells [20]. Here, we have applied this procedure to isolate MoPhabs binding to blood $CD33^{dim}CD14^-CD16^-$ precursor DCs and $CD33^+CD14^-CD16^-$ mature DCs. We demonstrate that these MoPhabs can be used as a tool to investigate lineage and subpopulation relationships between DCs in different lymphoid organs.

MATERIAL AND METHODS

Media and reagents

The medium used in all cell cultures was RPMI 1640 supplemented with (2 mM) L-glutamine and 25 mM HEPES buffer (Gibco BRL, Breda, The Netherlands), and with 1000 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (Integro, Zaandam, The Netherlands).

Antibodies

We used the following mouse IgG monoclonal antibodies: FITC- or PE- conjugated CD3, CD4, CD11c, CD13, CD14, CD16, CD19, CD33, CD56, and CD71 (all from Becton Dickinson, San Jose, CA), PE- conjugated CD1a (CLB, Amsterdam, The Netherlands), PECy5-conjugated antibody against CD33, PE-conjugated antibody against CD83 (all from Immunotech, Marseille, France), FITC-conjugated antibody against CD40 (Monosan, Uden, The Netherlands), FITC-conjugated antibody against CD86 and PE-conjugated antibody against CDw123 (both from PharMingen, Hamburg, Germany), TC-conjugated antibody against CD38 (Monosan), FITC-conjugated antibody against IgD (SBA, Birmingham, USA), an APC-conjugated antibody against CD4 (Becton Dickinson), monoclonal antibody 9E10 against the Myc tag (American Type Culture Collection) and a monoclonal antibody against M13 that was biotinylated in house (Pharmacia Biotech Uppsala, Sweden). In this study also sheep anti-M13 polyclonal antibody (Pharmacia Biotech), PE-conjugated donkey anti-sheep

polyclonal antibody (Jackson ImmunoResearch, West Grove, PA), streptavidin-FITC (SBA), streptavidin-PE (Immunotech), streptavidin-PercP (Becton Dickinson), and rabbit anti-mouse Ig HRP conjugate (Dako, Glostrup, Denmark) were used.

Phage antibody library

The semi-synthetic phage antibody display library of human scFv antibody fragments was similar to that described in detail elsewhere [18;19]. Briefly, germline V_H genes were fused to semi-randomized synthetic heavy chain CDR3 regions, varying in length between 6 and 21 amino acid residues. The resulting products were inserted into a phagemid vector, containing light chains of both kappa and lambda subclasses.

Cell isolation procedures

Peripheral blood was obtained from healthy donors. Heparin (50 U/ ml) or EDTA (0.47 M; Venglect) was used to prevent clotting. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation. Granulocytes, visualized by flow cytometry, were gated based on typical forward and side scatter, after hypotonic lysis of peripheral blood. Human tonsils were cut into small pieces and digested with 1 mg/ml collagenase IV (Sigma Chemical Co., St.Louis, MO) and 0.1 mg/ml deoxyribonuclease (Boehringer Mannheim) for one hour at 37°C. The single cell suspensions were washed twice. Single cell suspensions of adult bone marrow were obtained from healthy donors and mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation, diluted 1:1 in medium.

Selection of phage antibodies by cell sorting

The selection of phage antibodies and propagation of the selected phage antibodies was done essentially as described [18]. Briefly, 40 x 10^6 PBMCs were depleted of T cells by rosetting with sheep red blood cells as described [21]. A 500 µl portion of the phage library, containing approximately 10^{13} antibody bearing phage particles per ml, was blocked for 15 minutes in 250 µl of PBS/ 5% (w/v) milk powder. Subsequently, the T cell-depleted PBMCs were added to the blocked phages and the mixture was slowly rotated overnight at 4°C, the following day, the cells were washed twice with ice-cold PBS/1% (w/v) BSA. The pelleted cells were suspended in 20 µl PE-conjugated antibody CD33 and 20 µl FITC-conjugated antibody against CD14 to visualize different cell populations by flow cytometry. After 20

minutes incubation on ice, the cells were washed once with PBS/1% BSA and resuspended in 4 ml of PBS/1% BSA. Cell sorting was performed on a FACStar^{PLUS} fluorescence activated cell sorter (Becton Dickinson). For each cell population of interest 10^4 to 10^5 cells were sorted. Phages were eluted from the sorted cells, 1.5 times the volume of the cells of 76 mM citric acid (pH 2.5) was added followed by incubation for 5 minutes at room temperature. The mixture was neutralized with 0.5 times the volume of the mixture of 1 M Tris HCl (pH 7.4). The eluted phages were used to infect *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA) and plated out on TYE medium [22] containing the appropriate antibiotics and glucose. Bacterial colonies were counted, scraped form the plates, and used as an inoculum for the next round of phage rescue.

Preparation of MoPhabs and scFv fragments

After three rounds of selection, MoPhabs were rescued from single ampicillin-resistant colonies of infected XL1-Blue cells[22] and cultured in a final volume of 4 ml of 2TY containing the appropriate antibiotics and glucose. Helper phages were added to each culture and phages were harvested from the supernatant by precipitation using polyethylene glycol/NaCl, and resuspended in 400 µl of PBS/1%BSA, as described [18]. Binding to different cell populations was verified by immunofluorescence analysis. ScFv's were produced in the *Escherichia coli* non-suppressor strain SF110, which is deficient in the proteases DegP and OmpT. This results in the production of stable scFv fragments. Induction of protein synthesis and isolation scFv fragments from the periplasmic space was performed as described [18]. Expression of the scFvs was analyzed by Western blotting [18].

Immunofluorescence staining procedures

For staining of cells, 100 µl of MoPhab was blocked by adding 50 µl of PBS/ 4% milk powder for 15 minutes at room temperature. 5 x 10^5 cells in 50 µl of PBS/ 1%BSA were added and incubated on ice for 1 hour. To detect cell-bound phages, the cells were incubated in 10 µl of a 1:400 dilution of sheep anti-M13 polyclonal antibody for 20 minutes on ice, washed, and incubated in 10 µl of a PE-conjugated donkey anti-sheep polyclonal antibody solution (20 :g/ml) for 20 min on ice. The cells were washed and incubated with conventional monoclonal antibodies of interest. After a single final wash, the cells were suspended in 400 µl of PBS/ 1%BSA. Flow cytometric analyses were performed using a FACScan fluorescence-activated cell analyzer (Becton Dickinson).

Western blot analysis

Samples were separated on 10% SDS-polyacrylamide gels followed by electroblotting to nitrocellulose. ScFv proteins were visualized by staining with undiluted hybridoma supernatant containing the myc-tag specific antibody 9E10, followed a HRP-conjugated rabbit anti-mouse Ig diluted in PBS/4% milk powder. After a final wash, blots were developed with diaminobenzidene.

Screening and nucleotide sequence analysis of phage antibodies

The diversity of the phages with binding activity was assessed by BstNI DNA fingerprint analysis of clones[22]. The nucleotide sequence of the selected clones was determined by automated sequencing to establish V_H and V_L gene identity and heavy and light chain CDR3 composition.

Cell lines and transfectants

T cell line Jurkat, pre-B cell lines Nalm-6 and Reh, mature B cell lines Raji and Daudi, plasmacytoid cell line U266, myeloid cell lines, HL60, U937, KG1-A, MonoMac and THP-1, megakaryocyte cell line DAMI, hepatoma cell line Hep-G2, human umbilical cord endothelial cells, Hodgkin's cell lines L428 and HDLM-2, erythroid precursor cell line K562, cervical carcinoma cell line HeLa, colon carcinoma cell line LS174T and lung carcinoma cell line GLC-8 were cultured in complete medium. Baby hamster kidney cells transfected with the GM-CSF receptor (GMR) α subunit, the GMR β subunit and the GMR α/β subunits were cultured in DMEM with 10% FCS and 1% pencillin/streptomycin, with supplement of respectively 200 μM methorexate, 4 μM G418 and, 200 μM methorexate and 4 μM G418 together. Binding of MoPhbas to Jurkat transfected cell lines with immunoglobulin-like transcripts (ILT) 3, 4 and 5 respectively was kindly performed by Dr. Marco Colonna. The CD14 transfectant and the CD64 transfectants were a kind gift of drs R. Ulevitch and J. van de Winkel respectively.

RESULTS

Experimental strategy

We have employed a semi-synthetic phage display library of human scFv fragments to isolate antibodies that bind to DC subpopulations present in human peripheral blood. PBMC were isolated and stained with a combination of FITC-labeled CD14 and PE-labeled CD33 antibodies. Previous studies have demonstrated that with this staining protocol, blood DCs can be subdivided into a mature $CD14^-CD33^+$ population and a precursor $CD14^-CD33^{dim}$ population [6;7]. In this scheme, monocytes can be identified as a distinct $CD14^+CD33^+$ cell population, whereas lymphoid cells such as B cell, T cells and NK cells are $CD14^-CD33^-$ (Fig. 1).

PBMCs were depleted of T cells and 3-9 x 10^7 cells were incubated with the phage library. Subsequently, the cells with bound phages were incubated with PE-labeled CD33 and FITC-labeled CD14. Approximately 6 x 10^4 cells mature DCs and 1.3 x 10^5 precursor DCs were sorted; the phages were eluted from the cell surface and propagated in bacteria for the next round of selection. After the first round of selection 1 x 10^5 colonies were obtained for the selection with mature DCs and 2.3 x 10^5 colonies for the selection with precursor DCs.

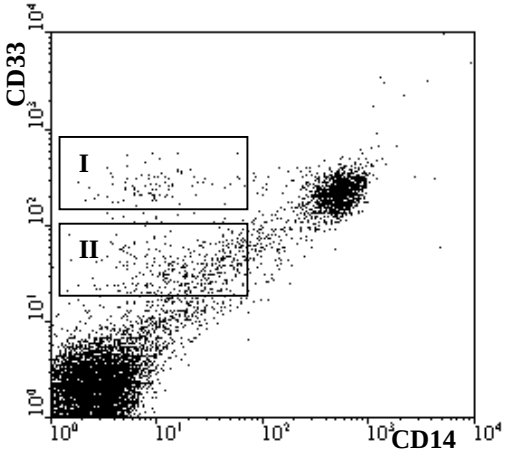

Figure 1 Identification of precursor and mature blood DCs. PBMCs were stained with PE-labeled anti-CD33, followed by staining with FITC labeled anti-CD14. The dot-plot depicting CD33 versus CD14 fluorescence intensity is shown. Blood DCs can be subdivided into a mature $CD14^-CD33^+$ DC population (I) and a precursor $CD14^-CD33^{dim}$ DC population (II). Rectangles represent sorted cell populations. Monocytes can be identified as a $CD14^+CD33^+$ population, whereas lymphoid cells reside within the $CD14^-CD33^-$ population.

Phages selected for binding to precursor DC

After three rounds of selection on $CD14^-CD33^{dim}$ precursor DC a total number of 70 bacterial clones was picked, MoPhabs were prepared and analysed in flow cytometry in double-staining with anti-CD33 for binding to PBMC. A total of 11 phages did not bind to any subpopulation in the PBMC fraction. The remaining 59 phages bound to subpopulations of $CD33^+$ cells and 39 of these displayed additional binding to $CD33^-$ cells. Phages that displayed binding to CD33- PBMC were not considered for further study. The remaining 20 MoPhabs were subjected to BstNI fingerprint and immunofluorescence staining analyses in

combination with lineage-specific monoclonal antibodies. Based on the cell distribution patterns of the molecules identified by independent MoPhabs in this collection and the intensity of immunofluorescence staining, no MoPhabs from this selection were pursued for further analysis.

Phages selected for binding to mature DC

A total of 90 MoPhabs derived from the selection on the $CD14^{-}CD33^{+}$ population was analysed by flow cytometry; 42 MoPhabs in this collection bound exclusively to $CD33^{+}$ cells or displayed additional binding to small subpopulations of $CD33^{-}$ cells. After BstNI fingerprinting, 5 MoPhabs, named MatDC11, MatDC16, MatDC27, MatDC51 and MatDC64 were propagated for further analysis.

Reactivity of MoPhabs with cells in peripheral blood

The binding of MoPhabs to subpopulations of PBMC was assessed by triple staining experiments with FITC-labeled CD14 and CD16, PECy5-labeled CD33 monoclonal antibodies and PE-labeled MoPhabs. The CD16 antibody was included to separate the $CD33^{dim}$ cells in $CD33^{dim}/CD16^{+}$ monocytes and $CD33^{dim}/CD16^{-}$ DCs[23]. For each experiment, 10^3 cells within the $CD14^{+}CD16^{-}CD33^{+}$ monocyte gate, the $CD14^{-}CD16^{-}CD33^{+}$ mature DC and the $CD14^{-}CD16^{-}CD33^{dim}$ precursor DC gates were analyzed. In addition, we performed double staining experiments with MoPhabs and fluorochrome-labeled lineage-specific monoclonal antibodies including CD3 (T lymphocytes), CD19 (B lymphocytes) and CD56 (natural killer cells). Binding of MoPhabs to granulocytes was analyzed based on forward and side scatter profile. As a control, PBMC were stained with MoPhab Mono-14. This MoPhab was obtained by phage selections on the $CD14^{+}CD33^{+}$ monocyte population and recognizes the CD14 molecule, as determined by specific staining of CHO cells transfected with the human CD14 cDNA (results not shown). As a negative control in staining experiments, a MoPhab specific for thyroglobuline[19] was used. The results are shown in figure 2 and summarized in table 1.

All five MoPhabs stained mature DC; with a bright staining for MatDC 11, MatDC 16 and MatDC 51. MatDC 11 bound to all $CD14^{-}CD33^{dim}$ precursor DCs, while the other four MoPhabs appeared to recognize only a subpopulation of the precursor DCs. All five MoPhabs displayed binding to the $CD14^{+}CD16^{-}CD33^{+}$ blood monocytes. No binding to blood $CD3^{+}$ T cells or $CD56^{+}$ NK cells was observed for any of the MoPhabs. MoPhabs MatDC 11 and MatDC16 bound to $CD19^{+}$ B cells.

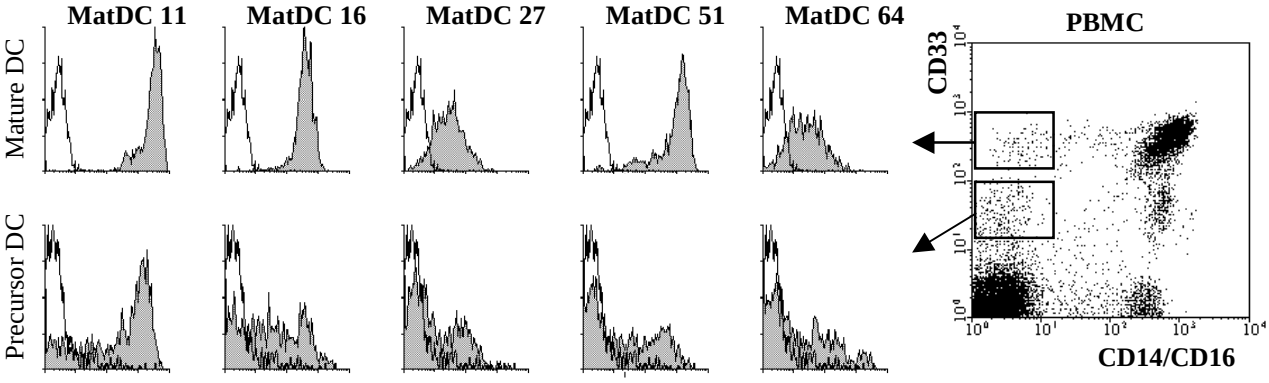

Figure 2 Binding of MoPhabs to different blood DC populations as assessed by three-color flow cytometric analysis. PBMCs were stained with individual MoPhabs, PECy5 labeled anti-CD33, and FITC labeled anti-CD14 and anti-CD16. The histograms show fluorescence values of the MoPhabs binding to the gated cells depicted in the dot-plot. Transparent histograms show control stainings with a negative control MoPhab; grey histograms show staining with the different MoPhabs as indicated.

Reactivity of MoPhabs with tonsil mononuclear cells

Human tonsils contain DCs that can be identified as a $CD3^-CD4^+$ cell population that lacks lineage-specific markers. A further division of this population is obtained by staining with antibodies to IL-3Rα (CDw123). Germinal center DCs, which consist of 65% of the $CD3^-CD4^+$ DCs, are only weakly stained with this antibody[14], whereas the remaining $CD3^-CD4^+$ DCs highly express this marker[9]. Staining of tonsil cell with APC-labeled CD4, PE-labeled CDw123 and FITC-labeled CD3 in combination with PercP-labeled MoPhabs, was used to examine the reactivity of the MoPhabs with the different DC populations in tonsil (Fig. 3).

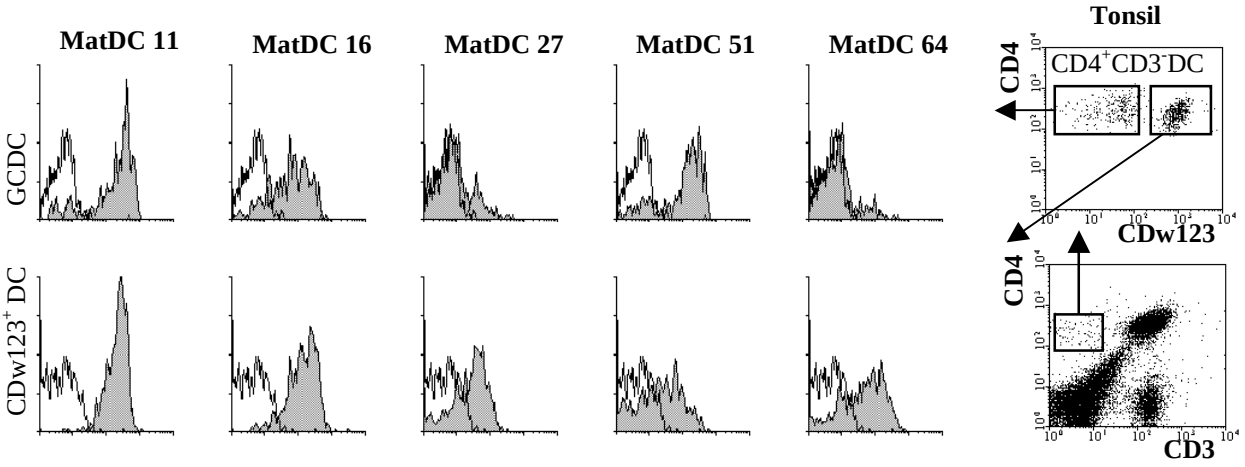

Figure 3 Binding of MoPhabs to DC subpopulations in tonsil as assessed by four-color flow cytometric analysis. Tonsil cells were stained with MoPhabs, APC-labeled anti-CD4, PE-labeled CDw123 and FITC labeled anti-CD3 antibodies. The histograms show fluorescence values of the MoPhabs binding to the gated cells depicted in the dot-plot. Transparent histograms show control stainings with a negative control MoPhab; grey histograms show staining with the different MoPhabs as indicated.

All MoPhabs stained the CDw123$^+$ DC, whereas only MatDC 11, MatDC 16 and MatDC 51 were positive on germinal center DCs. The CD3$^+$CD4$^+$ helper T cells and the CD3$^+$CD4$^-$ cytotoxic T cells were not recognized by any of the MoPhabs. The CD3$^-$CD4$^-$ population in tonsil consists predominantly of B lymphocytes that may be subdivided in four different subpopulations by staining with antibodies specific for IgD and CD38[24]. Triple-staining with these antibodies in combination with the MoPhabs, revealed that MatDC 11 and MatDC 16 stained the IgD$^+$CD38$^-$ naive B cells, the IgD$^-$CD38$^+$ germinal center B cells and the IgD$^-$CD38^{++} plasma blasts. However, no staining of the IgD$^-$CD38$^-$ memory B cells was observed.

Table 1 Reactivity of MoPhabs with different cell populations

	MatDC 11	**MatDC 16**	**MatDC 27**	**MatDC 51**	**MatDC 64**
PBMC:					
Mature DC	+++	++	+	++/+++	+
Precursor DC	++/+++	++*	+/++*	++*	++*
Monocytes	++	++	++	++	++
CD16$^+$ Monocytes	-/+	-/+	-/+	++/+++	-/+
CD3$^+$ T cells	-	-	-	-	-
CD19$^+$ B cells	+	+	-	-	-
CD56$^+$ NK cells	-	-	-	-	-
Granulocytes:	+$^{\#}$	+$^{\#}$	-	+	-
Tonsil:					
CDw123^{dim} DC	++	+/++	-(+)	++	-(+)
CDw123$^+$ DC	++	++	+	-/+	+
CD3$^+$CD4$^+$	-	-	-	-	-
CD3$^+$CD4$^-$	-	-	-	-	-
CD3$^-$CD4$^-$	+	+	-	-	-
Synovial Fluid:					
Mature DC	+	+	-	-/+	-
Precursor DC	+*	+*	-	-(+)	-
Monocytes	+	+	+	+	+
CD33$^-$CD14$^-$ cells	-	-	-	-	-
Adult BM:					
CD3$^+$ cells	-	-	-	-	-
CD10$^+$ cells	+	-	-	-	-
CD14$^+$ cells	+	+	+	+	+
CD15$^+$ cells	+	-/+	-	-/+	-
CD19$^+$ cells	+	-	-	-	-
CD34$^+$ cells	+	-/+	-	-/+	-

Cells were stained as described in legends to Figs. 2-4 or with other antibodies as indicated. Mean fluorescence intensity (MFI) levels for the different populations are shown as -, indicating the highest MFI in the first decade on a four log scale which corresponds to negative control levels. The +, ++ and +++ indicate MFI in the second, third and fourth decades, respectively. A slash (/) indicates that the MFI is on the border between two decades. Parenthesis indicate that less than 5% of the population is positive. *Approximately 50% of the population is positive for this MoPhab. #10-15% of the granulocytes are positive for this MoPhab.

Reactivity of MoPhabs with hematopoietic progenitor cells

In adult bone marrow cells, MatDC 11 brightly and MatDC 16 and MatDC 51 weakly stained $CD34^+$ hematopoietic progenitor cells. All five MoPhabs recognized $CD14^+$ cells in bone marrow that were mostly monocytes from contaminating blood in the aspirates. MoPhabs MatDC11, MatDC 16 and MatDC 51 stained the $CD15^+CD14^-$ myeloid progenitor cells. MatDC 11 also reacted with $CD19^+$ B-lymphoid cells and $CD10^+$ cells. CD10 is a marker expressed early during lymphoid development. None of the MoPhabs stained $CD3^+$ T lymphocytes.

Reactivity of MoPhabs with synovial fluid mononuclear cells of patients with rheumatoid arthritis

Synovial fluid (SF) from affected joints of patients with rheumatoid arthritis have been shown to contain increased numbers of DCs that may be involved in the prolongation and/or exacerbation of local immune-based inflammatory reactions[25;26]. DCs and monocytes in SF may be identified based on the same characteristics as DCs in peripheral blood. As in blood, all five MoPhabs stained monocytes present in SF (not shown). MoPhabs MatDC 11, MatDC 16 and MatDC 51 stained the mature DCs in SF, whereas a subpopulation of the precursor DCs was positive for MatDC 11and MatDC 16. MatDC 27 and MatDC 64 did not stain mature DCs in SF and appear to stain a small fraction of the precursor DCs (Fig. 4).

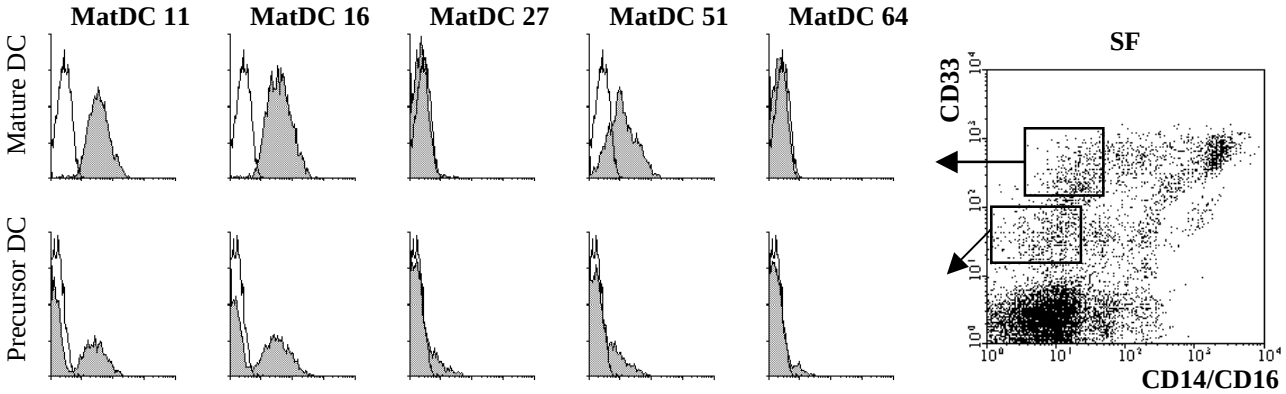

Figure 4 Binding of MoPhabs to DC subpopulations in SF as assessed by three-color flow cytometry analysis. Synovial cells were stained as described in the legend to Fig. 2. The histograms show fluorescence values of the MoPhabs on gated cells in the rectangles depicted in the dot-plot. Transparent histograms show control stainings with a negative control MoPhab, grey histograms show stainings with the different MoPhabs as indicated.

Reactivity of MoPhabs with cell lines

To identify MoPhabs that bound to known molecules, we developed the following strategy. Binding of the MoPhabs to a panel of 20 cell lines with known CD expression

Table 2 Reactivity of MoPhabs with cell lines

	MatDC 11	MatDC 16	MatDC 27	MatDC 51	MatDC 64
Cell lines:					
Jurkat	+	+	-	-	+/-
Nalm-6	nt	-	-	+*	-
Reh	+	+	-	-	-
Raji	+	+	-	-	-
Daudi	+	+	-	-	-
U266	nt	-	-	-	-
HL-60	+	+	-	+	-
U937	+	+	+/-	-	-
KG1a	nt	+	-	-	-
MonoMac	nt	+*	-	-	+
THP-1	+	+	-	+	-
DAMI	nt	-	-	-	-
Hep-G2	nt	-	-	-	-
HUVEC	+	+/-	-	-	-
L428	+	+	-	-	-
HDLM-2	-	-	-	-	nt
K562	-	-	-	-	-
HeLa	+	+	-	-	-
LS174T	+	+	-	-	-
GLC-8	+	+	-	-	-

+/-, weak staining; nt, not tested; *, a subpopulation of cells is positive

patterns was determined (table 2). Subsequently, MoPhabs with expression patterns that resembled known CD antigens were tested for binding to cell lines transfected with the corresponding cDNA including transfectants for CD14, CD31, CD40, CD64, GM-CSF Rα, GM-CSF Rα/β and ILT 3, 4 and 5. From this analysis, we concluded that none of the MoPhabs bound to the panel of transfectants or appeared to match distribution patterns compatible with known CD antigens. In this analysis, it also became apparent that MatDC 27 and MatDC 64 do not recognize the same molecule. MatDC 64 stains cell lines MonoMac and Jurkat, while MatDC 27 is negative on these cell lines. Conversely, MatDC 27 reacted with the U937 cell line while MatDC 64 did not.

DISCUSSION

In search for DC-specific antibodies, we have applied a recently developed method using a combination of a semi-synthetic phage antibody display library and fluorescence-activated cell sorting. We have previously shown that this approach can be used to obtain antibodies against rare populations of cells present in a heterogeneous mixture. DCs represent a rare and phenotypically and functionally heterogeneous population of cells with complex developmental relationships. DCs show the essential properties of antigen presenting cells: antigen uptake, processing and presentation. Monoclonal antibodies against subpopulations of DCs should provide valuable tools to gain insight in the developmental relationships and functional differences between DC subpopulations.

We did not find MoPhabs directed against molecules uniquely expressed by DCs. All 5 MoPhabs selected for further study also bound to blood monocytes and, in some cases, to

other subpopulations of hematopoietic cells. Among the five MoPhabs, MatDC 27 and MatDC 64 displayed the most restricted distribution pattern. Both MoPhabs bound to all mature DCs and a subpopulation of precursor DCs in blood. In addition, both MoPhabs bound to the $CD4^+/CD3^-$ DCs in tonsil that express high levels of the IL-3Rα, whereas few $CD4^+CD3^-$DCs in the tonsil expressing low levels of IL-3Rα were positive. Whether the tonsil $CD4^+CD3^-$IL-3Rα^{high} DCs are of lymphoid or myeloid origin is still a matter of debate [9;10]. Our observation that the tonsil $CD4^+CD3^-$IL-3Rα^{high} DCs expressed a marker that is also present on all monocytes in blood, is suggestive of a myeloid origin. Both MatDC 27 and MatDC 64 did not appear to bind to bone marrow cells including the $CD34^+$ and the $CD15^+CD14^-$ cells representing early myeloid progenitors. Thus, expression of the molecules detected by MatDC 27 and MatDC 64 is restricted to the more mature stages of monocyte and DC development. Strikingly, although monocytes in SF from patients with rheumatoid arthritis stained with both MoPhabs, no staining of precursor or mature DCs was observed in SF. Apparently, precursor and mature DCs in SF, defined as $CD33^+CD14^-$ and $CD33^{dim}CD14^-$ respectively are phenotypically different from their peripheral blood counterparts. It is temping to speculate that this phenotypic difference is related to their entry into the inflamed tissue and/or related to the cytokine-rich environment of the SF.

The epitope recognized by MoPhab MatDC 11, MatDC 16 and MatDC 51 displayed a broader distribution pattern on hematopoietic cells and cells of some other lineages. MoPhab MatDC 11 stained blood and tonsil DCs and B lymphocytes blood monocytes and a subpopulation of cells with forward scatter/side scatter properties of granulocytes. In bone marrow, the epitope recognized by MatDC 11 was found on both myeloid and B lymphoid progenitor cells. A common marker shared by myeloid cells and B lymphoid cells suggests a developmental relationship between these cell populations. It has recently been shown that normal murine pro-B cells can undergo a 'lineage-switch' to DCs in vitro by exposure to cytokines [27]. In addition, although malignancies involving DCs are rare, some Hodgkin lymphoma's of B cell origin have distinct DC features[26]. Indeed, MoPhab MatDC 11 bound to the Hodgkin cell line L428. DCs obtained during in vitro culture of pro-B cells progressively loose B cell markers such as CD19. In contrast, the molecule detected by MoPhab MatDC 11 appears to be shared by both lineages in vivo. The staining pattern of MatDC 16 on freshly-isolated cells closely resembled that of MatDC 11. Analysis of the hematopoietic progenitor cells revealed different staining patterns, suggesting that both Mophabs recognize different molecules.

In blood, MatDC 51 displayed binding to DC, monocytes, including the $CD16^+$ monocytes, and all granulocytes. In bone marrow, this MoPhab bound to most $CD34^+$ cells and to $CD15^+/CD14^-$ myeloid progenitor cells. This indicates that the epitope recognized by this MoPhab is present on all myelomonocytic cells.

We demonstrate that flow cytometric selection of small populations of freshly isolated DCs that have bound phages from a phage display library of scFv antibody fragments yields MoPhabs that can be used as a tool to detect phenotypic differences between DCs present in various lymphoid organs and in SF from patients with rheumatoid arthritis. In addition to their application as a research tool, it may be envisaged that these scFv antibody fragments can be used in clinical applications. DCs are unique leukocyte populations, that control the primary immune response, and targeting of molecules to DCs *in vivo* is considered a potentially valuable vaccination strategy to evoke for example anti-tumor responses. Ample data from *in vitro* studies suggest that DCs loaded with tumor antigens or nucleic acids encoding tumor antigens are able to induce anti-tumor responses[28-31]. In vitro studies further show that antibodies against molecules expressed on the surface of DCs such as Fc receptors and MHC class I and II molecules can mediate targeted delivery of antigens that are correctly processed and presented to T Lymphocytes[32;33;34]. The scFv antibody fragments described here may be converted to complete human antibodies[35] genetically fused to antigens or chemically coupled to nucleic acids for targeted delivery to subpopulations of DCs in immunotherapeutic applications.

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CHAPTER 2B

PHAGE ANTIBODIES SELECTED ON DENDRITIC CELLS IN HUMAN TONSIL

Annemarie Lekkerkerker, Michel de Weers and Ton Logtenberg

ABSTRACT

We have used a phage antibody display library in combination with multiparameter flow cytometry and cell sorting to isolate phage antibodies that bind to subpopulations of dendritic cells (DCs) in human tonsil. Two phage antibodies, TN55 and TN141, were further characterized. TN55 exclusively bound to the $CD11c^+$ and $CD11c^-$ DC subpopulations in tonsil, whereas in peripheral blood binding to all myeloid populations was observed. TN141 displayed a more DC-restricted reactivity pattern as it stained the $CD11c^+$ and $CD11c^-$ DC subpopulations in tonsil, and predominantly the $CD33^{high}$ DC subpopulation in blood. An additional dim staining of TN141 on $CD33^{dim}$ DCs and monocytes was observed. These antibodies can be used for further analysis of subpopulations of human DCs as well as for targeted delivery of antigens to DCs for vaccination purposes.

INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells that play a central role in initiating and modulating T-cell mediated immune responses [1]. Located in tissues throughout the body, DCs act as sentinels to capture antigens. Following antigen uptake and processing, DCs migrate to the lymphoid tissues where initiation of the immune response takes place. DC lineage relationships and functional properties are based mainly on *in vitro* models of DC generation. *In vivo*, morphological, phenotypical and functional differences exist between DC subsets, depending on their anatomical localization [2]. Whether these subsets represent distinct cell lineages or different maturation states is the subject of many current investigations [3-7].

Within tonsil, three fresh DC subsets have been identified: plasmacytoid DCs, interdigitating DCs (IDCs), and germinal center DCs (GCDCs) [8-10]. The major subpopulation, plasmacytoid DCs, is localized around the high endothelial venules and in T cell areas. They form the main source of type I interferon during inflammation [11]. These plasmacytoid DCs have also been identified in blood [12], cord blood [13] and thymus [6] and are considered lymphoid related [14;15]. The second subset consists of IDCs, which also populate the T cell areas of lymphoid tissues. These cells are characterized by high expression of MHC class II, CD40, CD80 and CD86 and have migrated from the periphery to lymph nodes where they interact with T cells [16]. The third subset, the GCDCs, is localized to B cell areas and has limited antigen-capturing capacity but has stong antigen-presenting capacity [9]. These

DCs are suggested to promote the T cell - B cell interaction with germinal centers and may regulate B cells.

Since DCs are central to induction of an immune response, they have gained interest in the area of cancer vaccines [17;18]. DC-based vaccine approaches rely on the generation of DCs *ex vivo* from precursor cells that are subsequently loaded with antigen and re-infused into patients to evoke an anti-tumor response. Alternatively, targeting antigens to directly DCs *in vivo* may induce anti-tumor responses. Antibodies to which antigens are chemically coupled or genetically fused antigen-antibody fusion proteins can be used as vehicles for targeted delivery of antigen, and may improve anti-tumor responses. As a prelude to these experiments, we set out to generate antibodies specific for subpopulations of human DCs.

We have used a semi-synthetic phage display library and flow cytometry to generate phage antibodies specific for DCs in the tonsil. The three different subsets of DCs in tonsil express CD4, whereas they lack T cell marker CD3 and B cell marker CD19. Hence, tonsil DCs can be identified by flow cytometry based on these three markers. Tonsil mononuclear cells were incubated with the phage antibody library and subsequently stained with fluorochrome-labeled antibodies against CD3, CD19 and CD4. The $CD4^+CD3^-CD19^-$ DCs and attached phages were isolated by cell sorting, whereby the remainder of the mononuclear cells served as absorber population for phages recognizing more ubiquitously expressed molecules. After two rounds of selection, monoclonal phage antibodies (MoPhabs) were isolated and immunofluorescent analyses revealed their binding specificities.

Three categories of MoPhabs were isolated, based on binding specificity of which one reacted uniquely with tonsil DCs. This category of MoPhabs consisted of two MoPhabs that stained both the $CD11c^+$ and $CD11c^-$ DC subsets in tonsil, though more intense on $CD11c^+$ DC. Staining of DC and monocyte subsets in peripheral blood revealed distinct differences between those MoPhabs. In conclusion, using phage antibody display, we identified putative novel markers on DCs in tonsil that can be used for identification and isolation of tonsil DC and may provide a tool in DC-based vaccines.

MATERIAL AND METHODS

Media and reagents

The medium used in all cell cultures was RPMI 1640 (Gibco BRL, Breda, The Netherlands), supplemented with, 25mM HEPES, 2mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin 10% (vol/vol) heat-inactivated fetal calf serum (Integro, Zaandam, The Netherlands), hereafter referred to as RMPI-S.

Antibodies

The following monoclonal and polyclonal antibodies were used for immunofluorescent analysis: phycoerythin (PE)-labeled CD1a (CLB, Amsterdam, The Netherlands), fluorescein isothiocyanate (FITC)-labeled CD3, PE-labeled CD4, allophycocyanin (APC)-labeled CD4, PE-labeled CD11c, FITC-labeled CD14, FITC-labeled CD16, FITC-labeled CD19, peridinin chlorophyll (PerCP)-labeled CD19 (all from Becton Dickinson, San Jose, CA), PerCP Cyanine 5.5 (PECy5)-labeled CD33, PE-labeled CD83 (Immunotech, Marseille, France), and PE-labeled CD123 (PharMingen, San Diego, CA) antibodies. Sheep-anti-M13 polyclonal antibody (Pharmacia Biotech, Uppsala, Sweden) was biotinylated using Sulfo-NHS-biotin according to the manufactures protocol (Pierce, Rockford, IL). PE-labeled streptavidin (Immunotech) and PerCP-labeled streptavidin (Becton Dickinson) were used to detect the biotinylated sheep-anti-M13 antibody.

Phage antibody library

For the selection procedure, equimolar amounts of two semisynthetic phage display libraries of human single-chain Fv (scFv) antibody fragments were used. The first library is described in detail elsewhere [19]. In brief, a collection of 49 different germ line heavy chain variable fragment (V_H) genes fused to synthetic CDR3 regions were combined to seven different light chain variable fragments (V_L) of both κ and λ subclasses and cloned in the pHEN1 vector containing a Myc-tag [19]. This resulted in a repertoire of $3.6x10^8$ different human scFvs displayed on filamentous phage particles. For the second library, $1x10^6$ light chain variable fragments PCR-amplified from peripheral blood of healthy donors were fused to the semi-synthetic V_H regions from aforementioned library, cloned in vector pPVT containing a VSV-tag, which resulted in a repertoire of $2x10^9$ different clones (J. de Kruif

and M. Tijmensen, unpublished results). The pPVT vector is similar to pHEN1. It was constructed using standard molecular biology techniques, resulting in a phagemid containing the pUC19 backbone, the pelB leader sequence, an scFv cloning site, the VSV-tag and a truncated geneIII sequence.

Cell isolation procedures

Tonsils were obtained from children undergoing routine tonsillectomy. Tonsils were minced and digested for 1 hour at 37°C with collagenase IV (1mg/mL; Sigma, St Louis, MO) and deoxyribonuclease I (100ng/mL, Boehringer Mannheim, Mannheim, Germany). The tissue was subsequently filtered though an open-filter chamber (NPBI, Emmer-Copascuum, The Netherlands) and the collected cells were subjected to Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation, to obtain tonsil mononuclear cells (MNCs).

Peripheral blood was obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation. Blood leukocytes were prepared by removal of erythrocytes from human peripheral blood by hypotonic shock.

Selection of phage antibodies by cell sorting

Phage selection on cells was performed as described elsewhere [20;21]. In brief, 10^{13} phage particles were blocked for 15 minutes in PBS containing 5% low-fat milk powder. 10^{8} tonsil MNCs were added to the blocked phages and the mixture was slowly rotated overnight at 4°C. The following day, the cells were washed twice with ice-cold PBA (PBS containing 1% BSA and 0.05% sodium-azide), and subsequently stained for 30 minutes on ice with PE-conjugated CD4 and FITC-conjugated CD3 and CD19 antibodies. The cells were washed with PBA and resuspended in RPMI-S; 10^{4} cells $CD4^{+}CD3^{-}CD19^{-}$ cells were isolated by cell sorting on a FACStarPLUS (Becton Dickinson). Phages were eluted from the sorted cells by incubation for 5 minutes at room temperature with 1.5 volume of 76 mM critic acid, pH 2.5, and neutralized by addition of 0.5 volume of 1M Tris-HCl, pH 7.4. The eluted phages were rescued by infection of *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA), and propagated for a second round of selection, employing the same procedure.

Preparation of MoPhabs and immunofluorescent analysis

After the second round of selection, MoPhabs were prepared from individual ampicillin-

resistant bacterial colonies [20] and analyzed for binding specificity in immunofluorescent analysis. For immunofluorescent analysis, MoPhabs were blocked for 15 minutes with PBS containing 4% milk powder. Cells were added and incubated for 1 hour on ice. Cell-bound phages were detected using biotinylated sheep anti-M13 polyclonal antibody for 20 minutes on ice, followed by either streptavidin-PE or streptavidin-PercP. Where indicated, cells were subsequently stained with appropriate FITC, PE, PercP, PECy5 or APC-labeled antibodies. Stained cells were analyzed on a FACSCalibur (Becton Dickinson).

Screening and nucleotide sequence analysis of MoPhabs

To establish V_H and V_L gene utilization and heavy chain CDR3 composition, the nucleotide sequences of selected MoPhabs were determined by automated sequencing using primers LINKSEQ and PHENSEQ [4].

Generation of monocyte-derived DC

DCs were generated from monocytes as previously described [22;23]. Briefly, PBMCs were isolated from blood by Ficoll-Hypaque density centrifugation, and allowed to adhere for 2 hours at 37°C. The adherent monocytes were cultured at $1x10^6$ cells/ml in RPMI-S, in the presence of recombinant human (rh) interleukin (IL)-4 (500 U/ml; Strathmann, Hannover, Germany) and rh granulocyte-macrophage colony-stimulating factor (GM-CSF; 800 U/ml, Schering-Plough, Amstelveen, The Netherlands) for 5 days. Fresh cytokine-containing culture medium was added at day 3. After 5 days, the immature monocyte-derived DCs were harvested. To generate mature DC, a combination of 800U/mL of rhGM-CSF (Schering Plough), 1000 U/ml of rhIL-4 (Strathmann), 20 ng/ml of rh tumor necrosis factor (TNF) α (Intergen Company, Purchase, NY) 10 ng/ml of rhIL-1β (Strathmann), 1000 U/ml of rhIL-6 (Roche Diagnostics, Almere, The Netherlands), 1 µg/ml of prostaglandin E2 (PGE2; Sigma, St. Louis, MO) were added to the immature DC for another 48 hours.

Cell lines

The following cell lines were used in this study: T cell lines CEM and Jurkat, B cell lines Raji and Daudi, myeloma cell lines RPMI8226 and Fravel, myelomonocytic cell line HL60, promonocytic cell line U937, monocytic cell line THP-1, erythroid/megakaryocytic precursor cell line K562.

RESULTS

Experimental strategy

To select MoPhabs specific for markers on DCs in human tonsil, a single cell suspension made from tonsil was incubated with a combination of two semi-synthetic phage antibody display libraries. Tonsil DCs were identified as $CD4^+CD3^-CD19^-$ cell population comprising about 0.8% of the tonsil MNCs (Fig. 1). The excess of non-selected cells in the mixture was hypothesized to absorb the MoPhabs that recognize molecules shared by selected and non-selected cells [20;24]. In the first round, $15x10^3$ $CD4^+CD3^-CD19^-$ cells with attached phages were sorted. Rescue of the phages yielded 10^4 single colonies, which were propagated and subsequently used for a next round of selection. For the second round, $20x10^3$ $CD4^+CD3^-CD19^-$ cells were sorted, which resulted in 5000 single colonies. After two rounds of selection, single colonies were picked for production of individual MoPhabs.

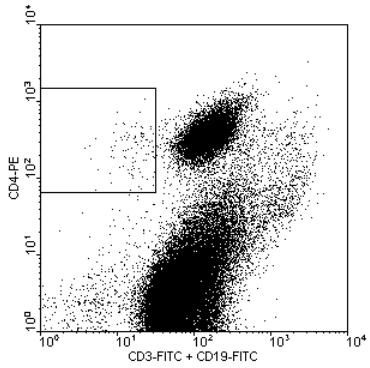

Figure 1 Identification of DCs in tonsil. Tonsil MNCs were stained with FITC-labeled CD3 and CD19, followed by PE-labeled CD14. Tonsil DCs can be identified as a $CD4^+CD3^-CD19^-$ population. A rectangle represents the sorted cell population.

Phages selected for binding to tonsil DC

After the second round of selection, 168 individual colonies were picked to produce MoPhabs. Comparative flow cytometric screening of tonsil MNCs, established the reactivity of MoPhabs for DCs. As a negative control in staining experiments, a MoPhab specific for dinitrophenyl (DNP) was used [19]. 8 % (14/168) of the MoPhabs were not reactive in flow cytometry and these MoPhabs were not pursued in further studies. Based on different staining patterns, the MoPhabs could be grouped in three categories. The majority of the MoPhabs reacted with all B cells in tonsil (category I). MoPhabs in category II stained DCs and in addition, a subpopulation of B cells in tonsil (Fig. 2). Two MoPhabs bound exclusively to DCs in tonsil (category III; Fig. 2). Subsequently, nucleotide sequence analysis was used to establish the V_H and V_L gene utilization and heavy chain CDR3 composition of the different MoPhabs. This revealed that MoPhabs of category I were derived from the same clone based on CDR3 sequence, V_H and V_L identity. This MoPhab

was isolated previously, recognizes the β-chain of CD22 [20;25] and is expressed on all mature B cells.

The MoPhabs in category II had different CDR3 sequences, though their staining patterns were similar. A similar staining pattern has been described by Van der Vuurst de Vries for MoPhab IV-7 [24]. MoPhab IV-7 is derived from selections on memory B cells in tonsil and binds to most memory B cells, a subpopulation of naïve B cells as well as all DC subsets in tonsil. The two MoPhabs in category III had different CDR3 regions and used different V_H genes and were hence different MoPhabs. Since TN55 and TN141 both uniquely stained tonsil DCs, further studies concentrated on these two MoPhabs. All MoPhabs in categories I, II, and III contained a Myc-tag as demonstrated by dot-blot (data not shown), indicating that they derived from the phage antibody display library as described by De Kruif *et al.* [19].

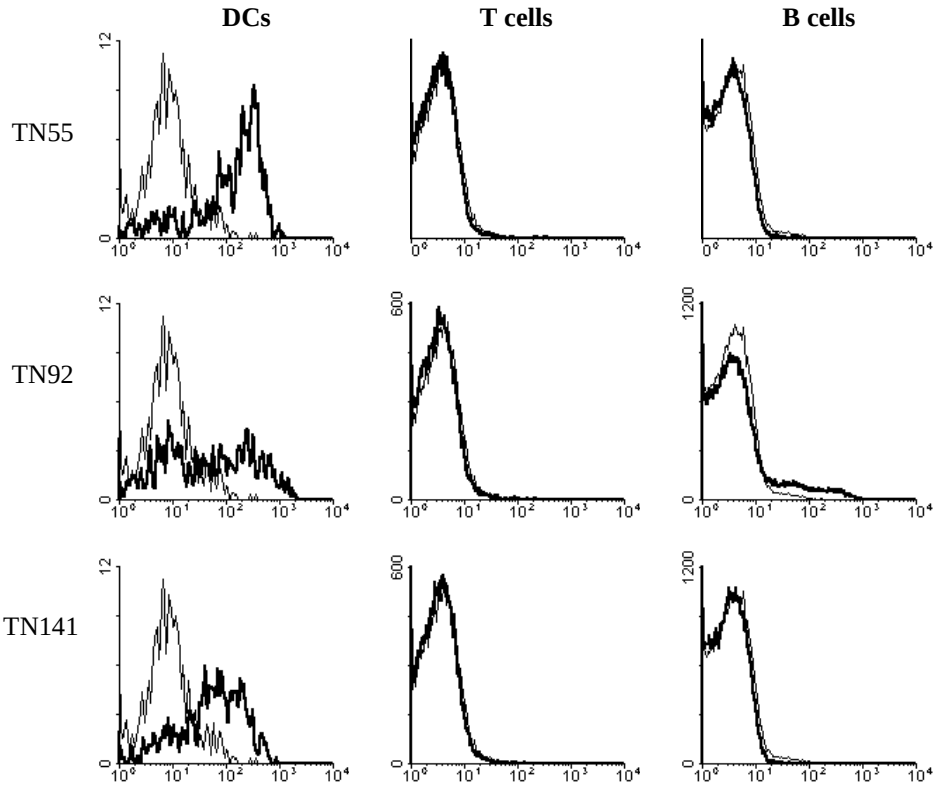

Figure 2 Staining pattern of MoPhabs of in tonsil. Triple immunofluorescent staining was performed with the MoPhabs in combination with FITC-labeled CD3, PercP-labeled CD19 and APC-labeled CD4 monoclonal antibodies. The histograms depict the staining patterns of the individual MoPhabs with DC ($CD4^+CD3^-$), T cell ($CD3^+$) and B cell ($CD19^+$) populations. The thin line represents staining with a control MoPhab specific for DNP, whereas the bold line represent staining with the MoPhab of category II (TN92) and III (TN55, TN141).

Staining patterns of TN55 and TN141 on DC subsets in tonsil and blood.

Tonsil DCs can be separated based on staining of the IL-3Rα (CD123). Germinal center DCs as well as IDCs have a low expression of IL-3Rα, whereas plasmacytoid DC, have a high expression of the IL-3Rα [9;10;12]. Reciproquely, CD11c stains the myeloid DCs, comprising GCDCs as well as IDCs, whereas it is negative on plasmacytoid DC. As shown in figure 3, staining patterns of TN55 and TN141 on $CD11c^-$ and $CD11c^+$ DCs subsets are similar. Both MoPhabs stained all tonsil DCs though the staining was more intense on $CD11c^+$ DCs.

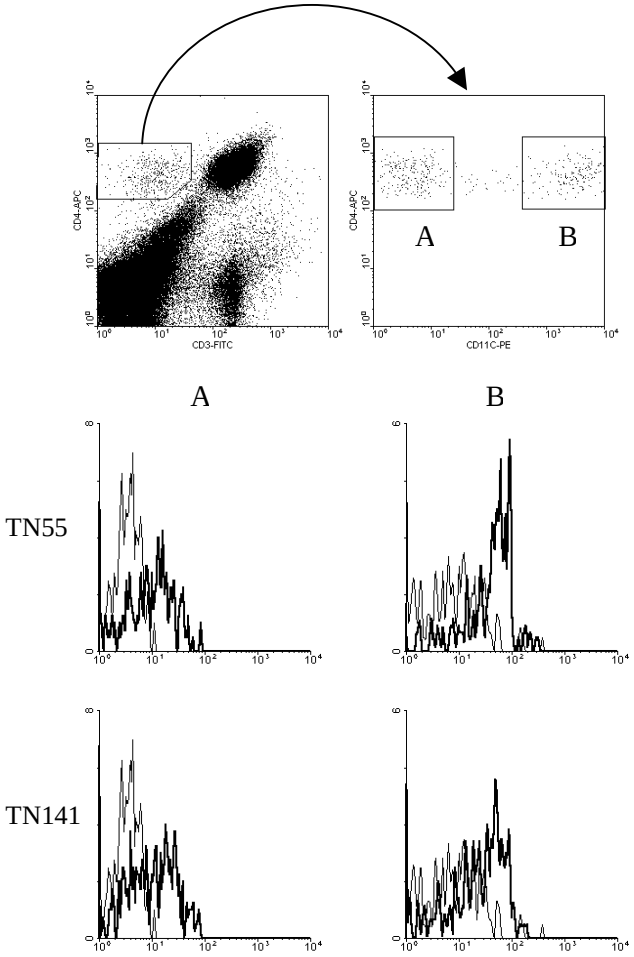

Figure 3 Staining patterns of MoPhabs TN55 and TN141 on different DC populations in tonsil. Tonsil MNCs were stained with individual MoPhabs, FITC-labeled CD3, PE-labeled CD11c and APC-labeled CD4 antibodies. The histograms show fluorescence values of the MoPhabs binding to the gated cells depicted in the dot plot: A, $CD11c^-$ DC; B, $CD11c^+$ DC. The thin line represents staining with a control MoPhab specific for DNP, whereas the bold line represent staining with the MoPhab as indicated.

In blood, two subsets of DCs can be identified by triple staining of markers CD14, CD16 and CD33 (Fig. 3) [26]. Accordingly, a more mature DC can be seen as a $CD33^{bright}CD14^-CD16^-$ population, known as the $CD11c^-$ population, which can induce a potent immune response [27]. The other DC subset can be seen as a $CD33^{dim}CD14^-CD16^-$ population, or $CD11c^-$ population. This population has recently been shown to represent the plasmacytoid DC [12]. In addition to $CD33^{bright}CD14^+$ monocytes, a subpopulation of $CD33^{dim}CD16^+$ monocytes is observed [26;28-30]. Binding specificity of different MoPhabs on DC subsets in

blood was examined. Whereas TN55 and TN141 gave similar staining patterns on tonsil subpopulations, a clear difference was observed on DC subsets in peripheral blood.

TN55 reacted with CD33^{bright} and CD33^{dim} DC subsets as well as CD14^{+} monocytes and CD16^{+} monocytes. TN55 did not stain lymphocytes in blood, while a bright staining on granulocytes was observed (Table 2).

TN141 specifically stained CD33^{bright} DC, whereas it was dim to negative on CD33^{dim} DC. In addition, a dim staining of the CD14^{+} monocytes was observed. However, TN141 did not stain either lymphoid cells or granulocytes in peripheral blood. In conclusion, TN141 demonstrated a staining pattern restricted to DCs and CD14^{+} monocytes.

Table 2 Reactivity of MoPhab TN55 and TN141with different cell populations

	TN55	TN141
Tonsil		
CD11c^{+} DC	+*	+
CD11c^{-} DC	±	-
B cells	-	-
T cells	-	-
PBMC		
CD33^{bright} DC	++	++
CD33^{dim} DC	++	±
Monocytes	++	+
CD16^{+} Monocytes	++	-
NK cells	-	-
Lymphocytes[a]	-	-
Granulocytes[a]	++	-
Mo-derived DC		
Immature	±	-
Mature	+	-

-, negative; ±, variable, +, positive; ++, strong positive; a, gated on forward and side scatter

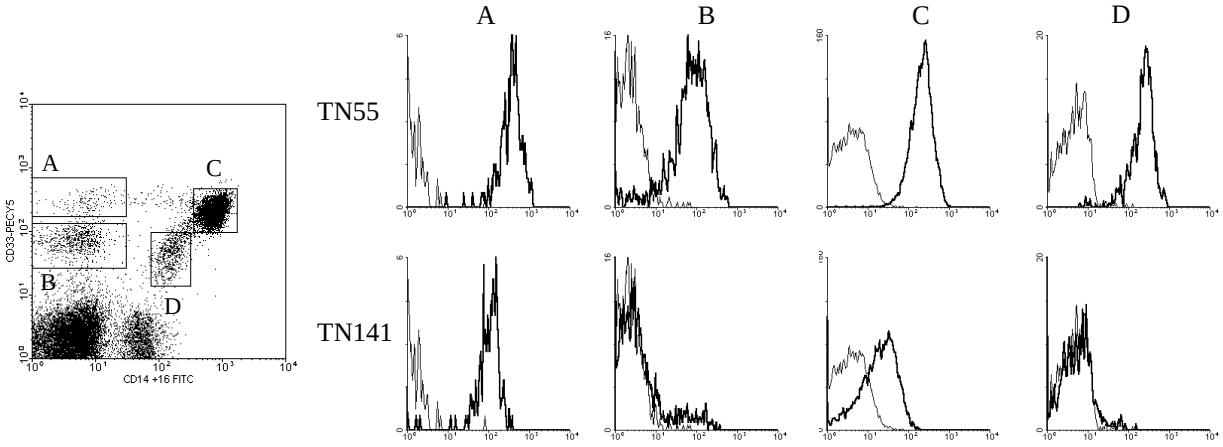

Figure 4 Staining patterns of MoPhabs TN55, TN92, and TN141 to different DC and monocyte populations in blood. PBMCs were stained with individual MoPhabs, PECy5-labeled CD33, and FITC-labeled CD14 and CD16 antibodies. The histograms show fluorescence values of the MoPhabs binding to the gated cells depicted in the dot plot. A, CD33^{high} DC; B, CD33^{dim} DC; C, CD14^{+} monocytes; D, CD16^{+} monocytes. The thin line represents staining with a control MoPhab specific for DNP, whereas the bold line represent staining with the MoPhab as indicated.

Reactivity on cultured monocyte-derived immature and mature DC

Monocyte-derived immature and mature DC were generated to evaluate the staining patterns of the MoPhabs on *ex vivo* cultured DCs. Immature DCs characterized by expression of CD1a, were generated by culturing monocytes in the presence of GM-CSF and IL-4 for 5 days [31]. Subsequently, these immature DC were stimulated using a combination of cytokines and PGE2 [23], resulting in mature DC that expressed high levels of CD83. TN55 stained both immature and mature DC, however TN141 was negative on both populations (Table 2).

Reactivity of MoPhabs TN55 and TN141 on different cell lines

Table 3 Reactivity of MoPhab TN55 and TN141 on different cell lines.

	TN55	TN141
Jurkat	-	-
CEM	-	+
Raji	-	-
Daudi	-	nt
RPMI8226	+	-
Fravel	-	-
HL60	+	+
U937	-	-
THP-1	+	nt
K562	-	-

* -, negative; +, positive; nt, not tested

Binding to different cell lines with known CD expression patterns was assessed in order to identify whether MoPhabs bound to known molecules (Table 3). From this analysis, we concluded that neither TN55 nor TN141 staining patterns matched distribution pattern of known CD antigens. Though staining of TN55 resembled expression pattterns of myeloid markers such as CD13, CD15 and CD33, these molecules are both expressed on U937. In contrast, staining of TN55 was lacking on these cell lines.

DISCUSSION

We employed a semi-synthetic phage display library in combination with multiparameter flow cytometry and cell sorting to select phage antibodies binding to tonsil DCs. We have previously used this method to identify phage antibodies that recognize putatively novel target antigens on DCs in human peripheral blood [21]. This method is rapid and independent of immunogenicity of the target antigen, and results in the selection of antibodies directed against native cell-bound antigens.

Tonsils from routine surgery provide the most readily accessible source of human lymphoid tissue. The tonsils play an important role in immunologic surveillance. As a route of pathogen entry, tonsils are considered a possible site of entry and/or replication of the HIV-1 virus [32]. At least three DC subsets in tonsil have been described, the plasmacytoid

DCs, the IDCs and the GCDCs. These subsets have a distinct phenotype and anatomical localization and are hypothesized to have a specialized function. To date, no cell surface markers specific for tonsil DCs have been described, mainly due to the scarcity of these cells *in vivo*. In our strategy, isolation of DCs prior to the selections was not necessary. The presence of cells other than DCs in the selection procedure is even crucial for the absorption of phage antibodies directed against commonly expressed antigens. It should be noted that follicular dendritic cells present in the germinal center do not derive from a bone marrow precursor and belong to a separate lineage as compared above-mentioned DC subsets. They lack expression of CD4 and were not included in this study.

In this study, a suspension of tonsil mononuclear cells was incubated with a combination of two semi-synthetic phage antibody display libraries. Tonsil DCs were identified as the $CD4^+CD3^-CD19^-$ cell population during cell sorting. After two rounds of selection, 168 individual clones were tested for binding to $CD4^+CD3^-$ DCs. More than 90% of these clones tested positive. Although our selection method was aimed at isolation of phage antibodies directed uniquely against DCs in tonsil, most of the isolated phage antibodies recognized antigens expressed by both DCs and B cells. This may not be very surprising, since both cell types are involved in antigen-presentation and are expected to share molecules associated with this function. In this context, it is noteworthy that we previously identified phage antibodies selected on blood DCs that also showed additional binding to B cells [21].

MoPhabs were grouped in three categories based on staining patterns. Phage antibodies in the first category were derived from the same clone, which was previously identified to bind to CD22. It is known that CD22 is expressed on all mature B cells, however its expression on DCs in tonsil has never been reported. Detailed analysis of tonsil suspensions of different donors revealed that this phage antibody rarely stained DCs. Since the sorted cell fraction is 95 to 98% pure, we could not exclude the possibility that this phage antibody was isolated because of contamination of B cells in the sorted DC fraction.

Phage antibodies from the second category actually contained all different antibodies based on nucleotide sequence analysis of V_H regions, though their staining patterns were similar. Interestingly, a similar staining pattern is seen for a MoPhab called IV-7, which is selected on memory B cells [24]. Notably, all MoPhabs with this staining pattern, including MoPhab IV-7, make use of a Vκ1 light chain in combination with a DP31 or DP32 V_H segment. The significance of this restricted V_H usage is not known.

The most interesting third category exclusively stained DCs in tonsil. This category contained only two phage antibodies, denoted TN55 and TN141. Further analysis revealed that MoPhabs TN55 and TN141 stained myeloid $CD11c^+$ DC subsets, the IDCs and GCDCs, and the $CD11c^-$ subset, the plasmacytoid DCs, although their targets antigens were expressed higher on the myeloid DCs. To conclude, we successfully isolated two MoPhabs that recognized unique antigens present on DCs in tonsil. The isolation of two tonsil DC specific MoPhabs without prior isolation of this small population clearly underlies the strength of the absorption effect of phage antibodies to structures shared by cells other than DCs.

TN55 uniquely bound to DCs in tonsil however further characterization binding to all myeloid cells, including granulocytes, in peripheral blood. In addition, binding to immature and mature monocytes derived DCs was demonstrated. From the staining pattern of TN55, we concluded that the target antigen is expressed on all myeloid cells. However, expression of the target antigen in an early stage of the myeloid lineage is not expected, since staining on the $CD33^+$ cell line K652, also displaying erythroid and megakaryocytic markers, was not observed. Despite extensive efforts, we were unable to identify the target antigen of TN55. Presumably, TN55 did not bound to common myeloid markers such as CD13, CD15 and CD33, based on staining patterns on cell lines.

TN141 showed a more restricted reactivity pattern in peripheral blood. Bright staining of the $CD33^{high}$ DCs was observed while staining of $CD33^{dim}$ DCs was almost lacking. In addition, TN141 bound to the $CD14^+$ blood monocytes, whereas binding to *in vitro* generated monocyte-derived DCs was absent. In general, TN141 preferentially bound to fresh myeloid DCs. This staining pattern is distinct from that of MoPhabs MatDC 27 and MatDC64 derived from blood DC selections. While in blood a similar staining of these MoPhabs can be seen, in tonsil only binding to plasmacytoid DC was observed for MoPhabs MatDC27 and MatDC64.

To identify the target antigen of MoPhab TN141, cDNA expression cloning using a commercially available Hela cDNA library transfected in 293T cells was carried out as described [33]. Unfortunately, this approach was not successful. ScFv fragments derived from MoPhabs TN55 and TN141 did not perform in immunohistochemistry. Whether this is caused by a low affinity of the scFv fragments or the nature of the antigen remains to be resolved.

In conclusion, we demonstrate that a subtractive approach to select antibodies specific for a small subpopulation of cells, yielded phage antibodies that bind exclusively to the cells of interest, tonsil DCs. Further characterization of these phage antibodies demonstrated a more broad reactivity pattern in peripheral blood. DCs represent a heterogeneous population

of cells, potentially derived from independent lineages. Therefore, it is likely that this selection method yields antibodies against antigens associated with the differentiation stage or anatomical localization of DCs, than antigens uniquely associated with DCs in general. In order to isolate more general DC-specific phage antibodies, one could envisage selection strategies whereby fresh DCs from different anatomical localizations, such as peripheral blood and tonsil, are used in alternating selection rounds. Another possibility is that DCs are not defined by unique antigens, but rather are distinguished from other cell types by expression of a unique combination of otherwise more commonly expressed cell surface antigens.

The MoPhabs, isolated during this study, can be converted to complete human antibodies. An effective method for this was developed in our laboratory [34] and has previously been used to generate a functional and clinically applicable huMab against a tumor-associated antigen [35]. As fully human antibodies, the MoPhabs described here could serve as tools for *in vivo* targeting to DCs in vaccination protocols. For example, chemical or genetic coupling of human DC-specific antibodies to tumor antigens may be explored to develop cancer vaccines.

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CHAPTER 3

REACTIVITY PROFILES OF HUMAN IgG4 MONOCLONAL ANTIBODIES ON NATURALLY OCCURRING AND CULTURED DENDRITIC CELL SUBSETS

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Submitted

ABSTRACT

Interpretation of published data concerning the phenotype of dendritic cell (DC) subsets in different human tissues is hampered by the ambiguous definition of the DC population. In this report, we exploited multiparameter flow cytometry for detailed immunophenotypic analysis of cultured DCs and DCs obtained from blood, tonsil, spleen, thymus and skin. The panel of antibodies used for this study consisted of murine monoclonal antibodies, including the recently described BDCA-1-4 antibodies, and of four human monoclonal antibodies isolated from a phage display library. Using this set of antibodies in combination with flow cytometry allowed us to further define the heterogeneous DC population in humans. Furthermore, the analysis of the binding characteristics of the panel of human antibodies forms the basis of the selection of human antibodies for the targeted delivery of antigens to DCs *in vivo*, an important step towards the design of new vaccination strategies.

INTRODUCTION

Dendritic cells (DCs) are a heterogeneous population of cells, with extraordinary immunostimulatory capacity, including the ability to prime naive $CD4^+$ T cells and $CD8^+$ T cells towards T helper cells and CTLs, respectively [1]. They are continuously produced from hematopoietic stem cells in the bone marrow and are widely distributed throughout the body as immature DCs [2]. Following encounter with pathogens, DCs mature and migrate from peripheral tissues to the secondary lymphoid organs, where they initiate an immune response. The high antigen capture and presenting capacity that results in extremely efficient induction and maintenance of immune responses have led to increasing interest in the use of DCs as vaccines to induce anti-tumor immunity. DCs cultured *ex vivo* from precursor cells, loaded with tumor antigens in the form of peptides, whole antigens or tumor cell lysates are beginning to enter clinical trials with some encouraging results [3-7]. However, several issues to ensure the optimal outcome of DC vaccines have to be evaluated further, among them the source of DCs in relationship tot the quality of the immune response. DCs used in clinical trials have been cultured and manipulated ex *vivo*, a tedious process that may alter the properties of DCs. An attractive alternative may be the targeting of antigens to DCs *in vivo.* Because of their exquisite specificity, monoclonal antibodies may be developed that, upon injection, act as targeting devices for the delivery of tumor antigens to DCs [8;9]. Given their presumed role in polarization of the immune response, it may even be envisaged that

targeting of tumor antigens to different DC subsets may permit better control of the quality of the immune response.

Different DC subsets have been described based on phenotype, function or anatomical localization. DCs can modulate of the development of T cell-mediated immune responses by influencing T helper (T_H) cell differentiation into either T_H type 1 (T_H 1) or T_H type 2 (T_H 2) [10]. It has been proposed that distinct DC subsets may have different specialized functions and can direct T_H responses differently [11;12]. However, there is increasing evidence that the type of response induced by a DC subset in T_H cells depends also on the kinetics of activation, the microenvironment and nature of the pathogen that is encountered. Taken together, this implies considerable plasticity of DC functions within a subset [13-17].

In a previous study, we have employed a semi-synthetic antibody phage display library, in combination with subtractive selection and flow cytometry to generate monoclonal phage antibodies against DCs in human blood [18] and tonsil (Lekkerkerker, A., De Weers, M., Logtenberg, T., Chapter 2B). Four of these phage antibodies, denoted MatDC16, MatDC27, MatDC64 and TN141, were found to preferentially bind to DCs and monocytes and chosen for further characterization. Here, we report the conversion of the phage antibodies to fully human monoclonal antibodies (huMabs) [19]. Therapeutic monoclonal antibodies currently used in the clinic contain mouse protein sequences and have the potential to trigger for the patient unwanted immune reactions. These problems can be circumvented by the use of fully human antibodies [20;21] hence human antibodies (huMabs) are the most desirable antibody format for clinical application. The IgG4 isotype was chosen because: (a) it has low affinity for FcγRI, and does not bind other Fcγ receptors and (b) it does not activate complement [22]. These characteristics make the IgG4 huMab an ideal vector for DC targeting strategies.

In light of this application, a detailed phenotypical analysis of these IgG4 huMabs on *in vivo* DC subsets of different tissues is critical. Although the analysis of DC subsets in human tissues has been hampered by the availability of suitable reagents and unambiguous definitions, progress has recently been made. In human peripheral blood, in addition to $CD14^+$ monocytes, two subsets of DC precursors were originally identified, based on the differential expression of CD11c and absence of lineage markers [23]. The $CD11c^+$ subset expresses myeloid-related surface molecules and is dependent on GM-CSF for growth and survival. In tissue culture, $CD11c^+$ DCs spontaneously develop into mature DCs, even when cultured without any exogenous cytokines [24]. The $CD11c^-$ subset expresses the IL-3 receptor (CD123) at high levels and has so-called plasmacytoid morphology [25;26]. This population depends on IL-3 for its survival and differentiation into mature DCs with typical dendritic

morphology and potent T cell stimulatory function [11;27]. Importantly, the plasmacytoid DCs are responsible for type I interferon (IFN) secretion in response to viral stimuli [28;29]. It appears that $CD11c^+$ DCs are of myeloid derivation, while the lineage commitment of plasmacytoid $CD11c^-$ DCs is less clear.

In secondary lymphoid organs, such as tonsil and spleen, DCs interact with other cells of the immune system, resulting in the induction of an immune response. In tonsil, three DC subsets have been defined: first, the plasmacytoid DCs, located near high endothelial venules, correspond to the $CD11c^-$ population in blood. Second, germinal center DCs (GCDCs) are $CD11c^+$ and are located in the germinal center, and presumably stimulate $CD4^+$ T cells and play a role in germinal center B cell proliferation and differentiation [30;31]. Third, the $CD11c^+$ interdigitating DCs (IDCs) are found in the T cell areas of tonsils in close contact with T cells [32]. Recently, $CD11c^+$ DCs were identified in human spleen, at three distinct regions, the marginal zone, the T cell and B cell zones, the latter resembling tonsil GCDCs [33].

The thymus is a primary lymphoid organ and of crucial importance in the development and maturation of T cells. Thymic DCs are implicated in elimination of self-reactive T cells. Human thymus contains two myeloid DC subsets, a $CD11c^+$HLA-DR^{high} subset that expresses markers of fully mature DCs such as CD40, CD83 and CD86, and a minor $CD11c^+$HLA-DR^+ population, resembling tonsil GCDCs [34;35]. In addition, the $CD11c^-$ DCs corresponding in phenotype to plasmacytoid DCs described in tonsil and blood, are the most abundant population found in thymus. Recently, significant levels of pre-TCR α-chain transcripts in plasmacytoid DCs in thymus as well as tonsil have been demonstrated, indicating that they may have a lymphoid origin [36].

Human skin, a non-lymphoid tissue, contains two distinct DC subsets: first, Langerhans cells (LCs) within the epidermis, characterized by the expression of CD1a and Birbeck granules [37;38]. Secondly, interstitial DCs in the dermis, lack Birbeck granules, and are also found in other non-lymphoid tissues all over the body.

The physiological differentiation pathways that yield the different DC subsets *in vivo* are not well known. In contrast, the origin and differentiation pathways of DCs *in vitro* have been extensively studied. *In vitro*, human hematopoietic $CD34^+$ progenitors from bone marrow or cord blood differentiate into DCs when cultured with GM-CSF and TNFα via two independent pathways, resulting in cells with either a dermal or interstitial DC phenotype or with a LC phenotype [39;40].

Human peripheral blood monocytes cultured with GM-CSF and IL-4 generate immature DCs that can be driven into a mature state using different maturation mediators such as TNFα, LPS and CD40L [41-43]. In addition, by *in vitro* crossing of an endothelial barrier in the ablumenal-to-lumenal direction, as occurs during entry into lymphatics, monocytes can differentiate into DCs [44]. Nonetheless, there is still limited understanding of the relationships between *in vitro* generated DCs and DC subsets *in vivo*; hence the question concerning the developmental origin of the subsets *in vivo* remains under discussion [45].

In the current study, we used a multi-parameter flow cytometry strategy to identify naturally occurring DC subsets and determine the phenotype of the distinct DC subsets found at different anatomical sites. Furthermore, in search of physiological counterparts of *in vitro* generated DCs, we compared the immunophenotype of freshly isolated DC subsets with DCs generated in well-defined culture systems. We provide extensive surface marker analyses using conventional monoclonal antibodies (Mab), including four recently described Mabs, BDCA-1 (CD1c), BDCA-2, BDCA-3, and BDCA-4, which identify three distinct blood DC subsets [46]. We show differential staining patterns of the huMabs on DC subsets *in vivo* and *in vitro*. Conversion of DC-specific phage antibodies to IgG4 huMabs along with their reactivity profiles on naturally occurring DC subsets represent an important step towards their possible application in direct *in vivo* targeting in DC-based vaccination strategies.

MATERIAL AND METHODS

Culture medium

Unless stated otherwise, medium consisting of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Integro, Zaandam, The Netherlands), 25 mM HEPES, 2 mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (referred to as RPMI-S) was used for culturing cells.

Antibodies

The sources of conjugated Mabs used for flow cytometry are listed in table 1. Alexa Fluor 633 streptavidin conjugate (Molecular Probes, Eugene, OR) or PE-streptavidin conjugate (Becton Dickinson, Erembodegem, Belgium) was used to detect biotinylated antibodies in flow cytometry. Goat anti-human IgG unlabeled and HRP-labeled (SBA, Birmingham, AL) was used for ELISA, HRP-labeled goat anti-human IgG4 antibody was used for immunoblotting (CLB, Amsterdam, the Netherlands).

Table 1 Monoclonal antibodies used for flow cytometry.

Antigen	Clone	Conjugate	Source
BDCA-2	AC144	PE	M
BDCA-3	AD5-14H12	PE, FITC	M
BDCA-4	AD5-17F6	PE	M
CD1a	BL 6	PE	I
CD1c	AD5-8E7	PE	M
CD3	SK7	PE	BD
CD4	SK3	PE	BD
CD11b	D12	PE	BD
CD11c	S-HCL-3	APC, PE	BD
CD13	L138	PE	BD
CD14	MfP9	FITC	BD
CD16	3G8	FITC	BD
CD19	SJ25C1	FITC	BD
CD20	L27	FITC	BD
CD25	2A3	PE	BD
CD40	Mab89	PE	BC
CD54	84H10	PE	BC
CD56	NCAM16.2	FITC	BD
CD64	M22	PE	W
CD80	L307.4	PE	BD
CD83	HB15a	PE	BC
CD86	FUN-1	PE	P
CD123	7G3	PE	P
HEA	HEA125	FITC	M
HLA-DR	L243	PercP	BD
Lin 1*		FITC	BD

*Lineage Cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56). Antibodies were obtained from: BC, Beckman Coulter, Marseille, France; BD, Becton Dickinson, Erembodegem, Belgium; I, Immunotech, Marseille, France; P, BD Pharmingen, Erembodegem, Belgium; M, Miltenyi Biotec, Bergisch Gladbach, Germany; W, dr. J. van de Winkel, UMC Utrecht, The Netherlands.

Construction and production of human IgG4 monoclonal antibodies

The phage antibodies used in this study were previously described [18]. To construct huMabs of the IgG4 istoype, the heavy and light chain variable regions encoding the phage antibodies of interest were excised and recloned into vectors for stable expression of complete IgG4/κ molecules in eukaryotic cell lines. The construction of the expression vectors and the production of huMabs has been described in detail elsewhere [19]. Briefly, the heavy and light chain variable regions were cloned in a vector containing the T cell receptor α chain HAVT20 leader peptide sequence and a splice donor site. Next, the fragment containing the heavy or light chain variable region with attached leader sequence and splice donor site were subcloned in the pNUT-Cγ4 or pNUT-Cκ expression vectors. Using the appropriate restriction sites, the complete heavy or light chain was recloned in the pCDNA3.1 vector (Invitrogen, Carlsbad, CA).

The different huMabs IgG4 were produced by stable transfection of human embryonic kidney (HEK) 293 cells. Cells were maintained in Iscove's modified Dulbecco's medium

(IMDM) containing 10% FCS, 2mM glutamine and $5x10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco). Cells were transfected at a density of 70-80% confluency using calcium chloride precipitated DNA of the heavy and light chain, for 5 hours at 37 °C, followed by a 15% glycerol shock for 1 minute. Cells were washed and after 48 hours and selection was initiated by adding 500 μg/ml of zeocin (Invitrogen). After 2 weeks, colonies of resistant cells were picked and cultured in zeocin-containing medium. Production of recombinant huMabs IgG4 was determined in the supernatant by sandwich ELISA using standard procedures and an IgG standard (CLB) as control. Large quantities of huMabs were produced in ULTRA-CHO medium (BioWhittaker, Walkersville, MD) using triple-flasks (Invitrogen). HuMabs were purified by protein A sepharose column chromatography (Biorad Laboratories, Munchen, Germany). SDS-PAGE and subsequent Coomassie brilliant blue staining of gel demonstrated the integrity of purified huMabs. The IgG4 isotype of the huMabs was confirmed by Western blot, stained with HRP-conjugated goat anti-human IgG4 antibodies, detected with the substrate diaminobenzidine containing 0.01% H2O2. Concentration of purified huMabs was determined by spectrophotometry at a wavelength of 280 nm using the Biorad protein assay (Biorad Laboratories). For detection in flow cytometry, the huMabs were biotinylated using a Biotin Labeling Kit according to the manufacturers instructions (Roche Diagnostics, Mannheim, Germany).

Flow cytometry

Cells were blocked for 20 minutes on ice in phosphate- buffered saline containing 1% BSA and 0.05% sodium azide (PBA) containing 10% human pooled serum and subsequent incubated with the biotinylated huMabs for 30 minutes on ice. Next, visualization of the huMabs was obtained using Alexa Fluor 633 streptavidin conjugate, in combination with the directly fluorochrome-conjugated antibodies as indicated in the RESULTS section. Cells were washed twice between incubation steps in PBA. After a final wash, cells were fixed in PBA containing 1% paraformaldehyde, and analyzed using a FACSCalibur (Becton Dickinson). For each experiment compensation was set using unstained ("autofluorescent"), FITC-only, PE-only, PercP-only and APC-only samples to eliminate fluorchrome crossovers between FL1, FL2, FL3 and FL4 channels.

Preparation of single cell suspensions:

Blood

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from healthy donors, by density centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Blood leukocytes were prepared by removal of erythrocytes from human peripheral blood by hypotonic shock.

Spleen

Spleen was obtained from organ transplant donors. Mononuclear cell suspensions were obtained by mechanical dissociation of spleen tissue, followed by digestion with type VII collagenase at 20 U/ml (Sigma) and deoxyribonuclease I (Dnase I; Boehringer Mannheim, Mannheim, Germany) at 20 U/ ml in medium supplemented with 2% FCS for 30 minutes at 37°C. The tissue was subsequently filtered though an open-filter chamber (NPBI, Emmer-Copascuum, The Netherlands) and the collected cells were subjected to Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation, to obtain spleen mononuclear cells (MNCs).

Thymus

Normal human thymuses from 15-day to 7-year-old children undergoing corrective cardiovascular surgery were cut into small pieces and subsequently sieved trough a gauze, to obtain a single cell suspension.

Tonsil

Tonsils were obtained from children undergoing routine tonsillectomy. Tonsils were cut into small pieces and digested for 1 hour at 37°C with collagenase IV (1mg/ml; Sigma, St Louis, MO) and Dnase I (100ng/ml, Boehringer Mannheim, Mannheim, Germany). The tissue was subsequently sieved through an open-filter chamber (NPBI, Emmer-Copascuum, The Netherlands) and the collected cells were subjected to Ficoll-Hypaque (Pharmacia Biotech) density gradient centrifugation, to obtain tonsil mononuclear cells (MNCs).

Epidermal cell suspension of the skin

Full-thickness human skin specimens were obtained from healthy donors undergoing plastic surgery of the breast. These specimens were shaved into slices of 0.2-mm thickness by

using a dermatome. To enable separation of the epidermis from the dermis, the slices were incubated with 0.2% dispase II (Boehringer Mannheim, Mannheim, Germany) in phosphate-buffered saline over night at 4 °C. The next day, slices were incubated for another 5 minutes at 37° C. The epidermis was collected in IMDM (Gibco) supplemented with 10% FCS and 5 U/ml Dnase I and sieved through a gauze to yield a single cell suspension.

Generation of monocyte-derived DCs

DCs were generated from monocytes as previously described [41;47]. Briefly, PBMCs were isolated from blood by Ficoll-Hypaque density centrifugation, and allowed to adhere for 2 hours at 37 °C. The adherent monocytes were cultured at $1x10^6$ cells/ml in RPMI-S, in the presence of recombinant human (rh) interleukin (IL)-4 (500 U/ml; Strathmann, Hannover, Germany) and rh granulocyte-macrophage colony-stimulating factor (GM-CSF; 800 U/ml, Schering-Plough, Amstelveen, The Netherlands) for 5 days. Fresh cytokine-containing culture medium was added at day 3. After 5 days, the immature monocyte-derived DCs were harvested. To generate mature DCs, a combination of 800 U/ml of rhGM-CSF (Schering Plough), 1000 U/ml of rhIL-4 (Strathmann), 20 ng/ml of rh tumor necrosis factor (TNF) α (Intergen Company, Purchase, NY) 10 ng/ml of rhIL-1β (Strathmann), 1000 U/ml of rhIL-6 (Roche Diagnostics, Almere, The Netherlands), 1 µg/ml of prostaglandin E2 (PGE2; Sigma, St. Louis, MO) were added for another 48 hours to the immature DCs.

Generation of $CD34^+$-derived DCs

Hematopoietic $CD34^+$ progenitor-derived DCs were prepared as described previously [39;40]. Briefly, mononuclear cells were isolated by Ficoll-Hypaque density centrifugation (Pharmacia) from adult bone marrow samples. $CD34^+$ cells were enriched using the MACS $CD34^+$ progenitor cells system according to manufacturers instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD34^+$ cells were seeded at $2x10^5$ cells per well in a six wells plate in 4 ml RPMI-S supplemented with rhGM-CSF (Scheringh Plough), 2.5 ng/ml (50U/ml) of rhTNFα (Intergen Company), 25 ng/ml of rh stem cell factor (SCF; Amgen, Thousand Oaks, CA) and 2.5% AB^+ pooled human serum. At day 3 fresh RPMI-S supplemented with rhGM-CSF and rhTNFα was added to each well. Hematopoietic $CD34^+$ progenitor-derived DCs were collected at day 6 of culture for analysis.

RESULTS

Conversion of phage antibodies to IgG4 huMabs

Recently, we generated phage antibodies selected on human DC subpopulations in peripheral blood [18] and tonsil (Lekkerkerker, A., De Weers, M., Logtenberg, T., Chapter 2B) using a phage antibody display library. Four phage antibodies that bound to DCs and also monocytes, denoted MatDC16, MatDC27, MatDC64, and TN141 were chosen for further characterization. A phage antibody directed against the human epithelial marker EpCAM, UBS54 [48], was used as a negative control.

The phage antibodies were converted to complete human antibodies of the IgG4 isotype using previously described eukaryotic expressions vectors [19]. The heavy and light chain variable regions coding the phage antibodies of interest, MatDC16, MatDC27, MatDC64, TN141 and UBS54, were recloned in vectors containing the genomic IgG4 heavy chain constant domain and the genomic kappa light chain constant domain, respectively. Stably transfected clones were made by co-transfection of the heavy and light chain constructs. The huMabs were purified from culture supernatants of the clones and integrity and purity of the huMabs was confirmed by Coomassie brilliant blue staining of a SDS-PAGE gel run under reducing and non-reducing conditions (Fig. 1). An immunoblot using goat antihuman IgG4 antibodies confirmed the IgG4 isotype (data not shown).

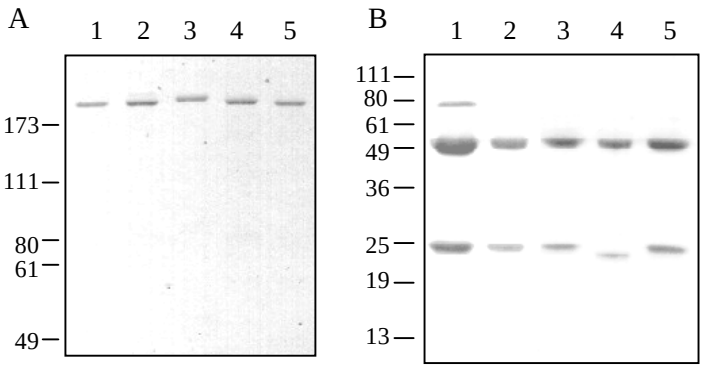

Figure 1 SDS-PAGE analysis of purified IgG4 preparations under (A) non-reducing and (B) reducing conditions. Lane 1, IgG4 MatDC16; lane 2, IgG4 MatDC27; lane 3, IgG4 MatDC64; lane 4, IgG4 TN141; lane 5, IgG4 UBS54. The molecular weight markers are shown in kilodaltons on the left.

Phenotypic identification of in vivo DC subsets in different tissues

We examined the expression of target antigens of the huMabs on different DC subsets *in vivo* and *in vitro*. DC subsets from blood and different tissues of the body were identified using four-color flow cytometry analysis, using the following antibodies: APC-labeled CD11c, PercP-labeled HLA-DR, a FITC-labeled cocktail of lineage markers (Lin: CD3, CD14, CD16, CD19, CD20, CD56) versus a panel of PE-labeled monoclonal antibodies on

single cell suspensions. Typically, DCs are characterized by expression of HLA-DR and the absence of lineage markers (Fig. 2). Therefore, $Lin^{-}HLA\text{-}DR^{+}$ cells were gated (Fig. 2B) and analyzed for CD11c expression in combination with other makers to establish the DC phenotype (Fig. 2C).

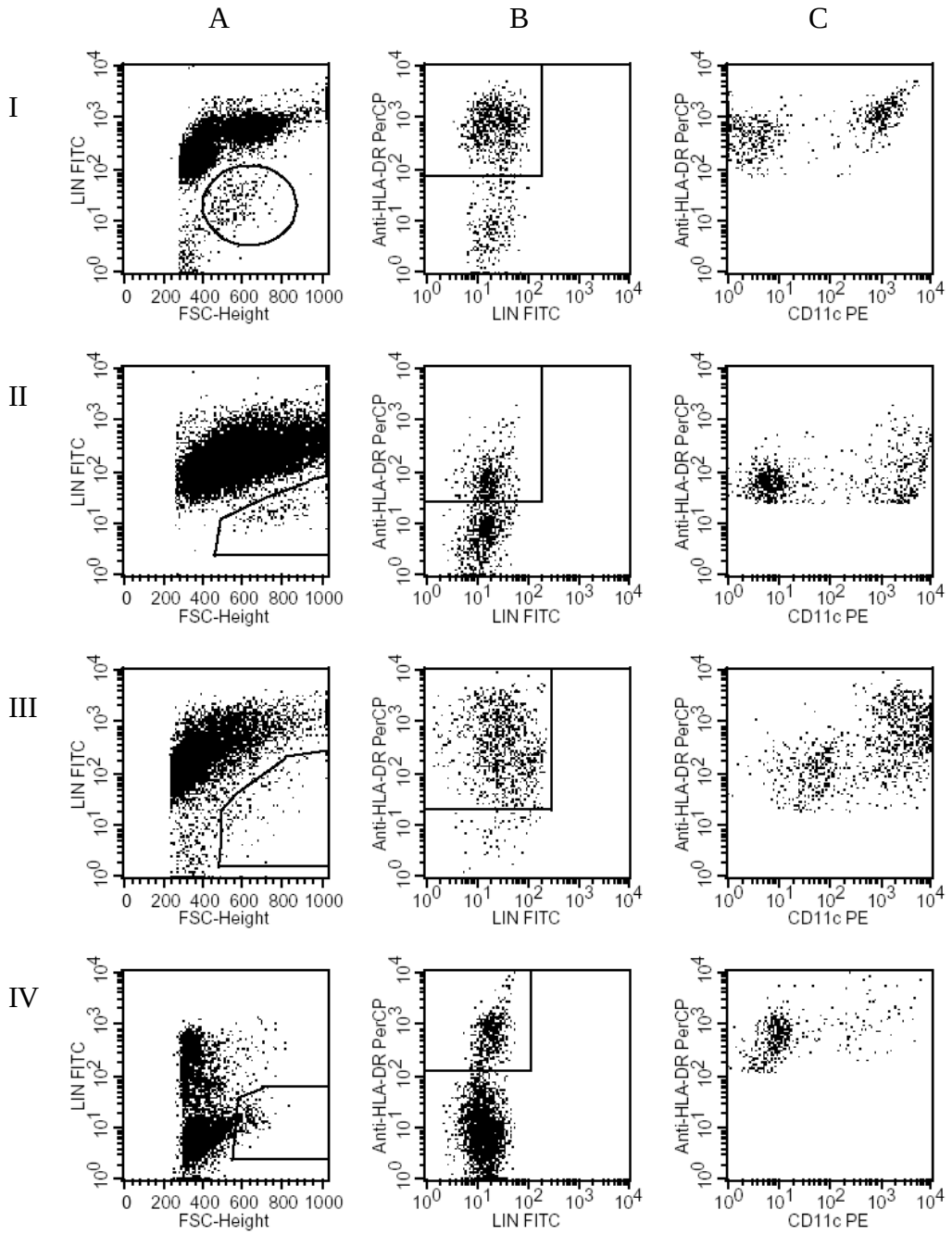

Figure 2 Identification of $CD11c^{-}$ and $CD11c^{+}$ DC subsets *in vivo* in (I) peripheral blood, (II) tonsil, (III) spleen and (IV) thymus. Isolation of single cell suspensions was carried out as described in MATERIAL AND METHODS. To identify DCs (A) Lin^{-} cells were gated, and subsequent (B) the $HLA\text{-}DR^{+}$ cells within this gate were analyzed, resulting in (C) segregation of subsets based on CD11c expression.

LCs in the epidermal cell suspension were gated based on a high expression of HLA-DR (Fig. 5). Monocytes are a possible precursor pool of DCs [44], they were gated as $CD14^+$ cells in a separate experiment and also subjected to detailed immunophenotyping (Table 2). A representative compilation of results of the different subsets is shown in table 2 and figure 3.

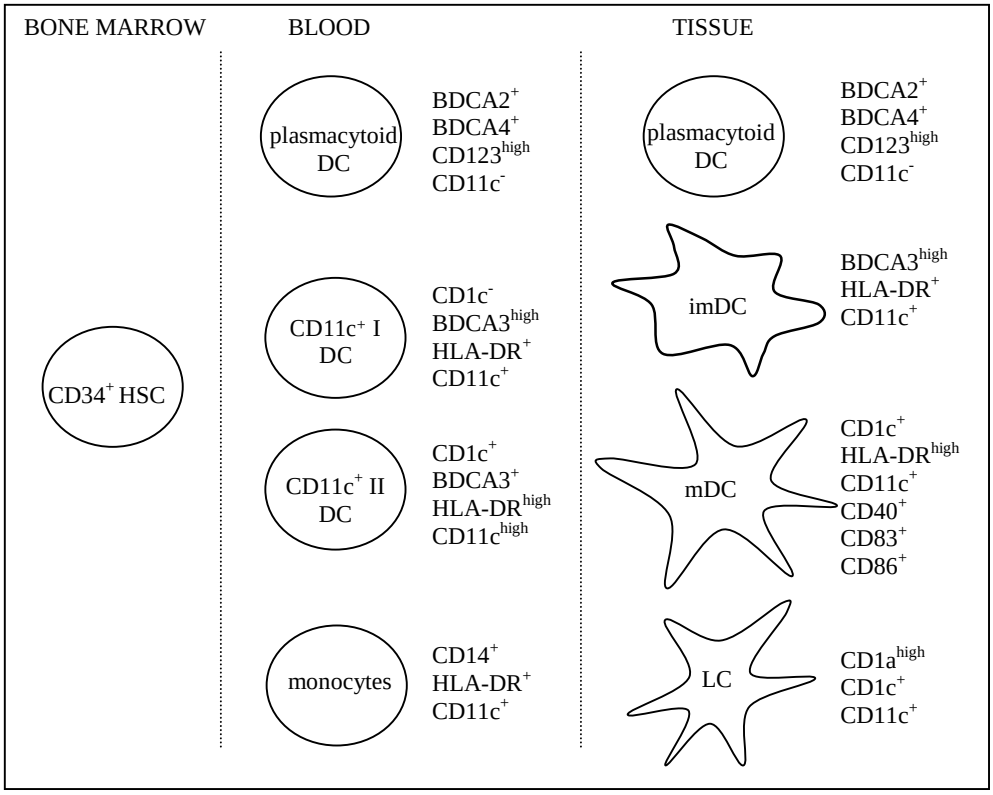

Figure 3 Schematic representation of several DC subsets, possibly derived from separate pathways, found at different anatomical sites *in vivo*. Discriminating surface markers of the different DC subsets are indicated, based on data obtained in this study, see Table 2. Abbreviation: $CD34^+$ HSC, hematopoietic stem cell.

Plasmacytoid DCs

As shown figure 2, DC subsets in the examined tissue suspensions segregated based on CD11c expression. Based on the previously described subsets of DCs in blood, the $CD11c^+$ cells corresponded to myeloid DCs and the $CD11c^-$ cells to plasmacytoid DCs [23;49]. All DC subsets, including LCs, expressed HLA-DR, CD4, CD40 and CD54 (ICAM-1). The $CD11c^-$ DC subsets demonstrated a uniform expression of markers in different tissues. This $CD11c^-$ DC subset was $CD123^{high}$, and expressed the recently described markers, BDCA-2 and BDCA-4 [46]. These two markers specifically recognize human $CD11c^-CD123^{high}$ plasmacytoid DCs in blood, confirming the identity of this $CD11c^-$ subset. Myeloid associated markers, such as CD64, and CD11b were absent on $CD11c^-$ DCs. Although also a low expression CD11c was present in spleen, this $CD11c^{dim}$ subset expressed high levels of CD123, BDCA-2 and BDCA-4, indicative of the plasmacytoid DC subset.

Table 2 Immunophenotype of different DC subsets *in vivo* .

	Blood				Tonsil			Spleen			Thymus				Epiderm
Surface Antigen	CD11c$^-$	CD11c$^+$ I	CD11c$^+$ II	monocytes	CD11c$^-$	imDC	mDC	CD11c$^-$	imDC	mDC	CD11c$^-$	imDC	mDC	CD11c$^+$Lin$^+$	LC
DC-associated															
CD1a	-	-	±	-	-	-	-	-	-	±	-	+	+	+	+++
CD1c	-	-	++	-	-	(+)	+	-	(+)	±	-	+	+	++	++
CD83	-	-	-	-	-	-	+	-	-	++	-	-	±	±	(+)
BDCA-2	++	-	-	-	++	±	±	++	-	-	+	-	-	-	±
BDCA-3	-	+++	+	±	±	++	++	±	++	++	-	++	+	+	-
BDCA-4	++	-	-	-	++	±	±	++	±	±	++	+	+	+	-
Myeloid															
CD14	-	-	-	+++	-	-	-	-	-	-	-	-	-	nt	-
CD64	-	-	+	++	-	±	±	nt	nt	nt	-	±	±	++	-
Lymphoid															
CD4	++	++	++	+	++	++	++	++	++	++	++	++	++	++	+
Adhesion															
CD11b	-	±	++	+++	-	+	+	nt	nt	nt	-	±	±	+++	±
CD11c	-	++	+++	++	-	+++	+++	-	+++	+++	-	++	++	+++	++
CD54	++	++	++	++	++	++	++	+	++	+++	++	++	++	++	+
Co-stimulation															
CD40	+	+	+	+	+	++	+++	+	++	++	++	++	+++	++	+
CD80	(+)	-	-	-	-	+	+	-	+	++	-	±	+	±	-
CD86	+	+	+	+	±	+	++	-	+	++	±	+	++	+	+
Cytokine receptors															
CD25	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD123	+++	±	+	+	+++	+	+	+++	+	++	+++	±	++	+	+
MHC															
HLA-DR	++	++	+++	++	+	+	++	++	++	+++	++	++	+++	+++	++

The levels of staining on the various cell populations are indicated as follows: -, negative; ±, variable; each + corresponds to an increase in fluorescence intensity of one logarithm; nt, not tested

In blood, BDCA-4 was uniquely expressed on plasmacytoid DCs, however in tonsil, spleen and thymus, expression was also observed on $CD11c^+$ DCs. BDCA-2 was expressed only on the plasmacytoid DCs in the tissues. In contrast to a recent paper [50], a low expression of BDCA-2 on $CD11c^+$ DCs in tonsil was observed. This might be caused by the relative sensitivity of flow cytometry for small subpopulations compared to either RT-PCR or immunohistochemistry. A heterogeneous expression of CD80 was observed for the $CD11c^-$ subset in blood, while the $CD11c^+$ subset was negative; in addition both subsets expressed CD86. Differences in expression levels of co-stimulatory molecules CD80 and CD86 have been reported [26;49;51]. A low expression of BDCA-3 was seen on the $CD11c^-$ subsets in tonsil and spleen while absent in blood and thymus. In thymus, also low levels of CD1a were observed on the $CD11c^-$ subset.

Myeloid DCs

A more diverse staining pattern was observed for the $CD11c^+$ DCs at different anatomical sites. While high expression of CD123 is the hallmark of plasmacytoid DCs, low level expression was always also observed on $CD11c^+$ subsets. In peripheral blood about 3-4% of the $HLA\text{-}DR^+Lin^-$ DCs ($CD11c^+$ I) showed a differential expression of certain markers compared to the major $CD11c^+$ DC subset ($CD11c^+$ II). The $CD11c^+$ I DC subset had a slightly lower expression of HLA-DR, CD11c, CD4, and CD86 and lacked expression of CD64, CD1c, CD11b. Noteworthy, the $CD11c^+$ I DC subset expressed high levels of BDCA-3 [46]. How this minor $CD11c^+$ BDCA-3^{high} DC subset relates to the $CD11c^+$ II DC subset is not known. In addition, low levels of BDCA-3 were also observed on the $CD1c^+CD11c^+$ II subset in contrast to literature [46].

Within the $CD11c^+$ DC subset of tonsil, spleen and thymus, a small subset with a higher expression of HLA-DR was observed. This $CD11c^+$ DC subset generally expressed higher levels of for CD40, CD80, CD83 and CD86, indicating that these cells are matured and activated, therefore this subset was denoted mDC (mature DC). Presumably in tissues, the other $CD11c^+$ DCs comprised immature GCDC-like and splenic marginal zone DCs and were denoted imDC (immature DC). The mDC subset constituted presumably IDCs [30;32-34;52]. The mDC subset had a somewhat higher expression of CD123. BDCA-3 expression on the mDC subset was lower compared to the imDC subset. Based on *in situ* identification of tonsil DC subsets, Summers *et al.* [53] propose that $CD11c^+$ $CD13^-$ are GCDCs, whereas the $CD11c^+CD13^+$ as well as the $HLA\text{-}DR^{high}$ $CD11c^+$ DC subsets are IDCs present in the T cell areas of the tonsil. Indeed the mDC subset expressed CD13, though our staining strategy did

not allow discriminating between different anatomical localizations of IDC subpopulations.

A CD11c^{high}HLADR^{high}Lin^{+} subset in thymus

The intriguing population of HLADR^{high}Lin^{+} cells in thymus was more closely examined and a distinct CD11c^{high} subset was observed (Fig. 4). This CD11c^{high}HLADR^{high}Lin^{+} subset expressed DC-associated marker CD1a and in addition, CD4, CD40, CD54, CD80 and CD86, indicative of a DC phenotype. Notably, this subset showed a distinct, high expression of CD64 and CD11b. This population resembled the thymic CD11b^{+} DC subset expressing the myeloid markers M-CSFR, CD11c, CD14 and CD64 that was described recently [35]. The Lin-cocktail we used, contained antibodies against CD14, which could explain why this CD11c^{high} DC subset was Lin^{+}.

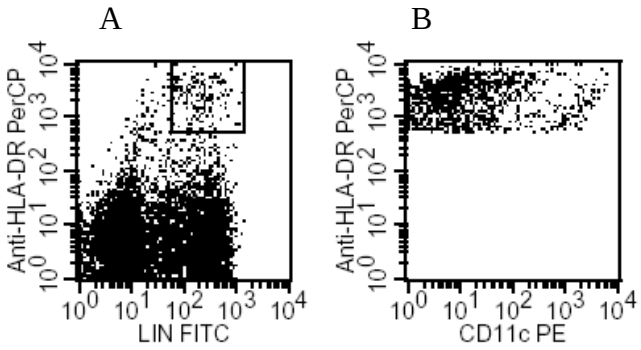

Figure 4 Identification of a CD11c^{high} subset within theHLA-DR^{high}Lin^{+} cells. Cells were gated as (A) HLA-DR^{high}Lin^{+} cells and (B) CD11c staining revealed the presence of a CD11c^{high} subset.

Identification of Langerhans cells in the skin

LCs, epidermis-resident DCs, were distinguished from other cell types in the skin by a high expression of HLA-DR (Fig. 5). These cells were also CD1a^{high}, confirming their LC identity (Table 2). The LCs expressed CD11c and CD1c^{high} and had a low expression of CD11b and CD86. They lacked expression of CD64, CD80, BDCA-3, and BDCA-4, and only few cells expressed CD83. However, expression of CD123 and low levels of expression of BDCA-2 were found on the LCs.

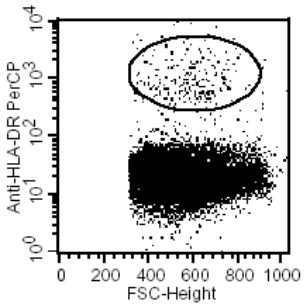

Figure 5 Identification of LCs in the skin, by staining of an epidermal cell suspension with HLA-DR PercP.

Staining patterns of the huMabs on in vivo DCs

To determine whether the huMabs retained their specificities, PBMCs were stained with the biotinylated huMabs in combination with different lineage markers for B cell (CD19), T cells (CD3), NK (CD56) cells and monocytes (CD14) and compared with staining patterns obtained using phage antibodies as previously described [18]. In peripheral blood, huMab MatDC16 stained in addition to DCs, monocytes and a subset of granulocytes, also a small

subset of T cells (<5%), the latter staining was not observed with the phage antibody. In addition to DCs and monocytes, huMab MatDC27 showed a weak staining of T cells, the latter staining was not observed with the phage antibody. Reactivity of huMabs MatDC64 and TN141 was restricted to DCs and monocytes as demonstrated for the phage antibodies.

HuMab MatDC16 stained preferentially plasmacytoid DCs in different tissues (Table 3). In addition, in blood the $CD11c^{+}$ II DC subset and monocytes were stained more intensely than the $CD11c^{+}$ I DC subset. For tonsil and spleen, staining on the imDC and mDC subsets was comparable, while in thymus staining of the mDC subset was lacking. However, bright staining of the $CD11c^{high}HLADR^{high}Lin^{+}$ subset was observed.

HuMab MatDC27 followed the staining pattern of MatDC16 in human peripheral blood although in general the reactivity was less intense. Also for the secondary lymphoid tissues, MatDC27 staining patterns resembled that of MatDC16, albeit less strong on both plasmacytoid and myeloid DCs. Staining of thymic plasmacytoid DCs was present, though absent on the $CD11c^{+}$ DC subsets. Similar to MatDC16, staining was also observed on the $CD11c^{high}HLADR^{high}Lin^{+}$ subset in thymus.

HuMab MatDC64 stained the different DC subsets and monocytes in blood. In tonsil and spleen staining of plasmacytoid DCs was observed, while staining of the $CD11c^{+}$ DC subsets was lacking. In thymus, MatDC64 stained merely the $CD11c^{high}HLADR^{high}Lin^{+}$ subset. Overall, while a more broad staining was observed in blood, MatDC64 in secondary lymphoid organs was restricted to the plasmacytoid DCs. In addition, MatDC64 reacted with the $CD11c^{high}HLADR^{high}Lin^{+}$ subset in thymus.

HuMab TN141 stained both the $CD11c^{+}$ I and II DC subsets in peripheral blood. In addition, a dim staining on both monocytes and plasmacytoid DCs was observed. While a distinctive staining of the imDC subset in tonsil and spleen was observed, this staining was persistently lacking on the $CD11c^{+}$ mDC subset. This staining pattern resembled that of BDCA-3, though intensity was much lower. Double staining of an antibody against BDCA-3 and TN141 revealed that TN141 specifically stains the 0.03% of PBMCs that is $BDCA\text{-}3^{high}$ (Fig. 6). In tonsil and spleen, staining of TN141 on the plasmacytoid DCs was

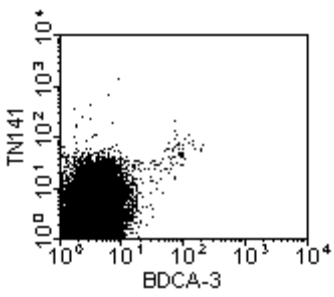

Figure 6 Double staining BDCA-3 and TN141 on PBMCs. Shown is a two-color staining of PBMC with an FITC-conjugated antibody against BDCA-3 and a biotinylated TN141 followed by Alexa Fluor 633 streptavidin

dim and of the CD11c^{high}HLADR^{high}Lin^{+} subset in thymus. None of the huMabs reacted with skin LCs.

Generation of DCs in vitro

To examine the phenotypic similarities and differences between fresh DCs and *in vitro* generated DCs, we cultured DCs using two well-defined systems. Culturing monocytes in the presence of GM-CSF and IL-4 generated CD83^{-}CD1a^{+} immature Mo-derived DCs that were collected at day 5 of culture. Immature Mo-derived DCs were stimulated with a mix of cytokines consisting of IL-1β, IL-4, IL-6, GM-CSF, TNFα and PGE2, resulting in mature Mo-derived DCs that expressed high levels of CD83 and CD1a. Interestingly, CD64 was not observed on either immature or mature DCs. Cytokine receptors CD25 and CD123 were only expressed on mature DCs.

In agreement with published data, BDCA-4 was also expressed at high levels on immature DCs and low levels on mature DCs, whereas BDCA-2 was absent on both populations (Table 3). Low levels of expression of BDCA-3 on both immature and mature Mo-derived DCs were detected [46]. CD40, CD86 and CD11b expression was upregulated on mature DCs, and *de novo* expression of CD80 and CD83 was observed, confirming previous findings [41].

Hematopoietic CD34^{+} progenitor cells differentiate *in vitro* along two unrelated DC pathways. The first pathway involves the generation of CD1a^{+}CD14^{-} cells with features of epidermal LCs. The second pathway includes a CD1a^{-}CD14^{+} intermediate that can differentiate into interstitial DCs that lack LC markers. At day 6, two populations characterized by exclusive expression of CD1a and CD14 emerged independently, with similar percentages as previously reported, the major subpopulation being CD14^{+}, the minor CD1a^{-} [39]. Both CD14^{+} and CD1a^{+} DC subsets expressed comparable levels of HLA-DR, CD4, CD11c, CD54, CD80 and CD86, whereas no CD83 was expressed. Only the CD14^{+} population expressed low levels of BDCA-3 and CD40.

High levels of expression of BDCA-4 were observed on the CD14^{+} population while weakly expressed on the CD1a^{+} population. Although CD1c expression has previously been reported, we did not observe CD1c on either subpopulation.

Staining patterns of the huMabs on in vitro DCs

Previously, we showed that phage antibody MatDC16 was weakly expressed on the surface of bone marrow CD34^{+} progenitor cells [18]. Up-regulation of the antigen was observed

Table 3 Reactivity of huMabs MatDC16, MatDC27, MatDC64 and TN141 on different DC subsets *in vivo* .

	Blood				**Tonsil**			**Spleen**			**Thymus**				**Epiderm**
huMab	$CD11c^-$	$CD11c^+$ I	$CD11c^+$ II	monocytes	$CD11c^-$	imDC	mDC	$CD11c^-$	imDC	mDC	$CD11c^-$	imDC	mDC	$CD11c^+Lin^+$	LC
MatDC16	++	+	++	++	++	±	±	++	+	+	++	±	-	++	-
MatDC27	+	+	+	+	+	±	±	++	±	±	+	-	-	+	-
MatDC64	+	±	±	+	±	-	-	+	-	-	-	-	-	±	-
TN141	±	+	+	±	±	++	-	±	++	-	-	±	-	±	-

Streptavidin Alexafluor 633 followed staining of biotinylated huMabs, in combination with PE-labeled CD11c, PercP-labeled HLA-DR and a FITC-labeled cocktail of lineage markers.The levels of staining on the various cell populations are indicated as follows: -, negative; ±, variable; each + corresponds to an increase in fluorescence intensity of one logarithm.

Table 5 Reactivity of huMabs MatDC16, MatDC27, MatDC64 and TN141 on different DC subsets *in vitro*.

	Mo-derived DC		**$CD34^+$-derived DC**	
	immature	mature	day 6	
huMab			$CD14^+$	$CD1a^+$
MatDC16	++	±	++	++
MatDC27	+	-	++	+
MatDC64	-	-	-	-
TN141	-	-	-	-

The levels of staining on the cell populations are indicated as follows: -, negative; ±, variable; each + corresponds to an increase in fluorescence intensity of one logarithm.

Table 4 Immunophenotype of different DC subsets *in vitro*.

	Mo-derived DC		**CD34$^+$-derived DC**	
	immature	mature	day 6	
Surface Antigen			CD14$^+$	CD1a$^+$
DC-associated				
CD1a	++	++	-	+++
CD1c	+	+	-	-
CD83	-	++	-	-
BDCA-2	-	-	-	-
BDCA-3	+	+	±	-
BDCA-4	++	+	++	±
Myeloid				
CD14	-	-	++	-
CD64	-	-	nt	nt
Lymphoid				
CD4	±	±	++	+
Adhesion				
CD11b	++	++	nt	nt
CD11c	+++	++	++	++
CD54	++	++	++	++
Co-stimulation				
CD40	++	+++	±	-
CD80	±	++	±	±
CD86	++	++	+	+
Cytokine receptors				
CD25	-	++	nt	nt
CD123	-	±	+	±
MHC				
HLA-DR	++	++	++	++

The levels of staining on the various cell populations are indicated as follows: -, negative; ±, variable; each + corresponds to an increase in fluorescence intensity of one logarithm; nt, not tested

during culture with a combination of SCF, GM-CSF and TNFα. On day 6, flow cytometric analysis demonstrated that MatDC16 stained the CD1a$^+$ and CD14$^+$ intermediates at comparable levels. MatDC16 stained immature Mo-derived DCs strongly but expression of the target antigen was down-regulated during maturation. MatDC27 stained immature Mo-derived DCs weakly. Staining on both the CD1a$^+$ intermediate and the CD14$^+$ intermediate, was present, tough preferentially on the CD14$^+$ intermediate. Staining of MatDC64 and TN141 on *in vitro* generated Mo-derived DCs or CD1a $^+$ and CD14 $^+$ intermediates was absent.

DISCUSSION

In this report, we demonstrate that phage antibodies can be rapidly converted to whole human antibodies of the IgG4 isotype. The huMabs were correctly assembled and retained their specificities. We assessed the staining patterns of huMabs MatDC16, MatDC27, MatDC64 and TN141 by flow cytometry on different DC subsets found in the human tissues, using a multiparameter flow cytometry strategy to identify *in vivo* DCs without the need for extensive cell separation procedures. The identification relies on the absence of lineage markers and the presence of HLA-DR, as previously demonstrated [49;54]. Expression of CD11c was employed to segregate DC subsets. Distinct $CD11c^-$ and $CD11c^+$ DC subsets were identified reliably in different tissues in the body, enabling us to compare expression of DC discriminating markers on different subsets *in vivo*.

While MatDC16 essentially bound to all *in vivo* DC subsets, in particular the plasmacytoid DCs had a high level of expression of the target antigen of MatDC16. MatDC27 bound preferentially to plasmacytoid DCs, though a weak staining was observed for myeloid subsets in tonsil and spleen.

More restricted staining patterns were observed for the other huMabs. MatDC64 uniquely reacted with plasmacytoid DCs in the secondary lymphoid organs, while in thymus only the $CD11c^{high}HLADR^{high}Lin^+$ subset was positive. The high level of expression of CD11b and CD64 observed in this subset and the reactivity of MatDC64 on monocytes in peripheral blood, would argue in favor of a monocyte derivation of this subset. In summary, while a broad staining pattern on DC precursors was observed in blood, the staining pattern of MatDC64 was strictly confined to plasmacytoid DCs in secondary lymphoid organs.

TN141 demonstrated a broad staining pattern in blood. In contrast, TN141 stained preferentially the myeloid DCs in tissues. Within the myeloid DC subset, expression of the target antigen of TN141 was restricted to the imDC subset, while expression was lacking on the mDC subset. Although the staining pattern of TN141 was reminiscent of expression of BDCA-3, we were not able to block binding of a antibody against BDCA-3 using huMab TN141 to $CD11c^+$ DCs in peripheral blood (unpublished results).

In thymus, negative selection of T cells takes place, a process that likely involves DCs. The different function of DCs in the thymus may account for the lack of staining of MatDC64 on the $CD11c^-$ and $CD11c^+$ DC subsets and absence of staining of the huMabs MatDC16 and MatDC27 on the mDC subset.

LCs are thought to be immature because of their ability to efficiently take up and process

antigens and their low cell surface expression of co-stimulatory molecules [38]. The low expression of CD40 and CD86 and the absence of CD80 confirmed this. LCs are considered myeloid DCs as they also express CD11c and CD1c. However, in addition to the expression of CD123, also a weak expression of BDCA-2, a plasmacytoid DC marker, was observed. Notably, staining of huMab UBS54, an antibody directed against the epithelial marker EpCAM, was observed (Table 2). This staining was confirmed using the conventional monoclonal antibody HEA against the human epithelial marker. Expression of the murine homologue of EpCAM has been demonstrated on mouse LCs [55], however this is the first report of EpCAM expression on human LCs. Staining of the other huMabs on LCs was not observed. We could not exclude that on LCs, the target antigens of the different huMabs were trypsin-sensitive, as trypsin was used in the cell separation procedure.

We compared the phenotype of the *in vivo* DC subsets with DCs generated *in vitro*. Differential staining patterns of the huMabs on DC subsets *in vitro* were demonstrated. DCs derived from $CD34^+$ hemopoietic progenitors *in vitro* have been shown to develop along two independent pathways giving rise to DCs with distinct phenotypes and functions. MatDC16 stained both the $CD14^+$ and $CD1a^+$ intermediates while staining of MatDC27 on the $CD14^+$ intermediate was higher than on the $CD1a^+$ intermediate. This is unexpected in view of the absence of staining on $CD1a^+$ LCs *in vivo*. MatDC64 and TN141 were negative on both the $CD14^+$ and $CD1a^+$ intermediates. Target antigens of MatDC64 and TN141 were downregulated during the differentiation of monocytes into immature Mo-derived DCs, around day 3 of culture. In this context, it is noteworthy that immature DCs obtained from reverse-transmigrated monocytes [44] were stained with phage antibodies MatDC16, MatDC27 and MatDC64 (unpublished results). Staining of phage antibody TN141 on immature DCs was absent. Neither of the phage antibodies reacted with reverse-transmigrated mature DCs that had phagocytosed zymosan particles. Collectively, 'immature' DCs from cultures of reverse-transmigrated monocytes differ from immature Mo-derived DCs generated in cultures of monocytes supplemented cytokines with respect to MatDC64 staining, suggesting substantial difference between different *in vitro* generated DC subsets.

The finding that TN141 preferentially bound the small $CD11c^+$ I DC subset but to none of the *in vitro* generated DCs indicates that no counterparts of these particular DCs found *in vivo* are present in any of the DC culture systems. In general, it appears that *in vitro* generated DCs may perhaps not have physiological counterparts and vice versa. This finding underscores the need to use *in vivo* DC subsets to determine the physiological generation and specific functions of DCs.

The fact that huMabs did not perform in immunohistochemistry, western blotting or immunoprecipitation studies using peripheral blood or positive cell lines, if available, hampered further characterization of the target antigens of these huMabs. Attempts to clone target antigens using cDNA expression libraries, including commercially available and a cDNA expression library made from monocytes and Mo-derived DCs (M. van Meijer, unpublished results) were unsuccessful.

Using the four recently described antibodies against DCs, CD1c (BDCA-1), BDCA-2, BDCA-3, and BDCA-4 [46], we demonstrate that the newly identified markers BDCA-2 and BDCA-4 form a reliable tool to identify plasmacytoid DCs in blood. In tissues, BDCA-2 showed a more restricted staining pattern than BCDA-4. In this respect, BDCA-2, recently identified as a novel plasmacytoid DC-specific type II C-type lectin, proves a more valuable tool for identification of plasmacytoid DCs [50]. In our hands, BDCA-3 expression was not restricted to the small $CD11c^+$ I DC subset in blood, but was also observed on the $CD1c^+$ $CD11c^+$ II DC subset, although staining was less intense.

In peripheral blood, part of the $CD11c^+$ II DC subset expressed CD1a, confirming previous data by Ito *et al*. [51]. Dzionek *et al.* [46] questions this observation by reasoning that the Mab used, B-B5, binds to CD1c. Dzionek *et al.* use anti-CD1a Mab BL-6 and do not observe CD1a expression on the $CD11c^+$ DC subset. However, we repeatedly observed staining of $CD11c^+$ cells using this same Mab BL-6, confirming the data of Ito *et al.*.

The goal in DC-based cancer vaccination protocols is to elicit an effective immune response to the tumor by generation of large numbers of tumor-reactive effector T cells. CTLs can directly kill tumor cells [56], whereas T_H cells are necessary to engage several effector mechanisms. Myeloid DCs can be induced to produce large amounts of IL-12 [57] and cause T_H1 differentiation. The T_H1 response, characterized by IFNγ and IL-2 producing T_H1 cells, is correlated with an efficient CTL response [58]. Therefore, targeting to myeloid DCs *in vivo* seems advantageous. Plasmacytoid DCs are said to preferentially promote a T_H2 response, however their action is still far from clear and data are contrasting in available studies [11;13;29;59]. Considering the different features of plasmacytoid DCs compared to myeloid DCs, a potential beneficial role of the engagement of the plasmacytoid DCs in DC-targeting strategies seems more intricate.

Taken all this into consideration, we presume that MatDC27 is less attractive for targeting to DCs. HuMabs MatDC64 and TN141 had more with restricted staining patterns and may well be valuable tools for further investigation and delineation of DC subsets. TN141 might be valuable for targeting purposes as this huMab predominantly reacted with

myeloid DCs. MatDC16 may also prove valuable for *in vivo* targeting of DCs, on account of its broad reactivity, possibly engaging different DC subsets. Chemical or genetic coupling of tumor antigens to a huMab may provide a vector to target tumor antigen directly to DCs *in vivo*. To ensure an optimal outcome, adjuvants that provoke migration and maturation of DCs might be necessary. In this respect, CpG oligonucleotides are of special interest since they can activate DCs *in vivo* and polarize T cells toward T_H1 [60;61]. Ongoing research will provide evidence concerning the efficacy of this approach.

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CHAPTER 4

BINDING AND CROSSLINKING OF HUMAB MATDC 64 PREVENTS THE *IN VITRO* DIFFERENTIATION OF HUMAN MONOCYTES INTO DENDRITIC CELLS

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ABSTRACT

We have previously generated a panel of four human antibodies (huMabs) against human dendritic cells (DCs) and performed a detailed phenotypical analysis of the reactivity of these huMabs on different DC subpopulations. In the present study, we assessed the functional effects of binding of these huMabs to monocytes and DC *in vitro*. None of the huMabs affected the allostimulatory capacity of PBMCs in an allogeneic mixed leukocyte reaction. A single antibody, huMab MatDC64, exerted a suppressive effect on the cytokine-driven differentiation of monocytes into DCs. This phenomenon was associated with the production of interleukin 6 (IL-6), known to redirect differentiation of monocytes towards macrophages. These results suggest that IL-6 production observed after crosslinking of MatDC64 might contribute to the prevention of the generation of DCs.

INTRODUCTION

Dendritic cells (DCs) are professional antigen presenting cells (APC), which are efficient in the activation of naïve T cells and the induction of primary T cell-mediated immune responses [1]. DCs reside in tissues throughout the body as immature cells that have high antigen capturing and processing capacities. Upon antigen uptake, DCs mature and concomitantly increase their antigen presenting functions.

DCs can be generated *in vitro* from peripheral blood monocytes [2]. Monocytes cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL) 4 acquire many morphologic, phenotypic and functional features of immature DCs. Loss of CD14 and the upregulation of costimulatory molecules, including CD80 and CD86 and the Langerhans cell marker CD1a characterize the transition of monocytes into DC. Alternatively, in response to macrophage colony-stimulating factor (M-CSF), monocytes can differentiate into $CD14^+$ macrophages[3;4]. Collectively, cytokines act as regulating mediators of monocyte differentiation towards DCs or macrophages.

Previous studies have suggested that binding of antibodies to monocytes may affect their capacity to differentiate into DCs in *in vitro* cultures. Binding of antibodies to inhibitory receptor LAIR-1 expressed on monocytes prevents the differentiation of monocytes into DCs by interfering with the GM-CSF receptor signaling pathway [5]. Binding of antibodies to CD33 expressed on monocytes inhibits the generation of DCs in cultures of monocytes supplemented with GM-CSF and IL-4 [6]. Both receptors are immune inhibitory receptors and

members of the immunoglobulin (Ig) superfamily. These inhibitory receptors can be identified by a consensus amino acid sequence, the immunoreceptor tyrosine-based inhibitory motif (ITIM), present in the cytoplasmic domain of these molecules. In addition, receptor engagement of FcεRI, containing a immunoreceptor tyrosine-based activation motif (ITAM), on monocytes from atopic donors has been shown to prevent their differentiation into DCs, mainly by production of the inhibitory cytokine IL-10 [7].

In a previous study, we have employed a semi-synthetic antibody phage display library, in combination with subtractive selection and flow cytometry to generate monoclonal phage antibodies specific for DCs in human blood [8]. Three of these phage antibodies, denoted MatDC16, MatDC27 and MatDC64, were found to bind DCs and monocytes in peripheral blood. MatDC16 showed additional binding to B cells. Phage antibody TN141 selected from the phage library for binding to human tonsil DCs shows additional binding to blood DCs and monocytes (Lekkerkerker A., De Weers, M., Logtenberg, T., Chapter 2B). These four phage antibodies were converted to complete human monoclonal antibodies (huMabs) of the IgG4 isotype, and a detailed phenotypical analysis of these huMabs on *in vitro* generated DCs and DC subsets found *in vivo* was carried out [9]. It was shown that huMab MatDC16 stained immature Mo-derived DCs, while a dim staining was observed for MatDC27. Expression of target antigens of MatDC64 and TN141 was downregulated and lost around day three of culture of monocytes with GM-CSF and IL-4.

In light of the possible application of these huMabs as a tool to target antigens to professional APCs, we analyzed whether engagement of the molecules recognized by these huMabs affects DC function or differentiation. In this study, we provide experimental evidence that crosslinking of MatDC64 inhibits the generation of DCs from monocytes, while it has no effect on the stimulatory capacity of PBMCs. HuMabs MatDC16, MatDC27 and TN141 showed no inhibitory nor activating properties.

MATERIAL AND METHODS

Culture medium

Unless stated otherwise, medium consisting of RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum (Integro, Zaandam, The

Netherlands), 25 mM HEPES, 2 mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin was used for culturing cells.

Antibodies

HuMabs used in this study were human IgG4 antibodies MatDC16, MatDC27, MatDC64 and TN141 directed against human DCs as previously described [8;9] and human IgG4 UBS54 directed against the epithelial cell marker Ep-CAM [10]. Biotinylated huMabs were used for flow cytometry. To detect biotinylated huMabs, Alexa Fluor 633 streptavidin conjugate (Molecular Probes, Eugene, OR) was used. The following directly fluorochrome-conjugated antibodies were used for flow cytometry: PE-labeled CD1a (Immunotech, Marseille, France), FITC-labeled CD14 (Becton Dickinson, Erembodegem, Belgium), PECy5-labeled CD14 (Immunotech), PE-labeled CD80 (Becton Dickinson), FITC-labeled CD86 (BD Pharmingen, Erembodegem, Belgium), PercP-labeled anti-HLA-DR (Becton Dickinson, San Jose, CA).

Cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood obtained from healthy donors, by density centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).

Flow cytometric analysis

Cells were blocked for 20 minutes on ice in phosphate- buffered saline containing 1% BSA and 0.05% sodium azide (PBA) containing 10% human pooled serum. For staining with the huMabs, cells were incubated with biotinylated huMabs for 30 minutes on ice and subsequent with Alexa Fluor 633 streptavidin conjugate. Directly fluorochrome-conjugated antibodies were used as indicated in the RESULTS section. Cells were washed twice with PBA between incubation steps. After a final wash, cells were analyzed using a FACSCalibur (Becton Dickinson).

Stimulation of monocytes in the presence of huMabs

$CD14^+$ monocytes were purified from PBMCs by magnetic cells sorting using CD14 MicroBeads (Miltenyi, Bergisch Gladbach, Germany). $CD14^+$ monocytes were blocked with 10% goat serum in medium, for 30 minutes on ice. Next, the cells were incubated with the different huMabs (25 μg/ml) for 1 hour on ice. Subsequently, the cells ($5x10^5$ per well) were

cultured in a 24-wells plate in the presence of goat F(ab')2 anti-human IgG (Southern Biotechnology Associates, Birmingham, AL), in medium supplemented with recombinant human (rh) GM-CSF (100 ng/ml, Schering-Plough, Amstelveen, The Netherlands) or GM-CSF and IL-4 (50 U/ml; Strathmann, Hannover, Germany) at 37 °C. On day 2, the cells were analyzed by flow cytometry and supernatants were harvested and stored at –20 °C until cytokine levels were measured.

Cytokine measurements

Measurements of IL-6, IL-10 and IL-12p70, levels in the culture supernatants were performed by specific solid-phase sandwich ELISA as described previously [11]. Measurements of IFNα were performed by ELISA using pairs of specific mAbs and recombinant cytokine standards obtained from Endogen (Woburn, MA). The limits of detection of these ELISAs were as follows: IL-6, 20 pg/ml, IL-10, 25 pg/ml, IL-12p70, 3 pg/ml; IFNα, 3 pg/ml.

Allogeneic mixed leukocyte reaction

Non-adherent cells from PBMCs, that were allowed to adhere for 1 hour at 37°C, were used for the isolation of allogeneic responder T cells, using Lympho-Kwik according to the manufacturer's instructions (One Lambda, Canoga Park, CA). Density centrifugation of PBMCs on a 52% Percoll gradient (Pharmacia) was performed to enrich for monocytes and DCs and these freshly prepared PBMCs were gamma irradiated at 25Gy and washed. Graded doses (100-1000-10000) of PBMCs in 96-well round bottom culture plates were incubated with huMabs (20 μg/ml) in complete RPMI with 5% human serum for 30 minutes at room temperature. Allogeneic responder T cells ($1x10^5$) were added and the cells were cocultured for 4 days. On day 4, each well was pulsed with 1 μCi of ^{3}H-thymidine (specific activity 5 Ci/mmol; Amersham, Buckinghamshire, England) for 18 hrs, and thymidine incorporation was quantified on a betaplate counter (Wallac, Freiburg, Germany). Results are given as mean counts per minute ± SD of triplicate cultures. Blocking anti-HLA-DR mouse antibodies (Leinco Technologies, St. Louis, MO) and isotype control mouse IgG2a (Rockland, Gilbertsville, PA) were included.

RESULTS

Crosslinking of huMab MatDC64 suppresses cytokine-induced differentiation of monocytes into DC

We analyzed whether binding and crosslinking of four previously described huMabs affect the differentiation of monocytes into DCs in cultures of monocytes stimulated with GM-CSF or with a combination of GM-CSF and IL-4. Expression of CD14 and CD1a were monitored during culture because GM-CSF rapidly down-regulates the expression of CD14 on monocytes, while expression of CD1a is induced [2]. First, blood monocytes were cultured with various concentrations of GM-CSF in combination with IL-4, to assess the number of $CD1a^+$ cells that was generated. Six combinations, 50 ng/ml or 100 ng/ml GM-CSF in combination with either 50 ng/ml, 250 ng/ml or 500 ng/ml of IL-4 were tested and yielded the same number of $CD1a^+$ cells on day 2 of the cultures (data not shown). A combination of 100 ng/ml GM-CSF and 50 ng/ml IL-4 was used in subsequent experiments.

Next, $CD14^+$ monocytes were incubated with the huMabs in the presence or absence of F(ab')2 goat anti-human IgGs to crosslink membrane-bound antibodies. After two days of culture, cells were analyzed for the expression of CD14 and CD1a by flow cytometry. In control cell cultures in the absence of antibodies, CD1a expression was induced upon stimulation with GM-CSF or GM-CSF and IL-4. Expression of CD1a was not altered in cultures supplemented with HuMabs MatDC16 and MatDC27 with or without F(ab')2 anti-human IgG. In contrast, upon crosslinking of MatDC64 bound to monocytes, a population that displayed low levels of CD1a was generated (Fig. 1). A reduction of 50-85% of the $CD1a^+$ cells compared to the control was observed in the presence of GM-CSF. A less profound effect, 35-45% reduction of $CD1a^+$ cells, was observed in the presence of GM-CSF and IL-4.

In the presence of huMab TN141, a small decrease of 15-30% of the $CD1a^+$ cell number was observed in the presence of GM-CSF. This effect was not detectable in cultures supplemented with GM-CSF and IL-4. Binding of HuMabs in the absence of the secondary crosslinker did not affect the differentiation of monocytes into $CD1a^+$ DCs. Crosslinking of huMabs on purified monocytes and subsequent culture in the absence of exogenous stimuli for 48 hours did not affect the monocytes, as they remained $CD14^+$. Monocytes rapidly lose expression of the CD14 during *in vitro* cytokine-stimulated differentiation into DC, and CD1a expression is induced. Expression of CD80, a marker not expressed by monocytes, is observed on day 5 of culture, whereas expression of HLA-DR and CD86, both present on monocytes, is augmented during differentiation of monocytes into DC [2]. We did not detect

CD80 expressing cells at day 2 in cytokine-stimulated control cultures without antibodies or in the presence of MatDC16, MatDC27 and TN141. In contrast, the presence of MatDC64 IgG4 antibodies induced a low but reproducible induction of CD80 expression. The expression of HLA-DR and CD86 on day 2 cytokine-stimulated monocytes was similar in presence or absence of huMabs. Staining of the cells with biotinylated MatDC64 at day 2 demonstrated that the cells had lost the antigen for MatDC64 (data not shown), suggesting internalization of the target antigen. Comparable results were obtained after 72 hours of incubation with either GM-CSF or GM-CSF and IL-4.

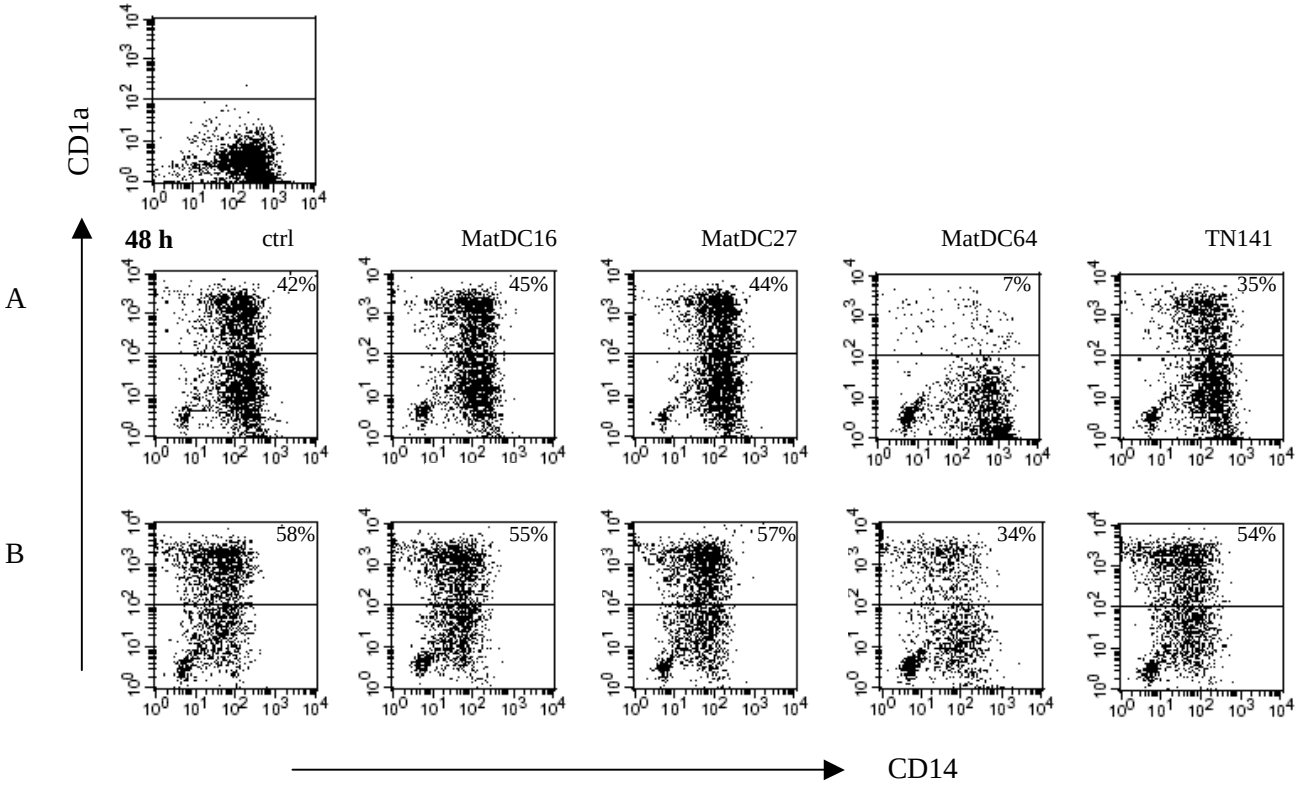

Figure 1 Phenotype of monocytes cultured in the presence of (A) GM-CSF or (B) GM-CSF and IL-4. Dot plots show CD14 vs. CD1a expression of the monocytes at the start and after 48 hrs culture in the presence of huMabs, as indicated; only crosslinker was added in control cultures (ctrl).

Crosslinking of huMab MatDC64 induces production of IL-6

As cytokines have been shown to modulate the differentiation potential of monocytes, we measured the cytokine production of cytokine-stimulated monocytes in the presence of either huMab MatDC16, MatDC27, MatDC64 or TN141 (Fig. 2 and 3). Compared with the control culture in the absence of antibodies, the supernatants of cultures of MatDC64-crosslinked cells showed an increased IL-6 production, while production levels of IL-10, bioactive IL12p70 and IFNα production were not altered.

Crosslinking of huMabs MatDC16, MatDC27 or TN141 did not result in increased production of the evaluated cytokines compared to the control. IL-6 is a pleiotropic cytokine that can inhibit the differentiation of hematopoietic $CD34^+$ progenitor cells into DCs and promote their commitment toward the monocytic/macrophage lineage [12]. Furthermore, IL-6 produced by fibroblasts can skew differentiation of monocytes into macrophages at the expense of DCs [13].

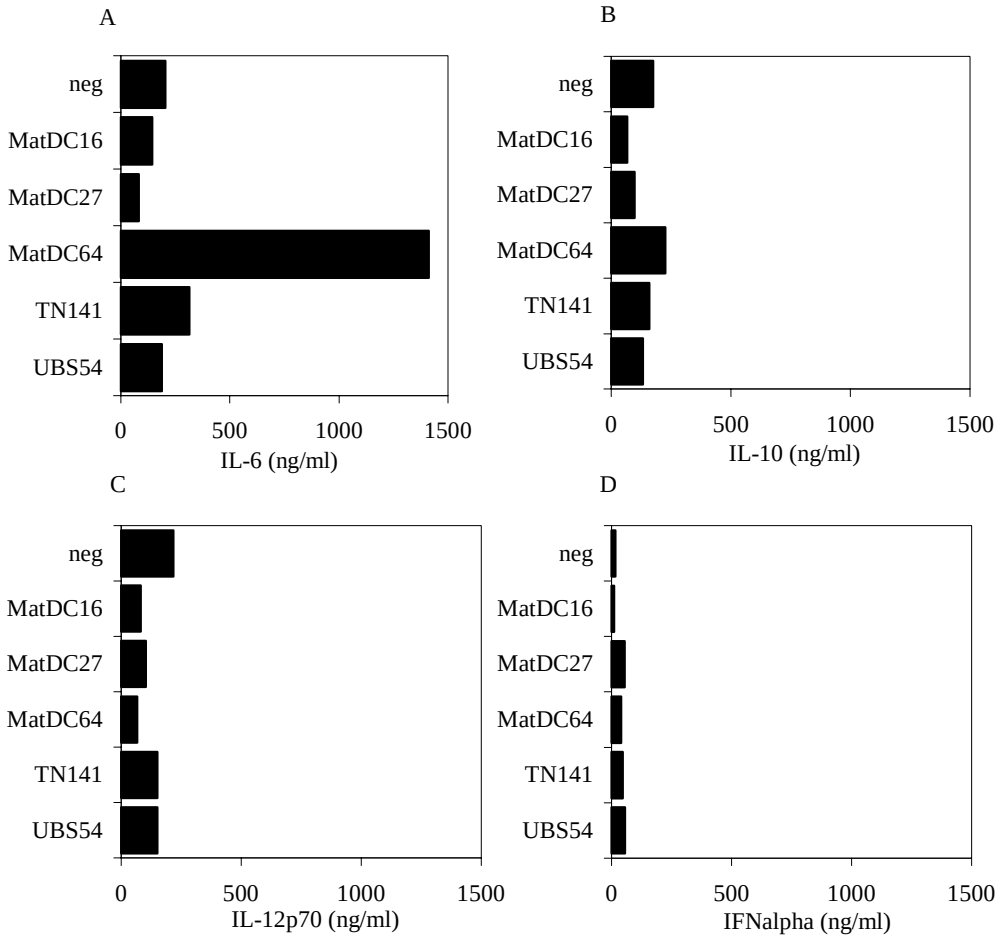

Figure 2 Monocytes were cultured in the presence of GM-CSF, crosslinked huMabs or crosslinker (neg). Supernatants were harvested after two days and secreted cytokines were measured by ELISA. A, IL-6; B, IL10; C, IL12p70; D, IFNα.

It has been shown that IL-4 can reverse the inhibitory effect of IL-6 on the differentiation of DC from hematopoietic $CD34^+$ progenitor cells [14]. Presumably, this antagonistic effect of IL-4 accounted for the lower IL-6 production of monocytes crosslinked with huMab MatDC64 cultured in the presence of IL-4 and GM-CSF (Fig. 2) compared to GM-CSF (Fig. 3). These findings indicate that, in addition to direct mechanisms, IL-6 produced by

monocytes after crosslinking of MatDC64 may contribute to the suppression of monocyte differentiation into DCs.

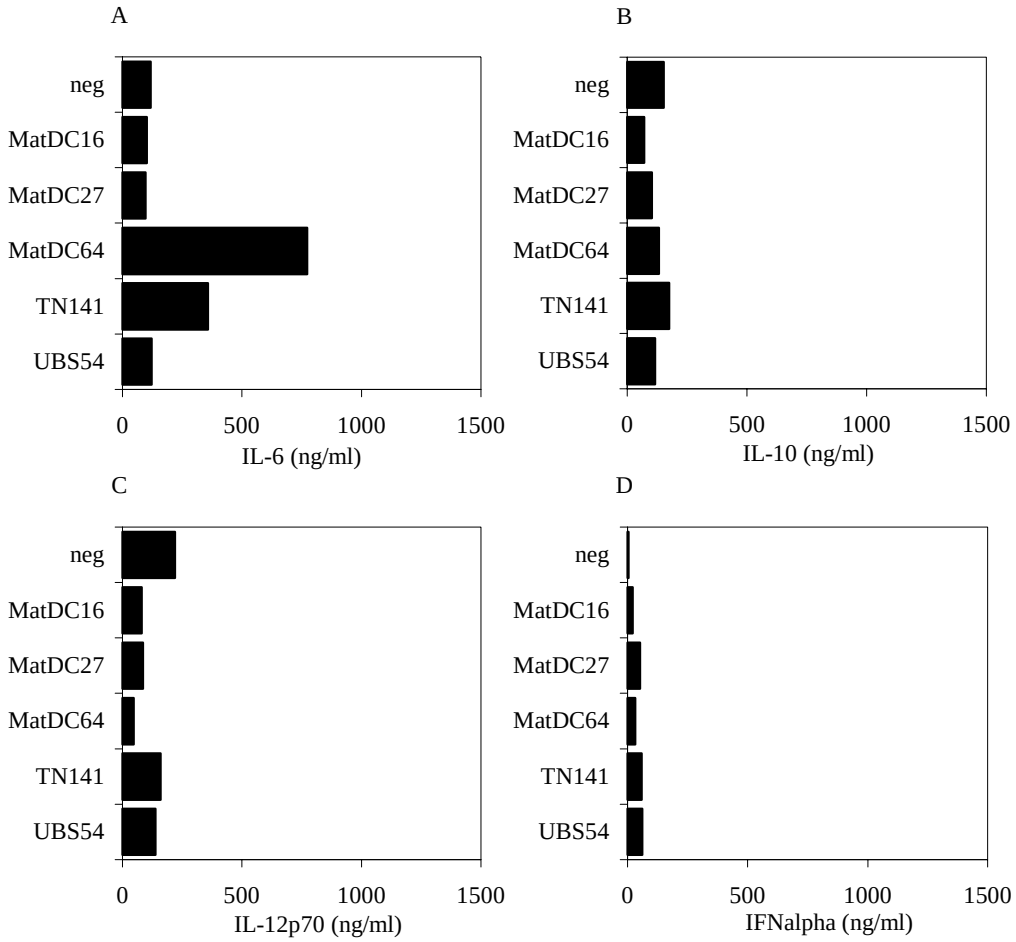

Figure 3 Monocytes were cultured in the presence of GM-CSF and IL-4, crosslinked huMabs or crosslinker (neg). Supernatants were harvested after two days and secreted cytokines were measured by ELISA. A, IL-6; B, IL10; C, IL12p70; D, IFNα.

Ligation of huMabs does not impair T cell stimulatory capacity in an allogeneic MLR

We assessed whether triggering with the different huMabs affected the stimulatory capacity of blood monocytes and DCs. Although monocytes and B cells are capable of stimulating the allogeneic mixed leukocyte reaction (MLR), blood DCs are considerable more effective [15-17]. Furthermore, a subset of $CD2^+$ monocytes also act as potent inducers of the allogeneic MLR [18]. Expression of MHC class II and accessory molecules by the stimulator cells is required for optimal stimulation of T cell proliferation. Accessory molecules, such as CD80/CD86 and adhesion molecules provide the necessary costimulatory signals. As the huMabs bound to DCs and monocytes, we decided to use PBMCs as a source of stimulator cells. The PBMCs were incubated with the huMabs and cocultured with

allogeneic T cells for 4 days. As a control, a blocking antibody against HLA-DR was included in the allogeneic MLR. HuMab UBS54, directed against the human epithelial marker Ep-Cam, was used as an IgG4 isotype control, to exclude the effect of engagement of Fc receptors. The anti-HLA-DR antibody efficiently blocked the capacity of the PBMCs to induce proliferation of allogeneic T cells (Fig. 4). Addition of the huMabs did not affect the stimulatory capacity of PBMCs in an allogeneic MLR. This suggests that the huMabs were not directed against epitopes involved in the stimulatory function of PBMCs.

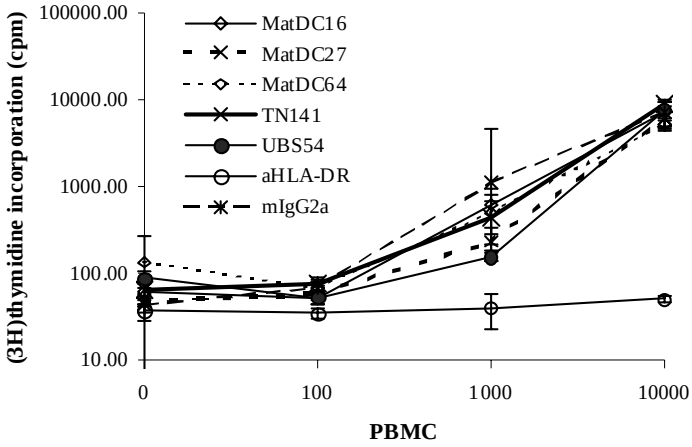

Figure 4 Ligation of huMabs on PBMC does not affect their stimulatory capacity in an allogeneic MLR. Allogeneic resting responder T cells were added to PBMCs with or without blocking huMabs. HuMab UBS54 was used as isotype control. Blocking mouse antibodies against HLA-DR together with a mouse IgG2a isotype control were included. The results are expressed as the mean percent of CPM from triplicate wells.

DISCUSSION

In this study, we assessed whether binding and crosslinking of a panel of huMabs to monocytes affected their capacity to differentiate into DCs *in vitro*. We demonstrate that crosslinking of monocyte-bound huMab MatDC64 suppresses the generation of $CD1a^+$ DCs upon culture with GM-CSF or GM-CSF and IL-4 for 2 or 3 days. However, other phenotypic alterations such as induction of HLA-DR and CD86 were not affected while a marginal induction of CD80 expression was observed. Based on these observations, we hypothesize that binding and crosslinking of the MatDC64 target partially blocks the DC differentiation pathway at the level of the generation of $CD1a^+$ DC. Crosslinking of huMabs MatDC16, MatDC27 and TN141 did not affect monocyte differentiation into DC. None of the antibodies interfered with the capacity of PBMCs to stimulate T cell proliferation, as determined in an allogeneic MLR, indicating that the target antigens are not involved in the primary immune response**,** dissimilar to e.g. the adhesion molecules CD11a, CD54 and CD58 [16].

Analysis of cytokine production revealed that in the cultures of monocytes crosslinked with huMab MatDC64 the production of IL-6 was approximately seven-fold, compared with the negative control. IL-6 is capable of inhibiting the differentiation of monocytes into DCs by promoting their differentiation towards macrophages [12;19]. Chomarat and co-workers [13] have shown that in the presence of IL-4 and GM-CSF, exposure of monocytes to IL-6 increases M-CSF receptor expression and M-CSF receptor internalization in response to autocrine M-CSF. We postulate that binding and crosslinking of huMab MatDC64 directly or indirectly triggered IL-6 production by monocytes, redirecting monocyte differentiation into the macrophage pathway.

We have so far not been able to identify the target antigen that is recognized by MatDC64, but in correlation with LAIR-1 and CD33, MatDC64 might bind to an inhibitory receptor. A large number of inhibitory receptors have recently been discovered, many of which are expressed by myeloid cells [20] Inhibition is as important as activation of the immune system for regulation of an immune response, and is controlled by a balance of inhibitory and activating signals. Different groups have report the presence on myeloid cells of ITIM-bearing molecules like ILT-3 [21], ILT-4 [22] and ILT-5 [23] belonging to a new group of Ig superfamily receptors named Ig-like transcripts (ILT) [24]. Very recently it was shown that upregulation of ILT-3 and ILT-4 on monocytes and DCs, renders them tolerogenic, demonstrating their physiological significance [25]. We previously showed that MatDC64 does not bind to stably transfected cells expressing ILT-3, ILT-4 or ILT-5 [8].

Another group of inhibitory receptors belongs to the family of sialic acid-binding Ig-like lectins (Siglecs) [26]. Members of the Siglec family contain two conserved ITIM-like motifs in their cytoplasmic tails and recently six CD33-related human Siglecs have been described. Based on staining patterns, we could exclude binding of MatDC64 to CD33 (expressed on monocytes and monocyte-derived DC), Siglec-5, Siglec-9, Siglec-10 (all expressed on monocytes and B cells), and Siglec-7 (expressed on monocytes, monocyte-derived DC and NK cells).

Similar to the inhibitory receptors CD33 and LAIR-1, the inhibitory activity of MatDC-64 may be related to the presence of cytoplasmic ITIMs in the target molecule. Upon receptor engagement, ITIMs can undergo phosphorylation and recruit tyrosine-phosphatases SHP-1 and/or SHP-2 [26;27], which are major cytosolic mediators of inhibition. Consequently, future studies on MatDC64 should focus on testing whether these phosphatases are involved in the signaling pathway engaged by MatDC64 crosslinking. Alternatively, analogous to FcεRI crosslinking, MatDC64 may activate signaling of a stimulatory receptor. Intracellular calcium

mobilization serves as a parameter of an activation signal transduction mechanism. In conclusion, our data provide evidence that MatDC64 recognizes a cell surface molecule regulating DC differentiation, possibly mediated by signals that include IL-6.

ACKNOWLEDGEMENTS

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CHAPTER 5

IN VITRO ANTIBODY-MEDIATED DELIVERY OF MELANOMA TUMOR ANTIGEN MAGE-1 TO IMMATURE DENDRITIC CELLS RESULTS IN ANTI-TUMOR T_H AND CTL RESPONSES

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*Ludwig Institute for Cancer Research, B1200 Brussels, Belgium

Submitted

ABSTRACT

We have fused the human antibody MatDC16 specific for dendritic cells, monocytes and B cells to the MAGE-1 tumor antigen and analyzed the capacity of the fusion protein to induce T cell responses in *in vitro* systems. Targeting the antibody-tumor antigen fusion protein to dendritic cells resulted in the induction of strong T helper and CTL responses compared to minimal response obtained with stimulation of dendritic cells with the MAGE-1 protein proper. Targeting to monocytes or B cells did not result in T cell stimulation. Another human antibody specific for a different molecule expressed by dendritic cells did not mediate the activation of T cells, suggesting that the uptake and presentation in class I and class II molecules, was receptor-dependent. These results suggest that targeted delivery of tumor antigens to dendritic cells via human antibodies may be used for the design of cancer vaccination strategies.

INTRODUCTION

The goal of cancer immunotherapy is the generation of an immune response against autologous tumors. Although there is abundant evidence for the presence of tumor antigens on a variety of tumors, they are seemingly unable to elicit an adequate anti-tumor immune response. Tumor antigens should be presented to the patients' immune system in such a way as to enhance both the cellular and humoral immune response. The efficient capture and presentation of antigens by dendritic cells (DCs) render these professional antigen presenting cells (APCs) central to the induction of an immune response, and appear efficient mediators for eliciting anti-tumor immunity [1-5].

Various strategies to deliver tumor antigens to DCs have been reported. These include the loading of DCs with soluble tumor antigens, tumor-derived RNA, defined peptides of known sequences, undefined acid-eluted peptides from the MHC class I molecules of tumor cells, tumor cell lysates, retroviral and adenoviral vectors encoding tumor antigens, fusion of DCs with tumor cells or the transfection of DCs with plasmid DNA encoding tumor antigens [6;7]. The most commonly used, clinically approved approach is based on loading empty MHC class I molecules with exogenous peptides.

The efficacy of current tumor vaccines could be improved by optimizing delivery of full-length native or recombinant protein as tumor antigen to DCs, which can 'decide' which epitopes will be presented to effector cells of the immune system. The antigen-processing and presenting machinery would direct responses to important and immune dominant epitopes

including both MHC class I and class II restricted epitopes, thereby exploiting the exceptional ability of DCs to induce $CD4^+$ T-helper (T_H) cells as well as $CD8^+$ cytotoxic T cells (CTLs). Antigens taken up via receptor-mediated internalization are processed in the endosomal pathway, and presented as exogenous antigen by MHC class II to activate T_H cells. Moreover, DCs are capable of presenting internalized exogenous antigens on MHC class I for activation of CTLs, a pathway called crosspresentation or crosspriming [8]. Peptide presentation by MHC class I as well as class II molecules is essential, in view of the critical contribution of T_H cells in the induction and maintenance of effective immunity against cancer [9].

Antibody-mediated targeting of tumor antigens provides an exciting approach for direct antigen delivery to DCs *in vivo*. Targeted vaccine strategies employing a variety of cell surface molecules have been described, including MHC class II, and FcγRI [10-13]. We describe a strategy that involves the construction of a eukaryotic expression vector encoding a fully human monoclonal antibody (huMab) that binds to DCs that is genetically fused to a tumor antigen at the C-terminus. Fully human antibodies are, by their very nature, minimally if at all immunogenic and therefore preferable for clinical application. Previously, we isolated monoclonal phage antibodies against human *in vivo* DCs [14]. In addition, the phage antibodies recognized a precursor pool of DCs, the monocytes. Two of these phage antibodies were chosen for recloning in the aforementioned expression vector, which resulted in conversion of the phage antibodies to complete human antibodies fused to a tumor antigen.

Melanoma-associated antigen A-1 (referred to as MAGE-1), a well-characterized tumor antigen already employed in clinical trials [15-17], was chosen for this study. In the past years, melanoma-specific CTL clones have served as tools to identify genes that code for a tumor antigen [18]. The MAGE gene family includes at least 17 related genes, namely MAGE-A1 to A12, MAGE-B1 to B4, and MAGE-C1. The MAGE genes are expressed by tumors of various histological types, but they are silent in normal cells, with the exception of male germ-line cells that do not carry MHC class I molecules and are therefore unable to present antigens to CTLs. Hence, antigens encoded by MAGE-A, -B, -C genes should be strictly tumor specific. Because the MAGE antigens are shared by many tumors and on account of their strict tumor specificity, they are of particular interest for cancer immunotherapy. The MAGE-1 gene was isolated as it encoded an antigen presented on HLA-A1 molecules to autologous CTLs of a melanoma patient [19].

The results of the current study demonstrated that the intracellular tumor antigen, MAGE-1, could be genetically fused to a fully human monoclonal antibody, and efficiently produced as a fusion protein that targets to DCs *in vitro*. This antigen delivery strategy led to

antigen processing and subsequent presentation of MHC class I and II -peptide complexes by DCs, as it was demonstrated that MAGE-1-specific T_H cells and CTL responses were induced by the targeted DCs. It can be envisaged that this approach offers new possibilities for tumor antigen delivery into DCs *in vivo* in an adjuvant-free manner, inducing efficiently both arms of the adaptive immune system. The approach described in this report may lead to more potent vaccines and immunotherapies.

MATERIAL AND METHODS

Cell lines, media, and reagents

Human recombinant interleukin (IL)-2 was purchased from Eurocetus (Amsterdam, The Netherlands), granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Novartis Pharma (Brussels, Belgium). Human recombinant IL-4 was produced in the Ludwig Institute for Cancer Research, Brussels, Belgium. The HLA-A*0101 MAGE-A1 and HLA-DR*1301 MAGE-A1 specific T cell clones, derived from melanoma patients [20;21], and the melanoma cell line MZ-2Mel MAGE-1^+ were cultured in Iscove's modified Dulbecco medium (IMDM) (Life Technologies,Gaithersburg, MD) supplemented with 10% fetal calf serum and 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml penicillin and 100 μg/ml streptomycin.

Vector construction

A eukaryotic expression vector pcDNA3.1+/zeo containing the genomic region that encodes the constant domain of the heavy chain of the IgG4 (Cγ4) [22], was fused in frame to the full-length cDNA of the MAGE-1 gene (acc nr P43355) and a poly-A tail. The heavy chain variable (V_H) regions from phage antibodies MatDC16, MatDC27, and Mono14 [14] were excised from the pHEN vector using restriction sites *NcoI* and *XhoI* and subcloned into the pLEADER vector [22] containing sequences coding for eukaryotic leader peptide HAVT20, and a splice donor site. Using restriction sites *BamHI* and *NotI*, a fragment containing the V_H region and flanking leader and splice donor sequences, was cloned into the pcDNA3.1 Cγ4-MAGE-1 vector. Transfection of this vector in combination with an expression vector containing the appropriate light chain would lead to a fusion protein comprising a complete human IgG4 with a MAGE-1 protein fused to the C-terminus of the heavy chain. The

eukaryotic expression vector pcDNA3.1 Cγ4-MAGE-1 vector also encoded for zeocin, a selective marker used for the generation of stable transfectants.

Northern blot analysis

Transient tranfections of constructs pCDNA3.1-Cγ4-MAGE-1 including a V_H fragment of MatDC16, pCDNA3.1-Cγ4 including a V_H fragment of MatDC16, and pCDNA3.1-MAGE-1 in the human embryonic kidney (HEK) cell line 293T were carried out using Lipofectamin according to the manufacturers instructions. After two days at 37 °C, the cells were harvested and washed twice with phosphate buffer. Total RNA was prepared using RNAzol B according to the manufacturers instructions (TelTest, Friendwoods, TE), followed by phenol-chloroform extractions and 2-propanol precipitation. 10 μg of total RNA was used for Northern analysis on 1% agarose containing 6% formaldehyde. RNA, transferred to hybond N^+ membrane (Amersham Pharmacia, Uppsala, Sweden), was hybridized with a MAGE-1 specific probe labeled using RadPrime DNA labeling system according to the manufacturers protocol (Life technologies).

Production of fusion proteins

Stably transfected cell lines for the production of fusion proteins, were established by co-transfection of pCDNA3.1-Cγ4-MAGE-1 including a V_H fragment of the phage antibody of interest, with the corresponding light chain construct, in HEK293 cells. $1.5x\ 10^5$ HEK293 cells were seeded per well in a 6-wells plate (Costar, Corning, NY). The next day, transfections were carried out at a cell density of 70-80% confluence using calcium chloride precipitated DNA for 5 hours at 37 ºC, followed by a 15% glycerol shock for 1 minute. Five μg of pCDNA3.1-Cγ4-MAGE-1 and 5 μg of the appropriate light chain construct were used. Cells were washed and after 48 hours 500 μg/ml zeocin (Invitrogen, Carlsbad, CA) was added as selective drug to obtain stable transfectants. When drug resistant colonies were large enough, 48 individual clones were picked and expanded, and tested in sandwich ELISA for production of fusion protein. Next, a fusion protein-producing clone was used for large-scale production in triple flasks (Invitrogen) using ULTRA-CHO medium (BioWhittaker, Walkersville, MD). Culture supernatant was harvested after 4 days, and fresh ULTRA-CHO medium was added to the triple flasks. After 4 days the culture supernatants were again harvested. Fusion protein was purified from the pooled culture supernatants by protein A chromatography. Concentration of purified proteins was determined by spectrophotometry at

a wavelength of 280 nm using the Biorad protein assay (Biorad Laboratories, Munchen, Germany).

ELISA

Supernatants from 48 stable clones per construct were screened for production of fusion protein by a sandwich ELISA. Briefly, microtiter plates (NUNC, Roskilde, Denmark) coated with anti-MAGE-1 antibodies (Ab-4; NeoMarkers, Fremont, CA) were incubated with culture supernatants of stable clones at room temperature for 2 hrs. After washing the plates with 0.05% Tween20/PBS, binding of IgG4-MAGE-1 fusion protein was detected by horseradish peroxidase (HRP) conjugated antibody against human IgG (Southern Biotechnology Associate, Birmingham, AL). As a substrate tetramethylbenzidine was used to detect bound conjugate. An ELISA-plate reader (Molecular Devices, Sunnyvale, CA) was used to measure absorbency at 450 nm.

Flow cytometric analysis

Cells were blocked for 20 minutes on ice in phosphate buffer containing 1% BSA and 0.05% sodium azide (PBA) supplemented with 10% human pooled serum. Next, the cells were incubated with the fusion proteins for 60 minutes on ice. Subsequently, the cells were incubated with a mouse anti-MAGE-1 antibody (Ab-4; Neomarkers) for 30 minutes. This step was followed by incubation with phycoerythrin (PE) conjugated goat anti-mouse antibodies (Southern Biotechnology Associate). Cells were washed twice between incubation steps in PBA. After a final wash, cells were resuspended in PBA, and analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA).

Western blot analysis

The purified proteins were separated by SDS-PAGE and electroblotted onto nictrocellulose membrane (Millipore, Bedford, MA). Proteins were detected with either mouse anti-MAGE-1 antibodies (Ab-4; Neomarkers), followed by HRP conjugated rabbit anti-mouse IgG antibodies (Pierce, Rockford, IL) or by HRP conjugated mouse anti-human IgG antibodies (SBA). Immunublotted proteins were visualized by chemiluminescence using enhanced chemiluminescence detection reagents (ECL; Amersham Pharmacia). A total protein cell lysate of human cells expressing MAGE-1 was used as a positive control (Neomarkers).

Preparation of DC

Blood cells were collected as buffy-coat preparations from HLA-A*0101 and HLA-DR*1301 positive donors. DCs were prepared as described [23]. In brief, PBMCs isolated by Lymphoprep (Nycomed, Oslo, Norway) were allowed to adhere for 2 hrs at 37 °C days in RPMI 1640 medium (Life Technologies) supplemented with AAG, antibiotics and 10% FCS (hereafter referred to as complete RPMI medium). Non-adherent cells were discarded and adherent cells were cultured in the presence of IL-4 (100 U/ml) and GM-CSF (100 ng/ml) in complete RPMI medium. Cultures were fed on days 2 and 4 by removing 1/3 of the volume and adding fresh complete RPMI medium with IL-4 (100 U/ml) and GM-CSF (100 ng/ml). On the day 5, the nonadherent cell population was used as a source of enriched immature DCs.

Induction of MAGE-1 specific T cell responses

Cultures were set up in 96-well round bottom plates (Nunc). 10^5 DCs per well were blocked in RPMI 1640 medium (Life Technologies) supplemented with AAG and 20% human serum for 30 minutes on ice. Next, the DCs were incubated with fusion proteins (10nM or 100nM) in RPMI medium supplemented with AAG and 10% human serum for either 24 hrs. Subsequently, the cells were washed and cocultures of 15 x 10^3 DC with 5 x 10^3 T cells and 25 U/ml IL-2 (Eurocetus, Amsterdam, the Netherlands) were set up in triplicate. Twentyfour hours later, supernatants were collected and assayed for interferon (IFN) γ, using the human IFNγ ELISA kit (BioSource, Camarillo, CA). Data are presented as picograms of IFNγ released /5 x 10^3/ ml/ 24 hrs (mean ± SD of triplicate cultures).

RESULTS

Construction of a vector for the production of a human IgG4-MAGE-1 fusion protein

To evaluate the feasibility of antibody-mediated targeted delivery of tumor antigen to DCs, a fusion protein was constructed using the constant region of the heavy chain of the human IgG4 gene and the entire coding region of the MAGE-1 molecule (Fig. 1). The IgG4 isotype was selected for this approach since it has low affinity for FcγRI and does not bind other Fcγ receptors. In addition, it does not activate complement, presumably preventing complement-mediated lysis of targeted DCs [24]. Melanoma tumor antigen MAGE-1 was

chosen since it is essentially tumor-specific and expressed in a variety of tumors other than melanoma.

cDNA encoding MAGE-1 was fused in-frame at the 3' terminus of the germline Cγ4 gene, cloned in a eukaryotic expression vector. Upon co-transfection of a construct containing the appropriate immunoglobulin light chain [22], a complete human antibody with the MAGE-1 protein fused to the C-terminus was produced (Fig.1). Different constructs were generated; each construct contains a different V_H, resulting in different antibody specificity. MatDC16, derived from phage display selections on human peripheral blood DCs, binds to blood DCs and immature monocyte-derived DCs [14;25]. In addition, binding to monocytes and weak binding to B cells was observed. MatDC27 also binds to blood DCs and weakly to immature monocyte-derived DCs. Mono14 recognizes the CD14 molecule expressed on monocytes [14].

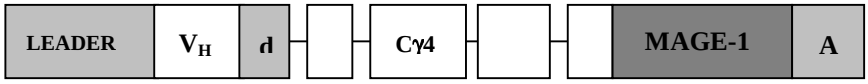

Figure 1 Schematic representation of the recombinant vector for production of IgG4-MAGE-1 fusion proteins. The IgG4-MAGE-1 construct was cloned into eukaryotic expression vector pcDNA3.1$^+$. LEADER, the HAVT20 leader sequence; V_H, the variable domain of the heavy chain; d, the donor splice site; Cγ4, the constant domain of the heavy chain of the IgG4 isotype; MAGE-1, cDNA encoding the MAGE-1 protein; A , poly-A signal.

Integrity of the constructs was confirmed by Northern blot analysis using total RNA derived from transient transfections of the constructs in the Human Embryonic Kidney cell line (HEK) 293T (Fig. 2). Probing RNA from cells harboring the MAGE-1 fusion construct yielded a specific band of the correct size (lane 3), while a specific band appears in lane 4 accounting for the non-fused MAGE-1 transcript. RNA from mock transfected HEK cells (lane 1) and RNA from cells containing the non-fused IgG4 MatDC16 (lane 2) were negative.

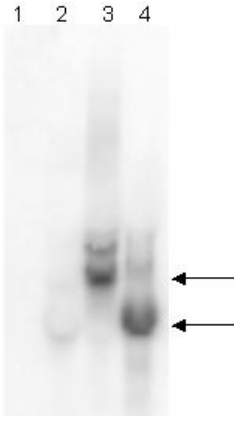

Figure 2 Northern blot analysis: 1) mock; 2) MatDC16Cγ4; 3) MatDC16Cγ4-MAGE-1; 4) MAGE-1, probed with a MAGE-1 specific probe.

Characterization of the recombinant fusion protein

To produce whole immunoglobulin fused to MAGE-1, stably transfected cell lines were established by co-transfection of Cγ4-MAGE-1 including a V_H domain, with a light chain construct, in the HEK cell line. Supernatants from stable clones were screened for production of fusion protein by a sandwich ELISA, using anti-MAGE-1 antibodies as a coating and anti-human IgG antibodies to detect the captured fusion proteins. The best producing clone was used for large-scale production. Recombinant fusion protein was purified from pooled supernatants using a protein A column.

The purified fusion proteins were further characterized by SDS-PAGE and immunoblotting. Under non-reducing conditions the fusion proteins migrated at an estimated molecular mass of ~230 kDa, indicating that the IgG4-MAGE-1 was expressed as a complete protein (data not shown). The molecular weight of the fusion protein was deduced from the known molecular weight of the IgG4 (146 kDa) and two times the MAGE-1 protein (40-45 kDa), as it is linked to each heavy chain. An additional band was observed at ~160 kDa, at the same height as an IgG4 control without MAGE-1. Under reducing conditions, Western blot analysis revealed a band of 100 kDa detected with the anti-MAGE antibody (Fig. 3), as well as with anti-human IgG antibodies, representing the heavy chain-MAGE-1 component of the fusion protein. A cell lysate containing MAGE-1 protein showed a band at ~43 kDa representing the MAGE-1 protein. Furthermore, an additional band appeared at ~50 kDa, representing heavy chain without MAGE, indicating that not all fusion protein that was produced contained MAGE-1.

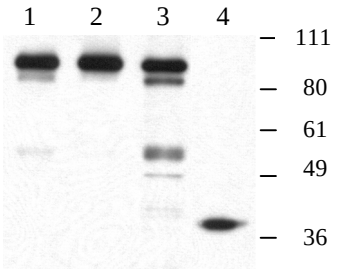

Figure 3 Western blot analysis of purified fusion proteins under reducing conditions, stained with a mouse anti-MAGE-1 followed by an HRP conjugated anti-mouse IgG antibodies, visualized by ECL. Lane 1) IgG4 MatDC16 MAGE-1; 2) IgG4 MatDC27 MAGE-1; 3) IgG4Mono14 MAGE-1; 4) cell lysate containing MAGE-1. The molecular weight markers are shown in kilodaltons on the right

Antibody specificity of the different IgG4 MAGE-1 fusion proteins was retained, as determined by flow cytometry (Fig. 4). Surface binding of the fusion protein to monocytes was detected by flow cytometry using a mouse anti-MAGE-1 antibody followed by PE conjugated goat anti-mouse Ig antibodies. A positive signal in this assay depends on the binding of intact IgG4 MAGE-1 fusion protein.

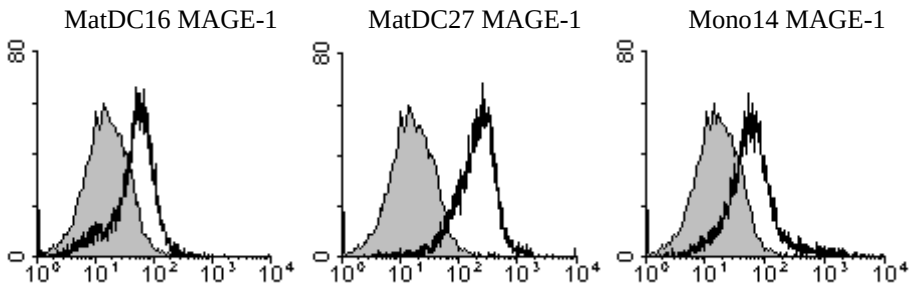

Figure 4 Flow cytometric analysis of fusion proteins using monocytes, identified by scatter characteristics. Unfilled histogram: indicated IgG4 MAGE-1 fusion protein, followed by mouse anti-MAGE-1 antibody and PE conjugated goat-anti-mouse antibodies; filled histogram: secondary mouse anti-MAGE-1 antibody followed by PE conjugated goat-anti-mouse antibodies.

MHC class I and II presentation of MAGE-1 peptides by immature DCs targeted with fusion protein

We determined whether the IgG4 MAGE-1 fusion proteins could be used for targeted delivery of the tumor antigen to immature DCs. Experiments were carried out with IgG4 MatDC16MAGE-1 and IgG4 MatDC27MAGE-1. As negative controls MatDC16 without MAGE-1 and non-fused protein MAGE-1 were used and in addition, IgG4 Mono14MAGE-1, a fusion protein that recognizes CD14 expressed by monocytes [14].

Immature monocyte-derived DCs from HLA.A1/DR1301$^+$ donors were incubated with the fusion proteins (10 nM or 100 nM) or control protein MAGE-1 (200nM) and cultured for 24 hrs. The molecular weight of the fusion protein (230 kDa), and the molecular weight of the IgG4 (146 kDa) and the MAGE-1 protein (43 kDa) were used for molar conversions of the proteins, using the concentration as determined by spectrophotometry. It must be taken into account that fusion protein preparations contained non-fused antibodies as well.

Subsequently, the DCs were replated and cocultured with either the anti-MAGE-1 HLA-DR13 T_H cell clone or the anti-MAGE-1 HLA-A1 CTL clone. Activation was assessed as IFNγ release in 24 hr supernatants. As a positive control, the stimulatory capacity of the DCs was assessed by exogenous peptide pulsing of the DCs with either an HLA-A1/MAGE-1 specific peptide, EADPTGHSY, in case of the CTL clone or an HLA-DR13/MAGE-1 specific peptide, LLKYRAEPVTKAE, in case of the T_H clone. The activation ability of the T cell clones was assessed by coculturing with the above-mentioned peptides in combination with the melanoma cell line MZ-2.

As shown in Figure 5, 100 nM IgGMatDC16MAGE-1 targeted to immature DCs was enough to stimulate a response from the CTL, resulting in a significant amount of IFNγ production. The non-stimulated CTL clone did not secrete detectable amounts of IFNγ (<30

pg/ml/24 h). No stimulatory activity can be seen for the negative control proteins IgG4MatDC16, IgG4Mono14MAGE-1 and MAGE-1. This excludes the possibilities that the response is a consequence of targeting via FcγRI [26] or by macropinocytosis. In addition, upon incubation with IgG4 MatDC27MAGE-1, no IFNγ was produced suggesting that the uptake, and subsequent processing and presentation were receptor-dependent.

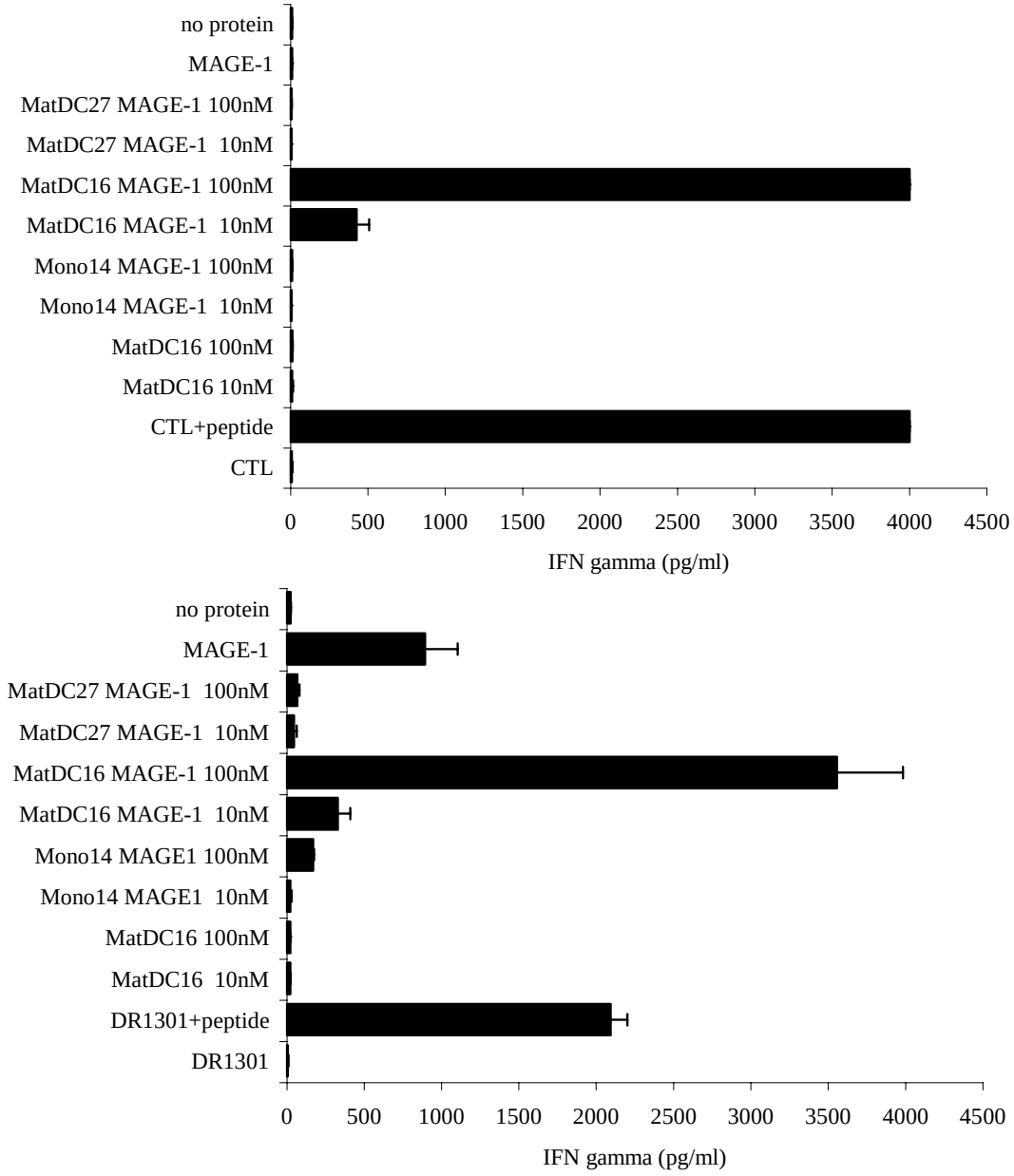

Figure 5 Tumor antigen presentation by immature DC incubated with fusion proteins. Immature DCs (10^5) derived from an HLA-A1$^+$/HLA-DR1301$^+$ donor were incubated with (fusion) proteins (10nM or 100nM) as indicated. After 24 hrs incubation, cocultures of DCs ($15x10^3$) with (A) CTL anti-MAGE-1.A1 or (B) T_H anti-MAGE-1.DR1301 ($5x10^3$) were set up. Activation was assessed as IFNγ release at 24 hrs. Data are presented as picograms of IFNγ released/$5x10^3$/ml/24 hrs (mean ± SD of triplicate cultures).

Upon addition of the MAGE-1 protein to immature DCs, IFNγ was also produced by the T_H clone. This is most likely the result of macropinocytosis by the DCs, ensuing in MHC class II presentation. Targeted delivery of MAGE-1 resulted in a two-fold up regulation of the IFN-γ production by the T_H clone. The stimulatory effect observed with the IgG4 Mono14MAGE-1 might be caused by residual expression of CD14 on the immature DCs [23;27]. Collectively, these data demonstrate a very efficient induction of MAGE-1-specific responses, using an IgG4 MAGE-1 fusion protein targeted to DCs.

Monocytes (Fig. 6) and B cells (Fig. 7) were also tested for their ability to stimulate T cell clones upon incubation with the fusion proteins. In these cultures no detectable IFNγ was produced, except in cultures with IgG4 Mono14 MAGE-1. This resulted in MHC class II presentation since T_H anti-MAGE-A1.DR1301 was activated. We conclude that the uptake, processing and presentation by DCs was very efficient compared to other populations of APCs.

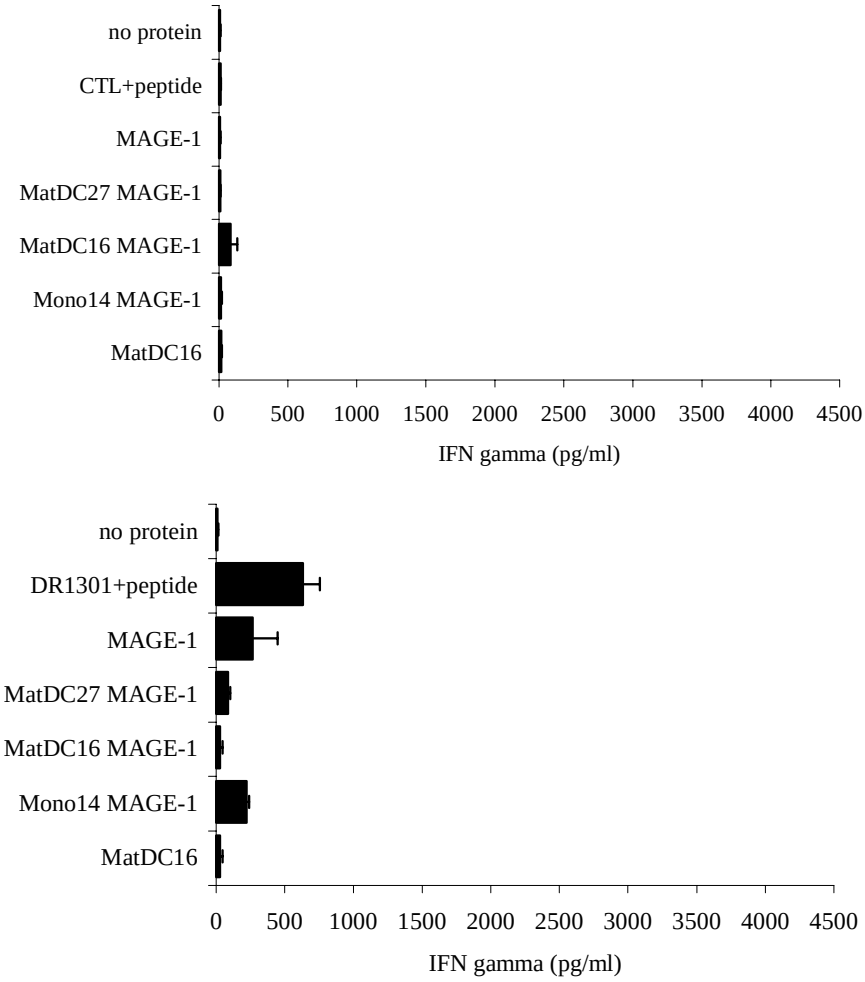

Figure 6 Tumor antigen presentation by monocytes incubated with fusion proteins. Monocytes (10^5) derived from an HLA-A1$^+$/HLA-DR1301$^+$ donor were incubated with (fusion) proteins (100nM) as indicated. After 24 hrs incubation, cocultures of monocytes (15000) with (A) CTL anti-MAGE-1.A1 or (B) T_H anti-MAGE-1.DR1301 (5000) were set up. Activation was assessed as IFNγ release at 24 hrs. Data are presented as picograms of IFNγ released/ $5x10^3$/ ml/24 hrs (mean ± SD of triplicate cultures).

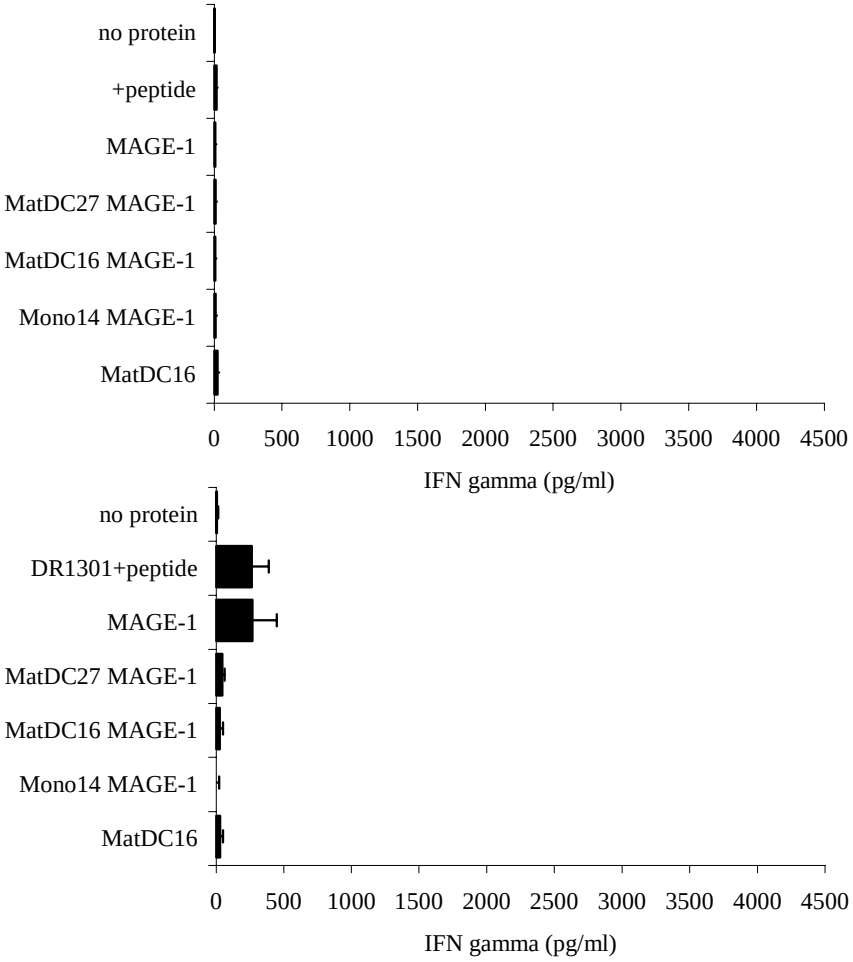

Figure 7 Tumor antigen presentation by B cells incubated with fusion proteins. B cells (10^5) derived from an HLA-A1$^+$/HLA-DR1301$^+$ donor were incubated with (fusion) proteins (100nM) as indicated. After 24 hrs incubation, cocultures of B cells (15000) with (A) CTL anti-MAGE-1.A1 or (B) T_H anti-MAGE-1.DR1301 (5000) were set up. Activation was assessed as IFNγ release at 24 hrs. Data are presented as picograms of IFNγ released/5x10^3/ml/24 hrs (mean ± SD of triplicate cultures).

DISCUSSION

We have evaluated the capacity of the DC-specific human antibody MatDC16 fused to the tumor antigen MAGE-1 to stimulate tumor antigen-specific T cells in an *in vitro* system. The results showed that immature monocyte-derived DCs targeted by the antibody-MAGE-1 fusion protein presented the tumor antigen, in all probability on MHC class II molecules via the endosomal pathway as well as on MHC class I molecules via the crosspresentation pathway.

Presentation of antigen by targeted DCs led to the induction of IFNγ secretion by MAGE-1 specific T_H and CTL clones. Efficient presentation was dependent on the antibody moiety because stimulation of DCs with the MAGE-1 protein did not result in CTL activation and only a marginal T_H activation. Our results confirmed previous findings that exogenous pulsing of DC with intact tumor proteins (not fused to an antibody) is very inefficient [28]. MAGE-1 fused to a control human antibody not binding to human DC, did not induce T_H or CTL activity, excluding the involvement of Fc-receptor-mediated uptake of the

MatDC16MAGE-1 fusion protein. MAGE-1 protein fused to another antibody, MatDC27 that binds to DCs, did not induce T cell responses, suggesting a receptor-dependent mechanism of fusion protein uptake and presentation. Targeting of the MatDC16MAGE-1 fusion protein to B cells did not lead to detectable activation of T_H and CTL clones, whereas targeting to monocytes induced a small but detectable T_H but no CTL response. These results confirm the unique capacity of DCs to present exogenous antigens for both induction of T_H and CTL responses.

The migratory function and strong antigen presenting capacities make DCs the cells of choice for transport of tumor antigens into secondary lymphoid organs and induction of anti-tumor immunity. Antigen-specific tumor immunotherapy can only be successful when high affinity tumor antigen-specific T cells are generated by appropriate immunization. Inducing both T_H and CTL activity is considered to be an important aspect of a successful vaccination approach. Generation *in vivo* of MHC class I-restricted CTL by crosspriming has been shown to be dependent on T_H cells [29-32]. The cross talk between the T_H and the APC and the spectrum of cytokines produced by the T_H cells will both contribute to the outcome of cellular immune response [9]. As a consequence, both CTL and helper epitopes need to be recognized on the same APCs [33].

In the current study, the antibody-tumor antigen fusion proteins were targeted to immature monocyte-derived DCs. The use of immature DCs as a target for delivery of tumor antigens is expected to result in an efficient uptake and processing of the tumor antigen [34]. However, in light of the strong the immunostimulatory capacity of mature DCs, induction of maturation of an immature DC upon uptake of the tumor antigen appears to be a desirable if not essential element of a successful vaccination approach. Indeed, it has previously been shown that immature DCs are able to silence an immune response [35;36]. *In vivo* maturation signals can be provided by T_H cells, which induce maturation of DCs through CD40 ligation further defining an essential role of T_H cells for initiating a CTL response [29-31]. In that light, our finding that targeted delivery of a tumor antigen to immature DC via the MatDC16 antibody activates both T_H and CTL responses is of pivotal importance.

Previous studies have shown that an antibody-mediated FcγRI-targeted fusion protein, containing a T_H cell epitope of tetanus toxoid, resulted in efficient presentation, and could potentially be used for delivery of peptides to APCs *in vivo* [12]. A drawback of this format is imposed by the presentation of a single T_H epitope and the lack of data in this study showing activation of CTL responses via the FcγRI receptor. FcγRI-PSA, a fusion protein targeting the

prostate tumor antigen also targeting to FcγRI, resulted in presentation of MHC class I-peptide complexes, however the delivery of antigen into the MHC class II pathway was not studied [26].

MAGE-1 is a defined tumor antigen expressed by subgroups of patients with metastasized melanoma, esophageal squamous cell carcinoma, head and neck squamous cell carcinoma, non-small cell lung carcinoma, and bladder carcinoma. Using a defined tumor antigen instead of intact tumor cells, tumor cell lysates or nucleic acids derived from tumor cells, has the advantage that the antigen is well-defined, preventing the induction of undesirable autoimmune responses against 'self' proteins in the undefined preparations [1]. The use of the full-length protein instead of MAGE-derived peptides poses a further advantage because the whole protein contains both T_H and CTL epitopes and allows the use of the antibody-MAGE-1 fusion protein for patients with different HLA haplotypes. Furthermore, other characteristics of peptides such as their very short half-life *in vivo* and the rapid turn over of peptide-loaded MHC class I molecules, render them a less optimal format. The half-life of a fusion protein consisting of a human antibody and a tumor antigen is unknown. Experiments with antibodies fused to cytokines have shown that the fusion protein acquires a long half-life, resembling that of the antibody moiety [37].

The antibody-mediated targeting approach we developed has several advantages. Different antibodies interacting with DCs can be simply tested, since the vector, as described in this report, allows rapid exchange of the immunoglobulin variable fragments. With these cassettes and the availability of a panel of DC-binding antibodies, the question whether targeting tumor antigen to different molecules on a DC or to different DC subsets may alter the type of response elicited can be evaluated. This would permit rapid evaluation of different antibodies for development of the most effective vaccine for induction of therapeutic anti-tumor immunity [5;38].

Recently developed DC-based vaccination strategies with *ex vivo* generated DCs loaded with tumor-derived materials are patient-specific and tedious for large-scale application. This drawback can be alleviated by procedure for efficient *in vivo* targeting of DCs, facilitating the broad application of this approach for the treatment of cancer patients [4;5]. Further investigation will demonstrate whether the fusion protein presented in this study targeted to blood DCs and monocytes can elicit a potent anti-tumor response *in vivo*. Studies on the effect of MatDC16MAGE-1 receptor-mediated uptake on maturation/activation state of the DC will elucidate whether additional signals are necessary to ensure maturation and migration of the targeted DCs to secondary lymphoid organs *in vivo*. We previously

demonstrated that IgG4MatDC16 did not inhibit differentiation of monocytes into DC nor inhibit the stimulatory capacity of PBMCs in an allogeneic MLR (Lekkerkerker, A., Chapter 4). Although numerous questions concerning *in vivo* DCs targeting still need to be answered e.g. the optimal route, dose and frequency of vaccination and the type of DC subset that should be targeted, these data give a first indication that this targeted vaccine can induce anti-tumor immunity *in vitro* and can potentially be used *in vivo* in cancer vaccination protocols.

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CHAPTER 6

LIPID-TAGGED SCFV FRAGMENTS INCORPORATED IN TUMOR CELLS MEDIATE RECEPTOR-DEPENDENT TARGETING TO ANTIGEN PRESENTING CELLS

Annemarie Lekkerkerker, Michel de Weers and Ton Logtenberg

ABSTRACT

We have constructed a panel of lipid-tagged scFv (LT-scFv) antibody fragments specific for dendritic cells and monocytes and inserted them in the membrane of the tumor cell line Jurkat. Tumor cells displaying LT-scFv were brought in contact *in vitro* with blood mononuclear cells and the uptake of tumor cells by monocytes and dendritic cells was monitored. The results showed that monocytes had taken up tumor cells loaded with an LT-scFv specific for the CD14 molecule but not tumor cells loaded with LT-scFv with other specificities. Blood dendritic cells were not capable of taking up LT-scFv-modified Jurkat cells. Induction of apoptosis of tumor cells did not affect the capacity of LT-scFv to mediate uptake of tumor cells by antigen presenting cells (APC). Phagocytosis of LT-scFv modified tumor cells did not appear to induce the maturation of monocytes into DCs. We conclude that some APC populations are capable of phagocytosing LT-scFv-loaded tumor cells and that this process is receptor-dependent.

INTRODUCTION

In cellular immunotherapy for the treatment of cancer, tumor cells are administered to induce an antitumor immune response in the patient. Immunity produced by cellular vaccines depends largely on the efficiency of antigen-presenting cells (APC) that process and present antigens to effector cells of the immune system. A variety of cell types including monocytes, macrophages and dendritic cells (DC) may function as APCs. Peripheral blood monocytes migrate into tissues and develop into macrophages, whereas they are also regarded as a precursor pool of DCs[1-3]. DCs are the most potent APCs with the ability to initiate primary immune responses[4] and are increasingly being explored for use in cellular vaccines[5-7]. Several strategies to deliver antigens to DC have been used in clinical trials[7]. The most frequently used approach is based on loading of MHC class molecules of *ex vivo* generated DCs with exogenous peptides derived from known tumor antigens. In the majority of human cancers, where tumor antigens are unknown, DCs pulsed with various tumor-derived preparations are employed as cancer vaccines. DCs can capture apoptotic tumor cells and elicit recall MHC class I restricted $CD8^+$ cytotoxic T cells (CTL) responses against tumor antigens [8-10] providing an alternative strategy for tumor antigen delivery. Another promising approach involves the immunization of patients with hybrids of DCs fused to the tumor cell. This approach has been used for patients with renal cell carcinoma, demonstrating significant clinical responses in a number of patients[11]. The approaches for antigen delivery that involve

whole tumor cells as source of antigens, allow DCs to process and present multiple antigens to both inducing $CD4^+$ T cells and CTLs, leading to a diverse immune response.

Previously, in our lab a procedure was developed that allows rapid and efficient insertion of lipid-tagged (LT) proteins into the membrane of prokaryotic and eukaryotic cells [12]. LT-single chain (sc) Fv antibody fragments, specific for molecules expressed by APC, such as CD14, CD32 and CD64, attached to the membranes of tumor cells mediated efficient phagocytosis of the tumor cells. It can be envisaged that targeting irradiated tumor cells to APC by membrane-bound LT-scFv provides an alternative strategy for delivering tumor antigens to APC in cellular immunotherapy.

In the present study, we expanded on these data by exploring whether insertion of other APC-specific scFv antibody fragments would induce phagocytosis. Specifically, four scFv antibody fragments, MatDC16, MatDC27, MatDC64 and TN141, previously generated by phage antibody display in combination with flow cytometry on human blood or tonsil DCs, with an APC-restricted reactivity pattern were converted to LT-scFvs. Unexpectedly, insertion of these LT-scFvs in the cell membrane of Jurkat cells did not result in phagocytosis by either human peripheral blood monocytes or DCs. We concluded that phagocytosis of cells with APC-specific membrane-anchored LT-scFvs requires engagement of specific receptors. Apparently, mere recognition of the lipid-modified cells did not suffice to trigger engulfment of the lipid-modified cells.

Previous experiments demonstrated that the insertion of anti-CD14 LT-scFvs into the membrane of cells mediated phagocytosis of these cells by monocytes. CD14 is expressed as membrane–anchored molecule on the surface of monocytes, macrophages, and granulocytes. Since CD14 does not have a membrane spanning domain it cannot transmit a signal into the cell [13]. Engagement of CD14 by ligands like bacterial lipopolysaccharide (LPS) results in a pro-inflammatory response. Toll like receptor (TLR) 2 and TLR4 function as co-receptors mediating cellular activation in response to LPS[14;15]. CD14 has also been implicated in recognition of apoptotic cells, which leads to phagocytosis without activation[16;17].

The unique properties of DCs make them the APC of preference for use in cancer vaccines. Along these lines, we examined whether phagocytosis of lipid-modified cells altered the monocytes phenotype into DC, as LPS, and other microbial products as well as apoptotic cells are implicated in activation of DCs [1;18;19]. However, phagocytosis did not appear to induce the maturation of monocytes into DCs. In conclusion, some APC populations were capable of phagocytosing LT-scFv-loaded tumor cells and this process appeared receptor-dependent.

MATERIAL AND METHODS

Medium

The medium used in all cell cultures was RPMI 1640 (Gibco BRL, Breda, The Netherlands), supplemented with, 25mM HEPES, 2mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin 10% (vol/vol) heat-inactivated fetal calf serum (Integro, Zaandam, The Netherlands).

ScFv fragments and conversion into LT-scFv

ScFv fragments used in this study were previously described [20]. The anti-CD14 scFv Mono14 was selected on monocytes and scFvs MatDC16, MatDC27 and MatDC64 were selected on $CD33^{high}$ blood DCs using flow cytometry [21]. ScFv TN141 was selected on tonsillar DCs (Lekkerkerker A., De Weers M., Logtenberg T., Chapter 2B). DNA encoding a scFv fragment was cloned into vector pLP2, as previously described [12].

Production and purification of LT-scFv

The resulting pLP2-scFv constructs were expressed in the *E. coli* strain SF110 F'. Bacteria were collected and the LT-scFv antibody fragments were purified as described. SDS-PAGE and Coomassie brilliant blue staining of the gels was carried out to examine production and purification.

Incorporation of LT-scFvs into cell membranes

Incorporation of LT-scFv into Jurkat or Ramos tumor cell lines was carried out as described [12]. Membrane-anchored LT-scFv antibody fragments were detected using monoclonal antibody 9E10 (ECACC, Salisbury, United Kingdom), specific for the myc-tag fused to the LT-scFv. A phycoerythrin (PE)-conjugated polyclonal goat antibody directed against mouse IgG (DAKO, Carpinteria, CA) was used to detect cell-bound 9E10 antibody against myc. Cells were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson, San Jose, CA).

Phagocytosis of cells displaying a membrane-anchored LTscFv fragment

Jurkat or Ramos cells were labeled with the lipophilic dye PKH-26 (Sigma), according to the manufacturer recommendations. The LT-scFv antibody fragments MatDC16, MatDC27,

MatDC64, TN141 and anti-CD14 or a control LT-scFv specific for denitrophenol, were incorporated into the cell membrane. Peripheral blood was obtained from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. For some experiments, enrichment of monocytes in the PBMC suspension was obtained by density gradient centrifugation on a 50% Percoll gradient (Pharmacia Biotech), resulting in 60% $CD14^+$ monocytes in the Percoll-enriched cell suspension. Approximately 1 x 10^5 modified Jurkat or Ramos cells were added to 1 x 10^6 PBMCs or 1.7 x 10^5 Percoll-enriched cell suspension in a total volume of 200 µl RPMI-S. This resulted in a Jurkat or Ramos: monocyte ratio of about 1:1. Apoptotic Ramos cells were used in some experiments. Apoptosis was induced by over night incubation of the Ramos cells with 50 µgr/ml campthotecin, dissolved in DMSO, as observed after staining with prodium iodide and annexinV. The cells were stained with an fluorescein isthiocyanate (FITC)-conjugated antibody against CD14 (Becton Dickinson) with or without an FITC-conjugated antibody against CD16 (Becton Dickinson) and a peridinin chlorophyll protein Cyanine 5 (PECy5)-conjugated antibody against CD33 (Immunotech, Marseille, France) and resuspended in PBS containing 1% BSA. Phagocytosis was analyzed by flow cytometry using a FACS Calibur (Becton Dickinson).

Phenotyping of monocytes

For phenotypic analysis, monocytes were stained after incubation with PECy5-conjugated antibody against CD14 (Immunotech) in combination with either an fluorescein (FITC)-conjugated antibody against CD83 (Immunotech) or FITC-conjugated antibody against HLA-DR (Becton Dickinson) and the cells resuspended in PBS containing 1% BSA. The cells were analyzed by flow cytometry using a FACS Calibur.

RESULTS AND DISCUSSION

Phagocytosis of LT-scFv modified cells is receptor-specific

ScFv antibody fragments MatDC16, MatDC27, MatDC64 and TN141 were previously selected from a phage library of human scFv antibody fragments and were found to preferentially bind to human DCs and monocytes [21] (Lekkerkerker A., De Weers, M., Logtenberg T., Chapter 2B). To determine whether these APC-specific scFv antibody

fragments coupled to tumor cells are capable of mediating phagocytosis by different populations of blood APC, the scFv antibody fragments were converted to lipid-tagged scFv, purified and incubated with Jurkat cells to anchor the lipid-modified proteins to the plasma membrane.

The LT-scFv modified Jurkat cells were labeled with the red lipophilic dye PKH-26 and incubated with unseparated PBMC containing both DCs and monocytes. Using a FITC-labeled antibody against CD14, monocytes could be identified as $CD14^{+}$ cells. Insertion of anti-CD14 LT-scFvs into the membranes of the Jurkat cells mediated phagocytosis by the monocytes, as visualized by the presence of red fluorescent monocytes. Insertion of a control LT-scFv specific for the hapten DNP, LT-scFv MatDC16, LT-scFv MatDC27, LT-scFv MatDC64 or LT-scFv TN141 did not lead to phagocytosis by monocytes (Fig. 1).

To visualize blood DC subsets, cells were stained with a PECy5-labeled antibody against CD33, a FITC-labeled antibody against CD14, and a FITC-labeled antibody against CD16, revealing a $CD14^{-}CD16^{-}$ $CD33^{high}$ mature DC population and a $CD14^{-}CD16^{-}CD33^{dim}$ precursor DC population [22]. Phagocytosis of the lipid-modified cells with either a control LT-scFv specific for the hapten DNP, LT-scFv MatDC16, LT-scFv MatDC27, LT-scFv MatDC64 or LT-scFv TN141 by the mature or precursor DCs did not occur given that no red fluorescent DCs were observed (data not shown).

These results confirm that membrane-anchored LT-scFv act as artificial receptors that may be capable of mediating phagocytosis of tumor cells by monocytes. This process appears to be dependent on the nature of the receptor molecule on the monocyte to which the tumor cells are targeted. CD14, CD32 and CD64 mediate the uptake of LT-scFv decorated tumor cells [12] whereas no phagocytosis was observed with tumor cells carrying LT-scFv MatDC16, MatDC27, MatDC64 or TN141. These results suggest that bridging tumor cells and monocytes through insertion of LT-scFv in the membrane is not sufficient for the induction of tumor cell phagocytosis. CD32 and CD64 are Fc receptors are involved in the uptake of antigen-antibody complexes and antibody-coated bacteria, hence phagocytosis mediated by these receptors could be anticipated [23]. Anti-CD14 LT-scFv mediated phagocytosis was less predictable, since CD14 cannot signal by itself. The role of CD14 as a macrophage receptor mediating phagocytosis of apoptotic cells has been demonstrated, however associated transmembrane proteins that mediate CD14 signaling which leads to phagocytosis of apoptotic cells have not been defined.

Gating on the $CD14^{-}CD16^{-}$ $CD33^{high}$ mature DC population and the $CD14^{-}CD16^{-}$ $CD33^{dim}$ precursor DC population demonstrated that none of the membrane-anchored LT-

scFvs mediated phagocytosis of Jurkat cells by these blood APC populations. These experiments show that blood DCs and monocytes differ in their capacity to phagocytose LT-scFv decorated tumor cells.

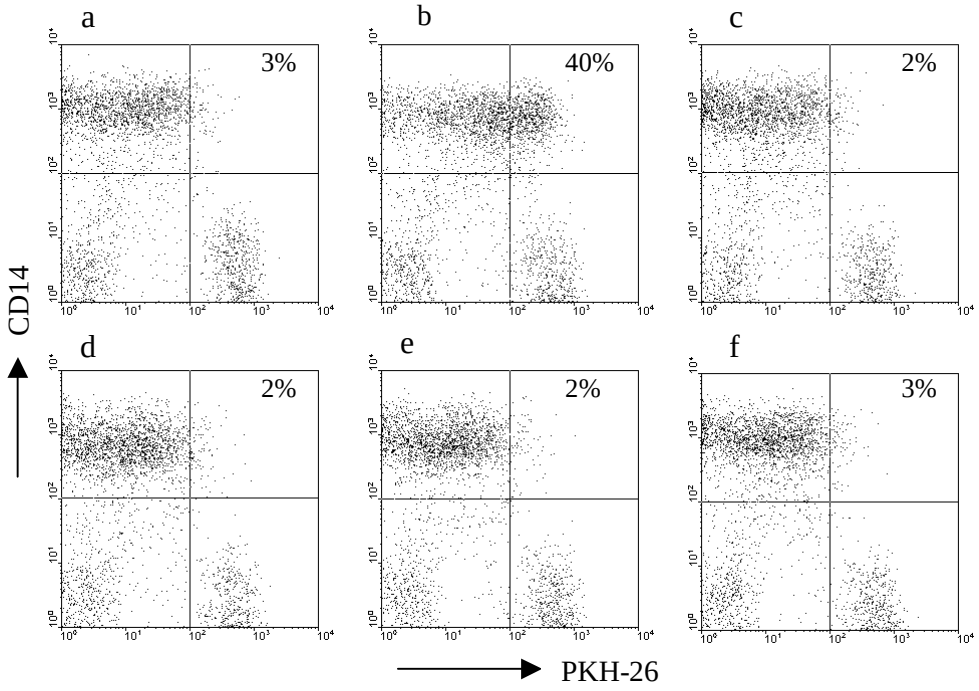

Figure 1

Phagocytosis of LT-scFV modified Jurkat cells by monocytes. PBMCs were mixed with Jurkat cells containing both a red fluorescent dye (PKH-26) and LT-scFv fragments. The cells were allowed to interact for 90 min at 37 °C, stained with FITC-labeled antibody against CD14 analyzed by flow cytometry. a, Jurkat cells displaying a control anti-DNP LT-scFV; b, Jurkat cells displaying anti-CD14 LT-scFv; c, Jurkat cells displaying LT-scFv MatDC16; d, Jurkat cells displaying LT-scFv MatDC27; e, Jurkat cells displaying LT-scFv MatDC64; f, Jurkat cells displaying LT-scFv TN141.

Induction of apoptosis of LT-scFv modified cells does not support phagocytosis

Recognition and processing of apoptotic cells in the human body is mediated by receptors that capture the apoptotic cells. A growing number of receptors are implicated in this clearance process, such as phosphatidylserine (PS) receptors and CD14 [16;24]. DCs appear to phagocytose apoptotic cells via $\alpha_v\beta_5$ and CD36 rather than via CD14 [25]. As a multiplicity of cell-surface changes take place during apoptosis, we reasoned that properties of the apoptotic-cell surface might support the recognition and phagocytosis of the LT-scFv-modified tumor cells. Viable Ramos cells, a B lymphocytic cell line, with incorporated LT-scFvs against CD14 and CD64 have previously been shown to be phagocytosed similar to Jurkat cells [12] (own observation). To induce apoptosis, Ramos cells were incubated with camptothecin and stained with annexin V and propidium iodide to determine the percentage of apoptotic cells that ranged from 25% to 40%. Incorporation of the red fluorescent dye PKH26 as well as the LT-scFv fragments was not affected by the induction of apoptosis (Fig. 2).

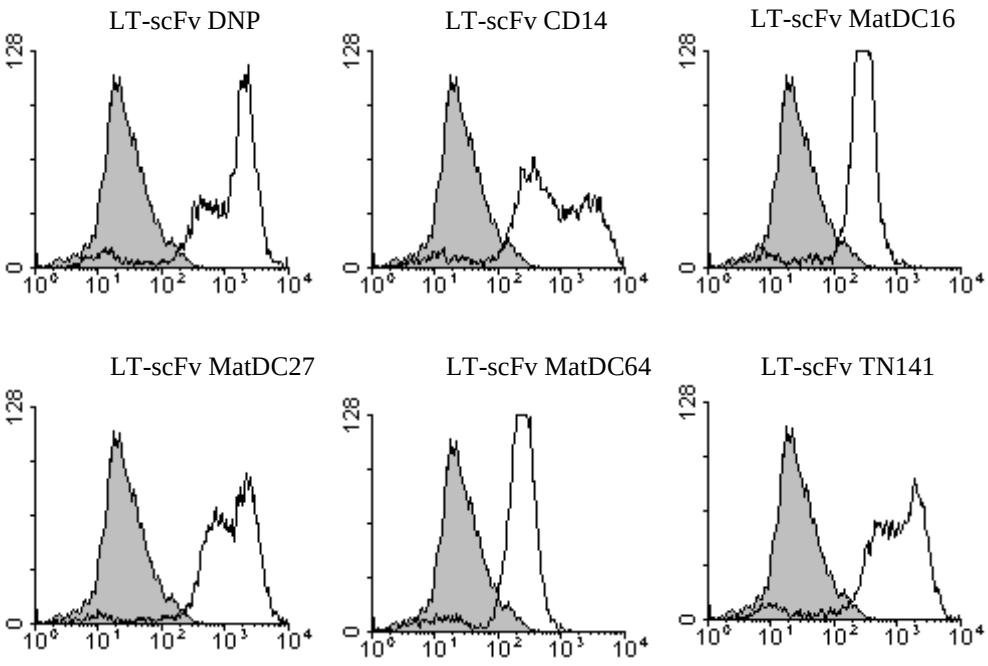

Figure 2 Incorporation of different LT-scFv into cell membrane of apoptotic Ramos cells labeled with a red fluoresenct dye (PKH26). Cells were stained using the antibody against myc tag and analyzed by flow cytometry. Filled histograms, cells incubated with buffer; Unfilled histograms, cells incubated with LT-scFv as indicated.

As shown in figure 3, phagocytosis of apoptotic Ramos cells with membrane-anchored LT-scFvs MatDC16, MatDC27, MatDC64, TN141 was not augmented compared to control LT-scFv specific for the hapten DNP. These data indicate that apopotosis of the cells that had membrane-anchored LT-scFvs MatDC16, MatDC27, MatDC64 or TN141 could not support phagocytosis by monocytes.

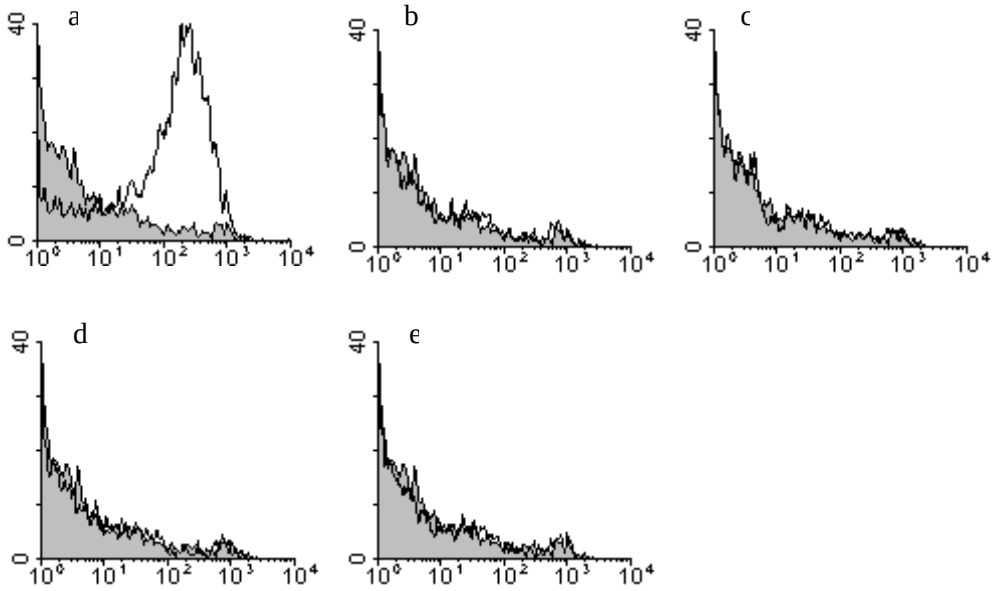

Figure 3 Phagocytosis of LT-scFv modified apoptotic RAMOS cells by monocytes. PBMCs were mixed with apoptotic RAMOS cells containing both a red fluorescent dye (PKH-26) and LT-scFv fragments. The cells were allowed to interact for 90 min at 37 °C, stained with FITC-labeled antibody against CD14 analyzed by flow cytometry. Transparent histograms show fluorescence value of red apoptotic RAMOS cells displaying a control anti-DNP LT-scFv in the gated $CD14^+$ monocytes. Filled histograms show apoptotic RAMOS cells displaying a, anti-CD14 LT-scFv; b, LT-scFv MatDC16; c, LT-scFv MatDC27; d, LT-scFv MatDC64; e, LT-scFv TN141.

Phagocytosis of LT-scFv modified tumor cells does not induce DC maturation of monocytes

To assess whether uptake of LT-scFv-modified tumor cells induced differentiation of monocytes into DCs, the induction of CD83, an early marker of DC activation/differentiation was monitored by flow cytometry. Human blood DCs induce expression of CD83 by overnight culture in media alone, and it was shown the $CD83^+$ cells are first detected after ~6 hours in culture [26;27]. These $CD83^+$ cells express high levels of HLA-DR.

Expression of CD83 and HLA-DR was monitored after 90 minutes, 3 hours and 18 hours of incubation of lipid-modified Jurkat cells and monocytes. After 90 minutes an induction of CD83 was observed on the $CD14^+$ monocytes, however this staining also observed in the absence of lipid-modifed Jurkat cells (Fig. 4). Similar CD83 expression was also observed if the serum used during experimenting was substituted for 1% bovine serum albumin. Expression of CD83 did not alter after 3 or 18 hours incubation, compared to 90 minutes incubation.

A similar pattern was observed for HLA-DR expression, since it was upregulated on the $CD14^+$ monocytes irrespective of the LT-scFv inserted in the Jurkat cells. This suggests that phagocytosis of Jurkat cells displaying anti-CD14 LT-scFvs did not induce an activated DC phenotype. Future experiments will concentrate on functional properties of monocytes upon phagocytosis, such as the capability to efficiently process and present antigens, as this is most important for use of this antigen delivery approach in immunotherapy.

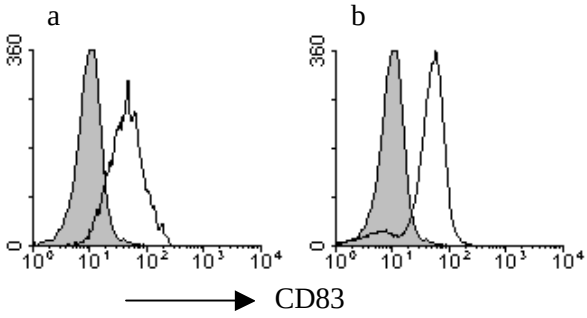

Figure 4 Staining of CD83 on $CD14^+$ monocytes before (filled histograms) and after (unfilled histograms) 90 minutes incubation on 37°C a, monocytes in the absence of lipid-modified Jurkat cells; b, monocytes in the presence of lipid-modified Jurkat cells displaying anti-CD14 LT-scFv.

We conclude that the uptake of LT-scFv-modifdied tumor cells by monocytes is dependent on the specificity of the scFv fragment inserted in the membrane of the tumor cells. ScFv specific for CD14, CD32 and CD64 mediate uptake whereas a panel of four scFvs binding to unknown targets on monocytes and DCs do not. We have not identified antibody fragments that mediate the uptake of LT-scFv modified tumor cells by blood DC. Phagocytosis of LT-scFv- modified tumor cells does not appear to signal monocytes to differentiate into DCs. Appropriate signals that would induce monocyte differentiation into

DC could be provided by incorporation of additional LT-scFv in the tumor cell membrane, e.g. scFv directed against CD40 [28].

Insertion of LT-scFv in tumor cell membranes provides a simple and rapid procedure for the modification of properties of tumor cells. It does not require gene transduction and culture of tumor cells, a method currently used to modify tumor cells prior to application in vaccine approaches. Further research is required to determine the optimal (combination of) scFv for the induction of an anti-tumor immune response.

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CHAPTER 7

SUMMARIZING DISCUSSION

The dendritic cell (DC) was named after its distinctive cell shape and also metaphorically the DC honors its name by extending its protrusions throughout the immune system. As reviewed in Chapter 1, DCs are key players in the immune system as they bridge the innate and adaptive immune system and are able to initiate a primary immune response. In addition, DCs might have roles in central and peripheral tolerance, but are also implicated in autoimmune diseases, as presentation of 'self' antigens is likely to play an important role in the initiation of autoimmunity.

The unique features of DCs lead to the hypothesis that, just like other cells in the immune system such as B, T and NK cells, unique cell surface molecules would exist for this cell type. Indeed, as DC research progresses and new techniques for the detection of markers have become available, several novel DC molecules have been discovered, paralleled by the discovery of different DC subsets based on anatomical localization and function [1-6]. The novel DC molecules are linked with the functional and/or anatomical features of DCs, rather than DC lineage. Although routes of DC differentiation are still a black box, the possibility of a DC lineage independent from the lymphoid or myeloid lineages remains and is supported by the recent description of a DC common progenitor [7].

The importance of DCs in generating an immune response has stimulated the exploration of these cells in cancer vaccines. Indeed, pilot DC-based vaccination studies have induced anticancer responses including some clinical responses [8-10]. In conclusion, understanding the complexity of the DC system is fundamental, enabling the construction of better cancer vaccines that are effective in eliciting a protective anti-tumor immune response.

The subject of the research described in this thesis is the identification of human antibodies that bind to human DCs, the use of such antibodies for further examination of DC subsets and the evaluation of their potential as targeting vehicles for the delivery of tumor antigens to DC for use in DC-based cancer vaccination strategies.

Phage antibody technology

The research described in this thesis exploited the versatility of phage antibody display technology (Fig. 1). First, isolating intact cells of interest with attached phage antibodies using flow cytometry combined with an excess of non-selected cells, that serve to absorb irrelevant phage antibodies, allows the selection of phage antibodies against cell surface markers in their native form [11]. In addition, selections can be performed on very small cell populations such as DCs, representing less that one percent of total mononuclear cells in blood and tonsil. Furthermore, the difficulty in obtaining antibodies to self-antigens that are

conserved between mouse and humans using standard hybridoma technology can be overcome by the use of phage display technology. The observation that MatDC16, one of the phage antibodies isolated and characterized in this thesis, also binds to HLA-DR^{+}Lin^{-} DCs in peripheral blood of macaques, as well as to splenic DCs in rat and mouse (data not shown) underlines this concept. It is evident that this property forms a major advantage for utilization of this antibody in *in vivo* models.

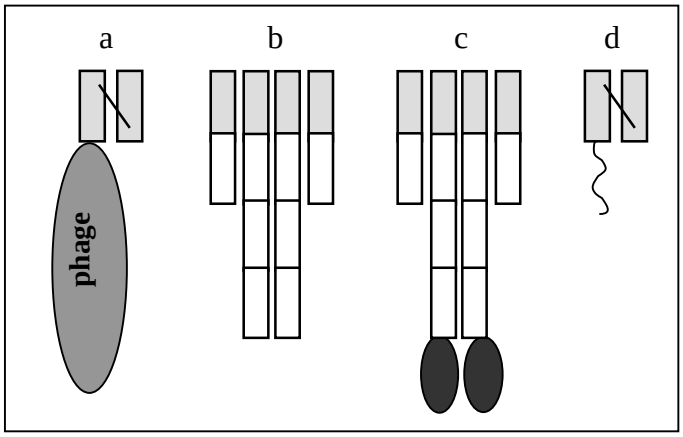

Figure 1 Schematic drawing of the engineering technology employed in the research described in this thesis. (a) Phage antibody comprised antigen binding antibody fragments (light gray) fused to the phage minor coat protein, expressed at the tip of the phage particle (dark gray). (b) The phage antibody was converted to a complete human antibody, containing constant domains (white), using a eukaryotic expression vector. (c) A fusion protein containing a human antibody linked to a tumor antigen was obtained by genetic fusion of coding sequences of a tumor antigen (black) to the constant domain of the IgG4 heavy chain. (d) Lipid-tagged antibody fragments were obtained by genetic fusion of the bacterial lipoprotein (line) to the antigen binding antibody fragment.

For immunotherapy in humans, the efficacy of mouse antibodies is hampered by the induction of human anti-mouse antibody responses [12]. This limitation can be overcome by the use of human antibodies. The engineering potential of phage display technology provides a direct route from antibody fragment to human antibodies. A eukaryotic expression vector system, that allows conversion of monoclonal phage antibodies to fully human monoclonal antibodies [13], was used to convert four phage antibodies to human antibodies (huMabs) of the IgG4 isotype (*Chapter 3*). In addition, the phage display engineering technology enabled the biosynthetic lipid tagging of phage antibodies into functional lipid-tagged antibody fragments that could be inserted in cell membranes [14] (*Chapter 6*).

Generation of phage antibodies against DCs

A large semi-synthetic phage antibody display library [15] was employed in combination with cell sorting to generate phage antibodies binding to cell surface molecules expressed on DCs. Selections were performed on DCs subsets found in human peripheral blood and tonsil (*Chapter 2A and 2B*). These selections yielded four phage antibodies that were chosen for

further characterization based on their restricted staining patterns. In summary, phage antibody MatDC16 bound to DCs, monocytes and weakly to B cells and a small subset of granulocytes in peripheral blood, whereas a unique reactivity on DCs in tonsil was observed. Phage antibodies MatDC27 and MatDC64 reacted with DCs and monocytes in blood and in tonsil only with DCs. Based on differential staining patterns on cell lines, we could exclude that MatDC27 and MatDC64 bound the same antigen. Phage antibody TN141, which was derived from the selections on tonsil DCs, stained uniquely DCs in tonsil, whereas in peripheral blood, DCs as well as monocytes were stained.

Analysis of phage antibodies

In light of the potential application of our huMabs as targeting vehicles for the delivery of tumor antigens directly to DCs *in vivo*, a detailed analysis of binding characteristics of the huMabs on naturally occurring DC subsets is essential. In due course, targeting to a specific DC subset may provide control of the quality of the immune response.

The four phage antibodies used in this study, MatDC16, MatDC27, MatDC64 and TN141 reacted with DCs as well as monocytes in peripheral blood. Monocytes and myeloid related DCs, such as the mature $CD33^{high}$ DCs used for selections, may arise from a common myeloid progenitor [16]. During the past years, it has become clear that monocytes can be regarded as immediate DC precursors. Monocytes are induced into DCs *in vitro* using the appropriate cytokines, while a subset of monocytes can differentiate into DCs after reverse transendothelial migration [17]. The latter *in vitro* system provides a more physiological context in which monocyte differentiation into DC can be studied. It is noteworthy that recent attempts to generate antibodies specific for blood DCs yielded four new antibodies of which two uniquely bound to plasmacytoid $CD11c^-$ DCs in blood [4]. However, such antibodies are still lacking for myeloid DCs, since the two other antibodies recognized myeloid $CD11c^+$ DCs plus either B cells (BDCA-1/CD1c) or monocytes (BDCA-3). We reason that the myeloid $CD11c^+$ DCs are a heterogeneous population in a flexible differentiation potential, closely related to other APCs. The plasmacytoid $CD11c^-$ DCs are much more mature, as they already exert their function in blood as IPCs [18;19].

Different DC subsets have been described based on tissue location, maturation stage or lineage. Originally, two DC subsets were identified in peripheral blood by the lack of lineage markers and expression of high levels of HLA-DR, and could be distinguished based on their ability or inability to express CD11c. In *Chapter 3*, these immunophenotypic properties were used along with analysis of discriminating cell surface markers to characterize these subsets.

This multiparameter flow cytometry could also be applied to single cell suspensions of different tissues, revealing $CD11c^+$ and $CD11c^-$ DC subsets. This enabled evaluation of the immunophenotype of DC subsets at distinct anatomical locations. A closer look at the DC subsets revealed two phenotypes within the $CD11c^+$ subset, an immature phenotype and a maturer phenotype with higher expression of MHC class II and costimulatory molecules. The recently described marker BDCA-3 was preferentially expressed on the more immature $CD11c^+$ DC in tissues. The staining pattern of huMab TN141 resembled that of BDCA-3, albeit at lower intensity. HuMab MatDC64 stained both DCs and monocytes in peripheral blood, however in secondary lymphoid organs staining was restricted to the plasmacytoid DCs.

The origin of DCs is still controversial, since *in vitro* lymphoid as well as myeloid precursors can differentiate into distinct DC subsets dependent on the cytokine environment[16]. It remains unclear how these differentiation pathways and the DCs that are generated parallel the *in vivo* situation. The fact that selected phage antibodies on fresh DCs did not react with DCs *in vitro* (this thesis) strengthens our idea that *in vitro* generated DCs may not have physiological counterparts. Additionally, MatDC64 staining was absent on monocyte-derived immature DCs, while immature DCs that had retraversed endothelium *in vitro* were positive, highlighting the differences between different DC culture systems. While MatDC16 was selected on myeloid DCs, this antibody stained plasmacytoid DCs brightly. This could argue for a resemblance of the myeloid and plasmacytoid DC subsets. However, the expression of markers might not be decisive evidence of DC relationships.

Furthermore, in view of the targeting device of our huMabs, we analyzed whether engagement of the molecules recognized by the huMabs exerted functional effects. We were not able to identify the target antigens of our four huMabs, in spite of extensive efforts that included cDNA expression cloning using a baculovirus-based cDNA library [20] as well as eukaryotic cDNA expression libraries, and immunoprecipitation studies (data not shown).
Therefore, the functional role of the target antigens remained unknown. The observation that expression of the target antigens of some huMabs that bound to monocytes, was downregulated during *in vitro* differentiation of monocytes into DCs, lead us to analyze the effect of crosslinking of huMabs on DC differentiation *in vitro* (*Chapter 4*). HuMab MatDC64 suppressed the differentiation of monocytes into $CD1a^+$ DCs, which could indicate that MatDC64 engaged an inhibitory receptor. A large number of inhibitory receptors have been discovered in the past few years, many of which are expressed by myeloid cells of the innate immune system [21]. The prevention of generation of CD1a+ DCs was accompanied

with the upregulated production of IL-6. This cytokine is able to redirect the differentiation of monocytes towards macrophages at the expense of DCs. As an alternative, this might point to the engagement of a presently unknown stimulatory receptor by MatDC64, eventually leading to the production of IL-6.

It is evident that future studies should focus on identification of the target antigens of these huMabs. What could be the nature of the target antigens recognized by the four huMabs? Many C-type lectins have been identified recently, some of them specific for DCs such as BDCA-2 [22] and the DC-specific adhesion receptor DC-SIGN [5]. Blocking the cells with mannan can inhibit specific antibody binding to C-type lectins. Preliminary data demonstrated that mannan could not inhibit the binding of MatDC16 or MatDC27 to cell line U937 (data not shown), suggesting that these huMabs did not recognize C-type lectins. Furthermore, staining using the four huMabs performed on transient or stable transfectants expressing ILT-3, ILT-4, ILT5, GM-CSF receptor, CD40, CD64 [23], OX40L and TREM-2, excluded the possibility that they bound to any of these molecules (unpublished data). In conclusion, further studies are required and currently undertaken to identify the target antigens of our four huMabs.

Use in vaccination

In a first step toward *in vivo* targeting of DCs in cancer immunotherapy, huMabs were employed as vector to target tumor antigens to DCs (*Chapter 5*). A eukaryotic expression vector was constructed encoding a complete huMab, genetically fused to the tumor antigen MAGE-1. Incubation of immature monocyte-derived DCs with the IgG4MatDC16MAGE-1 fusion protein resulted in presentation of MAGE-1 epitopes on MHC class I and II, as demonstrated by the activation of specific T helper and CTL clones. This uptake and subsequent processing and presentation appeared receptor-dependent, as IgG4MatDC27MAGE-1 and IgG4Mono14MAGE-1 could not induce CTL activation. In conclusion, fusion protein IgG4MatDC16MAGE-1 was able to target to DCs, resulting in stimulation of MAGE-1 specific T cells, providing a tumor antigen delivery vehicle that potentially can be targeted directly to DCs *in vivo*. In this respect MatDC16 might act as an 'intelligent missile' [24]. Ideally, an 'intelligent missile', containing a tumor antigen, should target to the desired DC subset, ensure efficient uptake and presentation by the DCs, and also DC activation. It will be exciting to further explore whether huMab MatDC16 can live up to the task.

Delivery of a broad spectrum of tumor antigens in DC-based cancer vaccination can be achieved when whole tumor cells are used for antigen delivery. Moreover, prior identification of tumor antigens is not necessary. In particular, lipid-tagged antibody fragments incorporated in the membrane of tumor cells can mediate phagocytosis by APCs, providing an alternatively strategy for tumor antigen delivery. Lipid-tagged antibody fragments MatDC16, MatDC27, MatDC64 or TN141 anchored in the membrane of tumor cells could not induce uptake by monocytes while an anti-CD14 lipid-tagged antibody fragment was able to induce phagocytosis, indicating that this process was receptor-dependent (*Chapter 6*). Future research is required to determine the efficacy of this simple and rapid procedure, for the induction of anti-tumor immunity.

Future perspectives

To further investigate the potential of the IgG4MatDC16 MAGE-1 fusion protein for induction of anti-tumor immunity, the use of animal models is imperative. Mice carrying a tumor expressing a human tumor antigen may provide an acceptable experimental model in which knowledge about vaccine strategies can be obtained prior to initiating clinical trials. Such a tumor model can for example be generated by injection of murine melanoma cells genetically engineered to express the human MAGE-1 molecule into mice [25]. Of great importance will be evaluation of the efficacy of this approach in elimination of pre-existing tumors. However it must be taken into account that the introduction of a xenogeneic protein into mice, such as the human MAGE-1, may induce a broad T cell response since no tolerance exists against this antigen. It may prove more difficult to induce anti-tumor immunity against naturally occurring, autologous antigens. Therefore, results obtained from animal models have to be interpreted with caution since successful strategies in mice may not be applicable in human.

Future studies in progress will elucidate the potential of *in vivo* targeting to DCs using huMab MatDC16 fused to a tumor antigen in cancer vaccination. It is tempting to speculate on the applicability of huMab MatDC16, not only in cancer vaccination but also in the treatment of other life-threatening diseases. For example, the infection of rhesus macaques with simian immunodeficiency virus (SIV) provides a useful animal model for experimental *in vivo* studies on HIV [26]. Since huMab MatDC16 binds to rhesus macaque DCs, this HuMab can be explored in an experimental HIV-1 vaccine. An appropriate experimental animal model can provide information on the efficacy, limitations, and safety of vaccination strategies before initiating clinical trials.

In conclusion, phage display antibody technology was successfully used to isolate four novel huMabs directed against DCs. These huMabs were used to further delineate DCs. In addition, a first step was made to evaluate the potential of modified forms of these huMabs as targeting vehicles for the delivery of tumor antigens. Targeting the antibody-tumor antigen fusion protein to DCs resulted in the induction of strong T helper and CTL responses. Future research on these huMabs in *in vitro* and *in vivo* models could open new and exciting avenues for cancer vaccination.

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NEDERLANDSE SAMENVATTING

Dendritische cellen (DC's) worden gezien als de waakhonden van het immuunsysteem. Wanneer DC's een binnendringer zoals een micro-organisme waarnemen, dan wordt deze opgenomen en in kleine stukjes eiwit ofwel peptiden afgebroken. De peptiden worden naar het oppervlak van de cel getransporteerd en gepresenteerd aan andere cellen van het immuunsysteem, zoals T-lymfocyten. De presentatie van peptiden, antigeen-presentatie genoemd, vormt een belangrijke stap in de activatie van het immuunsysteem die uiteindelijk leidt tot de eliminatie van de binnendringer.

DC's bevinden zich in onrijpe vorm in alle weefsels van het lichaam. Na opname van de indringer rijpen ze uit en migreren naar lymfoïde weefsels, waar presentatie door de uitgerijpte DC's aan de andere cellen van het immuunsysteem plaats vindt. DC's worden professionele antigeen-presenterende cellen genoemd en zijn als enige in staat naïve cellen van het immuunsysteem te activeren. Naast de initiatie van een immuunrespons kunnen DC's ook tolerantie induceren. Samenvattend kan gezegd worden dat DC's meerdere functies vervullen. Afhankelijk van het weefsel waar men DC's aantreft of het activatiestadium waarin ze zich bevinden, vertonen ze een ander fenotype, ofwel ze vormen een aparte subset. DC's vormen een heterogene populatie en de relatie tussen de verschillende DC subsets is niet exact bekend. Een overzicht is gegeven in hoofdstuk 1.

Antilichamen zijn in staat zeer specifiek te binden aan een bepaald molecuul. Hierdoor kunnen antilichamen die specifiek binden aan celoppervlakte moleculen van DC subsets meer inzicht geven in de heterogene DC populatie. Daarnaast kunnen antilichamen ook voor therapeutische doeleinden gebruikt worden. Aangezien DC's een centrale rol spelen bij de initiatie van een immuunrespons, zijn ze in de belangstelling gekomen bij het ontwikkelen van nieuwe vaccinatie strategieën ter behandeling van kanker, met als doel het immuunsysteem aan te zetten tot bestrijding van een tumor. Reeds uitgevoerde patiënten-studies demonstreren dat in het laboratorium of *in vitro* gekweekte DC's kunnen worden beladen met tumorantigenen; wanneer deze cellen worden terug toegediend aan de patiënt, kunnen ze een immuunrespons opwekken. Deze methode is echter tijdrovend en arbeidsintensief en maakt gebruik van gekweekte cellen die mogelijk niet dezelfde eigenschappen hebben als DC's die *in vivo* voorkomen. Een andere mogelijkheid zou kunnen zijn om in het lichaam een tumoreiwit naar de DC's te brengen met behulp van een DC-specifiek antilichaam als een doelgerichte drager.

Voor het genereren van zulke antilichamen gericht tegen DC's is gebruik gemaakt van een faag-antilichaam collectie die een groot aantal verschillende antilichaam fragmenten bevat. Hoofdstuk 2 beschrijft het selecteren van faag-antilichamen die gericht zijn tegen DC subsets, die men aantreft in bloed en keelamandelen, een bron van lymfoïd weefsel, door middel van sorteren met behulp van een flow cytometer. Er werden vier interessante antilichaam fragmenten

gevonden, MatDC16, MatDC27, MatDC64 en TN141, die bonden aan DC's en ook aan monocyten, een voorloper-populatie van DC's.

In hoofdstuk 3 is een inventarisatie beschreven van de fenotypes van natuurlijk voorkomende DC subsets in verscheidende weefsels. Deze fenotypes zijn onderzocht met behulp van de flow cytometer in combinatie met een groot aantal conventionele antilichamen en de vier geïdentificeerde antilichaam fragmenten. Met het oog op therapeutisch gebruik, is de specificiteit van de antilichaam fragmenten van belang aangezien aparte DC subsets mogelijk een verschillende immuunrespons kunnen opwekken. De overeenkomsten en verschillen tussen *in vitro* gekweekte DC's en de natuurlijk voorkomende DC subsets werden eveneens bestudeerd. De vier antilichaam fragmenten zijn eerst omgezet naar intacte en functionele humane antilichamen. In therapie hebben humane antilichamen de voorkeur aangezien deze als 'eigen' worden gezien in de mens en dus geen ongewenste afweerreactie opwekken.

In hoofdstuk 4 is beschreven hoe binding van het humane antilichaam MatDC64 op monocyten ervoor zorgde dat deze niet meer konden uitgroeien naar DC's. Deze cellen werden gekenmerkt door een verhoogde produktie van het cytokine IL-6, die mogelijk de oorzaak was van het waargenomen fenomeen.

In een eerste stap naar het gericht brengen van tumorantigenen naar DC's met behulp van een humaan antilichaam als drager, is er een fusie-eiwit gemaakt bestaande uit een humaan antilichaam met daaraan gekoppeld een geheel tumoreiwit. In hoofdstuk 5 is te zien dat incubatie van dit fusie-eiwit met gekweekte onrijpe DC's ervoor zorgde dat deze DC's anti-tumor specifieke T-lymfocyten activeerden. Dit impliceerde dat het fusie-eiwit gericht de DC's bereikte, aangezien een tumoreiwit zonder antilichaam dit effect niet gaf. Dit proces was eveneens afhankelijk van het antilichaam fragment dat werd gebruikt, aangezien enkel een fusie-eiwit met antilichaam fragment MatDC16 dit effect liet zien. Opname door DC's gevolgd door presentatie van tumorantigenen resulteerde in aktivatie van twee typen T-lymfocyten, die beide essentieel zijn voor een goede anti-tumor immuunrespons. Op lange termijn kan deze vaccinatie strategie mogelijk ingezet worden ter bestrijding van kanker.

Hoofdstuk 6 beschrijft het onderzoek aan antilichaam fragmenten met daaraan een vetstaartje gekoppeld voor mogelijk gebruik in anti-tumor vaccinatie. Deze lipide-gelabelde antilichaam fragmenten kunnen worden geïncorporeerd in de celmembraan van tumorcellen, waarbij vervolgens de opname van deze tumorcellen door antigeen presenterende cellen een alternatieve methode voor het gericht afleveren van tumoreiwitten kan vormen. Monocyten, eveneens antigeen presenterende cellen, waren in staat deze 'gelabelde' tumorcellen op te nemen. Dit gebeurde echter alleen wanneer het antilichaam fragment was gericht tegen CD14, een bekende receptor aanwezig op het celoppervlak van monocyten, en niet met een van de andere antilichaam fragmenten. Dit impliceert dat de opname door monocyten van 'gelabelde' tumorcellen receptor afhankelijk is. Aan het eind van dit proefschrift, in hoofdstuk 7, worden de belangrijkste resultaten van het beschreven onderzoek samengevat en bediscussieërd.

CURRICULUM VITAE

Annemarie Nicolette Lekkerkerker werd geboren op 6 december 1972 te Asse, België. Na één jaar op het Koninklijk Atheneum te Tervuren, België verhuisde zij in 1985 naar Nederland. In 1990 behaalde zij het VWO diploma (gymnasium-stroming) aan het Revius Lyceum te Doorn en begon zij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen.

In oktober 1995 vertrok zij voor een buitenlandse stage van 7 maanden naar de Verenigde Staten, waar zij onderzoek deed onder begeleiding van dr. G. Wiens in de groep van prof. dr. M. Rittenberg op de afdeling Molecular Microbiology and Immunology aan de Oregon Health Sciences University in Portland, Oregon.

In juni 1996 werd het ingenieursdiploma behaald in de chemisch-biologische oriëntatie met afstudeervakken Moleculaire Biologie (dr. J. Wellink en prof. dr. A. van Kammen), Virologie (dr. J. Wellink en prof. dr. R Goldbach), Experimentele Diermorfologie en Celbiologie (dr. B. Dixon en prof. dr. W. van Muiswinkel) aan de Landbouwuniversiteit Wageningen.

In september 1996 begon zij als assistent in opleiding bij de afdeling Immunologie van het Universitair Medisch Centrum Utrecht onder begeleiding van prof. dr. T. Logtenberg met als mede-promotor prof. dr. H. Clevers. Op deze afdeling werd onderzoek verricht aan humane dendritische cellen, zoals beschreven in dit proefschrift. Vanaf augustus 2002 gaat zij als post-doc werken bij prof. dr. Y. van Kooyk van de afdeling Moleculaire Celbiologie aan de Vrije Universiteit van Amsterdam.

LIST OF PUBLICATIONS

Annemarie Lekkerkerker, Joan Wellink, Ping Yuan, Jan van Lent, Rob Goldbach, Ab van Kammen. 1996. Distinct Functional Domains in the Cowpea Mosaic Virus Movement Protein. *Journal of Virology*, **70**: 5658-5661

Annemarie Lekkerkerker and Ton Logtenberg. 1999. Phage antibodies against human dendritic cell subpopulations obtained by flow cytometry-based selection on freshly-isolated cells. *Journal of Immunological Methods*, **231**: 53-63

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Annemarie Lekkerkerker, Liesbeth Bijl, Ton Logtenberg. Reactivity profiles of human IgG4 monoclonal antibodies on naturally occurring and cultured dendritic cell subsets. *Submitted*.

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NAWOORD

Zoals je hebt gelezen, dankt de dendritische cel haar naam aan de specifieke uitlopers die ze kan vormen. Dit lijkt op de manier waarop een boom groeit en kan vertakken in steeds kleinere twijgjes. Het Griekse woord ‘dendron’, waar het woord dendritisch vandaan komt, betekent dan ook boom. En welbeschouwd lijkt de totstandkoming van een proefschrift ook wel op het uitgroeien van een boom. Een boom kent dagen vol zonneschijn maar ook dagen met storm en wind. Gelukkig wordt een boom beschut door omringende bomen. Een boom vertakt zich voortdurend, er breekt wel eens een takje af, maar het blijft één geheel.

Vele mensen hebben me de afgelopen jaren gemotiveerd, geholpen, gezelligheid gegeven en gevoed (koekjes!), dank daarvoor. Ton, je bent een gemotiveerd wetenschapper (en ondernemer) en als geen ander kan je je enthousiasme voor het onderzoek overbrengen. Michel, bedankt voor de samenwerking en alle vrolijkheid. Lies, dank voor je inzet, jammer dat je wegging. Anne-Renée, bedankt voor je hulp en het beantwoorden van al mijn vragen. Labcollegae, bedankt voor de gezelligheid. Dank ook aan de Leidse DCers. Alle bloeddonoren wil ik bedanken voor hun gift. Je voudrais remercier Nathalie Demotte et Pierre van der Bruggen pour une collaboration fructueuse.

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Lieve, lieve Casper, nog steeds ‘head over feet’! Soms heeft zelfs de grootste prater geen woorden, dank je.

ANNEMARIE

LIEDJE

HET DUURT ALTIJD LANGER DAN JE DENKT,
OOK ALS JE DENKT
HET ZAL WEL LANGER DUREN DAN IK DENK
DAN DUURT HET TOCH NOG LANGER
DAN JE DENKT

HET IS ALTIJD VEEL DUURDER DAN JE DENKT,
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DAN DUURT HET TOCH
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JUDITH HERZBERG